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Regulation of apical-basal patterning during *Arabidopsis thaliana* embryo development

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biology

by

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ABSTRACT OF THE DISSERTATION

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The formation of the apical-basal axis during *Arabidopsis thaliana* embryogenesis is a crucial step that directs the overall body plan of the seedling and directs the establishment of stem cell populations at either end of the embryo that give rise to all of the adult structures. The establishment of the apical-basal axis occurs with the first division of the zygote and remains plastic until the transition stage, where apical-basal polarity becomes fixed.

The molecular mechanisms and genes responsible for the formation and fixation of the apical-basal axis remain largely unknown. This dissertation describes the advances made toward the elucidation of two major pathways that contribute to the formation and fixation of the apical-basal axis. Transcriptional repression plays a major role in developmental processes in general and this dissertation describes the role of the *TOPLESS* (*TPL*) gene in repression of the basal embryonic gene program in the developing apical domain of the embryo. We provide a framework for the function of *TPL* in transcriptional repression through chromatin remodeling. Furthermore, we show that the *PLETHORA* (*PLT*) family of transcription factors are direct targets for *TPL* mediated repression and necessary for the shoot to root transformation seen in the *tpl-1* mutant. Secondly, using the *tpl-1* mutant as a tool for forward genetics, the *HD-ZIP Class III* transcription factor family members are identified as determinants of apical fate in the globular stage embryo. Additionally, the *HD-ZIP III* genes are shown to have a mutually antagonistic relationship with the *PLT* gene family. Lastly, we show that the *HD-ZIP III* genes are true apical determinants and able to cause a homeotic conversion of the basal pole to a second apical pole.

CHAPTER 1

Introduction

ARABIDOPSIS EMBRYOGENESIS

Arabidopsis thaliana embryonic development has been well documented from a morphological and genetic prospective. The patterns of cell division in Arabidopsis early embryo development are largely invariant and thus provide an ideal framework for mechanistic study of pattern formation from a single cell.

Embryonic development proceeds immediately following the double fertilization event, which results in the triploid fertilized central cell, which gives rise to the endosperm, and the diploid zygote. The newly formed zygote elongates and divides asymmetrically to give rise to two cells with distinct composition and developmental fates. The larger basal cell is highly vacuolated and proceeds to divide horizontally to produce the filamentous structure of the suspensor. The smaller apical cell is densely cytoplasmic and will give rise to the embryo proper. This first division event generates the apical-basal axis, which persists throughout the development of the embryo. Following two rounds of longitudinal division from the apical cell, one round of transverse cell divisions creates the octant embryo. This round of transverse divisions takes place in a continuous plane that divides the embryo into apical

and basal domains of four cells each. This plane of cell division also creates the O' line, a morphological marker that can be followed throughout early development to delineates the apical and basal domains.

The cells of the octant embryo undergo a round of tangential divisions that generate the sixteen cell stage embryo with an inner and outer layer of cells along the newly formed radial axis. The outer eight cells form the protoderm, which will continue to divide in an anticlinal manor and remain separate from the inner cells to generate the entirety of the embryonic and seedling epidermis. At this stage the embryo is radially symmetric and denotes the beginning of the globular stage.

Immediately following the sixteen cell stage, the cells of the apical and basal domains exhibit drastically different patterns of cell division and elongation. The inner cells of the basal domain divide parallel to the apical-basal axis and elongate to generate the precursor cells to the vascular and ground tissue. As a crucial event in basal patterning, the uppermost cell of the suspensor, or hypophesis, is incorporated into the basal end of the embryo proper and gives rise to the cells of the quiescent center and root cap. In the apical domain patterns of cell division remain mainly isodiametric. Bilateral symmetry is subsequently initiated, marked by rapid cell divisions at the periphery, which direct the plane of outgrowth for the cotyledons and mark the start of the transition stage, when the embryo takes on a triangular shape.

Cell division in the apical-central domain of the transition stage embryo

slows dramatically during the outgrowth of the cotyledon primordia, generating the domain for the precursors of the shoot meristem. The emerging cotyledon develop along a new axis that defines top from bottom, or adaxial from abaxial. In *Arabidopsis,* the adaxial/abaxial notation is applied to all emerging lateral organs, such as leaves and floral organs, as well as vascular bundles. The adaxial pole is defined as being located proximal to the meristem, with the abaxial pole distal to the meristem. Cotyledons and leaves elongate as a laminar structure perpendicular to the adaxial/abaxial axis. Following the further outgrowth of the cotyledon primordia and growth of the basal domain the embryo enters the heart stage. At this stage, the precursors to the major organ systems of the embryo are morphologically distinct and the overall body plan of the embryo has been established. Embryogenesis culminates in the generation of two distinct stem cell populations, the shoot and root meristems at the apical central and basal embryonic poles, respectively, from which all above and below ground structures of the adult plant are derived.

THE ROLE OF AUXIN DURING EMBRYOGENESIS

Our current understanding of plant embryogenesis suggests that cell fate determination is largely, if not entirely, dependent on positional information rather than lineage-dependent fate restriction (Torres-Ruiz, Lohner et al. 1996). Due to the immobile nature of plant cells, the production, transport, and perception of small molecules is likely to play a central role in the

translation of positional information. Accordingly, the polar transport and localized accumulation of the phytohormone auxin has been shown to play a critical role in apical-basal axis formation, root patterning and cotyledon initiation during *Arabidopsis* embryogenesis (Weijers, Schlereth et al. 2006; Moller and Weijers 2009).

Auxin is the general name for a class of tryptophan derivatives, with differential activities and chemical properties, however, the most active endogenous auxin is indole-3-acetic acid (IAA). Auxin biosynthesis proceeds through multiple, parallel biosynthetic pathways, which are differentially regulated in response to developmental and environmental cues. The role of auxin biosynthesis in the generation of local auxin pools as a developmental mechanism has yet to be clearly defined. It is clear that the regulation of the family of YUCCA flavin monooxygenases contribute to the sustained auxin response following primordia initiation (Zhao 2008).

In contrast, the polar transport of auxin has been well studied and shown to be highly dynamic and during embryogenesis and crucial in generating local and discrete auxin maxima. Differential auxin accumulation in the cell is achieved through the coordinated action of influx and efflux carriers. While influx carriers clearly affect cellular auxin concentration, the directional flow of auxin is thought to be controlled by the polar distribution of the PINFORMED (PIN) family of auxin efflux carriers (Friml, Vieten et al. 2003; Kramer 2004; Feraru and Friml 2008).

In the two-cell embryo, the efflux carrier PIN7 is asymmetrically distributed to the apical edge of the basal cell and presumably results in the accumulation of auxin in the apical cell (Friml, Vieten et al. 2003). The PIN family of efflux transporters continue to direct auxin flow apically through the suspensor and into the embryo proper until the globular stage. This is corroborated by the expression of the synthetic auxin responsive promoter element reporter DR5 in the apical cells of early embryos. This PIN dependent apical accumulation of auxin is critical for proper apical-basal patterning, demonstrated by the *gnom* mutant, in which PIN proteins are no longer asymmetrically distributed and apical-basal polarity is disrupted, resulting in disorganized embryos lacking clear apical-basal patterning (Geldner, Anders et al. 2003).

At the mid-globular stage, the PIN proteins are redistributed within the embryo and direct auxin flow in an apical to basal direction. This switch in auxin flux is accompanied by the incorporation of the hypophesis into the embryo proper and a stronger observed expression of DR5 at the basal pole. This event is thought to be a crucial step in basal patterning and root meristem formation (Friml, Vieten et al. 2003).

Auxin-induced gene expression is achieved in large part through the degradation of AUX/IAA transcriptional repressors, which serve to repress auxin responsive genes in the absence of auxin. The AUX/IAA proteins bind to and repress the action of AUXIN RESPONSE FACTOR (ARF) transcription

factors (Gray, Kepinski et al. 2001). Auxin has been shown to bind to TRANSPORT INHIBITOR RESPONSE1 (TIR1) and the AUXIN SIGNALLING F-BOX (AFB) family of F-box proteins (Mockaitis and Estelle 2008). *TIR1* and *AFB* family genes are members of the *SKIP1-CULLIN-F-BOX (SCF)* ubiquitin ligase complex. Upon auxin binding the *TIR1/AFB* SCF complex greatly increases binding affinity for the *AUX/IAA* family of transcriptional repressors, which are subsequently targeted for ubiquitin-mediated protein degradation. Following the degradation of AUX/IAA repressors, ARF transcription factors proceed to activate their respective auxin-responsive gene transcription program.

To date, no *AUX/IAA* or *ARF* gene has been implicated in the initial apical-basal axis generation process in the two cell embryo. However, a large body of work has been dedicated towards the study of the role of auxin signaling during embryonic root formation. Most notably is the work that describes the actions of *ARF5/MONOPTEROS(MP)* and *IAA12/BODENLOS(BDL) (Hardtke and Berleth 1998; Hamann, Benkova et al. 2002; Weijers, Schlereth et al. 2006)*. Loss-of-function mutations in *mp* result in the lack of formation of the embryonic root and part of the hypocotyl. Conversely, gain-of-function mutations in *bdl* that render BDL resistant to auxin mediated degradation result in a similar phenotype. Additionally, *mp* and *bdl* both display additional developmental defects in the apical domain, resulting in fused and defective cotyledons and a reduced vascular system, highlighting

the multiple roles of auxin signaling during embryogenesis.

THE PLETHORA GENE FAMILY AND ROOT PATTERNING

In addition to *MP* and *BDL*, there have been a number of genes identified that contribute to patterning the basal domain and embryonic root. Most notably, the *PLETHORA* genes have been identified as master regulators of root formation (Aida, Beis et al. 2004; Blilou, Xu et al. 2005; Galinha, Hofhuis et al. 2007). Ectopic expression of *PLT2* has been shown sufficient to initiate root formation in both embryonic and post embryonic apical tissues (Aida, Beis et al. 2004). Conversely, segregants for loss of function mutations in *plt1 plt2 plt3 plt4/bbm* completely lack an embryonic root (Galinha, Hofhuis et al. 2007). The expression and function of the *PLT* genes has also been shown to be closely intertwined with auxin signaling and transport. *PLT1* and *PLT2* expression is dependent on the auxin response factor *MP* (Aida, Beis et al. 2004) and the *PIN* genes seem to focus the domain of *PLT* gene expression by controlling local auxin concentration (Blilou, Xu et al. 2005). The activation of *PLT* transcription, at least in lateral root formation is significantly later than the early auxin responsive set of genes, suggesting that there may be several levels of gene regulation that control *PLT* expression.

TOPLESS IS A REPRESSOR OF AUXIN SIGNALING AND ROOT FATE

One of the most informative modes of study in the field of development is the study of mutants or genetic backgrounds that give rise to homeotic transformations. The ability of *PLT1* and *PLT2* to cause homeotic transformations of vegetative tissues into root when misexpressed in those tissues provided great insight into their role in the generation and maintenance of root meristems. Similarly, the *topless-1* (*tpl*) mutant displays a homeotic transformation of the apical half of the embryo into a second root (Long, Woody et al. 2002). t*pl-1* is a semi-dominant and temperature sensitive mutant, which displays a range of phenotypes that disrupt apical patterning and cause partial transformation of shoot to root. These phenotypes, in order of least to most severe, include fused cotyledons and monocotyledony, seedlings which resemble a pin and lack cotyledons and the shoot meristem, and the double root phenotype. The severity and penetrance of these *tpl-1* phenotypes are temperature sensitive, both increasing with temperature. TPL is a protein of 1131 amino acid residues and contains a domain structure that resembles known corepressors from other model systems, including the Groucho/TUP1 family (Long, Ohno et al. 2006). In *Arabidopsis, TPL* belongs to a family of nine genes, for which it is the founding member, along with *TOPLESS RELATED1-8*. The *tpl-1* mutation is a single base pair mis-sense mutation within the TOP domain, which is highly conserved within all *TPL* homologs. The *tpl-1* mutation was found to function as a dominant negative

for at least four of the eight *TPR* genes. *TPL* was found to interact genetically with the chromatin modification enzymes *HISTONE ACETYLTRANSFERASE GNAT SUPERFAMILY1* (*HAG1*) and *HISTONE DEACETYLASE19* (*HDA19*), further suggesting that *TPL* plays a role in regulation of gene expression. The observation that loss of function mutations in *HAG1* (a transcriptional activator) suppress *tpl-1*, while loss of function mutations in *HDA19* (a transcriptional repressor) enhance *tpl-1*, further support the contention that *tpl-1* functions as a repressor.

TPL was subsequently found to function as a repressor through a series of *in planta* repression assays (Szemenyei, Hannon et al. 2008). Furthermore, TPL is recruited to chromatin by sequence specific transcription factors. This interaction is facilitated through the ERF-associated amphiphillic repression (EAR) domain residing in the transcription factor with the N-terminal portion of TPL containing lissencephaly type1-like homology (LiSH) and C-terminal to LiSH (CTLH) domains. From a yeast two hybrid screen for physical interaction partners with TPL, sixteen of the twenty three AUX/IAA proteins. Of these, *IAA12/BDL* was characterized in depth. *TPL* was found to play a central role in mediating the active repression of auxin responsive gene expression by *BDL.* This interaction fits nicely with the model of *TPL* as a repressor of root fate in the apical portion of the embryo, given that the *BDL/MP* pathway is essential for embryonic root formation. Thus, it is logical to hypothesize that the double root phenotype observed in *tpl-1* is a result of misexpression of root

program genes, repressed by *BDL* in the WT and downstream of auxin signaling.

APICAL EMBRYONIC PATTERNING

Apical embryonic patterning in *Arabidopsis* generally concerns the patterning of two organ systems, the shoot meristem and the cotyledons. Through the use of forward genetics, a large number of mutants have been identified that disrupt the develop of one or both of these structures. Shoot meristem formation is largely governed through two pathways, the *WUSCHEL* (*WUS*)/*CLAVATA*(*CLV*) and *CUP-SHAPED COTYLEDONS* (*CUC)/SHOOT MERISTEMLESS* (*STM*)/*ASYMMETRIC LEAVES1/2* pathways. Both *WUS* and *STM* are positive regulators of meristem development, and loss of function of either gene results in failure to produce a meristem. A central role for *WUS* is the regulation of meristem size through a negative feedback loop (Laux, Mayer et al. 1996; Fletcher, Brand et al. 1999; Schoof, Lenhard et al. 2000; Clark 2001). *WUS* acts in the organizing center of the meristem and signals the cells above to produce the *CLV3* secreted signaling peptide that binds to it's receptor pair *CLV1/2*, which in turn inhibit *WUS* expression.

STM is expressed much more broadly in the meristem than *WUS*, and serves to spatially define the meristem from the site of cotyledon primordia and promote the undifferentiated state (Long, Moan et al. 1996). *STM* helps to define the meristem-primordia border through a mutually antagonistic

relationship with *AS1.* *STM* expression is dependent on *CUC* genes (Aida, Ishida et al. 1999). Multiple loss of function combinations in *CUC1,2, and 3* result in fusion of the cotyledons and loss of the meristem (Vroemen, Mordhorst et al. 2003). The expression of the *CUC* genes is regulated by auxin, which acts to repress *CUC* expression in cotyledon primordia (Friml, Yang et al. 2004). Cotyledon specification is largely governed by local auxin concentration. DR5 reporter activity is detected very early in the cotyledon specification process in the site of incipient primordia and the network of *PIN* proteins have been shown to shuttle auxin to these sites. Mutations in auxin biosynthesis, transport, and signaling have all been shown to dramatically affect or abolish cotyledon formation (Moller and Weijers 2009).

The patterning of cotyledon and shoot meristem are closely interdependent, as demonstrated with the *CUC*/*STM*/*AS1* described above. This interplay is further exemplified through the study of the *HD-ZIP Class III* family of transcription factors. This family is composed of five members: *REVOLUTA (REV), PHABULOSA (PHB), PHAVOLUTA (PHV), INCURVATA4/CORONA (ICU4),* and *ARABIDOPSIS THALIANA HOMEOBOX-8 (ATHB-8)*. These genes have been best described in their role in promotion of adaxial polarity in the vegetative plant and contribute to patterning the polarity of all lateral organs, the vascular tissue, as well as ovule integument polarity (Bowman 2004; Schmitz and Theres 2005; Byrne 2006). The *HD-ZIP III* genes have also been described for their role in promotion of

adaxial fate and meristem formation in the embryo. Single loss of function alleles have no phenotype, but multiple combination with *rev* result in the loss of adaxial polarity in the cotyledons and the failure to for a meristem. Loss of *PHB, PHV,* and *REV* together results in the production of a pin shaped seedling with a single radialized cotyledon and no shoot meristem (Emery, Floyd et al. 2003). The expression of all members of this family is under the control of a family of microRNAs, *MIR165/166*, which is highly conserved among all flowering plants (Mallory, Reinhart et al. 2004; Floyd, Zalewski et al. 2006). Mutations in the microRNA recognition sequence lead to transcript over accumulation and misexpression, and semi-dominant mutants of this type have been identified for all members of the family with the exception of *ATHB-8*. This microRNA regulation serves to restrict the expression of the *HD-ZIP III* genes from the abaxial and peripheral zones of the embryo, as well as from the root meristem (McConnell, Emery et al. 2001). Recently, it was shown that the *HD-ZIP III* genes are also under negative feedback control in a subset of cells within the adaxial and meristem region by the *LITTLE ZIPPER* (*ZPR*) family of genes. The *HD-ZIP III* genes are obligate dimers, and the *ZPR* genes function by binding to the leucine zipper domain of the *HD-ZIP III* genes, preventing their dimerization and DNA binding capabilities . Lastly, the *HD-ZIP III* genes are involved in a mutually antagonistic relationship with the gene families associated with abaxial patterning, namely the *KANADI* and *YABBY* genes.

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CHAPTER 2

The role of *TOPLESS* in regulation of apical embryonic fate and identification of the *PLETHORA1* and *PLETHORA2* genes as direct targets of TPL mediated repression

SUMMARY

In this chapter contains two major sections. First, I present the paper titled "*TOPLESS* Regulates Apical Embryonic Fate in *Arabidopsis*". This work identifies the locus for the *tpl-1* mutation and describes some molecular properties of TPL, including it's nuclear localization, expression pattern, as well as describing some gene expression in the *tpl-1* mutant. Furthermore, the dominant negative nature of the mutation was elucidated and the *tpl-1* phenotype was recapitulated with a pseudo-quintuple mutant of *tpl-2 tpr-1 tpr-3 tpr-4* and RNAi knock-down of *TPR2*. The framework for the role of *TPL* in transcriptional regulation is established. *TPL* is postulated to be a corepressor, based on domain structure, as well as by the observation of the misexpression of basal genes in the apical domain of *tpl-1* embryos. Furthermore, two mutants are identified that interact genetically with *tpl-1*. In a suppressor screen performed in the *tpl-1* background, two loss of function alleles of *HISTONE ACETYLTRANSFERASE GNAT SUPERFAMILY1* (*HAG1*)

were identified that completely rescues the *tpl-1* embryonic phenotype. Furthermore, loss of function alleles of *HISTONE DEACETYLASE19* (*HDA19*) were found to enhance the *tpl-1* phenotype, as well as exhibit a similar phenotype to *tpl-1* when grown at high temperatures. This work placed *TPL* within a pathway that functions in the regulation of gene expression and chromatin states. Specifically, we hypothesized that *TPL* was necessary for repression of basal determinants, which are inappropriately activated in *tpl-1* and that activation is dependent on the activity of *HAG1.* Furthermore, we suggest that *HDA19* likely participates in this active repression of basal genes, possibly through it's recruitment by TPL. Both of these hypotheses are currently under investigation. The majority of this work was performed by Dr. Jeff Long during his postdoctoral fellowship in the laboratory of Dr. Elliot Meyerowitz. My contributions were three fold. I participated in the generation of the pseudo-quintuple mutant, *tpl-2 tpr-1 tpr-3 tpr-4* and RNAi knock-down of *TPR2*. I identified a second site mutation in *tpl-1* and contributed towards the identification of the causative lesion. However, my major contribution to this work was in the analysis of basal gene expression in *tpl-1* at permissive and non-permissive temperatures. This not only yielded informative expression patterns, but represented a technological advance in our embryonic confocal microscopy abilities as well as embryonic tissue collection strategies.

Secondly, I present work that I have performed towards the identification of direct targets for TPL repression that are causative of the

shoot to root homeotic conversion observed in *tpl-1.* Here the focus is on the study of the *PLETHORA* gene family and their genetic and molecular interaction with *TPL.* The expression patterns of *PLT1-4* are described and *PLT1, PLT2,* and *PLT3* are found to be misexpressed in the apical domain of young *tpl-1* embryos, suggesting that they may play a role in the shoot to root transformation. *PLT1* and *PLT2* were found to be necessary for this process, as loss of function alleles in *PLT1* and *PLT2* completely suppress double root formation. Lastly, chromatin immunoprecipitation (ChIP) followed by real-time qPCR on ChIP DNA from embryos harvested from a *TPLp::TPL-HA* transgenic line show that TPL is present on the promoter regions of the *PLT1* and *PLT2* genes. These lines of evidence suggest that repression of the *PLT* genes by *TPL* is crucial for proper apical embryonic development, and misregulation of the *PLT* genes is likely causative of the *tpl-1* double root phenotype.

REPRODUCTION OF THE MANUSCRIPT

TOPLESS regulates apical embryonic fate in *Arabidopsis*

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The embryos of seed plants develop with an apical shoot pole and a basal root pole. In *Arabidopsis*, the *topless-1* (*tpl-1*) mutation transforms the shoot pole into a second root pole. Here, we show TPL resembles known transcriptional co-repressors and *tpl-1* acts as a dominant negative for multiple TPL-related proteins. Mutations in the putative co-activator *HISTONE ACETYLTRANSFERASE GNAT SUPERFAMILY1* suppress the *tpl-1* phenotype. Mutations in *HISTONE DEACETYLASE19*, a putative co-repressor, increase the penetrance of *tpl-1* and display similar apical defects. These data point to a transcriptional repression mechanism that prevents root formation in the shoot pole during *Arabidopsis* embryogenesis.

The apical/basal axis of *Arabidopsis* embryos is established during the first cell division of the zygote and auxin accumulation and response have been shown to be important for early steps in axis establishment (Mansfield, Briarty et al. 1991; Hardtke and Berleth 1998; Steinmann, Geldner et al. 1999; Friml, Vieten et al. 2003; Blilou, Xu et al. 2005). As the embryo matures, specific cell types become apparent and a clear shoot/root axis is visible at the transition stage of development(Long and Barton 1998; Haecker, Grob-Hardt et al. 2004). Although several mutants have been isolated that affect the formation of specific patterning elements of the shoot at the transition stage of embryogenesis, only *topless-1* (*tpl-1)* so far switches the identity of the shoot into that of a root(Barton and Poethig 1993; Laux, Mayer et al. 1996; Aida, Ishida et al. 1999; Long, Woody et al. 2002). It is therefore likely that *TPL* is acting at a different level of control than those factors that have previously been isolated.

tpl-1 mutants are temperature sensitive and at the restrictive temperature (29°C), transform the embryonic shoot pole into a second root pole giving rise to a double-root seedling (Long, Woody et al. 2002)(Fig. 1A, B). At lower temperatures, *tpl-1*embryos fail to form a shoot apical meristem and show varying degrees of cotyledon fusion (Fig. 1C to E). We view these phenotypes as a result of partial apical to basal transformation during embryogenesis (Long, Woody et al. 2002)(Fig. S1). Previous work has shown that transition stage *tpl-1* embryos lack or have reduced expression of genes

associated with the apical half of the embryo, while the expression patterns of genes associated with the basal half of the embryo are expanded into the apical half and are ultimately duplicated. Pre-transition stage *tpl-1* embryos are morphologically indistinguishable from wild-type.

To examine the molecular organization of the apical half of *tpl-1* pre-transition stage embryos, we performed *in situ* hybridizations with the transcription factor *WUSCHEL* (*WUS*) (Laux, Mayer et al. 1996). *WUS* is initially expressed in a small group of cells in the apical half of 16-cell stage embryos. *WUS* mRNA accumulated normally in *tpl-1* globular stage embryos, but was absent in transition stage embryos at 29°C (Fig. 1F to H). This indicates that early *tpl-1* embryos have established an apical axis with the correct organization, but this fate is lost or masked at the transition stage.

tpl-1 was mapped to BAC F7H2 on chromosome 1 using PCR-based markers (*11*). We found two base-pair substitutions in At1g15750 that cosegregated with the *tpl-1* phenotype and result in a change of a lysine(K) to methionine(M) at amino acid 92 and an asparagine(N) to a histidine(H) at amino acid 176 of the predicted protein (Materials. Concurrently, we conducted a high temperature EMS suppressor screen in the *tpl-1* background and found 5 semidominant suppressors that mapped to the original *TPL* locus. We sequenced At1g15750 from these lines and found that each harbored a second site mutation that is predicted to reduce or abolish gene function (Fig. 2A). That second site mutations in the *tpl-1* mutant gene suppress the *tpl-1*

phenotype indicates that *tpl-1* is a gain-of-function allele. The semidominant nature of these loss-of-function alleles also implies a dosage requirement for the *tpl-1* protein.

TPL is predicted to encode an 1131 amino acid protein containing 11 WD40 repeats at the C-terminus (Fig. 2A). At the N-terminus, TPL has predicted lissencephaly type 1-like homology (LisH) and C-terminal to LisH (CTLH) domains that are thought to be important either for self-dimerization or for other protein-protein interactions (Emes and Ponting 2001). TPL also contains a 100 amino acid region rich in prolines (24/100). A similar domain organization is found in the TUP1/GROUCHO and LEUNIG family of transcriptional co-repressors, although there is little sequence identity between TPL and these proteins (Chen and Courey 2000; Conner and Liu 2000). Four other predicted proteins in *Arabidopsis* share extensive amino acid similarity with TPL and have been named TOPLESS-RELATED *(*TPR*)* (Fig. S2).

In situ hybridization experiments reveal that *TPL* mRNA accumulates in all cells of the embryo as well as in extra-embryonic tissues (Fig. 2B, C). *TPL* mRNA accumulates to higher levels in the embryo proper during early embryogenesis and the developing vasculature in later stages. A TPL-GREEN FLUORESCENT PROTEIN (GFP) translational fusion under the control of 4.1 KB of upstream genomic sequences rescues the *tpl-1* phenotype when homozygous and localizes to the nuclei of all cells in transgenic plants (Fig. 2D). This again indicates a dosage dependence for the *tpl-1* protein and

suggests that the wild-type version of the protein can outcompete the mutant form.

To determine if both of the two amino acid changes found in the original *tpl-1* allele were necessary for the *tpl-1* phenotype, we transformed a *tpl* T-DNA insertion line (*tpl-8)* with TPL-GFP fusion proteins containing either both mutations (*tpl-1)*, only the K92M mutation, or only the N176H mutation(Alonso, Stepanova et al. 2003). The *tpl-1* phenotype was observed in plants carrying either the *tpl-1* transgene or the N176H transgene (16 and 15 lines respectively). However, we did not observe any *tpl* phenotypes in 29 independent lines transformed with the K92M construct despite nuclear GFP accumulation comparable to lines with a phenotype. Therefore, the N176H mutation is necessary and sufficient to cause the *tpl-1* phenotype.

tpl loss-of-function alleles display no obvious phenotype when grown at the restrictive temperature (Fig. 2E). We therefore hypothesized that TPL may act redundantly with the other TPR proteins. We generated *tpl-2; tpr1; tpr3; tpr4* quadruple mutant lines and transformed them with a *TPR2* RNA interference (RNAi) transgene. We obtained 5 stable transgenic lines that displayed the original *tpl-1* phenotypes (Fig. 2F). This indicates that the *tpl-1* allele acts as a type of dominant negative for multiple *TPR* family members.

In the high temperature suppressor screen, we also isolated two alleles of a recessive extragenic suppressor of *tpl-1* designated *big top (bgt)*. At 24°C, the progeny of plants homozygous for *tpl-1* and heterozygous for *bgt-1*

segregated 24.1% wild-type seedlings (Fig. 3B)(n=513). This same combination with *bgt-2* yielded 19.3% wild-type seedlings (n=1746). We therefore characterized *bgt-1* in more detail. Morphologically, *tpl-1; bgt-1* embryos form cotyledons at the transition stage of embryogenesis, although they appear slightly stunted at later stages as compared to wild-type embryos (Fig. 3C, D). To examine the apical pattern of *tpl-1; bgt-1* embryos, we examined the expression of *WUS* in these double mutants at 29°C. At all stages tested, *tpl-1;bgt-1* embryos maintained the expression of *WUS* in the appropriate number of cells, indicating that the top half of these embryos had not lost their apical identity (Fig. 3E to G). The suppression of *tpl-1* is not mediated through WUS however, as *tpl; bgt; wus* triple mutants still display two unfused cotyledons (data not shown).

We mapped the *bgt-1* mutation and found it was tightly linked to marker TSA1 on chromosome 2 (0 recombinants/606 chromosomes). This genomic region contains the Arabidopsis homologue of the histone acetyltransferase GCN5 (*HAG1*)(also known as *atGCN5*) (Bertrand, Bergounioux et al. 2003; Vlachonasios, Thomashow et al. 2003). In other eukaryotes, GCN5 is recruited to specific promoters by DNA binding transcription factors and is thought to promote transcription by acetylating the N-terminal tail of histone H3(Kuo, Brownell et al. 1996). Sequencing revealed that both *bgt-1* and *bgt-2* carried lesions in *HAG1* (Fig. 3A). We therefore renamed these alleles *hag1-3* and *hag1-4.* 2 T-DNA insertions (*hag1-5, 1-6)* were also isolated and found

to suppress *tpl-1*(Fig. 3A, data not shown). All 4 *hag* alleles have no obvious embryonic phenotypes, although postembryonically they display pleiotropic phenotypes similar to a previously described allele (Vlachonasios, Thomashow et al. 2003). A translational fusion of a 4.3kb HAG1 genomic clone to GFP rescued the *hag1-3* mutant and the protein was found in the nuclei of all cells examined (Fig. 3H). The observation that a mutation in a co-activator suppresses the *tpl-1* phenotype is consistent with TPL acting as a co-repressor.

In eukaryotes, transcription from many promoters can be repressed through the activity of histone deacetylases. The RPD3 family of histone deacetylases can act as transcriptional co-repressors and in *Drosophila*, Groucho and an RPD3-like protein work together in the specification of anterior/posterior polarity (Chen, Fernandez et al. 1999). The *Arabidopsis* genome contains 4 class 1 RPD3-like proteins (Histone Deacetylase (HDA) 6, 7, 9, and 19)(Pandey, Muller et al. 2002). In a screen for mutants that affect floral organ identity, a T-DNA allele of HDA19 (*hda19-1*) (also known as *atHD1*and *RPD3a*) was isolated that displays floral phenotypes similar to those of *tpl-1* (Ohno and Meyerowitz; Wu, Tian et al. 2000; Tian and Chen 2001). A second T-DNA allele (*hda19-2*) was isolated from the Wisconsin *Arabidopsis* Knockout facility and found to show similar phenotypes (Fig. 4A). We therefore examined the role and expression of HDA19 more closely during embryogenesis.

HDA19, like *TPL* and *HAG1,* is broadly expressed throughout embryogenesis and a GFP fusion protein localizes to the nuclei of all embryonic cells (Fig 4B, C). Phenotypically, both *hda19-1*and *hda19-2* seedlings when grown at 24°C have narrow cotyledons as compared to wild-type (Fig. 4D). However, when mutants homozygous for either allele were grown at 29°C, mutant seedlings displayed several *tpl-1*-like phenotypes, including monocots, tubes and pins, indicating that these *hda19* alleles are temperature sensitive (Fig. 4E). These phenotypes were seen in 32% of *hda19-1* seedlings (n=397) and 28% of *hda19-2* seedlings (n=330). A morphological analysis of *hda19-1* embryos at 29°C showed that both the root and the shoot can be disorganized (Fig. 4F), indicating that HDA19 may play a broader role in embryogenesis than TPL.

We then examined the progeny of *hda19-1*$^{-/-}$*; tpl-1*$^{+/-}$ plants grown at 24°C, a temperature at which *tpl-1* segregates as a recessive (Long, Woody et al. 2002). We find that 45% of the resulting seedlings showed cotyledon fusion defects (n=804) instead of the expected 25%, indicating HDA19 may act on some of the same target genes as TPL during embryogenesis. In agreement with this hypothesis, we identified *tpl-1; hda19-1; hag1-3* triple mutant seedlings from plants grown at 24°C as well as 29°C and find that they display 2 narrow cotyledons like the *hda19-1* single mutant (Fig. 4G). Therefore, *hag1-3* mutants can still suppress *tpl-1* mutant phenotypes even in the absence of functional HDA19.

Recent work on embryonic polarity in *Arabidopsis* has focused on auxin transport and the first embryonic cell divisions in establishing the apical/basal axis (Hamann, Benkova et al. 2002; Friml, Vieten et al. 2003). Our studies have uncovered a set of proteins involved in a new step in axis formation, during the transition stage of embryogenesis, when shoot fate becomes fixed and distinct from root fate. We propose that at the transition stage of embryogenesis, TPL and other TPR proteins are necessary to repress the expression of root-promoting genes in the top half of the embryo to allow proper differentiation of the shoot pole. A histone deacetylase, HDA19, works in conjunction with TPL during this process, although it appears to have TPL-independent roles as well (Zhou, Zhang et al. 2005). HAG1 is necessary for the complete transformation of the apical half into a root, likely by activating the transcription of de-repressed root specific genes in the apical half of the embryo. However, HAG1 is dispensable for the formation of the basal "true" root. Conceptually, these two steps of polarity determination are similar to what has been reported in the brown alga *Fucus*, where axis formation and fixation are temporally distinct(Goodner and Quatrano 1993). In *Arabidopsis*, we propose that the axis formation step occurs during the first cell divisions of the embryo and likely relies on polar auxin distribution(Friml, Vieten et al. 2003). Only later does the axis become fixed, at the transition stage of embryogenesis, at which time the plant requires a chromatin-mediated transcriptional repression system for axis stabilization.

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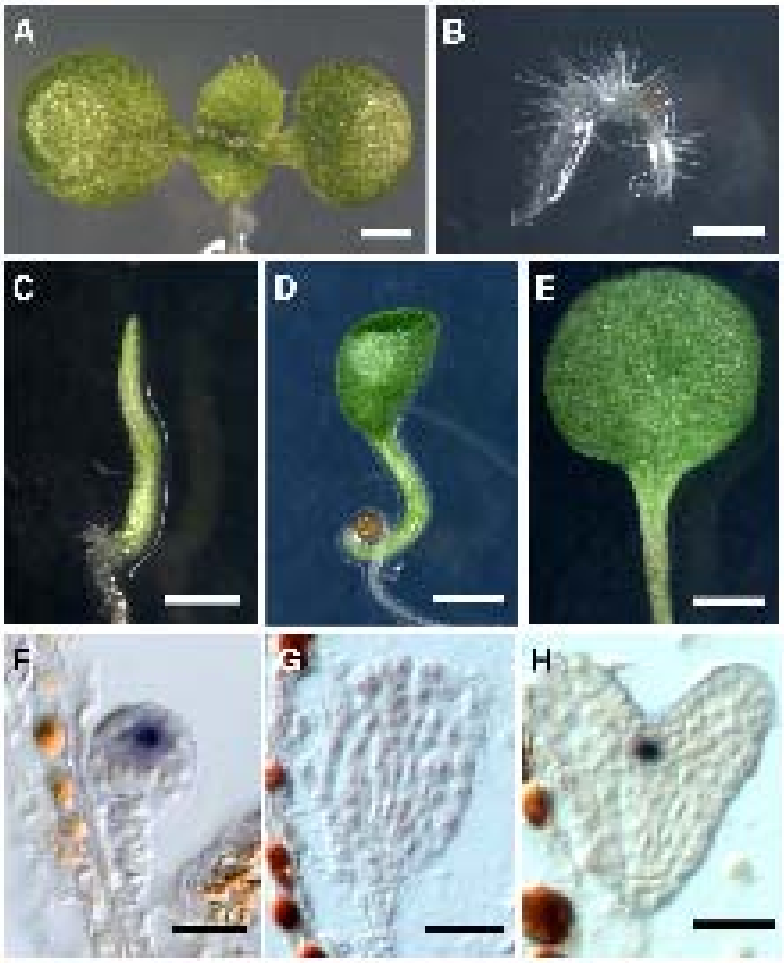

Figure 2.1 Effects of *topless-1* on embryonic polarity. (**A)**Wild-type 5 day old seedling. (**B)**A *tpl-1* double-root seedling. (**C)**A *tpl-1* pin seedling lacking cotyledons. (**D)**A *tpl-1* tube seedling. (**E)**A *tpl-1* monocot seedling with two fused cotyledons**. (F)***WUS* mRNA accumulation in a *tpl-1* globular stage embryo grown at 29°C. (**G)***WUS* mRNA does not accumulate in a *tpl-1* heart stage embryo**. (H)**Wild-type heart stage embryo accumulating *WUS* mRNA in a small group of cells in the developing meristem. Scale bars: 1mm (A-E), 25μm (F-H).

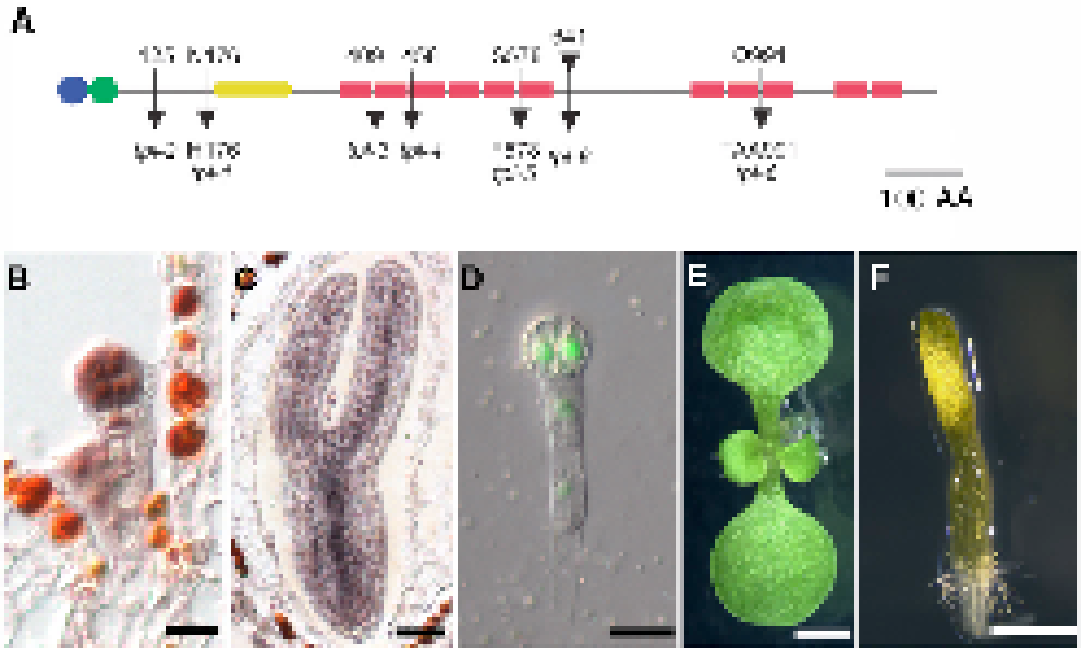

Figure 2.2 Molecular characterization of the *TPL* gene. (**A)**Diagram of the predicted structure of the TPL protein. TPL is predicted to have a LisH (blue circle) and CLISH (green hexagon) domain at the N-terminus, a 100 amino acid proline-rich domain (yellow box) and 11 WD40 repeats (red boxes). The *tpl-1* phenotype is caused by an asparagine to histidine substitution at amino acid 176. *tpl-2* and *tpl*-3 are splice acceptor site mutations while *tpl-4* is a splice donor mutation. *tpl-5* is a serine to phenylalanine substitution at amino acid 578 in the 6th WD40 repeat and *tpl-6* is a change of a glutamine at amino acid 991 to a stop codon (CAA to TAA). *tpl-8* is a T-DNA insertion allele (SALK_036566). Numbers represent the affected amino acid positions. **(B,C**)*TPL* mRNA accumulation in (B**)** a globular stage and (C) torpedo stage wild-type embryo. **(D**)A translational fusion of TPL to GFP localizes to the nuclei of all cells in a 4-cell stage embryo. **(E**)A *tpl-2* mutant shows no phenotype after developing at 29°C. **(F**)A *tpl-2; tpr1-1; trp3-1;tpr4-1* mutant carrying a *TPR2* RNAi construct displaying a pin phenotype. Scale bars: 25µm (B-D), 1mm (E,F).

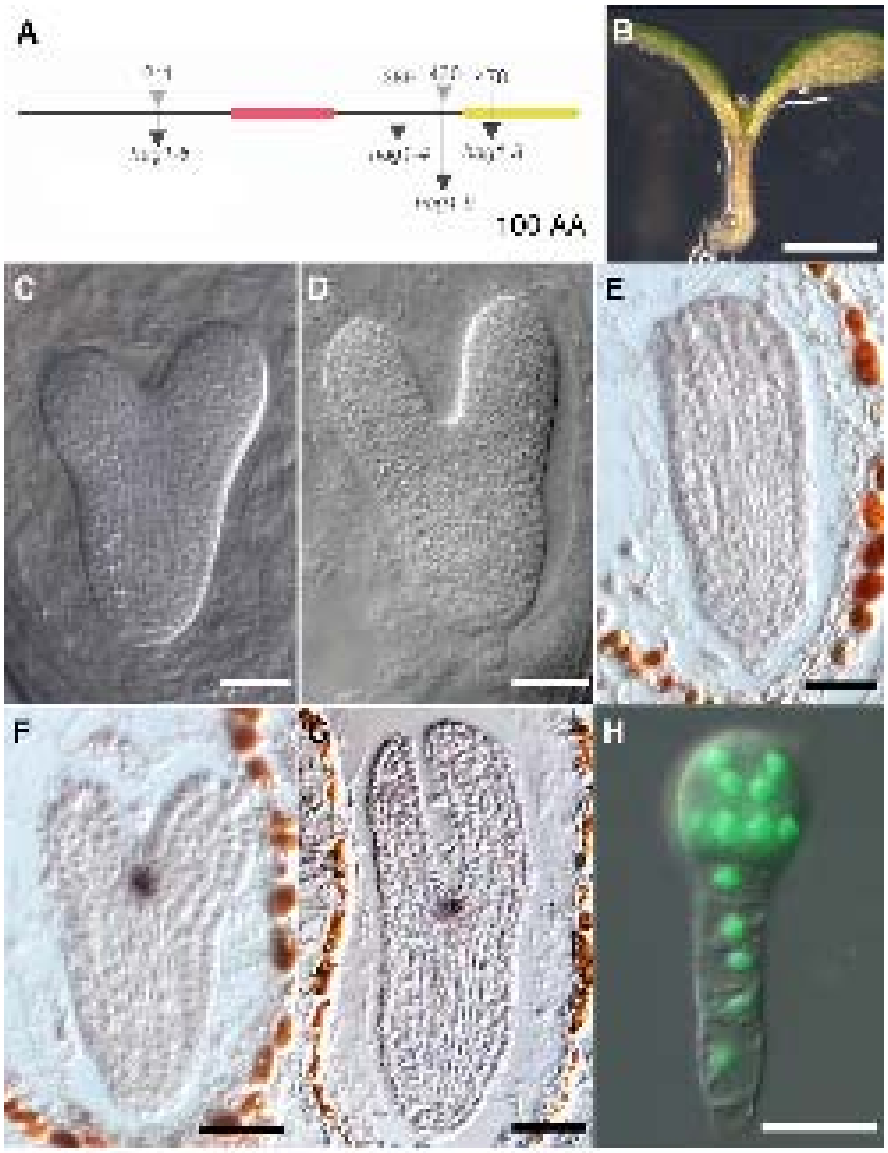

Figure 2.3 Characterization of *hag1* alleles and genetic interactions with *tpl-1*. **(A**)Diagram of the predicted structure of HAG1 that contains a conserved histone acetyltransferase domain (red box) and a bromo domain (yellow box). *hag1-3* contains a stop codon at amino acid 478 (TGG to TGA), *hag1-4* contains a splice donor mutation at amino acid 389, *hag1-5* is a T-DNA insertion in the 10th intron (SALK_048427), and *hag1-6* is a T-DNA insertion in the 1st intron (SALK_150784). **(B**)A *tpl-1;hag1-3* double mutant seedling grown at 24°C. **(C,D**)Cleared torpedo stage embryos of (C) *tpl-1*;*hag1-3* and (D) wild-type grown at 29°C. **(E,F,G**)WUS mRNA accumulation in (E**)** *tpl-1*, (F) *tpl-1;hag1-3*, and (G) wild-type 29°C grown torpedo stage embryos. (**H**)A HAG1-GFP fusion protein localizes to the nuclei of all cells of a 16-cell stage embryo. Scale bars: 1mm (B), 25µm (C-H).

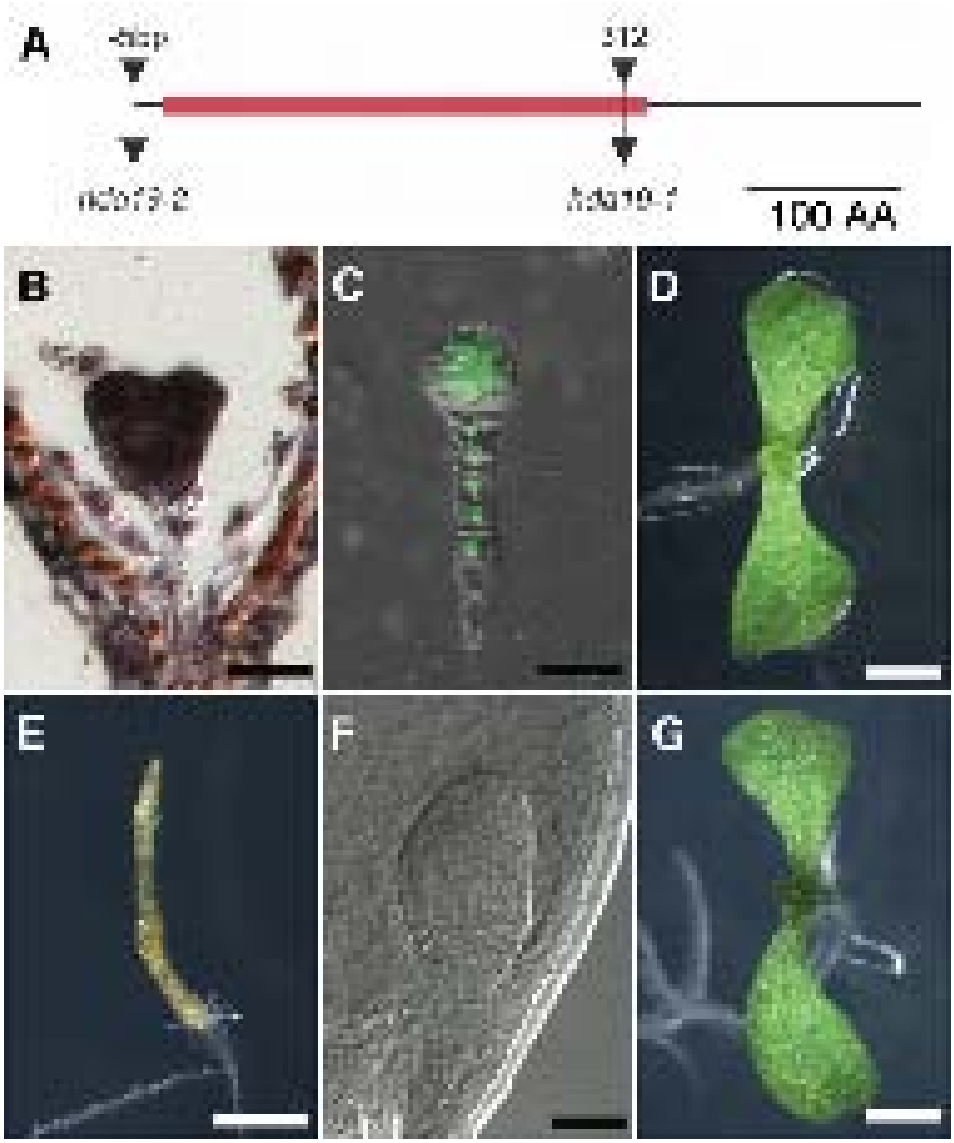

Figure 2.4 Characterization and genetic interactions of *HDA19*. (**A**)Predicted structure of HDA19. *hda19-1* contains a T-DNA insertion that disrupts amino acid 312 in the histone deacetylase domain (red box). *hda19-2* contains a T-DNA insertion 5 base pairs upstream of the start codon. (**B**)mRNA accumulation of *HDA19* in all cells of a early heart stage embryo. (**C**)A HDA19-GFP fusion protein localizes to the nuclei of all cells of a 16-cell stage embryo. (**D**)Seedling phenotype of *hda19-1* when grown at 24°C. (**E**)A *hda19-2* seedling displaying a pin phenotype when grown at 29°C. (**F**)A *hda19-1* heart stage embryo grown at 29°C showing both shoot and root defects. (**G**)A *tpl-1;hag1-3;hda19-1* triple mutant seedling grown at 24°C. Scale bars: 25μm (B,C,F), 1mm (D,E,G).

PLT genes are necessary for _tpl-1_ shoot to root transformation and direct targets for TPL repression.

Wild type (WT) *Arabidopsis* embryos pattern the embryonic structures along an apical-basal axis with the cotyledons and SAM at the apical end and the root apical meristem (RAM) at the basal end (Fig. 1a). *tpl-1* displays a range of phenotypes including defective cotyledon formation, pin shaped seedlings lacking cotyledon and shoot meristem, and the homeotic transformation of the apical shoot pole into a second root during embryogenesis (Fig. 1b, c, d). *tpl-1* is temperature sensitive and shows a high frequency of shoot to root transformation when embryos develop at the restrictive temperature of 29°C.

Figure 2.5 Wild type *Arabidopsis* seedling (a) and *tpl-1* seedlings displaying fused cotyledons (b), pin shaped (c), and double root (d) phenotypes. The *tpl-1 plt1-5 plt2-1* triple mutant never forms double roots.

The *PLETHORA* (*PLT*) genes have been shown to be essential for embryonic and post-embryonic root development. In WT, *PLT1, PLT2, PLT3,* and *PLT4/BBM* are expressed in the root meristem throughout embryo development (Fig. 2a, c, e, g). In *tpl-1* grown at 29^{o}C, *PLT1, PLT2,* and *PLT3* are misexpressed in the apical domain, beginning at the heart stage (Fig. 2b, d, f, h). Notably, *PLT4/BBM* is not misexpressed in *tpl-1* embryos even at the late heart stage, showing that there is some differential control of the *PLT* genes. It has been shown that the *PLT* genes are sufficient to initiate ectopic roots when driven from an embryonic promoter, suggesting that the misexpression seen in *tpl-1* is causative of the double root phenotype. In agreement with this, the *tpl-1 plt1-5 plt2-1* triple mutants never developed double roots at 29^{o}C (n>1000) (Fig. 1e) showing that the *PLT* genes are necessary for apical root formation in *tpl-1*.

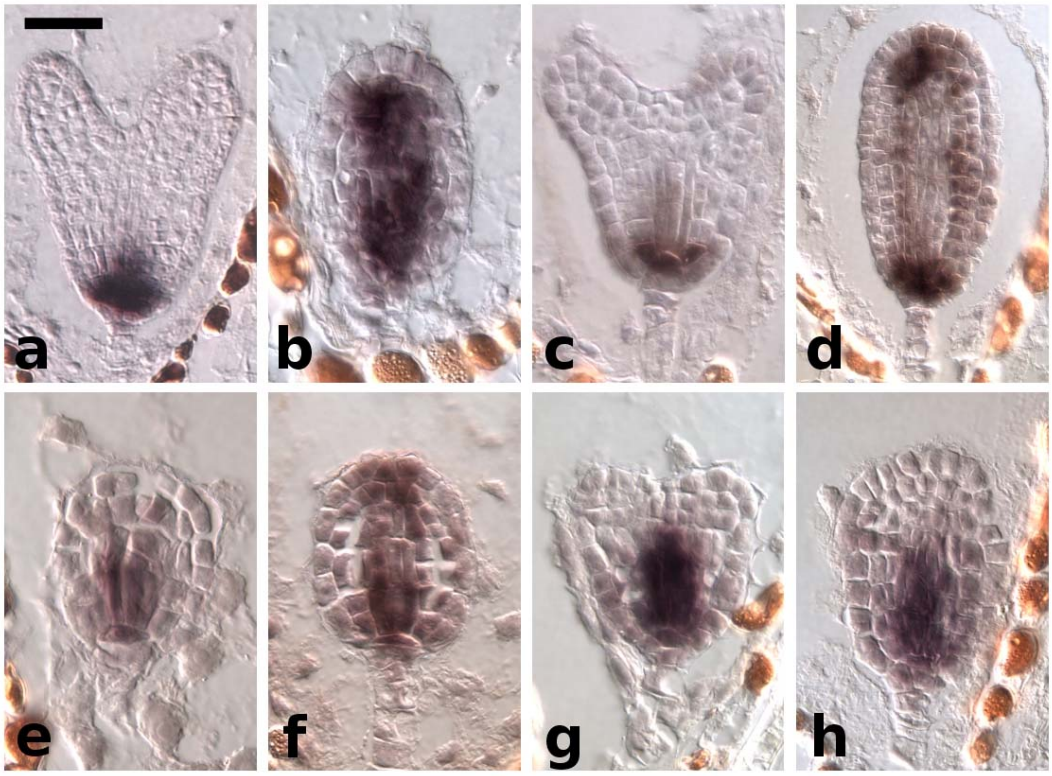

Figure 2.6 In *situ hybridization* with antisense probes against *PLT1* (a,b), *PLT2* (c,d), *PLT3* (e,f), and *PLT4* (g,h). The *PLT* genes are expressed in the basal pole of wild type heart stage embryos (a, c, e, g) and are misexpressed in the apical pole of *tpl-1* (b, d, f) with the exception of *PLT4/BBM* (h) which remains expressed in the basal half.

To assess whether the *PLT* genes are direct targets of TPL repression, we performed Chromatin Immunoprecipitations (ChIP) on dissected ovules containing globular to heart stage embryos from a *TPLp::TPL-HA* stable transgenic line. We observed enrichment of regions in both the *PLT1* and *PLT2* promoters in the TPL ChIP samples compared to controls (Fig.3a, b). Interestingly, these two enriched regions share no obvious sequence identity

suggesting TPL may be recruited to these promoters by different transcription factors. These data show that TPL controls apical embryonic patterning through direct repression of the *PLT* genes and loss of this repression results in the double-root phenotype observed in *tpl-1*.

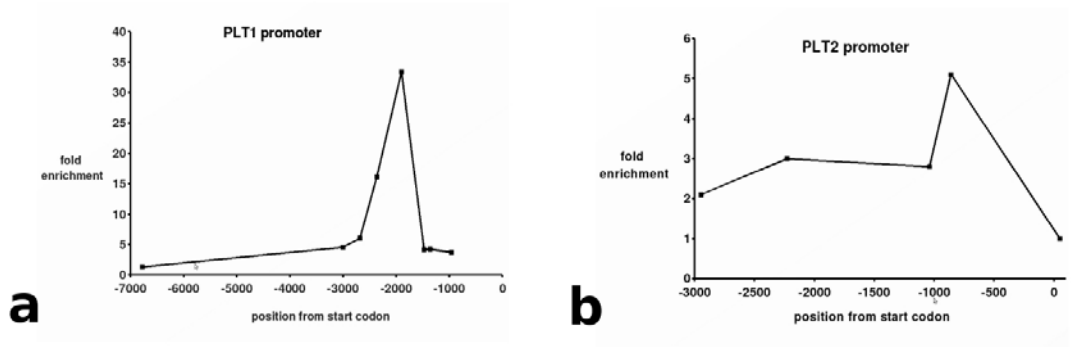

Figure 2.7 Relative enrichment levels of the *PLT1* (a) and *PLT2* (b) promoter regions determined by real-time qPCR from ChIP DNA between *TPLp::TPL-HA* and control embryo tissue.

APPENDIX

Materials and methods

Plant stocks and growth conditions

The isolation of *tpl-1* was described previously in the Landsberg *erecta* (Ler) ecotype(*4*). Plants were grown either on soil or Murashige and Skoog salts media. For controlled temperature experiments plants were grown in a Percival growth chamber. All other plants were grown under greenhouse conditions on a 16 hour light/8 hour dark cycle.

Genetic analysis

tpl-2 through *tpl-6* alleles and *hag1-3,4* were isolated as suppressors of *tpl-1* in a high temperature suppressor screen. *tpl-1* homozygous seeds were treated with 0.2% Ethylmethane Sulphonate and sown to soil at 24°C. Upon flowering, plants were shifted to 28°C and allowed to self. 4,000 individual T2 populations were then screened for suppressors. Quadruple mutants were generated using *tpl-2* in combination with Wisconsin Knockout facility alleles of *TPR1* and *TPR4* (Wassilewskija ecotype) and a Syngenta T-DNA allele of *TPR3* (Ler ecotype). Quadruple mutants were then transformed with a *TPR2* RNAi vector containing 200 base pairs upstream of the stop codon in a modified pHANNIBAL vector(*5*).

Cloning and sequencing

tpl-1 was mapped to chromosome 1 using PCR based CAPS markers (*4, 6*). A 30 base pair deletion was found in the Ler ecotype in the last intron of at1g15750 and used as a marker with primers TCATTAGCTCCAATCACTCATGC and GGATGTACGTTTGAATTGCTGCAC. 0/1200 chromosomes were found to be recombinant at this locus. At1g15750 was amplified from homozygous *tpl-1* plants using

primers ATTGCAGATCTGGTGATAGTTTG and AATCAGAGAGGTAGGTGGCTCT and sequenced using internal primers. Mutations in *hag1*-3 and *4* were identified by amplification of at3g54610 using primers CACCTTCACTATACTGCTTCGATGGACT and CATGTATTCCCAGTTCCAATG.

***In situ* hybridization**

TPL, *WUS*, and *HDA19* RNAs were detected with digoxigenin-labelled riboprobes using the method found at http://www.its.caltech.edu/~plantlab/protocols/insitu.htm. For *TPL*, 190 base pairs upstream of the start codon was used. For *HDA19*, a 490 base pair fragment starting at an internal HindIII site was used. For *WUS*, the entire coding region was used.

Microscopy

For morphological analysis, ovules were dissected and mounted in Hoyer's medium and imaged using a Leica DM5000B compound microscope. For GFP analysis, ovules were dissected into 0.5X LS media (Caisson Laboratories, Inc.; Rexburg, ID), vacuum infiltrated in 1% Fluorescent Brightener 28 (Sigma-Aldrich; St. Louis, MO), then washed 2X and mounted in 0.5X LS media. Embryos were imaged using a Leica DM IRE2 laser scanning confocal microscope. Fluorescent brightener was excited with the UV diode 405nm line, and emission was measured between at 420-470nm. GFP was excited with a 488nm argon laser line and emission was measured at 500-535nm. Images were overlaid using Leica Confocal Software (LCS) and overlapping signal is displayed as white.

Supplementary references

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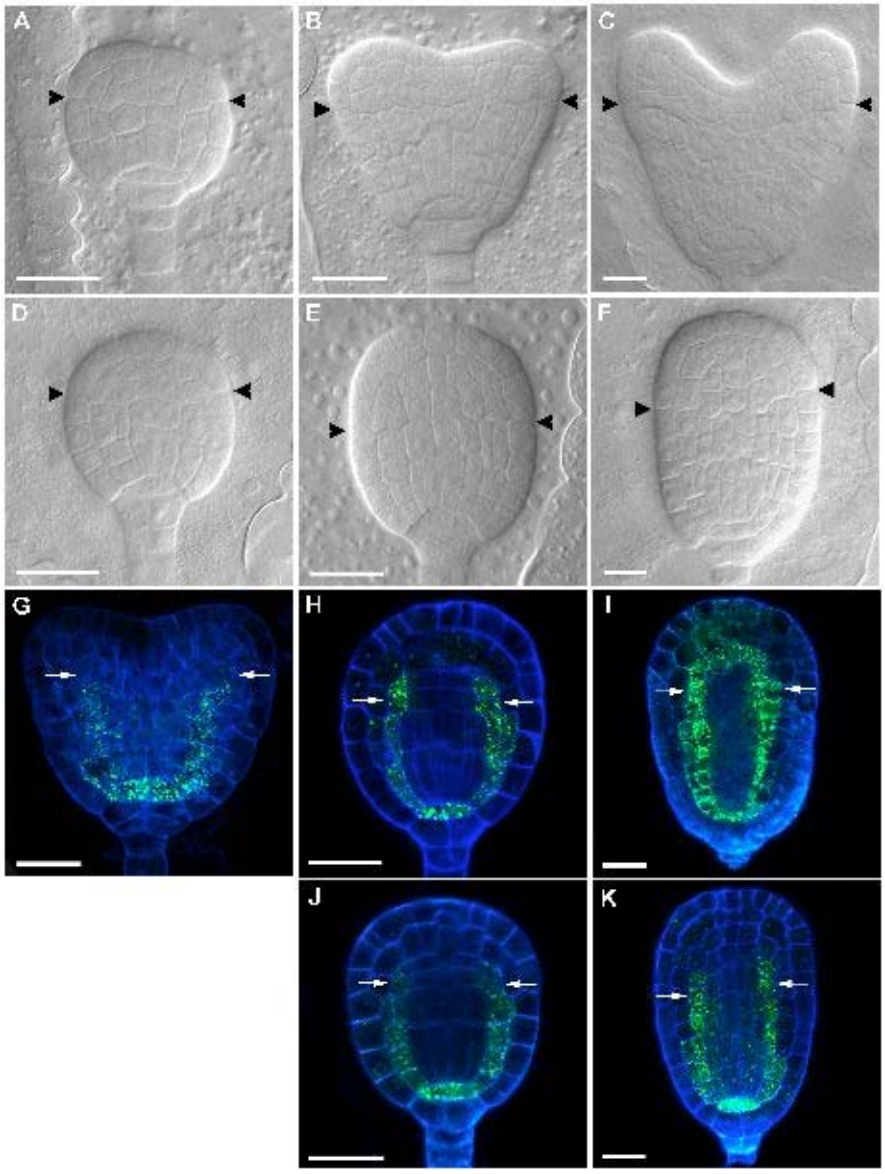
Figure S1. Expression of a reporter for the basal marker gene *SCARECROW* (*SCR*) is misexpressed in apical tissues in *tpl-1* embryos grown at both restrictive and permissive temperatures, visualized by *pSCR::ERGFP*. There is a clearly defined morphological boundary identified by a continuous line of cell walls that delineates apical versus basal lineages in a wild type (wt) embryo, designated the O' line. Cells above the O' line will only contribute to the apical structures of the cotyledon and shoot meristem, while cells below the O' line will only contribute to the basal structures of the hypocotyl and root(*1*). Cleared wt (**A** to **C**) and *tpl-1* embryos (**D** to **F**) grown at the permissive temperature (21°C) illustrate that the O' line can be identified throughout early embryo development. Black arrowheads point to the epidermal cell walls that define the O' line. *SCR* is expressed in the ground tissue of the root and hypocotyl in early embryos and excluded from tissues above the O' line that contribute to apical structures(*2, 3*). *pSCR::ERGFP* expression in wt at the heart stage (**G**). *tpl-1* embryos grown at the restrictive temperature (29°C) show expansion of *pSCR::ERGFP* expression into apical structures by the transition stage (**H**). During the heart stage *pSCR::ERGFP* forms a pattern similar to that in the root pole (**I**), the same stage at which *tpl-1* has been shown to misexpress root cap markers(*4*). Transition stage *tpl-1* embryos grown at the permissive temperature (21°C) show misexpression of *pSCR::ERGFP* (**J**) similar to those grown at the restrictive temperature. Later in the heart stage, *pSCR::ERGFP* continues to misexpress in cells that would contribute to apical structures in the wild type (**K**), however; it does not form a pattern that resembles the root pole. This is consistent with our observation that these embryos will develop into monocot or tube phenotype seedlings. White arrows point to

the first cell above the O' line, that does not express SCR in the wild type. Cell walls were counterstained with Fluorescent Brightener 28. Scale bars: 20μm.

Fig. S2. A clustalW alignment of TPL and TOPLESS-RELATED (TPR) proteins. TPL shows extensive similarity to 4 other proteins in *Arabidopsis*. TPR1=At1g80490, TPR2=At3g16830, TPR3=At5g27030, TPR4=At3g15880. Identical residues are shaded black, similar residues are shaded grey, black line marks the LisH domain, dotted line marks the CTLH domain, grey lines mark the WD40 repeats and the asterisk marks the position of the asparagine to histidine mutation found in *tpl-1*.


A
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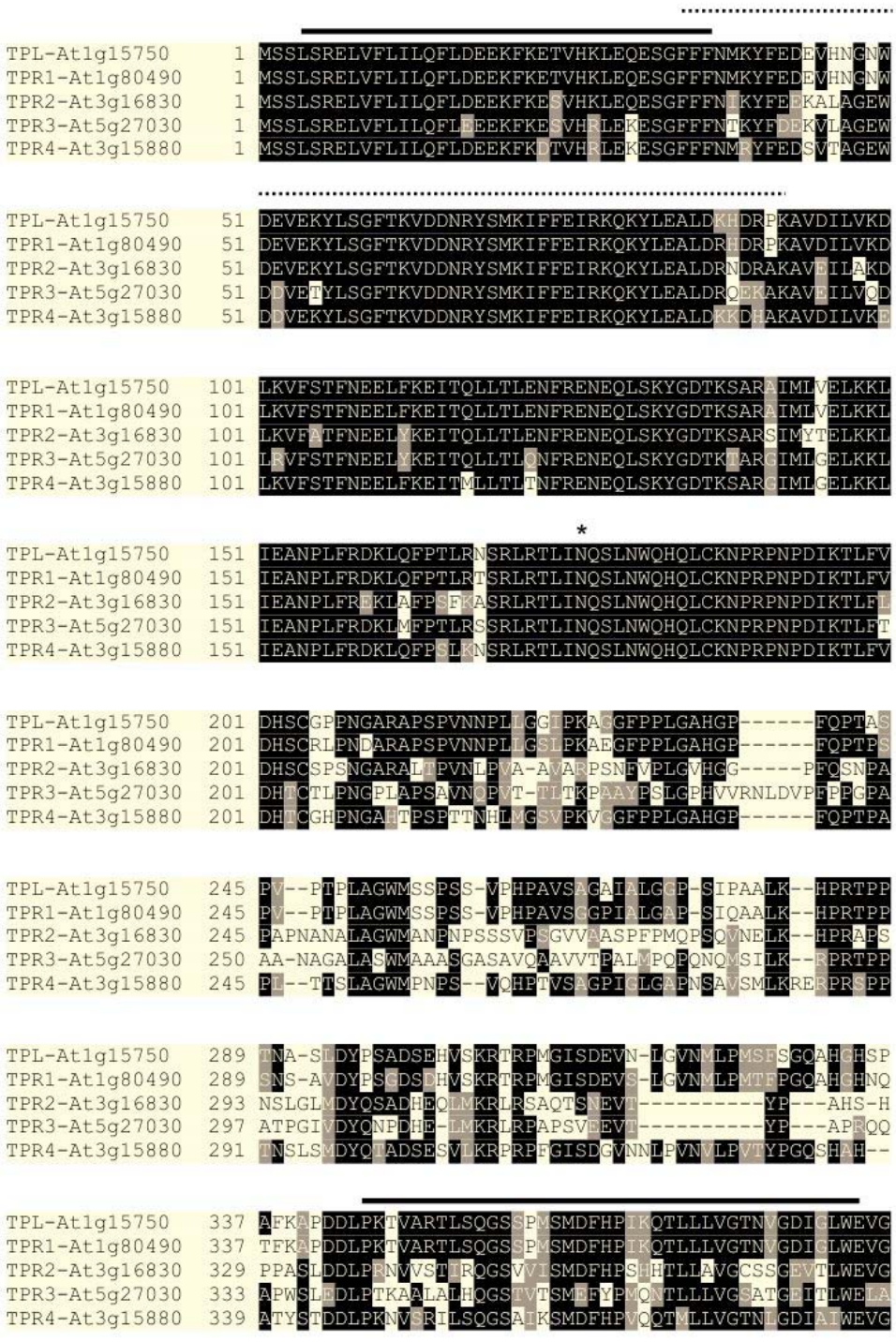
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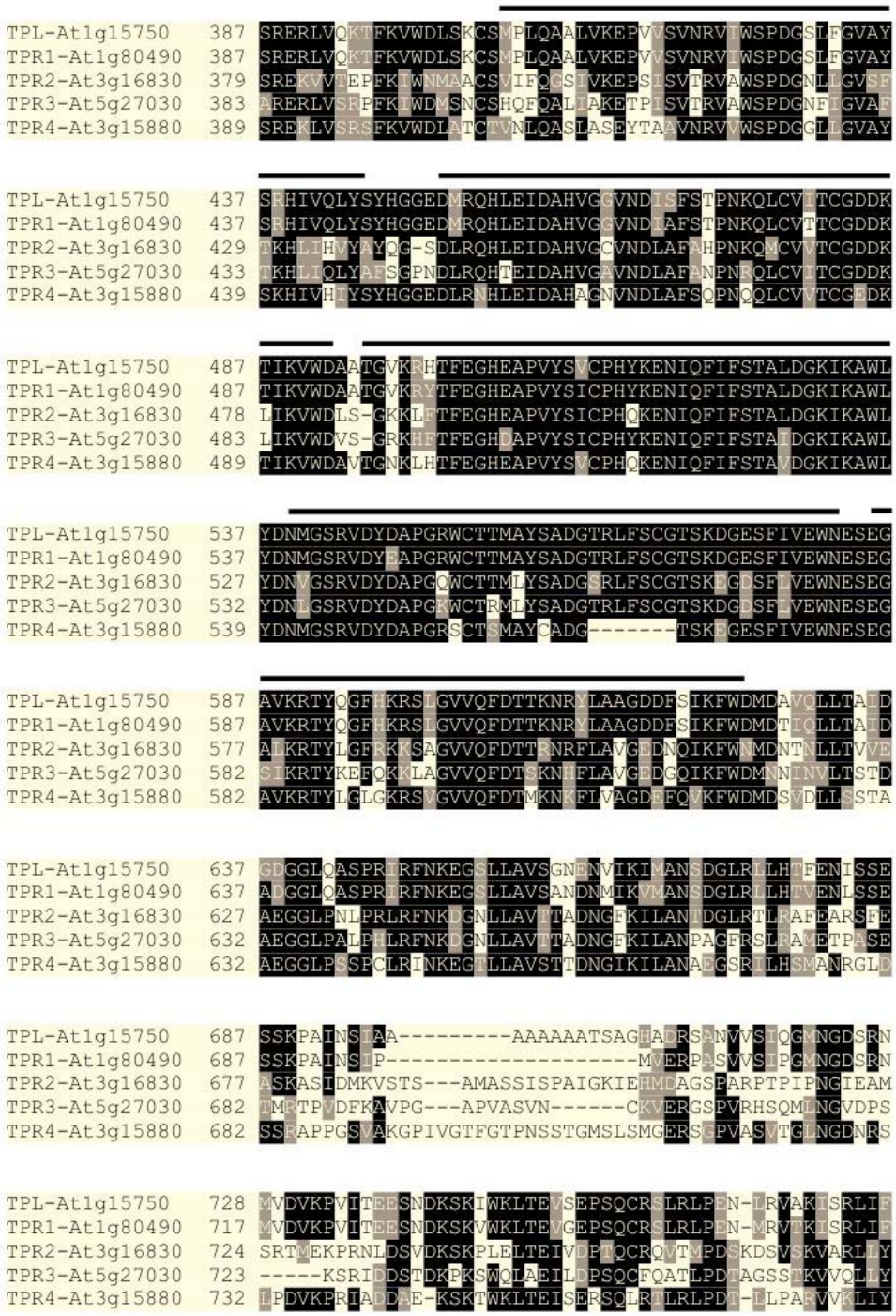
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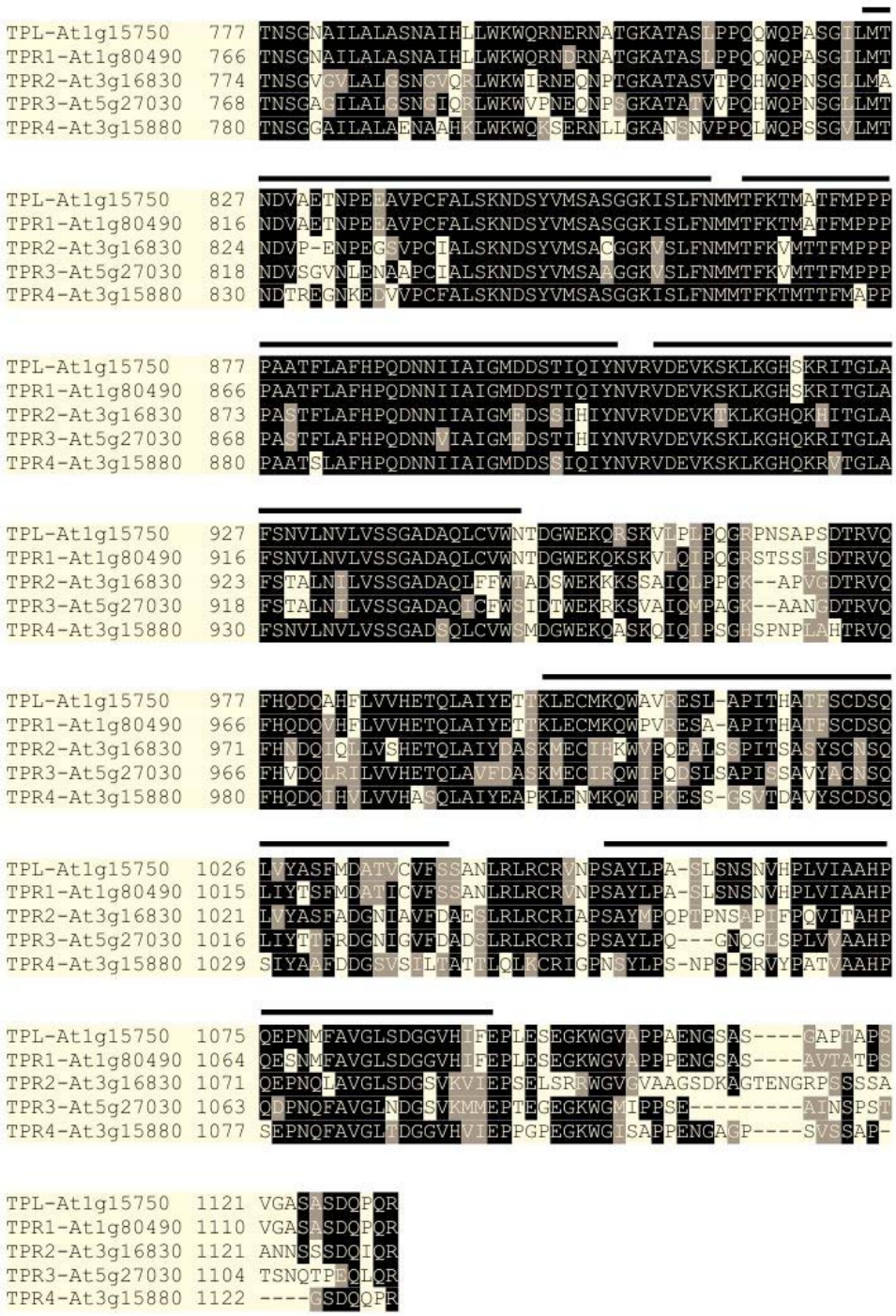
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Chapter 2 contains material from the publication: TOPLESS regulates apical embryonic fate in Arabidopsis. Long JA; Ohno C; Smith ZR; Meyerowits EM, Science 2006. The dissertation author was a co-author of this paper and the dissertation author's academic advisor was the primary author of this paper.

Chapter 3

Identification of *HD-ZIP Class III* transcription factors as master regulators of embryonic apical fate.

SUMMARY

The majority of this work is a reproduction of the material as it appears in the publication Control of Arabidopsis apical-basal embryo polarity by antagonistic transcription factors. Smith ZR and Long JA, Nature 2010. Here we identify the *HD-ZIP Class III* transcription factor family as dominant suppressors of the *tpl-1* double root phenotype. Furthermore, we show that they are master regulators of apical fate in the early embryo and are involved in a mutually antagonistic relationship with the *PLETHORA* family of root specifying genes.

INTRODUCTION

In *Arabidopsis*, pattern formation during embryogenesis follows a largely invariant series of cell divisions with distinct lineages, providing a highly tractable system to study complex pattern formation. During the first division of the zygote, the apical-basal axis is established, generating a small apical cell and a larger basal cell. Most of the embryonic structures are derived from the apical cell, while the larger cell gives rise to the extra-embryonic suspensor, as well as part of the root meristem and root cap. After several cell

divisions, bilateral symmetry is initiated from a radially symmetric globular stage embryo, directing the plane of outgrowth for the cotyledons (embryonic leaves), which subsequently establish a dorsal-ventral or adaxial-abaxial axis of polarity. Embryogenesis culminates in the generation of two distinct stem cell populations, the shoot and root meristems at the apical central and basal embryonic poles, respectively, from which all above and below ground structures of the adult plant are derived.

Polar transport and localized accumulation of the phytohormone auxin has been shown to play a critical role in apical-basal axis formation, root patterning and cotyledon initiation (Friml, Vieten et al. 2003). Auxin-induced gene expression involves degradation of AUX/IAA transcriptional repressors (Gray, Kepinski et al. 2001), and *IAA12/BODENLOS* was recently shown to require the transcriptional co-repressor *TOPLESS* (*TPL*) for its function during embryonic root development (Szemenyei, Hannon et al. 2008). In the dominant negative, temperature sensitive *tpl-1* allele the embryonic shoot pole is transformed into a second root pole, indicating that root specifying genes must be actively repressed in the apical half of the embryo for normal apical/basal patterning to occur (Long, Woody et al. 2002; Long, Ohno et al. 2006). Recent studies have shown that the *PLETHORA* (*PLT*) genes are master regulators of root development and both their expression and function are closely intertwined with auxin signaling and transport (Aida, Beis et al. 2004; Blilou, Xu et al. 2005; Galinha, Hofhuis et al. 2007). Ectopic expression

of *PLT2* has been shown sufficient to initiate root formation in both embryonic and post embryonic apical tissues and segregants for loss of function mutations in *plt1 plt2 plt3 plt4/bbm* completely lack an embryonic root.

Many genes have been described that contribute to specific patterning events of apical embryonic structures. However, master regulators of apical fate have remained elusive. During cotyledon initiation, members of the *HD-ZIP III* family of transcription factors have been shown to specify adaxial polarity (McConnell, Emery et al. 2001). In post-embryonic development, the *HD-ZIP III* genes are important regulators of lateral organ polarity, shoot apical meristem (SAM) patterning, and vascular development (McConnell, Emery et al. 2001; Emery, Floyd et al. 2003). The *HD-ZIP III* family is comprised of five genes: *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), *REVOLUTA* (*REV*), *INCURVATA4*/*CORONA* (*ICU4*/*CNA*), and *ARABIDOPSIS THALIANA HOMEOBOX-8* (*ATHB-8*).

RESULTS

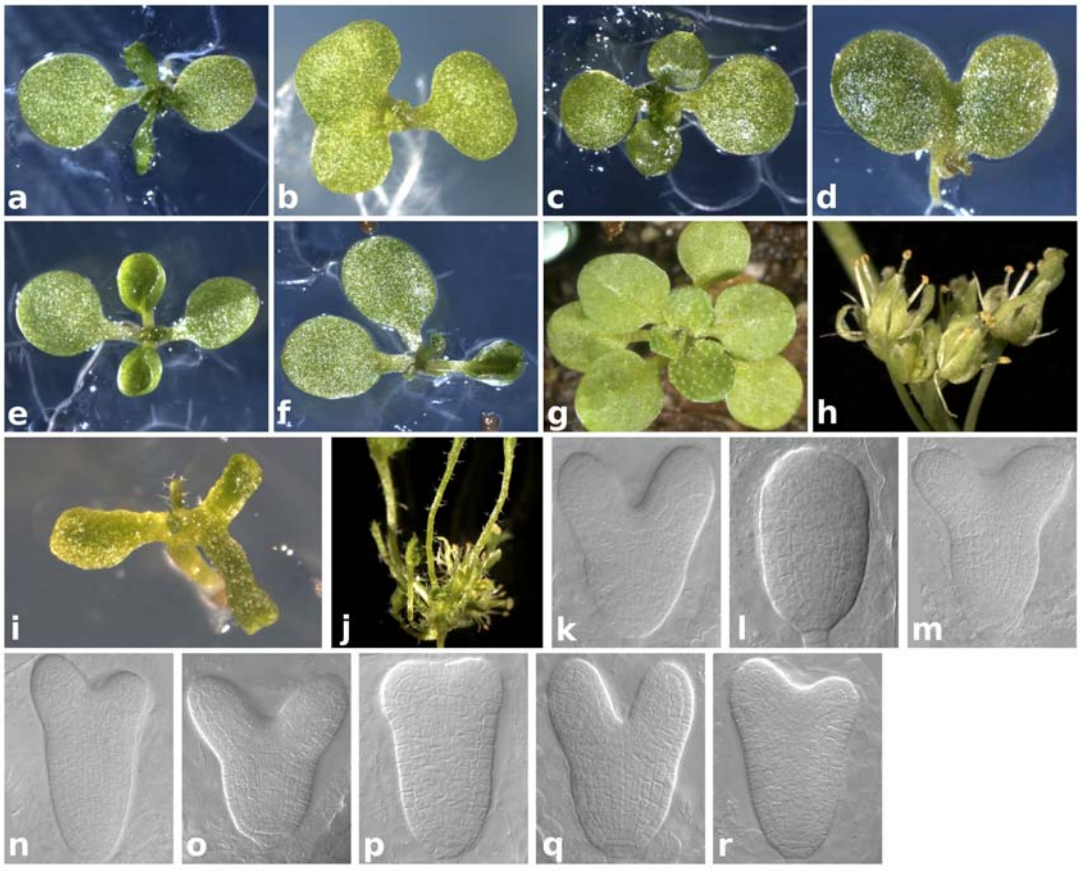
HD-ZIP III gain-of-function mutations suppress *tpl-1* double root formation.

tpl-1 embryos grown at 17^{o}C develop similarly to wild type embryos (Long and Barton 1998), allowing for propagation of homozygous *tpl-1* plants. From a high temperature suppressor screen on *tpl-1*, one semi-dominant mutant was isolated that completely suppressed the formation of double-root seedlings, which we temporarily named *topheavy* (*tph*). Using a map-based cloning approach, we found that the mutation was located between markers on BACs F19I3 and T1B8 on chromosome2. *tph* cDNA was isolated and candidate genes were sequenced, which identified a single mis-sense mutation within the coding region of *PHB* in this line and was renamed *phb-14d*. The *phb-14d* mutant alone shows no early embryonic phenotype, but by the late heart stage displays a weak phenotype where the cotyledon are slightly stunted (Fig.1m) and the seedling first leaves are often slightly adaxialized and down turned (Fig. 1a). The *phb-14d tpl-1* double mutant, while it never develops double roots, routinely produces fused cotyledons (Fig.1b). The mutation in *phb-14d* resides within a known *microRNA (MIR)165/166* family binding site and is predicted to result in a loss of *MIR165/166* mediated regulation (Tang, Reinhart et al. 2003; Mallory, Reinhart et al. 2004) (Fig. 1s, t). Previous studies have shown that this type of

mutation can result in an increase in transcript abundance and *in vitro* experiments have shown that the *phb-14d* mutation causes a less severe disruption in miR directed mRNA degradation than in other known *phb* alleles (Mallory, Reinhart et al. 2004). Consistent with this, the *phb-14d* mutant phenotypes are less severe than previously described *phb* alleles. *phb-1d* (McConnell, Emery et al. 2001) seedlings display completely adaxialized cotyledons as well as first leaves (Fig. 1i) and rosette leaves are either completely radialized or trumpet shaped (Fig. 1j), compared to *phb-14d* rosette leaves, which are laminar and similar to WT (Fig. 1g). The petals, however are severely affected in *phb-14d* (compare Fig. 1h to Fig. 1j), suggesting that there may be differential implementation of the microRNA regulation of *PHB* in the flower.

All five *HD-ZIP III* genes are predicted to be regulated by the *miR 165/166* family, and semi-dominant gain-of-function (GOF) mutations in the miR binding site of *ICU4*, *REV*, and *PHV* have been characterized (Zhong, Taylor et al. 1999; McConnell, Emery et al. 2001; Ochando, Jover-Gil et al. 2006). We therefore examined if GOF mutations in *ICU4* and *REV* could also suppress *tpl-1*. Although neither *rev-10d* nor *icu4-1d* displayed obvious embryonic patterning defects (Fig. 1c, e, o, q), they could completely suppress the shoot to root transformation seen in *tpl-1* when grown at 29°C (n>1000) (Fig. 1d, f, p, r). These suppressed double mutants also show the same cotyledon fusion defects seen in *phb-14d tpl* doubles. These results suggest

that the *HD-ZIP III* genes play an important role in promoting apical fate in early embryogenesis.



s

PHB	ATT	GGG	ATG	AAG	CCT	GGT	CCG	GAT
	I	G	M	K	P	G	P	D
phb-14D	ATT	GGG	ATG	AAG	CCT	GGT	CTG	GAT
	I	G	M	K	P	G	L	D
icu4-1D	CCT	GGA	ATG	AAG	CCT	GAT	CCG	GAT
	P	G	M	K	P	D	P	D
rev-10D	CCT	GGG	ATG	AAG	CCT	GGT	CTG	GAT
	P	G	M	K	P	G	L	D

t

Figure 3.1 Seedling phenotypes of *phb-14d* (a), *tpl-1 phb-14d* (b), *rev-10d* (c), *tpl-1 rev-10d* (d), *icu4-1d* (e), *tpl-1 icu4-1d* (f). Rosette (g) and floral (h) phenotypes of *phb-14d*. Seedling (I) and rosette (j) phenotypes of *phb-1d*. Embryo phenotypes of wt (k), *tpl-1* (l), *phb-14d* (m), *tpl-1 phb-14d* (n), *rev-10d* (o), *tpl-1 rev-10d* (p), *icu4-1d* (q), *tpl-1 icu4-1d* (r). The mutations which suppress *tpl-1* reside within the microRNA recognition sequence of the *HD-ZIP III* genes (s). The domain structure of the *HD-ZIP III* genes is shown in (t).

Gene expression analysis of polarity and apical genes in *tpl-1* and *tpl-1* double mutants with *HD-ZIP III* gain-of-function alleles.

Consistent with the observation that the GOF mutations in *HD-ZIP III* genes restore apical fate to *tpl-1* embryos, *PHB, REV, ICU4, and PHV* are all expressed in an apical/central domain of the globular embryo (Prigge, Otsuga et al. 2005) (Fig. 2a, g, m, s). By the heart stage, the expression of all four genes expands to the adaxial domain of the cotyledons and throughout the provascular tissue (Fig. 2b, l, o, u). In *tpl-1* embryos grown at 29^{o}C, *PHB*, *REV*, *ICU4, and PHV* expression is identical to WT at the globular stage, but is lost from the apical domain at the heart stage (Fig. 2c, j-l, p-r, v-x). These results are consistent with our previous reports that early embryogenesis is not compromised in *tpl-1*. We then examined *PHB* expression in *tpl-1 phb-14d* embryos grown at 29^{o}C, and found that *PHB* expression was not lost from the apical domain (Fig. 2d). This suggests that increasing *HD-ZIP III* transcript abundance through disrupting their regulation by *MIR165/166* is sufficient to restore apical fate in *tpl-1*. In the *phb-14d* single mutant, *PHB* mRNA remains expressed similar to the WT pattern (Fig. 2e) in contrast to the *phb-1d* mutant where *PHB* mRNA spreads throughout most of the embryo (Fig. 2f). Therefore, *miR165/166* regulation of *PHB* is only partially compromised in *phb-14d*.

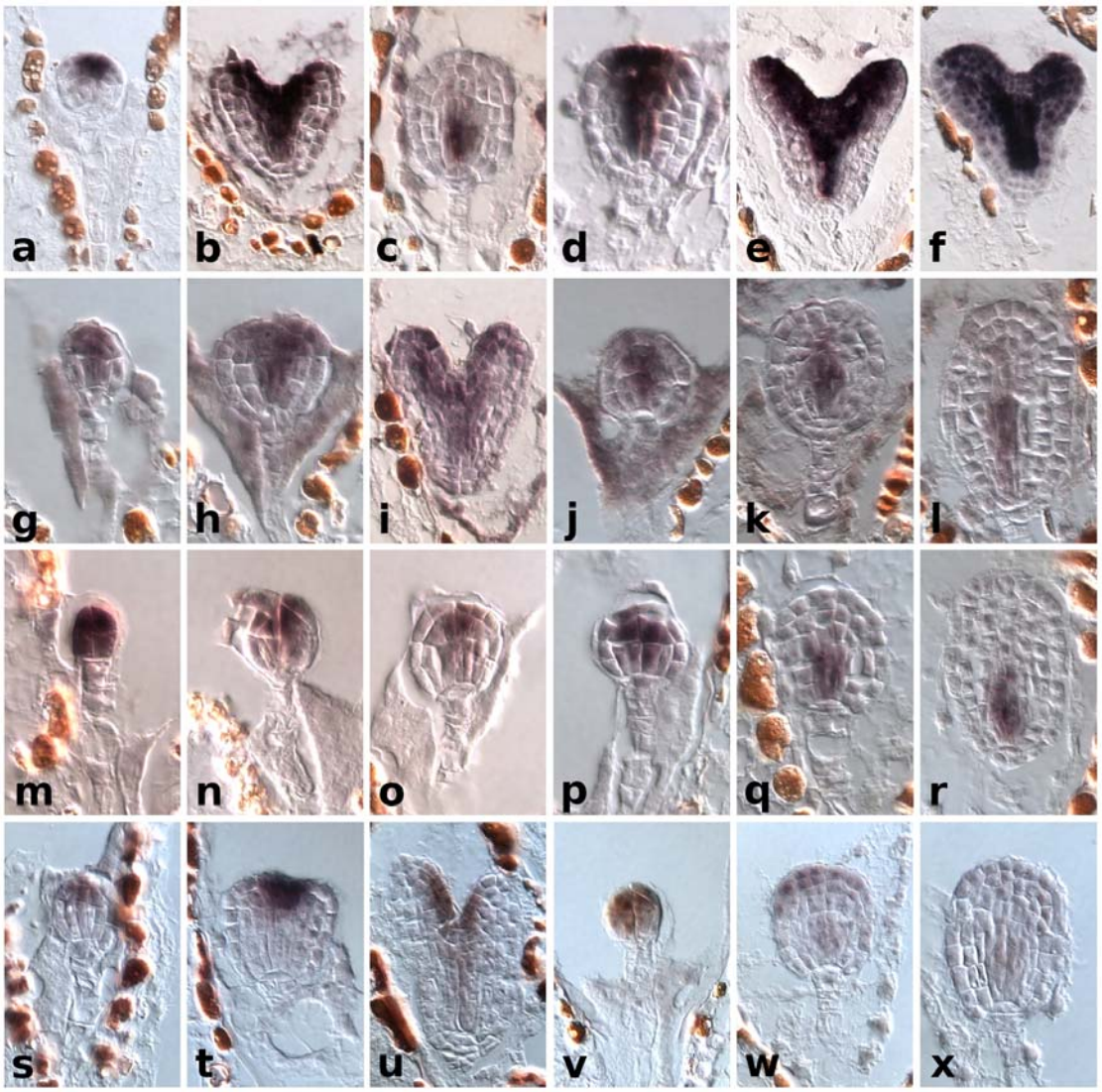


Figure 3.2 Gene expression patterns of the *HD-ZIP III* genes with antisense probes against *PHB* (a-f), *REV* (g-l), *ICU4* (m-r), and *PHV* (s-x). Expression patterns in the wild type (a, b, g-i, m-o, s-u), *tpl-1* (c, j-l, p-r, v-x), *tpl-1 phb-14d* (d), *phb-14d* (e), and *phb-1d* (f).

These findings provide a new link between the adaxial polarity pathway and early apical-basal patterning of the embryo. We therefore examined the radial organization of the apical domain of these embryos at 29°C by examining the expression patterns of the *FILAMENTOUS FLOWER* (*FIL*), *WUSCHEL* (*WUS)* and *SHOOT MERISTEMLESS (STM)* genes. *FIL* is expressed peripheral to the meristem in globular stage WT embryos and is further restricted to the abaxial cells of the cotyledons in heart and later stage embryos (Fig. 3a-c). Consistent with our finding that the *HD-ZIP III* transcripts are cleared from the apical domain of *tpl-1* embryos, we found that *FIL* expression expanded throughout the entire apical domain in *tpl-1* from late globular to heart stage (Fig. 3d, e). Past the heart stage, *FIL* expression was subsequently lost in *tpl-1* embryos (Fig. 3f). However, in *tpl-1 phb-14d* embryos *FIL* expression is restored to the abaxial domain and restricted from the central meristem region (Fig. 3g-i).

WUS plays a critical role in SAM initiation and maintenance, and serves as a central, apical marker throughout embryogenesis (Mayer, Schoof et al. 1998) (Fig3m, n). In *tpl-1*, *WUS* is expressed correctly through globular stage but is subsequently lost (Fig. 3o). In the *tpl-1 phb-14d* double mutant, *WUS* expression is maintained throughout embryogenesis (Fig. 3p), showing that apical fate is never lost. *STM* is a second marker for apical fate and shoot meristem identity. *STM* is expressed in a stripe of cells in the shoot meristem perpendicular to the plane of cotyledon emergence (Fig. 3s). In *tpl-1*, *STM* is

either lost entirely or expressed in the central domain of the embryo in small patches at the periphery (Fig. 3t). Interestingly, this is a pattern similar to that found in monocot embryonic *STM* expression. This expression is likely to be non-functional, however, because *tpl-1* grown at 29^{o}C very rarely produces functional shoot meristems. In the *tpl-1 phb-14d* double mutant, *STM* expression is observed in the meristem region, however the expression is weak and does not occupy all three layers of the meristem (Fig. 3u). This shows that the rescue of *tpl-1* by *phb-14d* is only a partial rescue, and that *STM* is dispensable for specificaiton of apical fate.

The triple loss-of-function mutant *rev-9 phb-6 phv-5* develops as a pin shaped seedling, similar to one of the less penetrant phenotypes seen in *tpl-1* (Fig. 3v, w). The patterns of *FIL* and *WUS* misexpression in *tpl-1* are identical to what is seen in *rev-9 phb-6 phv-5* (Fig. 3j-l, q, r). This further shows that the apical half of *tpl-1* embryos lose adaxial identity during the shoot to root transformation.

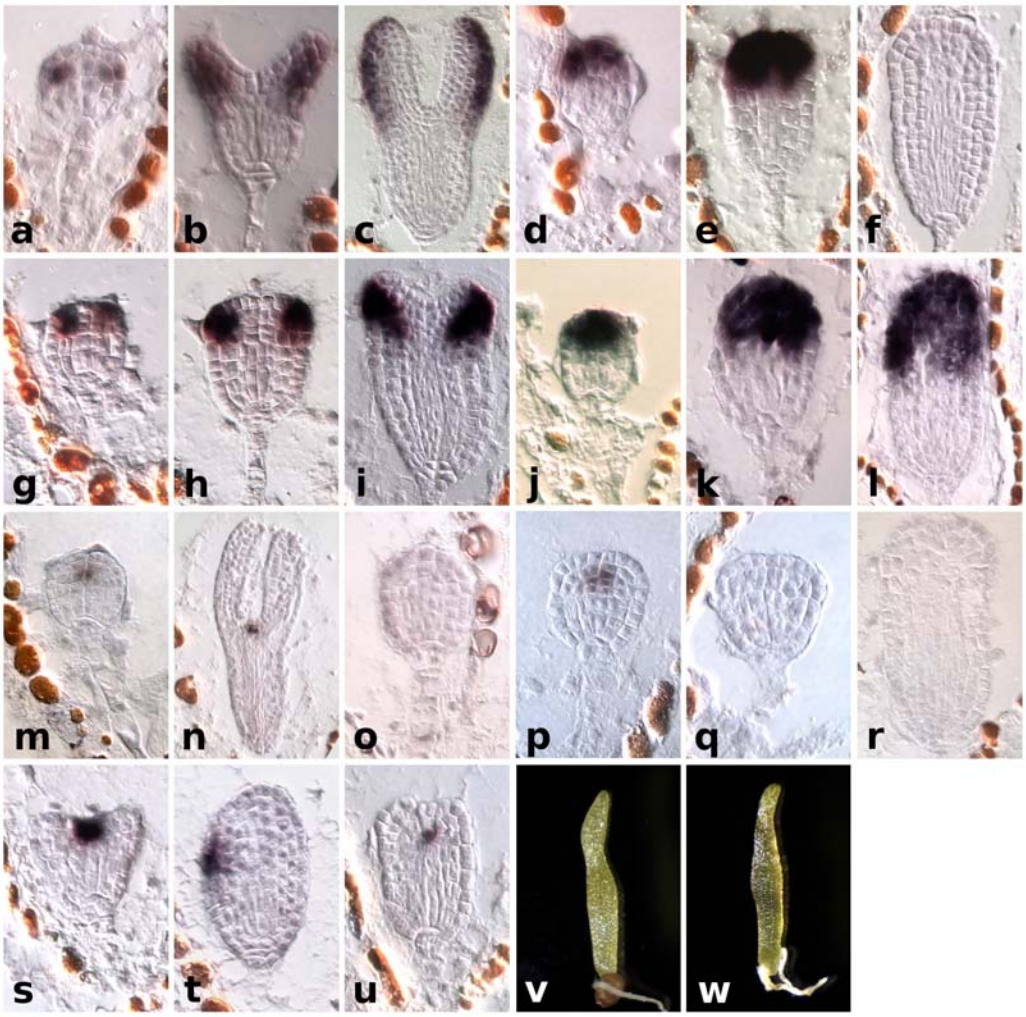


Figure 3.3 *In situ* hybridization patterns with antisense probes to *FIL* (a-l), *WUS* (m-r), and *STM* (s-u) in wild type (a-c, m, n, s), *tpl-1* (d-f, o, t), *tpl-1 phb-14d* (g-i, p, u), and *rev-9 phb-6 phv-5* (j-l, q, r). Seedling pin phenotypes of *tpl-1* (v) and *rev-9 phb-6 phv-5* (w).

Investigation of *miR165/166* as possible downstream targets of TPL and analysis of embryonic expression patterns.

We then sought to explain the observation that *HD-ZIP III* expression was lost in the apical half of *tpl-1* embryos. It could be that the expression or activity of *MIR165/166* is expanded in *tpl-1*, leading to the clearance of *HD-ZIP* mRNA. To test this hypothesis, we first sought to measure the relative expression levels of *MIR165* and *MIR166* in *tpl-1* compared to the WT. Flowering plants were grown at 29^{o}C and transition to torpedo stage embryos were harvested by microdissection and subjected to small RNA northern blot analysis. For both *MIR165* and *MIR166*, there was no significant change in *MIR* abundance in *tpl-1* vs WT (Fig. 4a, b), 1.1 and 1.2 fold, respecitvely. This suggests that *MIR165/166* are not under the control of TPL mediated repression.

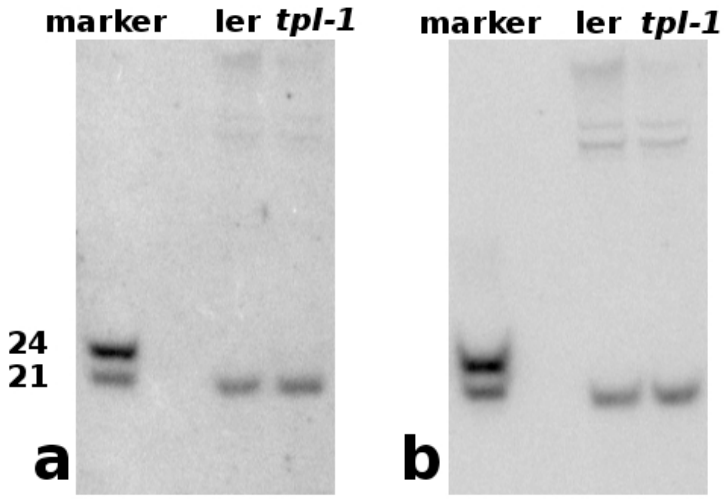


Figure 3.4 Northern blot analysis of the relative accumulation of *MIR165* (a) and *MIR166* (b) on small RNA isolated from *tpl-1* and wild type embryos grown at 29^{o}C.

Secondly, we used the ChIP method described in Chapter 2 to investigate whether *tpl-1* is recruited to the promoter regions of *MIR165/166.* *MIR165/166* are encoded at nine loci throughout the genome. Multiple regions of interest were chosen for each locus and RT-PCR primers were designed to amplify immunoprecipitated DNA from a *TPLp::TPL-HA* transgenic line. In this experiment, none of the selected regions showed enrichment in the IP versus control tissue. This further suggested that *MIR165/166* are not under TPL control.

Lastly, it was postulated that the levels of *MIR165/166* may not be significantly altered, however the pattern of expression might change in *tpl-1.*

Thus, we constructed Green Fluorescent Protein (GFP) based sensors for *miR165/166* activity during embryogenesis, in which the sensor is inactivated in all cells where *miR165/166* are active. The microRNA recognition sequence varies slightly between the members of the *HD-ZIP III* family, such that there are three variants. These variations, however occur in base pairs that are thought to be insignificant for miR recognition and predicted to be functionally equivalent. We decided to make sensor lines for all three variations, but only two were analyzed in depth: one sequence that is contained in both *PHB* and *PHV,* and the other which is contained in *REV* and *ATHB8*. We analyzed these lines by both confocal microscopy for GFP fluorescence and *in situ* hybridization against the GFP tag. In WT embryos these sensors accumulated in a pattern similar to that of the *HD-ZIP III* mRNA pattern and notably, the sensor is cleared from the root meristem organizing center from the globular stage on (Fig. 5a-c, f, g, k-m, q). Additionally, there was no observable difference between the two recognition sequence, suggesting that these differences in sequence do not have an effect on target recognition. It is possible that there are minor differences that are not observable under this resolution. If *miR165/166* were misexpressed in *tpl-1*, we would expect to observe clearance of the sensor similar to that of the mRNA of *HD-ZIP III* genes. However, in *tpl-1* we observe continued accumulation of the sensor in the apical domain of *tpl-1* embryos grown at 29^{o}C (Fig. 5d, e, h, n-p, r). These results show that the loss of apical *HD-ZIP*

III expression in *tpl-1* is due to a mechanism independent of *mir165/166* action and is likely at the level of transcriptional control. This represents a novel aspect of the control of *HD-ZIP III* gene expression and suggests that *HD-ZIP III* genes are excluded from the root by both transcriptional and post-transcriptional mechanisms.

Possibly, *miR165/166* independent loss of *HD-ZIP III* expression in *tpl-1* is caused by *PLT1/PLT2* misexpression in apical tissues. In support of this idea, *PHB* and *REV* expression was maintained in the apical domain of *tpl-1 plt1-5 plt2-1* triple mutants (Fig. 5s, t). Therefore, *PLT1/PLT2* appear to act as negative regulators of *HD-ZIP III* expression during embryogenesis. This is also consistent with what is observed in mutants where *PHB* mRNA is thought to be completely uncoupled from *miR165/166* regulation, such as *phb1-d* and *serrate* (*se*) (McConnell, Emery et al. 2001; Grigg, Galinha et al. 2009). Although *PHB* mRNA accumulates throughout a wide pattern in *phb1-d* and *se-5* embryos, it is still restricted from the descendants of the lenticular cell (Fig. 2f), an area of high *PLT1/PLT2* expression.

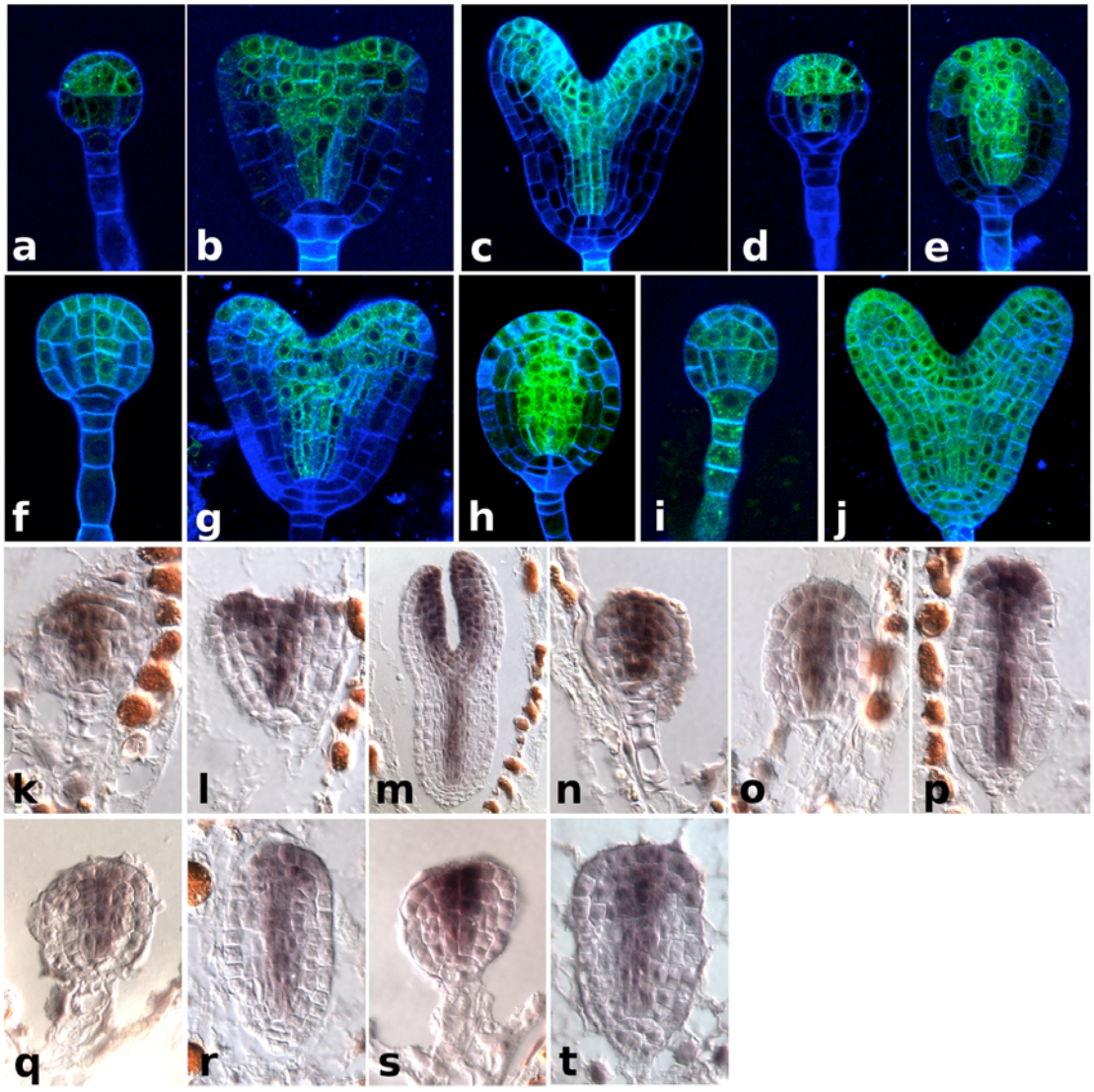


Figure 3.5 Confocal micorscopy *MIR165/166* sensor lines containing ER-GFP and the microRNA recognition sequence from *PHB* (a-e) and *REV* (f-h), and a control with three silent mutations in the *REV* micorRNA recognition sequence (I, j). Expression of these reporter lines in the wild type (a-c, f, g, i, j), *tpl-1* grown at 29^{o}C (d, e, h). *in situ* hybridization with antisense probe to GFP (k-r), *PHB* (s) and *REV* (t) in the *PHB* sensor (k-p), *REV* sensor (q-r), wild type (k-m, q), *tpl-1* (n-p, r), and *tpl-1 plt1-5 plt2-1* (s, t).

Analysis of the interaction of *PLETHORA* genes with *HD-ZIP III* genes.

Double root formation in *tpl-1* requires *PLT1*/*PLT2* misexpression and is suppressed by GOF *HD-ZIP III* mutations. To explore a possible effect of these mutations on *PLT1/PLT2* misexpression, we examined gene expression in *tpl-1 phb-14d* and *tpl-1 rev-10d* double mutants grown at 29°C. *PLT1* and *PLT2* were still misexpressed in the vascular tissue and abaxial regions of developing cotyledons (Fig. 6a-d). However, we never observed *PLT* gene misexpression in the cells that would give rise to the shoot meristem. This suggests that the ability of *PLT* genes to promote root meristem formation in *tpl-1* is dependent on misexpression in the meristem, and the GOF *HD-ZIP III* alleles are able to repress the *PLT* pathway in these cells.

Our genetic studies with the GOF *HD-ZIP III* alleles and *tpl-1* implicate the *HD-ZIP III* genes in specification of apical fate and antagonism of basal fate. Previous reports have shown that plants carrying loss-of-function alleles of *phb, phv,* and *rev* produce seedlings with a single abaxialized cotyledon and no shoot meristem (Emery, Floyd et al. 2003; Prigge, Otsuga et al. 2005). This phenotype is similar to the pin-shaped seedlings observed at low frequency in *tpl-1*, which we interpret as a partial loss of apical fate (Fig. 3). We therefore investigated whether loss-of-function alleles of *HD-ZIP III* genes could enhance the *tpl-1* phenotype at the permissive temperature of 24°C. Whereas only 2% of *tpl-1* seedlings make double roots at 24°C (n=682), the *tpl-1 rev-9* double mutant developed double roots at a frequency of 48%

(n=355). Additionally, loss of *PHB* and *PHV* in the *tpl-1 rev-9* background caused an increased frequency of the double-root phenotype (Table 1). At this temperature, *PLT1* and *PLT2* are not broadly misexpressed in the apical domain of *tpl-1* embryos (Fig. 6e, f). However, in *tpl-1 rev-9* embryos grown at 24°C, *PLT1* and *PLT2* are misexpressed similar to *tpl-1* at 29°C (Fig. 6g, h). This shows that at lower temperatures, the *HD-ZIP III* genes act to prevent the misexpression of *PLT1/PLT2* in a *tpl-1* background.

Table 3.1 Enhancement of *tpl-1* by loss of function alleles of the *HD-ZIP III* genes.

tpl-1 enhancement by *HD-ZIP III* loss-of-function alleles 21°C		
genotype	%double roots	number of seedlings counted
tpl-1	0	221
tpl-1 rev-9+/-	1.3	156
tpl-1 rev-9+/- phb-6+/-	5.8	325
tpl-1 rev-9+/- phb-6+/- phv-5+/-	14.5	145
tpl-1 enhancement by *rev-9* loss-of-function 24°C		
genotype	%double roots	number of seedlings counted
tpl-1	1.8	379
tpl-1 rev-9-/-	48	355

Given our observations that the GOF *HD-ZIP III* alleles have an antagonistic effect on apical root formation in *tpl-1*, we investigated the genetic interactions of GOF *HD-ZIP III* mutants with *PLT* loss-of-function mutants. The *phb-14d* and *rev-10d* mutants have no discernable root developmental defects during embryogenesis (Fig. 1) and *plt1 plt2* double mutants have only a minor defect in embryonic root formation (Aida, Beis et al. 2004), resulting in a properly organized seedling. However, *phb-14d plt1-5 plt2-1* triple mutant seedlings completely lacked a root and displayed only a rudimentary hypocotyl structure (Fig. 6i, Table 2), a phenotype similar to what has been reported for *plt1 plt2 plt3 plt4/bbm* quadruple mutant segregants (Galinha, Hofhuis et al. 2007). *rev-10d plt-5 plt2-1* seedlings showed an even more severe loss of both root and hypocotyl tissues (Fig. 6j, Table 2). In addition to root developmental defects, *rev-10d plt1 plt2* triple mutants show expansion of *REV* transcript into the root meristem region (Fig. 6k) further suggesting that the *PLT* genes play an active role in repression of the *HD-ZIP III* genes, in addition to negative regulation by *miR165/166*. These dramatic effects on basal patterning further exemplify the antagonistic relationship of *HD-ZIP III* genes and the *PLT* pathway. Given the difference in phenotype between *plt1 plt2* double mutants and the *plt1 plt2 plt3 plt4/bbm* segregants, it is likely that *PLT3* and *PLT4/BBM* retain some basal patterning function, antagonistic to *PHB* and *REV,* preventing more severe phenotypes in *phb-14d* and *rev-10d* single mutants.

Table 3.2 Enhancement of *plt1-5 plt2-1* by *phb-14d* and *rev-10d.*

parental genotype	percent lacking root/hypocotyl	number of seedlings counted
plt1-5-/- plt2-1-/-	0	>1000
phb-14d-/-	0	>1000
phb-14d-/- plt1-5-/- plt2-1-/-	25.9	220
enhancement of *plt1-5 plt2-1* by *rev-10d*		
parental genotype	percent lacking root/hypocotyl	number of seedlings counted
rev-10d-/-	0	>1000
rev-10d+/- plt1-5+/- plt2-1+/-	1.88	319

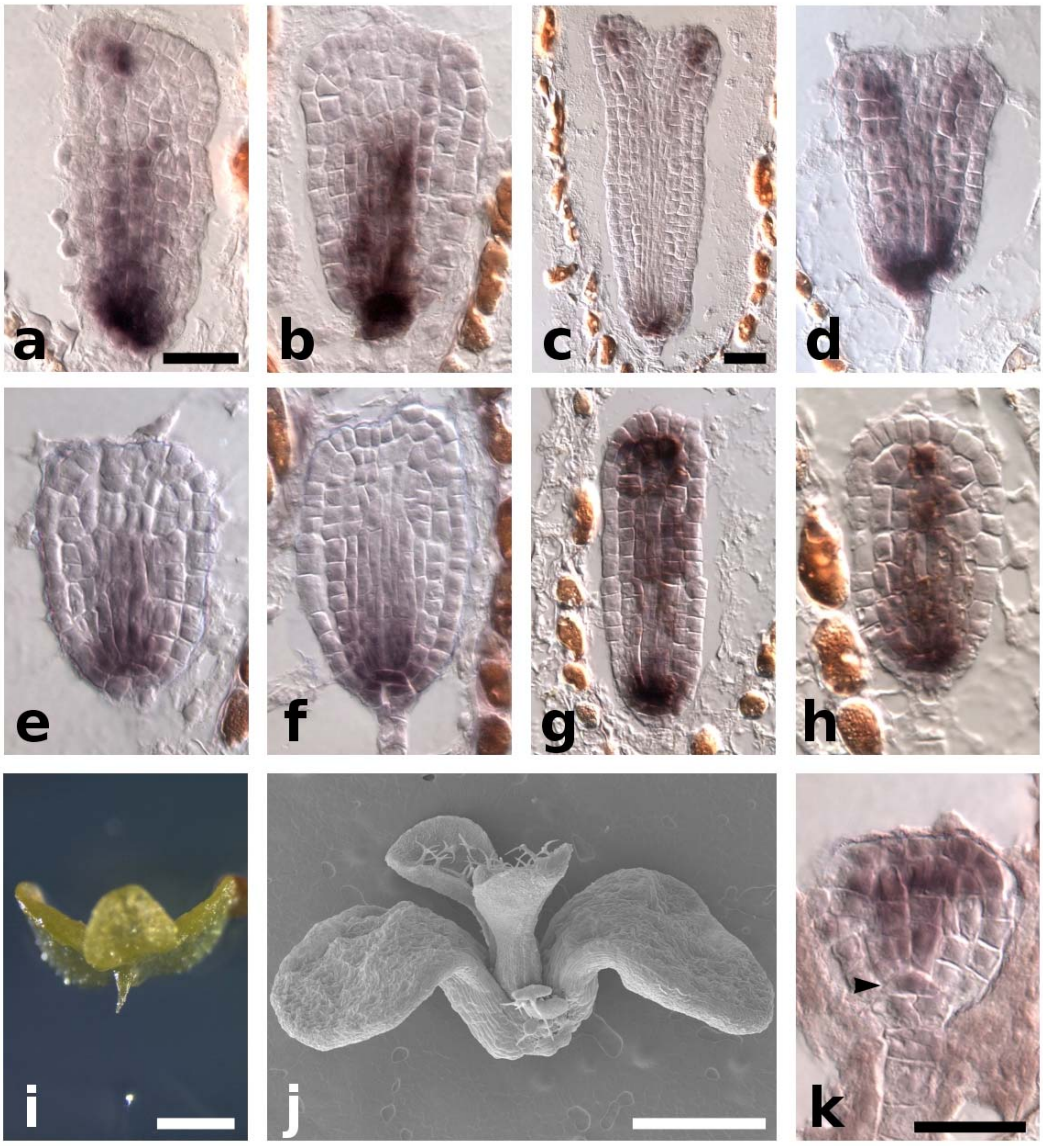


Figure3.6 a-c, *in situ* hybridizations with *PLT1* and *PLT2* anti-sense probes in 29°C grown embryos. a, *PLT1* expression in *tpl-1 phb-14d*. b, *PLT1* expression in *tpl-1 rev- 10d*. c, *PLT2* expression in *tpl-1 phb-14d*. d, *PLT2* expression in *tpl-1 rev-10d*. e-h, *in situ* hybridizations with *PLT1* and *PLT2* anti-sense probes in 24°C grown embryos. e, *PLT1* expression in *tpl-1*. f, *PLT2* expression in tpl-1. g, *PLT1* expression in *tpl-1 rev-9*. h, *PLT2* expression in *tpl-1 rev-9*. i, *phb-14d plt1-5 plt2-1* seedling. j, scanning electron microscope (SEM) image *rev-10d plt1-5 plt2-1* seedling. k, *in situ* hybridization with *REV* anti-sense probe in *rev-10d plt1-2 plt2-1*. Scale bars, 50 μm (a-h, k) and 1 mm (i, j).

***HD-ZIP III* genes are apical determinants**

We hypothesized that the *HD-ZIP III* genes are master regulatory genes that control apical fate in the early embryo and act antagonistically to the *PLT* genes. As such, we expected they could possibly impart apical polarity to basal tissue if misexpressed in the basal pole of the early embryo. We therefore expressed miR resistant cDNAs of the *HD-ZIP III* genes fused to the glucocorticoid receptor domain (GR) under the control of the *PLT2* promoter. When induced with dexamethasone during early embryogenesis, plants harboring either *PLT2p::REV∆miR-GR, PLT2p::PHB∆miR-GR,* or *PLT2p::ICU4∆miR-GR* transgenes produced seedlings that showed a complete transformation of the root pole into a second shoot pole (Fig. 7a-c, Table 2). In addition to the complete conversion of basal to apical fate, we also observed a range of phenotypes that represent a partial conversion or a mixture of apical and basal fate (Fig. 8a-c). Furthermore, in *PLT2p::PHB∆miR-GR* and *PLT2p::REV∆miR-GR* we observed phenotypes similar to *phb-14d plt1-5 plt2-1* and *rev-10 plt1-5 plt2-1*, respectively (Fig. 8d-f). Taken together, our data show that *HD-ZIP III* function is sufficient to specify shoot fate.

To better characterize these root to shoot transformations we examined the expression of *WUS* and *AINTEGUMENTA* (*ANT*), a cotyledon primordia marker, in induced *PLT2p::REV∆miR-GR* embryos. In globular stage embryos treated with dexamethasone, *WUS* became misexpressed in the basal region

corresponding to a subset of the *PLT2* domain (Fig. 7d). Later, *WUS* expression was frequently restricted to a distinct location in the presumptive second shoot, indicating that a shoot organizing center had formed (Fig. 7e). Likewise, *ANT* expression, which marks the cotyledon primordia in the WT embryo (Fig. 7f), could be detected in the lower half of transition stage embryos (Fig. 7g). In later embryos, multiple basal foci of *ANT* expression were seen (Fig. 7h). These results show that the alteration in embryo polarity began during the early globular stage of induced embryos. Furthermore, these results indicate that establishment of apical cell fate by the *HD-ZIP III* genes precedes *WUS* and *ANT* expression (and therefore meristem and cotyledon formation). In conclusion, the *HD-ZIP III* genes are true master regulators of shoot fate during embryogenesis.

Lastly, we examined the expression pattern of *PINFORMED4* (*PIN4*), which is important for establishing a localized auxin maximum in the developing root (Friml, Benkova et al. 2002) and the continued expression of *PIN4* in the root meristem is dependent on the activity of PLT1 and PLT2 (Blilou, Xu et al. 2005). In early WT embryos, *PIN4* is expressed in the embryo proper, as well as the cells of the suspensor adjacent to the embryo proper (Fig. 7i). During the globular stage, *PIN4* mRNA is restricted from the suspensor and expressed in the developing root meristem and provascular cells, where it remains expressed past the heart stage (Fig. 7j, k). In globular through heart stage *PLT2p::REV∆miR-GR* embryos, *PIN4* mRNA was only

detectable in aberrantly dividing suspensor cells (Fig. 7l, m). The loss of *PLT1/PLT2* dependant *PIN4* expression in cells ectopically expressing *REV* again illustrates the antagonistic action of these two classes of genes.

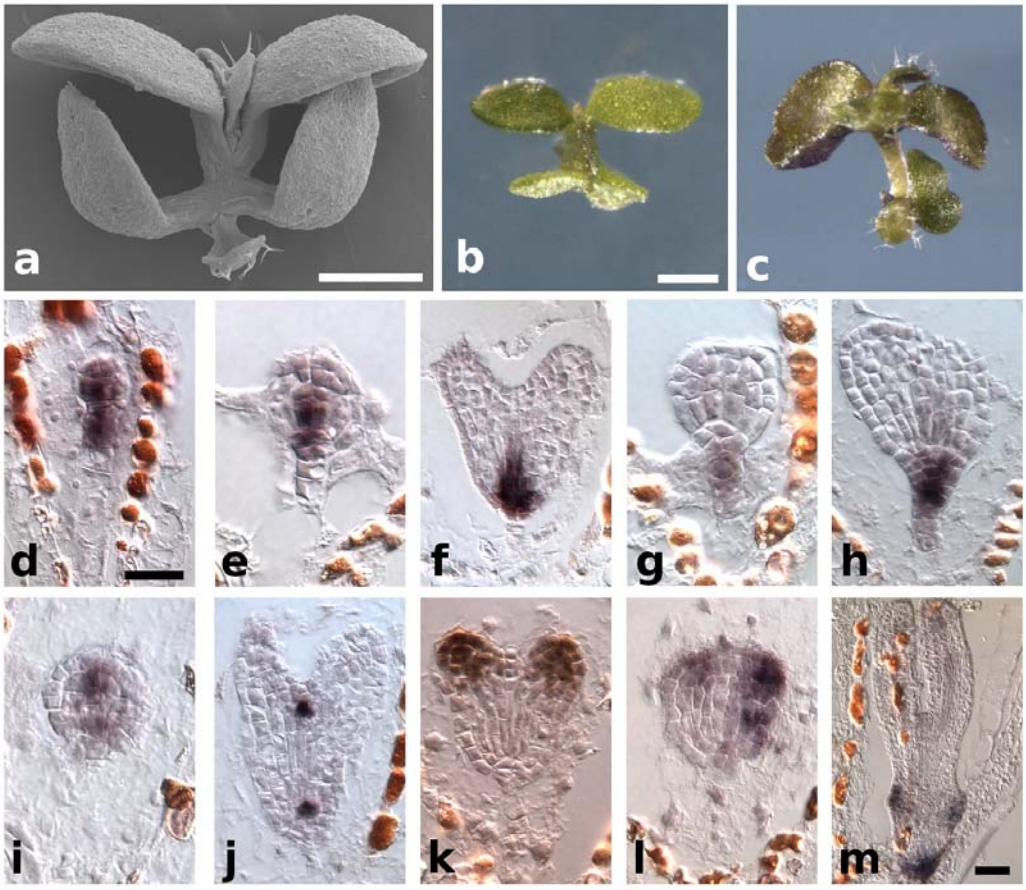


Figure 3.7 SEM image of *PLT2p:REV∆miR-GR* seedling (a). *PLT2p:PHB∆miR-GR* seedling (b). *PLT2p::ICU4∆miR-GR* seedling (c). d-e, *in situ* hybridizations with anti-sense *WUS* probe in *PLT2p:REV_miR-GR* globular (d) and heart (e) stage embryos after induction with dexamethasone. f-h, *in situ* hybridizations with anti-sense *ANT* probe in WT transition stage (f) and *PLT2p:REV∆miR-GR* transition stage (g) and torpedo stage (h) embryos after dexamethasone induction. i-m, *in situ* hybridizations with anti-sense *PIN4* probe. i-k, WT 16-cell (i), globular (j), and heart (k) stage. l-m, *PLT2p:REV∆miR-GR* globular stage (l) and late heart stage (m) embryos after dexamethasone induction. Scale bars, 1 mm (a-c) and 50 µm (d-m).

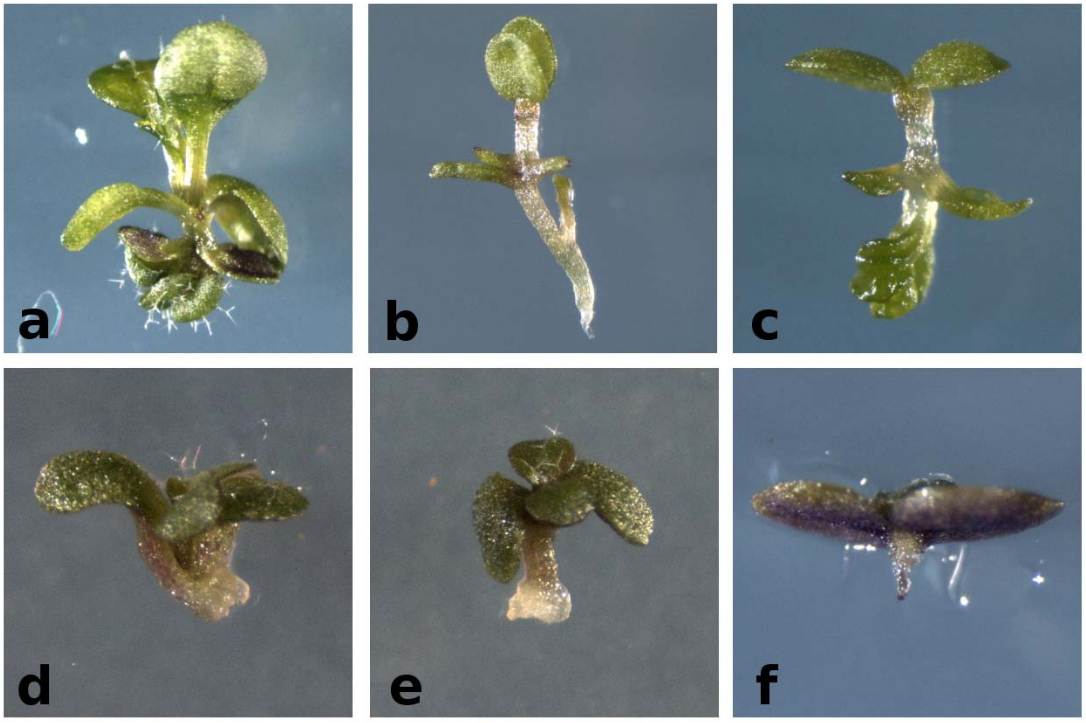


Figure 3.8 a-c and f, *PLT2p::PHBΔmiR-GR* seedlings that were induced with dexamethasone during embryogenesis. Double shoot seedlings frequently develop two functional SAMs that continue to produce leaves 3 weeks post-germination (a). Some seedlings display disorganized basal structures, with multiple cotyledon and a root-like structure (b). Additionally, some of the basal cotyledons are large and disorganized (c). Occasionally *PLT2p::PHBΔmiR-GR* produces seedlings that lack a root and resemble the basal peg of *phb-14d plt1 plt2* triple mutants (f). *PLT2p::REVΔmiR-GR* induced during embryogenesis occasionally produces seedlings that lack a hypocotyl and root (d) or that produce callus-like undifferentiated tissue in place of a root (e). These phenotypes are similar to *rev-10d plt1-5 plt2-1* triple mutants as well as the recently published phenotype of *plt1 plt2 plt3* triple mutants.

DISCUSSION

Arabidopsis embryo patterning has been extensively investigated through genetic, molecular, and biochemical methods. This has lead to the discovery of many genes responsible for patterning specific tissues during embryogenesis. Recently, the *PLT* genes have been described as master regulators of root fate, but their counterpart in the shoot has remained elusive. Here we have provided evidence that members of the *HD-ZIP III* family represent those apical /shoot master regulators during embryogenesis, as their misexpression in the developing root meristem can cause the formation of both a SAM and cotyledons.

Ectopic *PLT*2 expression was shown to induce root formation in postembryonic shoot tissue, confirming its role as a root meristem identity gene. Interestingly, misexpression of *HD-ZIP III* genes in any other tissue does not drive shoot formation but rather disrupts tissue polarity without affecting organ identity, indicating that they act in a spatial and temporal specific manner to provide positional information. Our observations that *PLT2p::REVΔmiR-GR* induced embryos show dramatic gene expression changes in the basal pole at the globular stage indicates that shoot specification by the *HD-ZIP III* genes precedes organ initiation. This suggests that the *HD-ZIP III* genes control shoot fate by specifying apical fate in the early embryo, preceding the establishment of bilateral symmetry and

independently from their role in cotyledon adaxial-abaxial polarity.

Our analysis of embryos undergoing either shoot to root transformation in *tpl-1* or root to shoot transformation in *PLT2p::REVΔmiR-GR* lines may point to a unique property of both stem cell populations. In *tpl-1* embryos, the highest level of *PLT2* misexpression in the apical half of the embryo occurs in the position that should have given rise to the SAM. Conversely, in induced *PLT2p::REVΔmiR-GR* embryos, a second shoot organizing center (as visualized by *WUS* expression) forms in a similar position to where the root organizing center should have formed (Fig. 7e). Therefore these cells might be predisposed to assume stem cell identity regardless of the underlying apical-basal polarity, pointing to a commonality between the shoot and root stem cell programs. This is consistent with the interchangeability of *WUS* and *WUSCHEL-RELATED HOMEOBOX 5* in the shoot and root stem cell organizing centers (Sarkar, Luijten et al. 2007).

Finally, our results show that there is a delicate transcriptional balance between members of the *PLT* and *HD-ZIP III* gene families during embryogenesis. In WT embryos, TPL prevents *PLT1* and *PLT2* from accumulating in the shoot pole, while the *HD-ZIP III* genes are excluded from the root pole at both the transcriptional and posttranscriptional level. There is a clear antagonism between these two classes of genes, as high expression of one restricts the expression of the other. Whether this transcriptional regulation is direct or is a more downstream consequence of fate change will

require further investigation. Given our observation that the expression patterns of the *HD-ZIP III* and *PLT* genes overlap in the central region of transition stage embryos and that increasing or expanding either *HD-ZIP III* or *PLT* gene expression leads to a loss of hypocotyl tissue, it is possible that the balance between these two classes of genes is necessary for proper hypocotyl formation following the root/shoot patterning process.

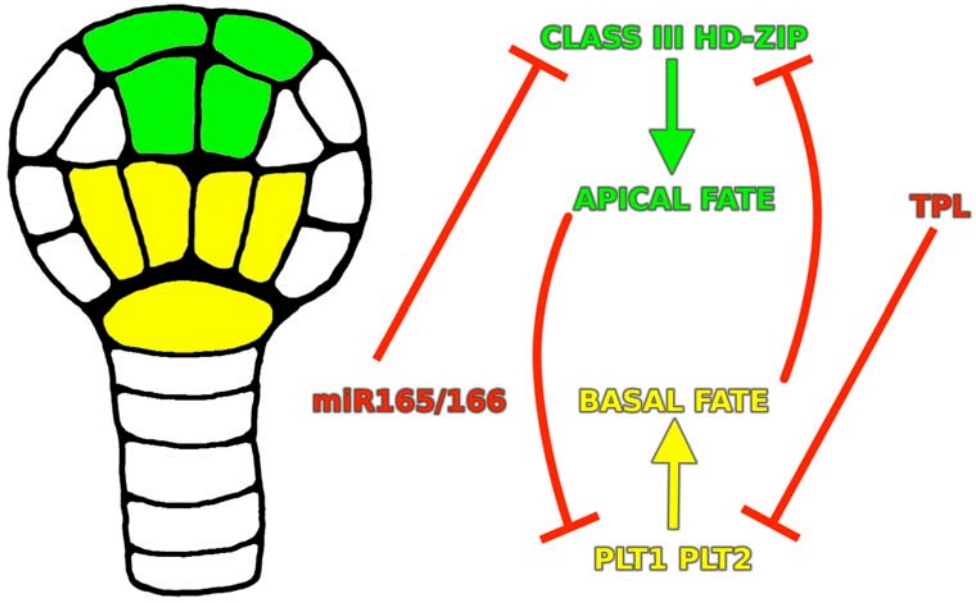


Figure 3.8 Model of the antagonistic interactions between *HD-ZIP III* family and *PLT1/2* in a globular stage embryo. During early embryogenesis, the *HD-ZIP III* genes are expressed in the apical, central portion of the embryo (green) and specify apical fate. Expression of the *HD-ZIP III* genes is directly restricted from the basal and peripheral regions by the microRNA family *miR165/166* at the post-transcriptional level. Expression of the *HD-ZIP III* genes is also negatively regulated by the action of PLT1/2 in the basal pole, at the transcriptional level and possibly downstream of basal fate specification. Conversely, *PLT1* and *PLT2* are expressed in the basal portion of the embryo (yellow) and promote basal/root fate. In the apical region of the embryo, expression of *PLT1* and *PLT2* is repressed by the recruitment of TPL to the *PLT1* and *PLT2* loci. Additionally, *PLT1* and *PLT2* expression is antagonized by the function of the *HD-ZIP III* genes.

Materials and Methods

Plant growth and mutant alleles

Plants were grown on either soil or petri dishes containing Linsmaier and Skoog salts medium. Percival growth chambers were used for controlled temperature experiments. All other plants were grown under greenhouse conditions on a 16 hour light/8 hour dark cycle. All mutants, with the exception of *icu4-1d* are in the Landsberg erecta (Ler) ecotype. Germplasm used were as follows: *plt1-5* and *plt2-1*[6], *rev-10d*[12], *phb-1d*[16], *phb-6 phv-5 rev-9*[12], *icu4-1d*[17]. *icu4-1d* was isolated in the Enkheim-2 (En-2) and back crossed to *tpl-1* four times.

in situ hybridization

in situ hybridizations were detected with digoxigenin-labeled riboprobes using the method found at http://www.its.caltech.edu/~plantlab/protocols/insitu.htm. *PHB, PHV, REV,* and *FIL,* probes were made generated using 300-700bp regions of coding sequence using the primers listed in Supplementary Table 2. *PLT1, PLT2, ICU4, WUS, ANT, PIN4* and *GFP* probes were generated using full length cDNAs.

Chromatin Immunoprecipitation

ChIP was performed as described[24] with the following modifications. Ovules were dissected from siliques containing transition to torpedo stage embryos. Tissue was fixed in 2% formaldehyde/PBS under vacuum for 2 hrs, replacing vacuum every 30 min. 500mg of starting material was used for each ChIP sample. The anti-HA monoclonal antibody HA.11 (Covance) and M-280 sheep anti-mouse IgG Dynabeads (Invitrogen) were used to immunoprecipitate TPL-HA fusion. Two negative controls were performed, including a no antibody sample and a ChIP reaction performed on wild type (no transgene) tissue.

Real-time PCR

The BIO-RAD MyiQ, single color, Real-Time PCR Detection System was used with the MyiQ Optical System Software for analysis. SYBR Green I was used as an intercalating fluorescent dye. The standard curve method was used to determine reaction efficiency for each primer pair and determine fold enrichment by comparing the CT (threshold cycle) values of IP and negative control which were normalized normalize by calculating input (IP)/input (control) when appropriate.

Plasmid Construction

The *miR165/166* sensor was generated using complementary 42 base pair primers encompassing the *miR165/166* recognition sequence in *PHB* and *REV,* which were annealed to generate double stranded fragments with EcoRI compatible sites at each end. These fragments were then treated with T4 polynucleotide kinase in T4 DNA ligase buffer and cloned into a unique EcoRI site in the *mERGFP5* sequence that lies between the endoplasmic reticulum (ER) localization signal and *mGFP5*. The modified *mERGFP5* were then cloned as a BamHI fragments into a *pBJ36* construct, 3' to a 925bp promoter fragment from the potato *UBI3* gene[25]. For the negative control, three silent mutations were introduced within the 3' end of the miR recognition sequence.

For construction of the *PLT2p::HD-ZIPIII-GR* constructs, a 4380 bp genomic fragment 5' to the *PLT2* start codon was cloned as a XhoI/SalI fragment into a SalI site of a *pBJ36* vector containing the hormone binding domain of the rat glucocorticoid receptor[26]. *HD-ZIP III* miR resistant cDNAs were generated by inducing three silent mutations within the 3' end of the miR recognition sequence by site directed mutagenesis.

Microscopy

Excised ovules were mounted in Hoyer's solution for analysis of embryonic morphology. Embryos were imaged using a Leica DM5000B microscope, seedlings using a Leica MZ FLIII microscope. For GFP analysis, ovules were dissected into 0.5X LS media (Caisson Laboratories, Inc.; Rexburg, ID), vacuum infiltrated in 4% paraformaldehyde, rinsed with water, vacuum infiltrated with 2% SCRI Renaissance 2200 (Renaissance Chemicals Ltd.; North Yorkshire, UK) and 4% DMSO, then washed 2X and mounted in 20% glycerol. Embryos were imaged using a Leica DM IRE2 laser scanning confocal microscope. SR2200 was excited with the UV diode 405nm line, and emission was measured between at 420-470nm. GFP was excited with a 488nm argon laser line and emission was measured at 500-535nm.

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Chapter 3 contains material from the publication: Control of Arabidopsis apical-basal embryo polarity by antagonistic transcription factors. Smith ZR and Long JA, Nature 2010. The dissertation author was the primary author of this paper.

CHAPTER 4

Conclusions and Perspectives for Future Research

The role of *TPL* in repression of the basal embryonic program.

The formation of the apical-basal axis during *Arabidopsis thaliana* embryogenesis is a crucial step that directs the overall body plan of the seedling and directs the establishment of stem cell populations at either end of the embryo that give rise to all of the adult structures. The establishment of the apical-basal axis occurs with the first division of the zygote and remains plastic until the transition stage, where apical-basal polarity becomes fixed. The molecular mechanisms and genes responsible for the formation and fixation of the apical-basal axis remain largely unknown. Much of Chapter 2 is work that was published in the paper titled "*TOPLESS* Regulates Apical Embryonic Fate in *Arabidopsis*". This work identifies the locus for the *tpl-1* mutation and describes some molecular properties of TPL, including it's nuclear localization, expression pattern, as well as describing some gene expression in the *tpl-1* mutant. Furthermore, the dominant negative nature of the mutation was elucidated and the *tpl-1* phenotype was recapitulated with a pseudo-quintuple mutant of *tpl-2 tpr-1 tpr-3 tpr-4* and RNAi knock-down of *TPR2*. The framework for the role of *TPL* in transcriptional regulation is established. *TPL* is postulated to be a corepressor, based on domain structure, as well as by the observation of the misexpression of basal genes in

the apical domain of *tpl-1* embryos. Furthermore, two mutants are identified that interact genetically with *tpl-1.* In a suppressor screen performed in the *tpl-1* background, two loss of function alleles of *HISTONE ACETYLTRANSFERASE GNAT SUPERFAMILY1* (*HAG1*) were identified that completely rescues the *tpl-1* embryonic phenotype. Furthermore, loss of function alleles of *HISTONE DEACETYLASE19* (*HDA19*) were found to enhance the *tpl-1* phenotype, as well as exhibit a similar phenotype to *tpl-1* when grown at high temperatures. This work placed *TPL* within a pathway that functions in the regulation of gene expression and chromatin states. Specifically, we hypothesized that *TPL* was necessary for repression of basal determinants, which are inappropriately activated in *tpl-1* and that activation is dependent on the activity of *HAG1.* Furthermore, we suggest that *HDA19* likely participates in this active repression of basal genes, possibly through it's recruitment by TPL.

While this paper represents a large body of work and an advancement in our understanding of *TPL* and the role of transcriptional repression in the fixation of apical fate, there remain many questions regarding the function of *TPL* and it's mechanism of action. Much of our knowledge regarding *TPL* has been provided through the study of the *tpl-1* allele, which we have found to be a dominant negative for at least 5 of the nine *TPL* and *TOPLESS RELATED* (*TPR*) family members. While the *tpl-1* allele has been indispensable in our studies, it is also confounding to some of our conclusions regarding the

function of *TPL.* The dramatic phenotypes seen in the *tpl-1* allele must be attributed to the entire *TPR* family as well, and we currently have little understanding how the disruption of the function of the individual *TPR* genes contribute to the *tpl-1* phenotype. Preliminary chromatin immunoprecipitation followed by sequencing (ChIP-seq) studies have shown that TPL is recruited to it's own promoter region and those of the *TPR* genes, suggesting that the expression of *TPL* in the *tpl-1* mutant may be relieved of it's wild type control mechanisms. Additionally, it is clear that the *tpl-1* phenotype is is highly dosage dependent as *tpl-1* is semi-dominant and extra copies of the *tpl-1* allele enhance the phenotype. Teasing out the differential recruitment of the *TPR* genes to apical targets during early embryogenesis may be informative towards understanding how repression programs are employed during early development. This could be pursued through the ChIP-seq approach in a comparative genomics project with one or more of the more prominent *TPR* genes and *TPL.* In this publication, we implicate *HDA19* as a component of the *TPL* repression pathway. Understanding if these proteins form a repression complex is a major and ongoing research interest.

Additionally in Chapter 2, I present work that I have performed towards the identification of direct targets for TPL repression that are causative of the shoot to root homeotic conversion observed in *tpl-1.* Here the focus is on the study of the *PLETHORA* gene family and their genetic and molecular interaction with *TPL.* The expression patterns of *PLT1-4* are described and

PLT1, PLT2, and *PLT3* are found to be misexpressed in the apical domain of young *tpl-1* embryos, suggesting that they may play a role in the shoot to root transformation. *PLT1* and *PLT2* were found to be necessary for this process, as loss of function alleles in *PLT1* and *PLT2* completely suppress double root formation. Lastly, chromatin immunoprecipitation (ChIP) followed by real-time qPCR on ChIP DNA from embryos harvested from a *TPLp::TPL-HA* transgenic line show that TPL is present on the promoter regions of the *PLT1* and *PLT2* genes. These lines of evidence suggest that repression of the *PLT* genes by *TPL* is crucial for proper apical embryonic development, and misregulation of the *PLT* genes is likely causative of the *tpl-1* double root phenotype.

While we have found what we think are the major contributors to the *tpl-1* phenotype, there are a number of aspects of this relationship that remain uncertain. First, we see that *PLT1, PLT2* and *PLT3* are misexpressed in *tpl-1* embryos. Furthermore, we see that *PLT1* and *PLT2* are directly bound by TPL through chromatin immunoprecipitation. It is unknown whether *PLT3* is also a target for TPL mediated repression and what role *PLT3* plays in the *tpl-1* phenotype. This could be assessed through further ChIP experiments with the *TPLp::TPL-HA* transgenic line. Secondly, we see that although the *tpl-1 plt1-5 plt2-1* triple mutant no longer displays the double root phenotype, there are major patterning defects in the apical region. These defects may be a result of continued *PLT3* misexpression, as we have seen misexpression of *PLT3* in *tpl-1 plt1-5 plt2-1* by *in situ* hybridization (data not shown). It is possible that

the majority of the apical patterning defects are mediated by the *PLT* genes and could be tested by the generation of a *tpl-1 plt1-5 plt2-1 plt3* quadruple mutant. If apical patterning defects remain in this genetic background, it would suggest that there are additional targets of *TPL* that require active repression during apical embryonic development that have yet to be identified. Secondly, it has been shown that *TPL* is recruited to it's target genes by sequence specific transcription factors. The identification of the transcription factors responsible for recruiting TPL to the *PLT1* and *PLT2* promoters would contribute to our understanding of pathway of apical repression of basal genes. The regions of ChIP enrichment for the *PLT1* and *PLT2* promoters is not conserved and suggests that TPL may be recruited to these sites through different mechanisms. It has been recently reported that *TPL* participates in the *AUX/IAA* repression of the *ARF* transcription factor pathway. These families of genes provide good candidates for mediating *TPL* dependent *PLT* repression, as it has been shown that *PLT* expression is dependent on the *MONOPTEROS/ARF5* transcription factor.

The *HD-ZIP III* genes are apical determinants.

Arabidopsis embryo patterning has been extensively investigated through genetic, molecular, and biochemical methods. This has led to the discovery of many genes responsible for patterning specific tissues during embryogenesis. Recently, the *PLT* genes have been described as master

regulators of root fate, but their counterpart in the shoot has remained elusive. Here we have provided evidence that members of the *HD-ZIP III* family represent those apical /shoot master regulators during embryogenesis, as their misexpression in the developing root meristem can cause the formation of both a SAM and cotyledons.

Ectopic *PLT*2 expression was shown to induce root formation in postembryonic shoot tissue, confirming its role as a root meristem identity gene. Interestingly, misexpression of *HD-ZIP III* genes in any other tissue does not drive shoot formation but rather disrupts tissue polarity without affecting organ identity, indicating that they act in a spatial and temporal specific manner to provide positional information. Our observations that *PLT2p::REVΔmiR-GR* induced embryos show dramatic gene expression changes in the basal pole at the globular stage indicates that shoot specification by the *HD-ZIP III* genes precedes organ initiation. This suggests that the *HD-ZIP III* genes control shoot fate by specifying apical fate in the early embryo, preceding the establishment of bilateral symmetry and independently from their role in cotyledon adaxial-abaxial polarity.

Our analysis of embryos undergoing either shoot to root transformation in *tpl-1* or root to shoot transformation in *PLT2p::REVΔmiR-GR* lines may point to a unique property of both stem cell populations. In *tpl-1* embryos, the highest level of *PLT2* misexpression in the apical half of the embryo occurs in the position that should have given rise to the SAM. Conversely, in induced

PLT2p::REVΔmiR-GR embryos, a second shoot organizing center (as visualized by *WUS* expression) forms in a similar position to where the root organizing center should have formed (Fig. 7e). Therefore these cells might be predisposed to assume stem cell identity regardless of the underlying apical-basal polarity, pointing to a commonality between the shoot and root stem cell programs. This is consistent with the interchangeability of *WUS* and *WUSCHEL-RELATED HOMEOBOX 5* in the shoot and root stem cell organizing centers.

Finally, our results show that there is a delicate transcriptional balance between members of the *PLT* and *HD-ZIP III* gene families during embryogenesis. In WT embryos, TPL prevents *PLT1* and *PLT2* from accumulating in the shoot pole, while the *HD-ZIP III* genes are excluded from the root pole at both the transcriptional and posttranscriptional level. There is a clear antagonism between these two classes of genes, as high expression of one restricts the expression of the other. Whether this transcriptional regulation is direct or is a more downstream consequence of fate change will require further investigation. Given our observation that the expression patterns of the *HD-ZIP III* and *PLT* genes overlap in the central region of transition stage embryos and that increasing or expanding either *HD-ZIP III* or *PLT* gene expression leads to a loss of hypocotyl tissue, it is possible that the balance between these two classes of genes is necessary for proper hypocotyl formation following the root/shoot patterning process.

While the identification of the *HD-ZIP III* genes as apical determinants represents an advance in our understanding of early apical-basal patterning mechanisms, much remains unknown regarding the mode of regulation of the *HD-ZIP III* genes. The *HD-ZIP III* genes are first expressed in very early globular stage embryos, and are expressed in a polar manor in the apical region of the embryo. We know from the study of our microRNA sensors that the expression of the *HD-ZIP III* genes is restricted from the basal domain by *MIR165/166* beginning at the globular stage. Secondly, we know that there is a microRNA independent pathway that restricts *HD-ZIP III* expression from the root and is *PLT* dependent. Understanding the mechanisms which control this polar distribution of the *HD-ZIP III* genes at the globular stage would provide great insight towards the understanding of how apical-basal polarity is established. We assume that cis-acting elements in the *HD-ZIP III* promoters are responsible for *PLT* dependent repression. We have develop a system that is capable of reporting repressive capabilities of DNA elements, where DNA fragments are cloned downstream of a ubiquitous promoter and upstream of a GFP reporter. This system could be utilized, inserting fragments of the *HD-ZIP III* regulatory regions and observing repression of the GFP reporter in the basal region. Secondly, yeast one hybrid approaches could be used to identify transcription factors that bind these regions.

The *HD-ZIP III* genes seem to function as integrators of positional information throughout the plant. They serve to both maintain stem cell

function in the shoot meristem as well as specify adaxial identity to the developing lateral organs at the flanks of the meristem. Misexpression of the *HD-ZIP III* genes throughout the plant does not result in homeotic conversions, but rather disrupts tissue polarity in individual organs. Understanding the mechanism of action of the *HD-ZIP III* genes would be of great interest. We have developed a system of transcriptional profiling of all five family members and should be highly informative towards the function of the *HD-ZIP III* genes in meristem maintenance, lateral organ polarity, and the integration of positional information between these developing structures.