A WUSCHEL-independent stem cell specification pathway is repressed by PHB, PHV and CNA in Arabidopsis

by

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CHAPTER ONE

Core pathways controlling shoot meristem maintenance

Abstract

Essential to the function of shoot meristems in plants to act as sites of continuous organ and tissue formation is the ability of cells within the meristem to remain undifferentiated and proliferate indefinitely. These are characteristics of the stem cells within meristems that are critical for their growth properties. Stem cells are found in tight association with the stem cell niche – those cells that signal to maintain stem cells. Shoot meristems are unique among stem cell systems in that the stem cell niche is a constantly changing population of recent stem cell daughters. Recent progress from Arabidopsis and other systems have uncovered a large number of genes with defined roles in meristem structure and maintenance. This review will focus of well-studied pathways that represent signaling between the stem cells and the niche, that prevent ectopic differentiation of stem cells, that regulate the chromatin status of stem cell factors, and that reveal intersection of hormone signaling and meristem maintenance.

Introduction

Since shoot apices were first observed in 1759, the shoot meristem has become a major focus of plant biology research. Investigations have focused on the diversity, morphology, histology, cell divisions and cell lineages of the shoot meristem [1-4]. A critical aspect of meristem function is the stem cell-like activity of a subset of cells of the meristem. Specifically, meristematic cells require a delicate balance of self-renewal and differentiation. This homeostatic characteristic is essential for the meristem to act as a site of continuous organogenesis. Our understanding of the regulatory control of meristem development advanced rapidly with the advent of Arabidopsis molecular genetic studies. During this period, an large array of genes and mutants have been identified that effect meristem development [5]. A number of these genes function in well-studied pathways that are critical for meristem homeostasis and are the focus of this review.

The Arabidopsis shoot meristem is one of a number of indeterminate sites of organogenesis and continuous growth (others include the flower, root and vascular meristems) [4, 6]. The shoot meristem specifically is the ultimate source of all organs above the ground: stems, leaves, and flowers. During the globular stage of embryogenesis, the precursors of the shoot apical meristem are established. By heart stage of embryogenesis, both the cotyledon primordia and the early shoot meristem have been established [7-10]. Post-embryonically, lateral shoot meristems repeatedly arise from the axils of leaves to form secondary and high-ordered shoots [6, 11, 12]. In addition, various Arabidopsis mutants that lack shoot meristems will undergo a poorly-understood process of adventitious meristem initiation [13, 14]. Once formed, indeterminate shoot meristems must be maintained through a process of homeostasis that can act over a period of weeks or decades, depending on the plant species. While not the focus of this review, the homeostatic controls must be highly sensitive to environmental and physiological conditions and adapt to changing rates of growth as well as periods of quiescence.

The shoot meristem contains a population of undifferentiated self-renewing cells at the central apex (Figure 1.1). These stem cells are the source of new organs and

tissues for the plant. Peripheral and basal daughter cells transition toward differentiation, divide much more rapidly, and organize into distinct organ primordia (Figure 1.1B). The central and peripheral regions of the meristem were initially identified based on their histological characteristics. The more slowly dividing central stem cells were termed the central zone (CZ), while the more rapidly dividing and cytoplamsically dense peripheral cells were termed the peripheral zone (PZ) [4]. While technically a description of cell morphology, CZ and PZ have become used as shorthand for the central stem cells and their peripheral differentiating daughters, respectively [3, 5, 6, 11, 15-17].

In addition of this zonal division, the shoot meristem is organized into distinct cell layers (Figure 1.1B). In Arabidopsis, the outer most L1 and L2 layers (the tunica) are normally maintained as clonally distinct because cell divisions in these layers are strictly anticlinal in the meristem center. The more internal L3 layer, on the other hand, divides both anticlinally and periclinally [18-21].

The stem cell population must be maintained while appropriately positioned peripheral and basal daughter cells are directed toward organ primordia and other differentiated tissues. The maintenance of these stem cells is influenced by their position, or niche. At the shoot meristem, the niche is driven by an Organizing Center (OC), which is located at the base of the central zone of the shoot meristem [14, 22, 23]. Critically, the OC is a constantly changing population of cells (see below). Because the L1, L2 and L3 layers are clonally distinct, each needs to maintain a population of stem cells in the very center during the process of cell division. In the L1 and L2 layers, it is the centrally-located daughter that remains a stem cell, while the peripheral daughter makes a transition toward differentiation. Within the L3 layer, cells that undergo periclinal divisions require the apical daughter to retain stem cell identity, while the basal daughters differentiate [18-21]

The balance between stem cell specification and differentiation is also involved in flower meristem function. Floral primordia are initiated on the flanks of the shoot meristem and a floral meristem is established. The floral meristem is a modified shoot meristem and thus also requires a process of stem cell maintenance; however, within the floral meristem, stem cells are transiently maintained [2, 4, 24]. Stem cell

maintenance at the shoot and flower meristems can vary considerably between different plant species [4].

The WUS-CLV pathway

A large number of factors influence and control shoot meristem maintenance. Rather than covering every gene with an effect on meristem development, this review will focus on the best-characterized pathways. The meristem homeostasis pathway explored in greatest detail is the WUS-CLV pathway. The essential nature of this pathway is exemplified by the mutant phenotypes from disruption of this pathway that range from shoots with no stem cells to shoots with only stem cells [14, 25-28]. This pathway appears to be central for signaling between the stem cells and the underlying organizing center.

WUS is a transcription factor and the founding member of the WOX homeodomain family found throughout plants [14]. *WUS* expression is initiated at the 16-cell stage of embryogenesis and becomes progressively restricted to the basal daughters of the L3 stem cells as the embryonic shoot meristem is formed [22]. In the active shoot meristem, the expression domain of *WUS* defines the OC which acts as the stem cell niche [14, 22]. The earliest studies on *WUS* observed: 1) *wus* mutants fail to establish or maintain stem cells at shoot meristems (*wus* mutants reiteratively form adventitious shoots which also fail to maintaining stem cells); 2) *WUS* is expressed in the OC; 3) *WUS* over-expression within the meristem drives ectopic stem cell accumulation [14, 22, 23]. The long-held model that evolved from this data was that *WUS* acts to specify the Organizing Center, which in turns signals to the overlying cells to maintain stem cell identity.

A potentially enlightening twist to this model was recently proposed based on data on the movement of WUS protein within the meristem. Reddy and colleagues observed movement of a GFP-tagged WUS protein into the stem cell layers of wild-type shoot meristems, presumably through the plasmodesmata [29]. Nuclear-targeted or double-GFP tagged WUS protein had restricted movement and failed to completely

rescue *wus* mutant plants, providing a correlation between movement and WUS function. While these experiments are not definitive as of yet, they open up a completely novel way at looking at the nature of stem cell/niche interactions.

One aspect of *WUS* transcriptional regulation has been extensively studied, that is the limitations of *WUS* expression by the CLAVATA (CLV) signaling pathway (Figure 1.2). *clavata* (*clv*) mutants have opposite phenotypes to *wus* mutant in that *clv* mutants have enlarged meristems, specifically accumulating stem cells [25-27]. Evidence suggests this is the result of *WUS* expression expanding into the L3 layer of stem cells in *clv* mutants so that WUS is expressed in both the apical and basal L3 daughters. The interpretation of these *clv* phenotypes is that the expanded OC represented by WUS expression leads to ectopic stem cell specification on the overlying cell layers [23, 30].

The CLV pathway is composed of a numbers of factors. These include the receptor-kinases CLV1, BAM1 and BAM2, the receptor-like protein CLV2, the transmembrane kinase-related protein (but, catalytically inactive) CRN, the secreted ligand CLV3 and the membrane-associated protein phosphatases POL and PLL1 [31-38].

CLV signaling starts with CLV3, which is specifically expressed in what appears to be the stem cells of the shoot meristem [33, 39] (Figure 1.2). CLV3 is a founding member of the CLE family of secreted peptides found throughout land plants [40-42]. CLV3 undergoes extracellular proteolytic maturation to release the short CLE peptide (likely 12 or 13 amino acids) from the precursor protein [43-47]. CLV3 may undergo further maturation through hydroxyproline modification and arabinosylation [44, 47]. Because the active CLV3 signal is a small peptide, no direct monitoring of CLV3 diffusion has been possible, but indirect evidence suggests it moves quite broadly throughout the meristem [48-50]. The mature CLV3 peptide binds to a number of plasma membrane receptors, the most important (based on mutant phenotypes) being CLV1 and CLV2. Interestingly, CLV3 binds to both of these receptors at similar affinities, and both binding events appear to be essential for CLV signaling [51-53]. Mature CLV3 also binds to the CLV1-redundant receptors BAM1 and BAM2 [52], and a potential parallel pathway involving the related receptor RPK2 [54]. How CLV3 binding

effects receptor activation and what activation means in terms of receptor function are unclear. Genetic analysis demonstrates that CLV3 acts upstream as a positive regulator of CLV1 and CLV2, thus CLV3 binding to these receptors presumably activates their ability to signal within the cell. Evidence from Arabidopsis shoot meristems supports the presence of two receptor complexes: CLV1/BAM multimers and CLV2/CRN heteromultimers [52] (Figure 1.2). Higher order receptor complexes can be detected in transient expression [51, 52, 55]. One detectable effect of CLV3 binding to CLV1 is its internalization within meristematic cells [49]. In other receptor systems, internalization can be linked to signaling, receptor recycling, and feedback inhibition.

POLTERGEIST (POL) is a critical target of CLV signaling. Originally identified in a *clv* mutant suppressor screen, POL and the related PLL1 are signaling intermediates between the CLV receptors and *WUS* regulation [28]. The *pol pll1* double mutant is seedling lethal due to loss of asymmetric cell divisions in the early embryonic hypophyseal and procambial cells [56]. However, grafting the apices of *pol pll1* seedling to wild-type roots revealed that the *pol pll1* tissue phenocopied *wus* mutants and failed to maintain *WUS* expression in the shoot. Thus, POL/PLL1 promote *WUS* transcription and are repressed by CLV signaling [28] (Figure 1.2). Interestingly, POL/PLL1 localize to the cytoplasmic face of the plasma membrane via dual acylation [57]. In addition, POL/PLL1 bind to, and are catalytically activated by, phosphatidylinositol 4-phosphate in vitro [57]. Considering that CLV1 endocytosis is triggered by CLV3 activation, there may be a link between receptor activation, phospholipids and cytoplasmic signaling.

Even though WUS has been studied as a major factor regulating stem cell specification and maintenance, how WUS functions to specify stem cells is not known in detail. A useful resource of meristem gene expression profiling was published by Reddy and co-workers [58]. In this study, cells of the meristem were sorted in one of three regions: the stem cell region (marked by *CLV3*), the OC (marked by *WUS*), and the periphery (marked by *FIL*). Each of the populations was then used for microarray analysis to provide a transcriptome profile for each region. This resource provides both a list of potential marker genes and effectors for the key aspects of meristem maintenance. Other recent advances in identifying WUS transcription targets have

been made. WUS has been shown to directly bind genes encoding ARR-A isoforms, which act in a negative feedback loop of cytokinin signaling [59]. Comparative microarray using plants with ethanol-inducible overexpression *WUS*, *STM*, and *LFY* was performed and 148 candidate genes were identified to responsive in *WUS* overexpression but not *STM* and *LFY* [59]. Four genes encoding ARR-A isoforms (*ARR5*, *ARR6*, *ARR7*, *ARR15*) were among 148 genes. Quantitative RT-PCR showed that the transcription level of ARR-A type genes is sensitively reduced by WUS induction. The results of chromatin immunoprecipitation (ChIP) and electrophoretic mobility-shift assays (EMSAs) indicated that WUS directly binds to the cis elements of *ARR7*. These results suggest a direct interaction between WUS/CLV signaling and cytokinin signaling for meristem formation and maintenance.

Busch and colleagues performed genomic and transcriptomic analysis to identify downstream targets of WUS using a suite of *WUS* loss-of-function and gain-of-function backgrounds [60]. By this analysis, more than 100 potential direct transcriptional WUS target genes were identified. Thus, WUS could affect a large number of downstream genes to maintain stem cell integrity. Interestingly, CLV1, which negatively regulates *WUS*, was identified as one of the direct targets of WUS, suggesting WUS may bind to *CLV1* and modulate its expression. Because *WUS* and *CLV1* expression domains extensively overlap [22, 31], this regulation would likely be one to modulate the levels, not region, of *CLV1* transcription. One aspect of these genomic studies that requires further investigation is the observation that the targets identified as WUS protein binding sites in the genome were largely independent of the genes identified as transcriptionally controlled by WUS activity.

A critical WUS target within the meristem is *CLV3*. This creates a feedback loop, whereby CLV signaling restricts *WUS* expression to the OC, while WUS induces *CLV3* expression in the overlying stem cells. The evidence for WUS induction of *CLV3* was based both on the loss of *CLV3* expression in *wus* mutants and the ectopic expression of *CLV3* driven by *WUS* over-expression [23, 30]. More recently a potentially direct mechanism for this induction has been proposed. In addition to providing evidence of WUS protein movement to the stem cells, Reddy and colleagues demonstrated that WUS binds to region of the *CLV3* cis elements in EMSA experiments [29].

In addition, WUS may have a direct or indirect role in controlling the rate of growth and proliferation in the PZ. Transiently induced *WUS* expression led to not only expansion of the stem cell region, but also an increase cell division rate in the PZ. Conversely, decreased WUS level result in smaller CZ and a reduction of cell division rate [61]. Whether this reflects direct WUS control of the PZ or an underlying compensatory mechanism between CZ and PZ growth remains to be determined.

Pre-division vs post-division model for CLV/WUS control

A variety of evidence suggests that the primary function of CLV signaling in shoot meristem homeostasis is to restrict *WUS* expression in the L3 stem cell layer. Two different models have been proposed for how this *WUS* repression is achieved. These models differ in the timing of CLV signaling relative to the division of the L3 stem cells.

One way to view L3 stem cell divisions is to see them as asymmetric. L3 cells undergoing periclinal (apical/basal) divisions have very different fates adopted by the two daughter cells. The apical daughter remains a stem cell and continues *WUS* repression. The basal daughter activates *WUS* expression and becomes part of the stem cell niche OC (see above).

In *clv* mutants, a key aspect of asymmetry is lost, namely, the differential *WUS* expression. Whether other aspects of asymmetry between apical and basal L3 daughters are lost in *clv* mutants has not been ascertained. Thus, one way to view *clv* mutants is that they lose L3 cell asymmetry. This could result from a role for CLV signaling in maintaining cell asymmetry either after or before cell division L3 cell division.

The post-division model, developed from the earliest evidence of CLV repression of *WUS*, hypothesizes that active CLV signaling is required continuously to prevent *WUS* expression from being activated in the L3 stem cell layer (i.e., in the apical daughters of divided L3 stem cells). An interesting challenge for this model is that it requires functional CLV signaling in the L3 stem cell layer, but no CLV signaling one cell away in the OC. A possible mechanism for this differential signaling is ligand sequestration, where sufficient levels of the receptor deplete the ligand from apoplasm.

Ligand sequestration essentially acts to dramatically increase the gradient of ligand concentration and has been observed in some animal signaling systems. Studies using *CLV3* over-expressed specifically in the L1 layer interrupted the ability of *CLV1* expression to block the effects of this ectopic *CLV3* expression as evidence of ligand sequestration [48]. However, a more recent study analyzing the internalization of CLV1 in response to activation by CLV3 reported no evidence of ligand sequestration [49]. The challenge for definitively establishing whether CLV1/CLV2/BAM receptors can limit the range of CLV3 diffusion is that there is currently no way to detect the active form of CLV3 within the meristem. This is because CLV3 is proteolytically processed with additional hydroxyproline and arabinosylation modifications (see above). Which form of mature CLV3 is produced and physiologically active within the shoot meristem is unknown. A further challenge for the model is that the L3 cells themselves express *CLV3*, at least at the transcriptional level [33]. This *CLV3* expression, if converted into active CLV3 ligand, would have to not activate CLV signaling in the immediately adjacent basal cell daughter.

A second model postulates that CLV signaling acts prior to division of the L3 stem cell. In this scenario, CLV signaling acts to polarize the L3 stem cell prior to division, with the differential fates, and corresponding differential *WUS* expression, being driven by cell asymmetry established earlier [56]. Here, CLV3 would act as a cell polarity signal to drive an apical domain with the L3 stem cells. In the absence of this signal, L3 stem cells fail to adopt an apical cell fate after division, leading to basal fate and *WUS* expression in both daughter cells. This model is inspired by the embryonic phenotypes of mutations in the CLV signaling intermediates POL and PLL1 (see above). During embryonic development, *pol pll1* double mutants lose asymmetric character to both the hypophyseal and procambial cells [56]. One advantage of this model is that CLV3 expression from the L3 cells would not disrupt polarity establishment as long as there is an apical-basal gradient of CLV3 across the L3 stem cell. One drawback is that there are currently no studies assessing whether L3 cells and their basal daughters have lost other aspects of cell asymmetry (potentially cell morphology, cell division rates, markers for OC identity) in *clv* mutants.

One critical feature that we must keep in mind when thinking about the mechanism of the CLV/WUS system in regulating stem cell homeostasis is that these signaling events take place over days. The cell cycle time of cells within the shoot meristem of Arabidopsis is on the order of 18-72 hours, depending on where the cell is located. CZ cells have a cell cycle length of 1.5 to 3 days [62], thus CLV signaling is a long-term maintenance program that may not act like other signaling events where the introduction of ligand leads to rapid changes in cell identity. L3 stem cells presumably are always detecting CLV3 ligand. Indeed, in studies on altering CLV3 activity within the meristem no changes are seen prior to many hours after induction [39, 63].

The STM pathway

A separate regulatory pathway that appears to function in parallel with the WUS-CLV3 pathway to regulate shoot meristem maintenance includes the critical meristem regulator SHOOTMERISTEMLESS (STM) [13, 64-67]. STM is a KNOX-class homeodomain transcription factor orthologous to the maize KNOTTED1 protein [68]. The repression of stem cell differentiation by STM occurs by preventing the expression of the organ-formation factors *ASYMMETRIC LEAVES1* (*AS1*) and *AS2* in the center of the shoot meristem [69, 70]. *AS1* is orthologous to *PHAN* Antirrhinum and *ROUGH SHEATH2* (*RS2*) from maize [69]. AS1 is a MYB domain transcription factor, while AS2 encodes a protein that contains an AS2/LATERAL ORGAN BOUNDARY (LOB) domain [69, 71]. In each species, these genes are involved in lateral organ initiation. While STM and related KNOX proteins (KNAT1/BP, KNAT2 and KNAT6) prevent ectopic *AS1*/*AS2* activation in the center of the meristem, AS1/AS2 in turn repress the *KNOX* genes in developing organ primordia [69, 70, 72-74]. The expression of *STM* typifies this regulation, with high expression throughout the center region of the meristem, but clearly down-regulated in nascent organ primordia [69]. The homologous and partially redundant *KNAT* genes are similarly down-regulated in organ primordial but exhibit complex and different expression patterns within the meristem [69, 70, 72-75]. Evidence suggests the AS1/AS2 repression of *KNOX* transcription occurs by the ability

of these interacting proteins to form a transcriptional repression complex on the *KNOX* gene cis elements through interaction with the histone chaperone HIRA [5, 76].

The STM pathway may also involve regulation of Gibberellin (GA) signaling. GAs comprise a large family of diterpenoid molecules that are involved in promoting organ expansion and morphogenesis in higher plants [77]. The repression of GA activity by KNOX transcription factors in the CZ is important to maintain meristem integrity while up-regulation of GA in the peripheral zone contributes the generation of lateral organ development [5, 17, 78]. Evidence for this comes from the *Nicotiana tabacum* homeobox15 (NTH15) KNOX homeodomain protein, which represses the GA biosynthesis by directly binding the first intron of GA 20-oxidase (Ntc12) to maintain the indeterminate state [5, 17, 79]. GA 20-oxidase is key enzyme in GA biosynthesis. In Arabidopsis, exogenous GA application suppresses phenotypes from *KNOX* mis-expression, such as highly lobed leaves [79]. Conversely, in Arabidopsis *KNOX* mis-expression suppresses GA 20-oxidase expression in the leaves [80]. In addition, the expression pattern of *AtGA20ox1:GUS* is complementary to *STM* expression [5, 17, 80].

miRNAs in the meristem

Shoot meristem maintenance is also affected by miRNAs. Evidence for a role in meristem development for a specific miRNA class, the factors involved in miRNA function, and the miRNA targets has emerged over the course of many studies.

A role for miRNAs in meristem maintenance came from the investigation of the miRNAs *miR165* and *miR166* [81-85]. Critically, the *jabba-1D* mutation, caused by constitutive over-expression of one of the genes encoding *miR166*, leads to defects in meristem maintenance, displaying enlarged shoot and floral meristem [85]. *miR166* along with the very similar *miR165* target the set of five transcription factors in Arabidopsis that make up the class III homeodomain-leucine zipper transcription factors (HD-zip IIIs) [81, 83, 84, 86]. *jabba-1D* mutants display enlarged shoot meristems, radialized leaves and defective vasculature. Furthermore, *WUS* expression in *jabba-1D*

is variable but expanded and *WUS* transcription levels are ~12 fold higher in *jabba-1D* compared to wild type [85].

The HD-zip III transcription factors that are targeted by *miR165/166* control critical aspects of meristem development. The HD-ZIP III family in Arabidopsis is composed of five members: REV, PHB, PHV, CNA, and ATHB8 [87, 88]. HD-zip III genes had been previously characterized through both loss-of-function and gain-of-function mutations with effects on meristem development as well as many other aspects of plant development [89-94]. The gain-of-function alleles are the result of silent substitutions leading to resistance to *miR165/166* repression [90, 91]. Understanding the function of these genes is complicated by their simultaneously redundant and antagonistic roles [93]. In establishing the embryonic shoot meristem, REV, PHB, PHV and CNA all appear to work in parallel. However, in shoot and flower meristem development, REV works antagonistically to PHB, PHV and CNA. Specifically, *rev* mutants lack or develop reduced meristems, while *phb phv cna* triple mutants exhibit enlarged meristems very similar to *clv* mutants [92, 93]. The effect of the *jabba-1D* mutation is similarly antagonistic: *REV* transcripts increase in the *jabba-1D* mutants, while *PHB*/*PHV*/*CNA* transcripts decrease [85]. Both of these changes would be consistent with an enlarged shoot meristem.

In addition to the direct evidence from *miR165/166* genetics, factors involved in miRNA processing and function also show evidence of roles in meristem regulation. Specifically, members of the ARGONAUTE (AGO) family show evidence of meristem function. The AGO family acts as central components of RNA-induced silencing complexes (RISC) [95, 96]. AGOs contain a variable N-terminal domain followed by conserved PAZ, MID, and PIWI domains. The PAZ and MID domain bind 3' and 5' ends of small RNAs, respectively, while the PIWI domain has a RNase H catalytic activity that allows the AGO/miRNA complex to cleave target mRNAs [95-97].

Among ten AGO members in Arabidopsis, ARGONAUTE1 (AGO1) and AGO10 (also known as ZWILLE or PINHEAD) have been shown to regulate shoot meristem development [98-103]. AGO1 is required for stem cell function. A portion of *ago1* mutants develop no embryonic shoot meristem seedlings [99]. However, post-embryonic shoot meristems of *ago1* mutants are larger than that of wild type. *ago1*

mutants fail to form axillary meristems and most of the mutant plants die within six weeks after germination [98, 99]. AGO1 has been shown to be required for *STM* expression, which has important role in meristem establishment and maintenance [101]. These variable and conflicting effects on meristem development in the *ago1* mutant may reflect the effect on *ago1* on the function of multiple miRNAs. AGO10 similarly has differential roles for in embryonic shoot meristem initiation compared to post-embryonic meristem maintenance. *ago10* mutants fail to form an embryonic shoot meristem with incomplete penetrance, while they fail to restrict to the size of the post-embryonic shoot meristem [102]. During the process of embryonic shoot meristem initiation, AGO10 acts non-cell autonomously and may potentiate WUS signals to the overlying stem cells [103]. The post-embryonic role for AGO10 is less well understood and the source of conflicting interpretations. One source of confusion is that *ago10* mutants appear to increase *miR165/166* activity. Normally, if AGO10 acts with *miR165/166* to target specific mRNAs for repression, then removing AGO10 by mutation would tend to impede *miR165/166* function. How does AGO10 carry out this non-intuitive role for repressing miRNA activity? A study by Chen and co-workers concluded that AGO10 directly or indirectly represses *miR165/166* transcription, consistent with a prior analysis [100, 104]. However, a separate study from Zhang and co-workers concluded that AGO10 holds the processed *miR165/166* in a non-functional complex away from AGO1 [105].

Cytokinin Signaling

Phytohormones have been known to regulate a variety of plant growth and developmental processes. Cytokinins are one of class of phytohormones regulating proliferation, differentiation of plant cells, tissue culture regeneration, leaf senescence, and more. Cytokinin biosynthesis, distribution, degradation and perception are closely associated with SAM formation and maintenance [5, 11, 78, 106].

The KNOX1 class of Arabidopsis transcription factors (STM, KNAT2 and BP) activates cytokinin signaling by the induction of cytokinin isopentenyl transferase

(AtIPT7), the enzyme catalyzing the first step of cytokinin biosynthesis [5, 107-109]. *LONELY GUY* (*LOG*), which was first identified in rice (*Oryza sativa*), encodes an enzyme with a phosphoribohydrolase activity that converts inactive biosynthetic cytokinin to its active form. Loss-of-function phenotypes in rice are characterized by a mild reduction in the size of the vegetative meristem, a dramatic reduction of inflorescence meristem activity and premature termination of flower meristems [110]. In rice, *LOG* is expressed at the tip of the first two or three layers of shoot meristem and is not expressed in the OC or differentiating regions, suggesting this *LOG* expression-region may have a role to define the maintenance of shoot meristem [5, 11, 110]. Subsequently, in Arabidopsis nine rice homolog *LOG* family members have been identified. Among them, seven LOG proteins (LOG1 to LOG5, LOG7, and LOG8) have variable phosphoribohydrolase activities [111]. Multiple LOG mutants (up to septuple) indicated that LOGs have overlapping and differentiated functions [111]. LOG4 and LOG7 are the only two LOG members expressed in the SAM proper [58, 112]. LOG4 is expressed in the L1 cell layer of SAM and floral meristem. The apically produced cytokinin along with CLV signaling is suggested to together form a positional cue for *WUS* expression in the stem cell niche [112]. LOG7 has been shown that it has important role in maintaining shoot meristem and root growth [113].

Cytokinin is perceived by the HISTIDINE KINASE (AHK) receptors and the signal is transmitted to the Arabidopsis Response Regulator (ARR) transcription factors ARR-A and ARR-B [59, 114]. While ARR-B isoforms activate cytokinin signaling, ARR-A isoforms down-regulate cytokinin signaling. ARR-A class genes appear to act to limit meristem size [59]' [115]. In addition, WUS directly represses the transcription of four ARR-As (*ARR5*, *ARR6*, *ARR7*, *ARR15*), thus enhancing cytokinin signaling [59]. When ARR7 and ARR15 transcript were silenced via microRNAs, *WUS* expression and shoot meristem size were mildly increased [115]. However, the observed reduction of *WUS* expression in the ARR-A class septuple mutant (*arr3 arr4 arr5 arr6 arr7 arr8 arr9*) might indicate a positive role of other ARR-As on WUS [59]. Furthermore, cytokinin suppresses the expression of *CLV1*, further complicating the role of cytokinins in meristem function [116, 117]. Thus it appears that cytokinin regulates *WUS* expression in a CLV-dependent and CLV-independent fashion [116, 117].

Cytokinin oxidase/dehydrogenases (CKX) irreversibly catalyzes cytokinin degradation [108]. *Arabidopsis* has seven CKX members and each gene has a different pattern of expression during plant development [118]. The *WUS* expression domain is expanded in the inflorescence meristem in *ckx3 ckx5* mutants [119].

In addition, *STIMPY* (*STIP*), which encodes a WUS-related homeodomain transcription factor also known as *WOX9*, acts with cytokinin signaling to promote continued proliferation of cells within the meristem [5, 120, 121]. The loss-of-function *stip* has reduced number of stem cells compared to those of wild-type after germination. While the wild-type seedling gradually forms a dome-shaped shoot meristem, *stip* has flat and differentiated shoot meristem and resembles *wus* mutant phenotype. This *stip* mutant phenotype can be fully rescued by exogenous sugar application. The sugar containing medium can induce the *CycD* expression, leading to increased cell division. This result shows that STIP acts by maintaining cell division and preventing differentiation in the shoot meristem [121]. In addition, STIP mediates cytokinin signaling during vegetative development of Arabidopsis. *STIP* expression in the meristem is induced by cytokinin. The cytokinin triple-receptor mutant (*ahk2-2 ahk3-3 cre1-12*) and ARR-B triple mutant (*arr1-3 arr10-5 arr12-1*) have significantly lower levels of *STIP* expression. When higher concentrations of exogenously cytokinin, which normally results in growth inhibition, is applied to wild type and *stip* seedlings, the *stip* are less sensitive. In addition, expression of *ARR5* (a type A ARR) is reduced in *stip* mutants and over-expressed *STIP* partially rescues shoot meristem defects in the cytokinin sensing mutants, indicating STIP works closely with cytokinin signaling [120].

Chromatin remodeling complexes impact *WUS* and *STM* expression

Despite the growing understanding of specific aspects of meristem function in general and WUS function in particular, a comprehensive understanding of shoot meristem control and stem cell function is lacking. In the case of *WUS* expression, the nature of transcriptional control is poorly understood. Because of the intimate and functional relationships between chromatin organization and transcription, one might expect a

critical contribution from chromatin regulatory factors in establishing, maintaining and repressing *WUS* transcription. Indeed, emerging evidence implicate chromatin remodeling factors such as chromatin assembly, ATP-dependent chromatin remodeling, and histone modifications have important roles in regulating the transcription of *WUS* and the equally important *STM* [122].

The *FASCIATA1* (*FAS1*) and *FASCIATA2* (*FAS2*) genes, which encode subunits of chromatin assembly factor-1, are required to maintain the organization of shoot and root meristems. *fas1* and *fas2* mutants display fasciated stems because of the shoot meristems become enlarged and disorganized [123]. In *fas1* and *fas2* mutants, *WUS* expression is not maintained in the OC, but exhibits variable lateral and apical expansion, indicating these chromatin factors are required for stable, OC-limited expression of *WUS* [5, 17, 122, 123].

BRUSHY1 (*BRU1*) also known as *MGOUN3* (*MGO3*) and *TONSOK* (*TSK*) has a function ensuring chromatin reconstitution during DNA replication or repair. *bru1* mutants display distorted *WUS* expression and altered shoot meristem development similar to *fas1* and *fas2* mutants [124]. However, *bru1 fas* double mutants show overlapping and non-overlapping function on the stability of epigenetic states and the corresponding proteins do not interact in vitro [124]. *BRU1* encodes a novel nuclear protein with two types of protein-protein interaction domains [5, 17, 122, 124-126].

SPLAYED (*SYD*), which encodes a SNF2 chromatin-remodeling ATPase, is recruited to the *WUS* cis elements and controls transcriptional levels of *WUS*. In *syd* mutants, *WUS* expression and meristem size are reduced [5, 17, 122, 127]. BRCA1-associated RING domain1 (BARD1) protein represses *WUS* expression by inhibiting SYD. *BARD1* encodes a protein containing two tandem BRCA1 C-terminal (BRCT) domains and a RING domain. Loss-of-function mutations in *BARD1* result in ectopic expression of *WUS* throughout the meristem, while over-expression of *BARD1* leads to a *wus*-like phenotype. The BARD1 protein directly binds upstream of the *WUS* promoter [5, 17, 122, 128].

The polycomb group (PcG) is important chromatin regulatory complex that silences gene expression by binding specific regions of DNA and inducing post-translational modifications of histones [129, 130]. PcG was originally identified in

Drosophila and subsequently found in other plants and animals. PcG proteins assemble into at least two complexes called Polycomb Repressive Complex1 (PRC1) and PRC2. PRC2 catalyzes H3K27 methylation and trimethylated H3K27 induces binding of Polycomb (PC), which is a central component of PRC1. PRC1 leads to stable repression by catalyzing monoubiquitination of histone H2A via its RING-domain subunits [122, 131, 132]. The four core components of PRC2 in animals are E(z), ESC, Su(z)12, and p55**.** 12 homologs of these four components are conserved in Arabidopsis. Among different possible PRC2 complexes, CURLY LEAF/SWINGER (CLF/SWN), EMBRYONIC FLOWER2 (EMF2)/VERNALIZATION2 (VRN2), FERTILIZATION INDEPENDENT ENDOSPERM (FIE) and MSI1 play important roles in different stages of the Arabidopsis life cycle [122, 133-135].

CLF and the closely related *SWN* encode histone methyltransferases [6, 113]. CLF binds to the *STM* cis elements and together with SWN redundantly represses *STM* expression [5, 122, 136]. *clf swn* double mutants lead to elevated *STM* expression and a reduction in H3K27me3 at the *STM* locus [136]. De-repression of *STM* is also observed in mutants of *LIKE-HETEROCHROMATIN PROTEIN1* (*LHP1*) and *Atring1a/Atring1b* mutants as well [137-140]. *LHP1* encodes a protein similar to heterochromatin regulators metazoans and *S. pombe*. LHP1 may play the role in plants that Polycomb plays in animals, which recognizes H3K27me3 and recruits the PRC1-like complex. AtRING1a and AtRING1b are similar to the animal PRC1 core component RING1 [140].

INCURVATA2 (*ICU2*), which encodes the catalytic subunits of DNA polymerase α, also restricts the *WUS* expression level. In *icu2* mutants, *WUS* expression is up-regulated [5, 122, 141]. Interestingly, ICU2 interacts with both CLF and LHP1, suggesting a link between multiple DNA replication and chromatin remodeling complexes in the controlling the expression critical factors in meristem maintenance [141]. Another putative DNA-related factor, MGOUN1, which encodes a putative type IB DNA topoisomerase, exhibit synergistic mutant interactions with chromatin remodeling factors SYD, CLF and LHP1 [142].

Conclusions

The large and rapidly growing list of genes with roles in meristem maintenance hints at the tremendous complexity of the process of maintaining active stem cells. In hindsight, this should be readily apparent from the critical importance of shoot meristem for plant growth and architecture and the requirement that these structures adapt to rapidly changing physiological and environmental conditions. Examining individual pathways in isolation is a necessary experimental technique that has taught us much about some of the key processes in meristem maintenance, but we should remain cognizant that each pathway acts as part of a complex web of interacting affects. One only need consider the dizzying array of factors shown to affect *WUS* transcription to appreciate the underlying complexity.

Considering the directions of future studies, it becomes apparent that despite the rapid progress made in dissecting the pathways controlling shoot meristem maintenance, fundamental aspects of meristem function are completely unknown. What makes a cell in the center of the meristem a stem cell? Specifically, what set of transcription factors, chromatin status and/or protein activities define a cell as a stem cell? Is it as simple as WUS protein moving into these cells or is WUS just the first step in a long pathway? Either way, what are the key WUS targets that make the stem cells different from every other differentiated cell in the plant? What is the primary effect of CLV signaling? Does it control WUS transcription directly or indirectly? What are the factors downstream of the plasma-membrane localized components? What is the overall role of chromatin remodeling in maintaining stem cells and governing the switch to differentiation?

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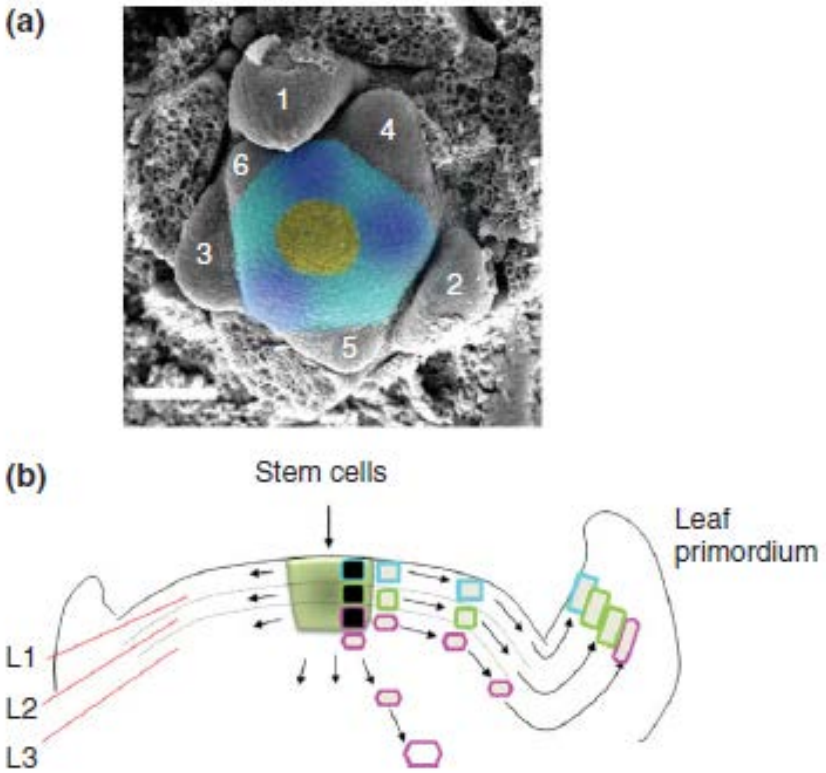

Figure 1.1. The shoot meristem. (a) A scanning electron micrograph of an Arabidopsis shoot apical meristem. Recently formed organ primordial are numbered from oldest to youngest. The CZ region, PZ region, and sites of nascent organ primordial are false-colored yellow, blue, and dark blue, respectively. (b) Diagrammatic cross section of a shoot meristem. The L1, L2, and L3 layers of cells in ther meristem center are indicated. The region of stem cells is indicated, and individual stem cells are shown (black cells) along with their predicted cell fates. Arrows indicate the fate of cells over developmental time, not the movement of cells.

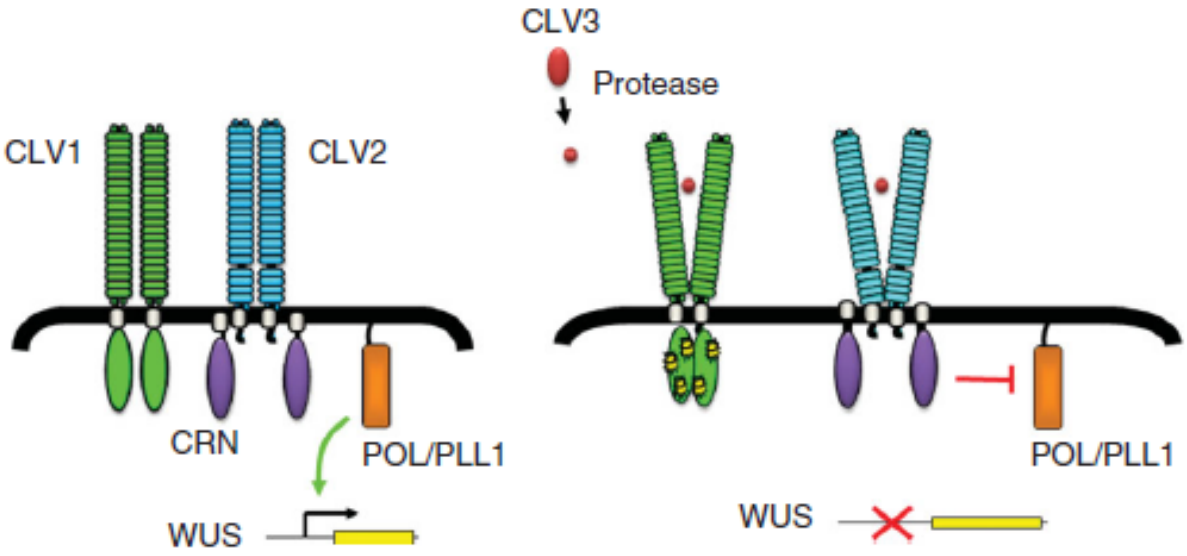

Figure 1.2. A model for CLAVATA (CLV) signaling. In the absence of CLV3 ligand, CLV1and CLV2/CRN complexes are inactive, allowing POL/PLL1 to (likely indirectly) promote *WUS* transcription. Mature and processed CLV3 activates the receptor complexes, repressing POL/PLL1. In the absence of POL/PLL1 activity, *WUS* transcription is not maintained. The CLV1-redundant BAM receptors are not included for clarity.

CHAPTER TWO

Identification of a novel *AGO10* allele through a *pol-6* enhancer mutagenesis

ABSTRACT

A tight balance between stem cell specification and differentiation is important to maintain a functional meristem and continuously initiate new organs throughout the lifespan of plants. Extensive classical mutagenic screens carried out by several labs have identified only a handful of genes that specifically regulate shoot meristem development, including *WUS*, *STM*, and *CLV*. To overcome this obstacle, enhancer/suppressor mutageneses have been performed and successfully identified additional regulatory components, including *POL*, *PLL1*, and *CRN.* The *Arabidopsis* WUSCHEL (WUS) transcription factor is essential for specification of stem cell identity at shoot and flower meristems. CLAVATA (CLV) signaling acts through POL/PLL1 to limit *WUS* transcription and thus promote stem cell differentiation. To further dissect the CLV signaling pathway, I performed a modifier mutagenesis on *pol*-6 seeds. Among the *pol-6* enhancers identified in the screen was a novel *ago10* allele.

INTRODUCTION

Plant development is characterized by limited organogenesis during embryogenesis. In addition to two leaf-like cotyledons formed by Arabidopsis embryos, the critical outputs of embryonic developmental patterning are the establishment of two stem cell populations at the apical and basal ends of the embryo, forming the shoot meristem and root meristems, respectively. These meristems are homeostatically maintained throughout the plant lifespan. The shoot meristem is the source of all above-ground organs and tissues such as leaves, axillary shoots, flowers, internodes and the vasculature. The root meristem is responsible for generating underground organs such as roots and a root cap [1-4].

The stem cells of the Arabidopsis shoot meristem are characterized by three distinct cell layers - the L1, L2, and L3 layers [5-8]. The L1 epidermal layer is the outer-most layer, followed internally by the subepidermal L2 layer and the corpus L3 layer (Figure 2.1). The L1 and L2 stem cells divide exclusively in an anticlinal orientation, whereas the L3 cells divide both anticlinally and periclinally. The central daughter of a dividing L1 or L2 stem cell remains a stem cell while the peripheral daughter makes a transition toward differentiation. The apical daughter of a dividing L3 stem cell remains a stem cell, while the basal daughter differentiates [1-3, 9, 10].

The stem cell population must be maintained while appropriately positioned peripheral and basal daughter cells are directed toward organ primordia and other differentiated tissues. The maintenance of these stem cells is influenced by their positional information, or niche. At the shoot meristem, the niche is driven by an Organizing Center (OC), which is composed of the immediate basal daughters of the L3 stem cells [11-13]. Interesting, the OC is a constantly changing cell population (Figure.2.1).

The balance between stem cell specification and differentiation is also involved in flower development. Floral primordia are initiated on the flanks of the shoot meristem and a floral meristem is subsequently established. The floral meristem is a modified shoot meristem [14-16]. Within the floral meristem, there is a tight equilibrium of floral stem cell specification and the promotion of cells from an undifferentiated state towards

a differentiated state. However, in contrast to the shoot meristem, floral meristems only maintain stem cells for a limited time and eventually all stem cell differentiate to form floral organs. In Arabidopsis, floral organs are initiated in a whorl pattern from the exterior to the center of the floral meristem. In wild-type Arabidopsis, the first whorl gives rise to four sepals, the second whorl gives rise to four petals, the third whorl gives rise to six stamens, and the fourth whorl gives rise to two fused carpels [14-16].

Since shoot systems were first observed in 1759, the shoot meristem has become one of the most studied aspects of plant developmental [14, 15, 17, 18]. The diversity, morphology, histology, cell division patterns and cell lineages of the shoot meristem have been extensively investigated [15, 17-20]. Insights into the molecular regulation of shoot meristem function have come primarily from studies in Arabidopsis. In the 1990s, genetic screens in *Arabidopsis thaliana* identified several genes that are key regulators of shoot meristem development: *SHOOT MERISTEMLESS* (*STM*), *WUSCHEL* (*WUS*), *CLAVATA1* (*CLV*), *CLV2* and *CLV3*. Many genetic and biochemical studies of these genes have expanded our understanding of a signal-transduction pathway that regulates stem cell specification and differentiation [12, 21-24].

The primary factor known to specify stem cells is *WUS* in Arabidopsis. Previous studies have indicated that *WUS* is both necessary and sufficient for stem cell specification within the shoot meristem [11-13, 25, 26]. *wus* mutants lack all stem cells at shoot and flower meristems, while *WUS* over-expression within the meristem gives rise to ectopic stem cell accumulation. *WUS*-expressing cells act as a stem cell niche/Organizing Center (OC) to specify overlying cells as stem cells. *WUS* encodes a WOX-class homeodomain transcription factor. *WUS* expression is initiated at the 16-cell stage of embryogenesis and becomes progressively restricted to a small group of cells in the developing shoot meristem. Eventually, *WUS* expression is limited to the basal daughter of the L3 stem cells, which is defined as the OC (Figure 2.1). *wus* mutants fail to maintain stem cells, leading the seedlings with no post-embryonic development. Adventitious shoots of unknown origin are later formed in *wus* mutants, but these shoots lack stem cells and form just a few organs. In *wus* flowers the organs formed are limited to the initial sepals and petals, with an occasional single central stamen.

clavata (*clv*) mutants have opposite phenotypes to *wus* mutant in that *clv* mutants have an enlarged meristems, extra floral organs, and extra whorls of floral organs. Plants homozygous for strong *clv1* and *clv3* alleles can accumulate over 1000-fold more stem cells than wild-type at the apical shoot meristem (Figure 2.2). The floral meristem of *clv* mutants is also enlarged, and produces more floral organs than wild type. In *clv* mutants, carpels are the most dramatically affected whorl where the mean number in *clv* mutants is often double or triple that of wild-type [27-29].

Genetic analyses indicate that the three *CLV* genes (*CLV1*, *CLV2*, and *CLV3*) act in the same genetic pathway to maintain shoot meristem activity by restricting stem cell accumulation, but *CLV2* has additional functions to regulate organ development [28, 29]. *clv1 clv3* double mutants have the same phenotype to the strong *clv1* and *clv3* single mutants, indicating that *CLV1* and *CLV3* function in the same pathway. *clv2* mutants also have similar shoot and floral meristem phenotypes to intermediate *clv1* and *clv3* mutants, while the *clv1 clv2* and *clv2 clv3* double mutants are phenotypically similar in meristem defects to the *clv3* null allele.

The *CLV* loci encode signal transduction components. *CLV1* encodes a leucine-rich repeat (LRR) receptor kinase, which carries an extracellular domain containing 21 LRRs, a transmembrane domain, and a functional cytoplasmic ser/thr kinase domain [22]. *CLV2* encodes a LRR receptor protein with a short cytoplasmic tail [23]. *CLV3* encodes a small extracellular secreted polypeptide with a conserved C-terminal domain called CLE domain [24, 30]. Mature CLV3 binds directly to the LRR domains of CLV1 and CLV2 [31-35].

WUS is a target of the CLV signaling pathway [13, 36]. CLV signaling limits OC activity by repressing *WUS*. *WUS* and *CLV1* are expressed in an overlapping pattern within the meristem. In wild type, *WUS* is expressed OC, while *CLV1* is expressed in the OC plus adjacent apical and lateral cells. *CLV3* is expressed in the central stem cells in the L1, L2 and L3 layers. The over-expression of *CLV3* has similar phenotype to *wus* if *CLV1* and *CLV2* are functional [36]. *WUS* expression expands both apically and laterally in *clv* mutants (Figure 2.3). Furthermore, *wus* mutants are epistatic to *clv* in that *clv wus* double mutant are phenotypically equivalent to *wus* single mutants [13]. These results indicate that WUS acts downstream of CLV signaling.

In the 1990s, several laboratories screened mutagenized Arabidopsis plants to identify genes that specifically regulate shoot and floral meristem development. However, these extensive efforts only yielded multiple alleles of *WUS, STM,* and *CLV*. One explanation for the failure to identify a broader array of regulators is that these factors may play essential roles for embryogenesis and are lethal or pleiotropically defective when mutated. Many chromatin-regulating factors that were subsequently characterized fall into this category [37]. Another explanation is that the gene activity may be redundantly encoded, such that a single mutant does exhibit meristem phenotypes. These obstacles, especially the latter, can be overcome by an enhancer/suppressor screen in a genetically sensitized background [20].

These types of modifier screens have been successful in identifying additional components of meristem development. For example, an enhancer/suppressor mutagenesis of *clv1* and *clv3* lead to the identification of *POLTERGEIST (POL)* [20, 38]. Because *POL* activity is redundantly encoded by the related *PLL1* gene, *pol* single mutants are nearly identical to wild type [39]. Nonetheless, *pol* mutants partially suppress the stem cell accumulation of *clv* mutants [40].

The *pol pll1* double mutant is seedling lethal primarily because a pair of key asymmetric cell divisions in the basal embryo is lost [39, 41]. This defect can be bypassed by grafting the apical portion of a *pol pll1* seedling onto a hypocotyl plus root of a wild-type seedling. The grafted *pol pll1* double mutants phenocopy *wus* mutants with terminated shoot and flower meristems. *pol pll1* mutants are epistatic to *clv3,* lack maintenance of *WUS* transcription, and are rescued by ectopic *WUS* expression. Taken together, POL/PLL1 act as signaling intermediates in the CLV pathway. CLV signaling represses POL/PLL1, while POL/PLL1 promote *WUS* transcription [41, 42].

Another component of CLV signaling, *CORYNE (CRN)*, was also identified by a sensitized genetic screen looking for Arabidopsis mutants resistant to CLV3 peptide treatment. *CRN* encodes a catalytically inactive transmembrane kinase-related protein that partners with CLV2 to form a receptor complex that responds to CLV3 [33, 43-46].

Our current model of CLV function is that CLV3 protein is secreted from stem cells [47]. Extracellularly, CLV3 is proteolytically processed to an active peptide ligand capable of diffusing to adjacent cells [30, 48-50]. Activate CLE peptide ligand binds to

CLV1 homodimers and CLV2/CORYNE heterodimers at the plasma membrane of the L3 stem cells [31-35]. This CLV signal transduction pathway represses POL/PLL1 protein activity [42]. POL/PLL1 repression then prevents *WUS* transcription in the apical daughters of the L3 stem cell via an unknown mechanism.

To expand our understanding of CLV pathway, I sought to identify additional CLV signaling components using *pol* single mutants as a sensitized genetic background for an enhancer/suppressor screen.

RESULTS

EMS mutagenesis

To identify novel components of stem cell homeostatic control, I performed ethyl methanesulfonate (EMS) mutagenesis on *pol-6* seeds. To optimize EMS mutagenesis, *pol-6* single mutant seeds were treated overnight in EMS concentrations ranging from 0.1% to 0.5% in 0.05% intervals. The resulting M1 plants were sown with the goal of collecting from the highest EMS concentration allowing for fertile progeny for modifier screening in the subsequent M2 generation. The 0.45% EMS-treated plants were almost completely sterile, presumably due to high levels of induced embryo-lethal mutations. I therefore collected M2 seeds from the next highest EMS treatment levels (0.35% and 0.40%), which developed viable progeny. Seeds from pools of 10 M1 plants were collected and 64 M2 seeds from each pool were sown and observed for meristem termination.

Seeds were collected in pools from 884 M1 individuals and the M2 population was screened for shoot and flower meristem termination. Nine putative enhancer candidates were identified (Table 2.1). Putative modifier mutants were crossed to wild-type L*er* to test if the meristem termination phenotype was reproducible and if it was dependent on the presence of the *pol* mutation. Based on preliminary analysis of the F2 generation from these crosses, I prioritized individual enhancer isolates for mapping.

Isolate #361

Isolate #361 displayed seedling shoot meristem termination that was largely dependent on the presence of the *pol* mutation. In the wild-type L*er* outcross for isolate #361, 22 of 369 plants displayed meristem defects, and 19 of these were homozygous for *pol-6*.

The F2 population of the L*er* cross was used for map-based cloning of the modifier mutant. Among a population of 3888 F2 plants, 144 enhanced mutants were identified and DNA was collected for analysis. Using SSRP, CAPS, and dCAPS markers, the modifier mutation was localized to a 440 kb region on chromosome 5 (Figure 2.5, 2.6A). In this region, 20 candidate genes were prioritized and sequenced based on the predicted gene function (Figure 2.5)

I identified an A-to-G transition mutation in the *NUTCRACKER* gene (At5g44160) compared to the wild-type sequence in The Arabidopsis Information Resource (TAIR: www.arabidopsis.org). This lesion is predicted to result in a missense substitution in the coding sequence in the 3rd exon (Figure 2.5). However, the meristem termination phenotype was not complemented by transformation of wild-type genomic At5g44160 DNA into the #361 mutant lines. The meristem termination phenotype was not created by expressing a *NUTCRACKER* RNAi transgene in *pol-6* and was not suppressed by introducing the *NUTCRACKER* RNAi into isolate #361. Finally, full complementation was observed in F1 crosses of the At5g44160 T-DNA insertion lines (129969C and 124222C) with #361 in the *pol* mutant background. Taken together, I concluded that the lesion in the *NUTCRACKER* gene was not responsible for the meristem termination phenotype I observed in the original isolate.

I identified a second DNA lesion among the candidate genes. In the *ARGONAUTE10* (*AGO10*) gene, also known as *ZWILLE*/*PINHEAD*, a G to A transition mutation in the 14th exon was identified, resulting in a nonsense mutation (Figure 2.6B). I numbered this new allele *ago10-15* following the nomenclature from a recent publication [51].

To confirm that the lesion within *AGO10* was responsible for the meristem termination in the original isolate, I transformed *AGO10* genomic DNA [51] into the #361 isolate and observed rescue of the meristem termination phenotype (Figure 2.6C). In

addition, among the F1 progeny of our *ago10-15 pol* isolate crossed to *ago10*$^{zll-3}$ and *ago10*$^{pnh-2}$ [52, 53], I observed meristem termination and an increase in the mean number of carpels per flower typical of *ago10* mutants (Figure 2.6C, 2.8).

ago10-15 Genetic and Phenotypic Analysis

Interestingly, the *ago10-15* allele I isolated differs from most published *ago10* alleles in that over 90% of the *ago10-15* plants exhibited no meristem termination, while published alleles typically have greater than 60% penetrance (Figure 2.7A) [52, 53]. This is despite the severe effect *ago10-15* should have on AGO10 protein and the fact that the enlargement observed in *ago10* flower meristems is as strong in *ago10-15* as in other alleles (Figure 2.6B). *ago10*$^{pnh-4}$ and *ago10*$^{pnh-11}$ also have weak penetrance of the seedling meristem termination, but the DNA lesions in these alleles have not been described [52]. However, *ago10-15* and *ago10*$^{pnh-4}$ may be different alleles since *ago10*$^{pnh-4}$ was described in the paper as having a very weak increase in the number of carpels per flower. The mean number of carpels per flower is 2.28 in *ago10*$^{pnh-4}$, 2.72 in *ago10*$^{pnh-2}$, and 2.95 in *ago10-15* (Figure 2.7C).

ago10-15 pol-6 seedlings displayed completely penetrant meristem termination, characterized by a differentiated flat apex, a filament, a solitary leaf, or at most the production of up to four leaves followed by termination (Figure 2.7A,B). Given that neither *pol-6* nor *ago10-15* exhibit significant meristem termination, this represents a synergistic interaction.

Most of the genetic, phenotypic, and molecular analyses of AGO10 have been performed in the embryonic stage [52, 53]. During embryonic development, AGO10 may potentiate WUS activity, resulting in shoot apical meristem termination in *ago10* mutants [54]. However, in the post-embroynic stage, *ago10* mutants were briefly described as exhibiting enlarged meristems and having increased number of carpels per flower. This suggests AGO10 has different roles in embryonic versus post-embryonic meristem development [53].

While *ago10-15* enhances *pol* embryonically, a different genetic interaction was observed post-embryonically. Presumably because of the antagonistic roles of *AGO10*

in the embryonic shoot meristem compared to the postembryonic shoot/flower meristems, *pol-6* suppressed the flower meristem enlargement of *ago10-15* (Figure 2.7C). This is similar to the ability of *pol* to suppress the flower meristem defects in *clv* mutants with a severity similar to *ago10-15* [40].

To further examine the *CLV/AGO10* genetic interactions, I generated double mutants between the previously characterized *ago10*$^{zll-3}$ and *ago10*$^{pnh-2}$ and *clv3-2* [28, 52, 53]. In these double mutants there was no enhancement of the shoot or flower meristem enlargement compared to *clv3-2* alone, with flowers either similar to those of *clv3-2* or occasionally replaced by filamentous structures (Figure 2.7C). Nor was there any observed increase in the size of the shoot meristem in the double mutants compared to *clv3-2* single mutants.

Other *pol* enhancer isolates

The second *pol* enhancer candidate, #29, was outcrossed to L*er*. In the F2 population from this cross, the expressivity of *pol*-enhanced plants varied considerably from very weak to severe. Very few plants among thousands of F2 individuals recapitulated the significant flower meristem-termination phenotype observed in the original isolate. In addition, preliminary molecular analysis of these plants indicated that some of the plants with terminated meristems were heterozygous for the *pol* mutation, raising questions about both the genetic basis of this enhancer and its dependence on *pol*. Because of the poor penentrance of the phenotype and the possible lack of dependence on *pol*, I decided not to further pursue this modifier.

Lindsey Gish, a fellow graduate student at the time, analyzed the F2 population I generated from isolates #171 and #33 (Table2.1) and mapped them. Using map-based cloning, Dr. Gish identified a G to A mutation in *AGO10* in the #171 isolate. This mutation alters the intron donor site after the 13th exon and should lead to a truncated AGO10 protein. Interestingly, this *ago10-16* allele exhibits a normal *ago10* seedling meristem termination penetrance similar to most *ago-10* alleles that are enhanced but

not dependent on *pol* [52, 53]. This lesion is identical to the previously described *zll-8* allele [53].

Using map-based cloning for isolate #33, Dr. Gish identified a nonsense mutation in codon 131 in the gene *TONSOKU* (At3g18730) [55, 56]. A re-analysis of the F2 population for this mutation revealed it was not dependent on *pol*. Mutations in TONSOKU have been identified in many different screens. The gene appears to play important roles in cell cycle progression.

Discussion

The most interesting *pol* modifier that I identified was the novel *ago10-15* allele. This allele exhibits shoot meristem termination in *pol*-dependent manner. This suggests a synergistic interaction with between *ago10-15* and *pol*, neither of which has extensive shoot meristem termination on their own. Finding this interaction prompts a consideration of the relationship between AGO10 and the CLV/WUS stem cell pathway.

Previous studies have shown roles for ARGONAUTE-related proteins in meristem development. The *ARGONAUTE* genes encode a central component of the RNA-induced silencing complex (RISC). AGO family proteins are composed of a variable N-terminal domain followed by conserved PAZ, MID, and PIWI domains [57-59]. The PAZ and MID domains bind to small RNAs, while the PIWI domain has RNase H catalytic activity. In Arabidopsis, the roles of AGO proteins in meristem development are both complicated and in some cases controversial. The most important AGO proteins for meristem regulation in Arabidopsis are AGO1 and AGO10. Despite their related sequences, these proteins play very different roles at both the biochemical and the developmental level. *AGO1* is broadly expressed, *ago1* mutants exhibits dramatic pleiotropic phenotypes, and the AGO1 protein appears to facilitate the ability of a wide variety of miRNA products to repress transcript accumulation for their targets [60-62]. *AGO10*, on the other hand, is very specifically expressed, exhibits almost exclusively meristem-related mutant phenotypes, and the AGO10 protein only acts on a very small group of miRNAs [63, 64]. Paradoxically, AGO10 blocks miRNA function, thus

protecting the mRNA targets for these specific miRNAs. Controversy surrounds this last aspect of AGO10 function, with the most favored current model proposing that AGO10 titers these specific miRNAs away from AGO1, preventing their function [65].

ago10 mutants fail to form an embryonic shoot meristem and display an empty apex, a filamentous structure, or a solitary leaf with incomplete penetrance [52, 53]. AGO10 may potentiate WUS activity non-cell autonomously in the stem cells of the embryo [54]**.** While the postembryonic phenotypes and function of AGO10 are much less characterized, *ago10* mutants appear to develop enlarged meristems, especially in the flower [53]. Thus, AGO10 has opposite roles in embryonic versus post-embryonic stem cell specification.

The miRNAs miR165 and miR166 are antagonistically acted on by AGO1 and AGO10. AGO10 specifically binds to miR165/166 [63-65]. Two miR165- and seven miR166-encoding genes have been identified in Arabidopsis genome. Mature miR165 and 166 have one nucleotide difference from each other [64]. Both miR165 and miR166 have near complete complementarity to a section of the mRNAs of the homeodomain-leucine zipper class III genes. Arabidopsis contains five HD-zip III members: PHABULOSA (PHB), PHAVOLUTA (PHV), CORONA (CNA), REVOLUTA (REV), and ATHB15. Arabidopsis HD-zip III genes encode transcription factors containing a homeodomain, a leucine zipper motif, and a sterol/lipid-binding domain (START) [66]. These HD-zip III proteins have independent, overlapping and antagonistic functions in many aspect of Arabidopsis development [67]. The convoluted genetic relationships between the HD-zip III genes has often complicated analysis of the function of these genes. HD-zip III genes have been linked to meristem development in many different studies. In the developing embryo, *REV*, *PHB*, *PHV*, and *CNA* all work in parallel to promote shoot meristem formation. Post-embryonically, *REV* is necessary for lateral shoot and flower meristem initiation [68]. Interestingly, *PHB*, *PHV* and *CNA* antagonize this post-embryonic role of *REV*, based on the observation that each mutant partially suppresses lateral meristem loss in *rev* mutants [67]. Consistent with this, the *phb phv cna* triple mutant has been superficially described and appears to develop enlarged post-embryonic meristems [67]. Furthermore, an unusual dominant-negative

cna allele, *cna-1*, was identified in a *clv3* enhancer screen. While, the null *cna* allele *cna-2* does not enhance *clv3* phenotypes, *cna-1* leads to an enhancement of the shoot meristem defects found in *clv3* mutants [69].

The relationship between the AGO10/miR165/166/HD-zip III pathway and the CLV/WUS pathway has remained unclear. The expression patterns for the two miR165 and seven miR166 genes are spatially and temporally different from each other [64]. Thus, the previous studies using *jba-1D* activation-tagged allele of miR166g and *men1* activation-tagged allele of miR166a lead to different meristem phenotypes, either an enlarged shoot meristem or a terminated shoot meristem, respectively. *jba-1D*, a miR166g over-expressing line, leads to an enlarged shoot meristem, filamentous siliques, fasciated stems, and abaxialized curled leaves [70]. The *WUS* expression level was also significantly expanded and increased up to ~12 fold higher in the *jba-1D* mutant compared to wild type. When *jba-1D* was crossed to *wus, wu*s was epistatic to *jba-1D*. RT-PCR using *jba-1D* seedlings showed that *PHB*, *PHV*, *CNA* transcripts were significantly reduced, *REV* transcripts were elevated, and *ATHB8* transcripts were not changed. In a separate study, when miR166a was overexpressed in the *men1* line, meristem termination phenotype in the seedlings, retarded growth, and transcript reductions for *PHB*, *PHV*, *CNA*, *REV* were all observed [64].

Thus, the relationship between AGO10-HD-ZIPIII and CLV3- WUS suffers from many complications. (1) The HD-zip III genes have both overlapping and antagonistic roles in meristem development. (2) The nine miR165/166-encoding genes exhibit spatially and temporally different expression patterns. (3) *wus men1* and *wus jba-1D* double mutants have different phenotypes, even though both mutants lead to miR166 over-expression. (4) *ago10-15* enhances the *pol* embryonic meristem phenotype, while *pol* suppresses the *ago10-15* post-embryonic phenotypes. On the other hand, *ago10* does little to modify the *clv3-2* phenotypes. In the end, it is not possible to make a consistent model for the observed genetic interactions with the data in hand. The genetic complications of the HD-zip III family clearly need to be unraveled before one can understand the relationships between HD-zip IIIs, AGO10, miR165/166 and the CLV/WUS pathway in meristem development.

MATERIALS AND METHODS

Plant growth and genetic analysis

Arabidopsis seeds were sown on a 2:1:1 mixture of top soil:perlite:vermiculite supplemented with fertilizer and imbibed for 7 days at 4˚C. Plants were grown under continuous cool-white fluorescent lights at 22˚C. Plants in petri dishes were grown on half-strength Murashige and Skoog salts (Sigma) with 0.8% (w/v) phytoagar. Seeds were imbibed for 4 days at 4°C and grown under continuous cool-white fluorescent lights at 22˚C.

zll-3 and *pnh-2* seeds were obtained from the Arabidopsis Biological Resource Center (ABRC, Ohio State University, Columbus, OH, USA). The *ago10-15* allele was generated from 0.35%-0.40% ethyl methanesulfonate (EMS) mutagenesis on *pol-6* single mutant seeds. EMS mutagenesis screening was performed as described [20]. Approximately 578 M1 plants from 0.35% EMS treated and 306 M1 plants from 0.40% EMS treated were collected as pools of 10 individual plants and 64 M2 seeds from each pool were sown and observed for meristem termination. Putative *pol-6* enhancers showing defective meristem phenotypes were crossed to L*er* to test if the putative enhancer was dominant/recessive, to test if it was dependent on *pol* mutant background.

Map-based Cloning

The F2 population of isolate #361 x L*er* was used for mapping and cloning the modifier mutant. Among a population of 3888 F2 plants, 144 mutants were identified and DNA was extracted. The primers for the SSLP and CAPS markers used for the mapping are shown in Figure 2.4 , 2.6A and listed in Table 2.2 and 2.3. While a few of the mapping markers were from prior studies, the majority were designed by using the Monsanto Arabidopsis Polymorphism Sequence Collection Database. All restriction enzymes used were obtained from New England Biolabs or Promega and PCR amplification was done using GoTaq (Promega, USA).

Complementation of *ago10-15 pol-6*

For the complementation of the *ago10-15 pol-6* double mutants, the pPZP211 vector containing *AGO10* genomic DNA was kindly provided by Xuemei Chen (University of California, Riverside). The provided vector was sequenced and confirmed to include ~2 kb upstream sequence, 4.6 kb *AGO10* genomic coding sequence, and 380 bp terminator sequences. The construct was transformed into the *Agrobacterium* strain GV3101, which was then used to transform *Arabidopsis* Col and *ago10-15 pol-6* as described [71]. Transgenic plants were isolated by selection on plates containing 50 µg/ml spectinomysin.

Allelism test

Allelism test were performed by crossing *ago10-15 pol-6* with $ago10^{pnh-2}$ and $ago10^{zll-3}$. Phenotypes were observed in the F1 generation. $ago10^{zll-3}$ was crossed to *pol-6* and $ago10^{zll-3}/ago10^{zll-3}$ *pol-6*/+ was isolated and observed to test if the *pol-6* heterozygous state was altering the allelism test.

Image Analysis

Images were collected with a Zeiss stemi sv11 microscope and captured with a Canon digital camera PowerShot S51S. Images were collated in Photoshop with occasional adjustments to brightness and contrast.

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FIGURE LEGENDS

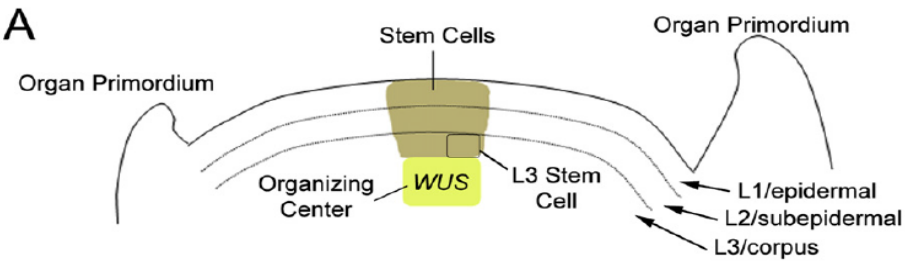

Figure 2.1. Longitudinal section of shoot meristem, showing 3 layers of stem cells, and the organizing center [41].

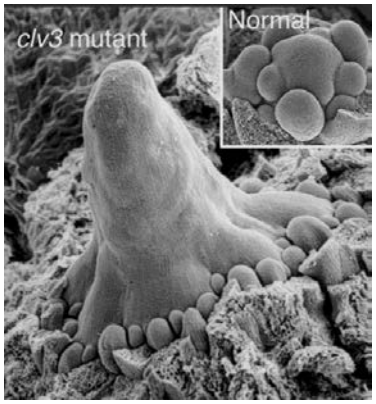

Figure 2.2. *clv3-2* and wild-type shoot meristem shown at the same magnification reveals a massive accumulation of undifferentiated cells in the mutant.

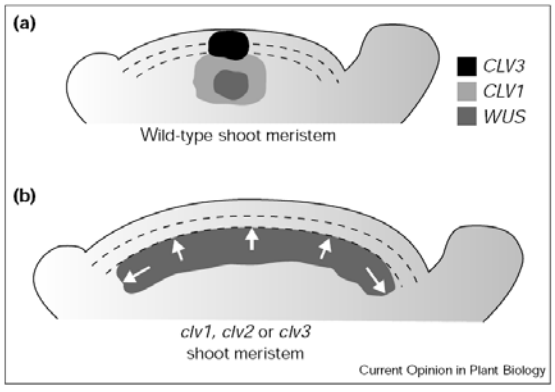

Figure 2.3. *CLV* loci regulate *WUS* expression (a) approximate mRNA expression domains for *CLV1, CLV3* and *WUS* within the shoot meristem. (b) The effect of *clv* mutations on the expression of *WUS* [3].

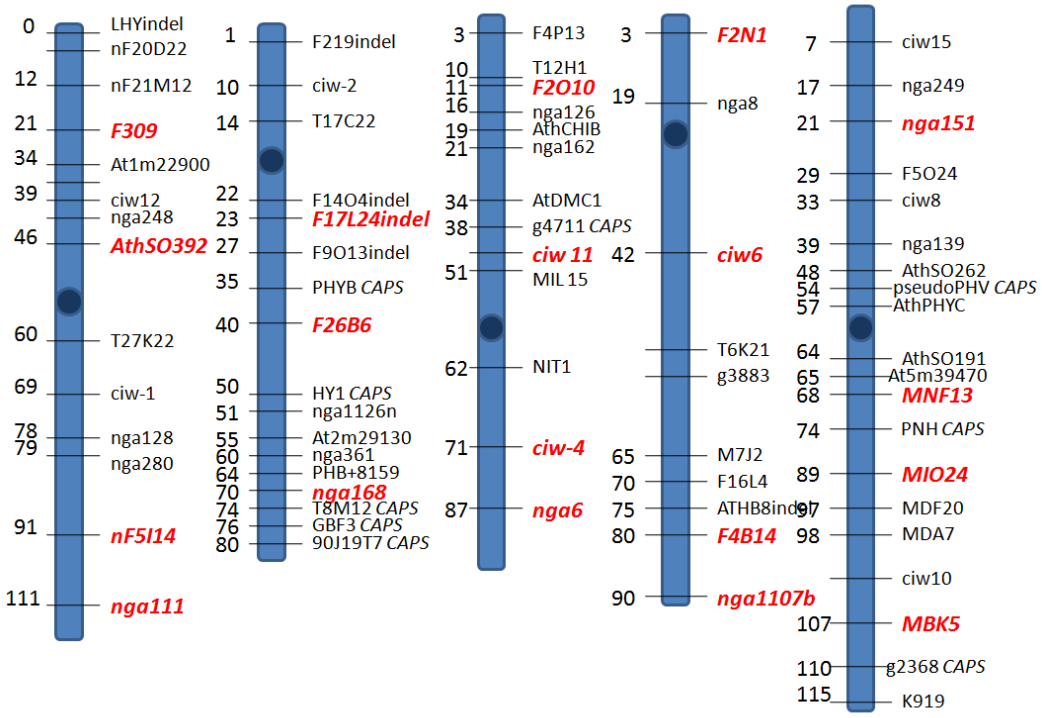

Figure 2.4. *Arabidopsis* genomic map with markers (markers written in italic and bold with red color were used for rough mapping). These markers are evenly distributed on the *Arabidopsis* chromosome (see Table 2.2).

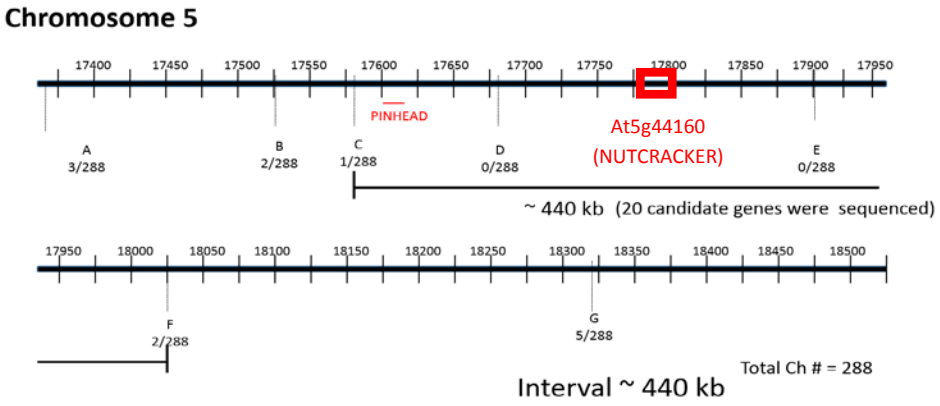

Figure 2.5. #361 was mapped in ~440 kb region on chromosome 5 and 20 candidate genes in the region were sequenced. At5g44160 (*NUTCRACKER*) was initially identified as missense mutation occurred in the 3rd exon.

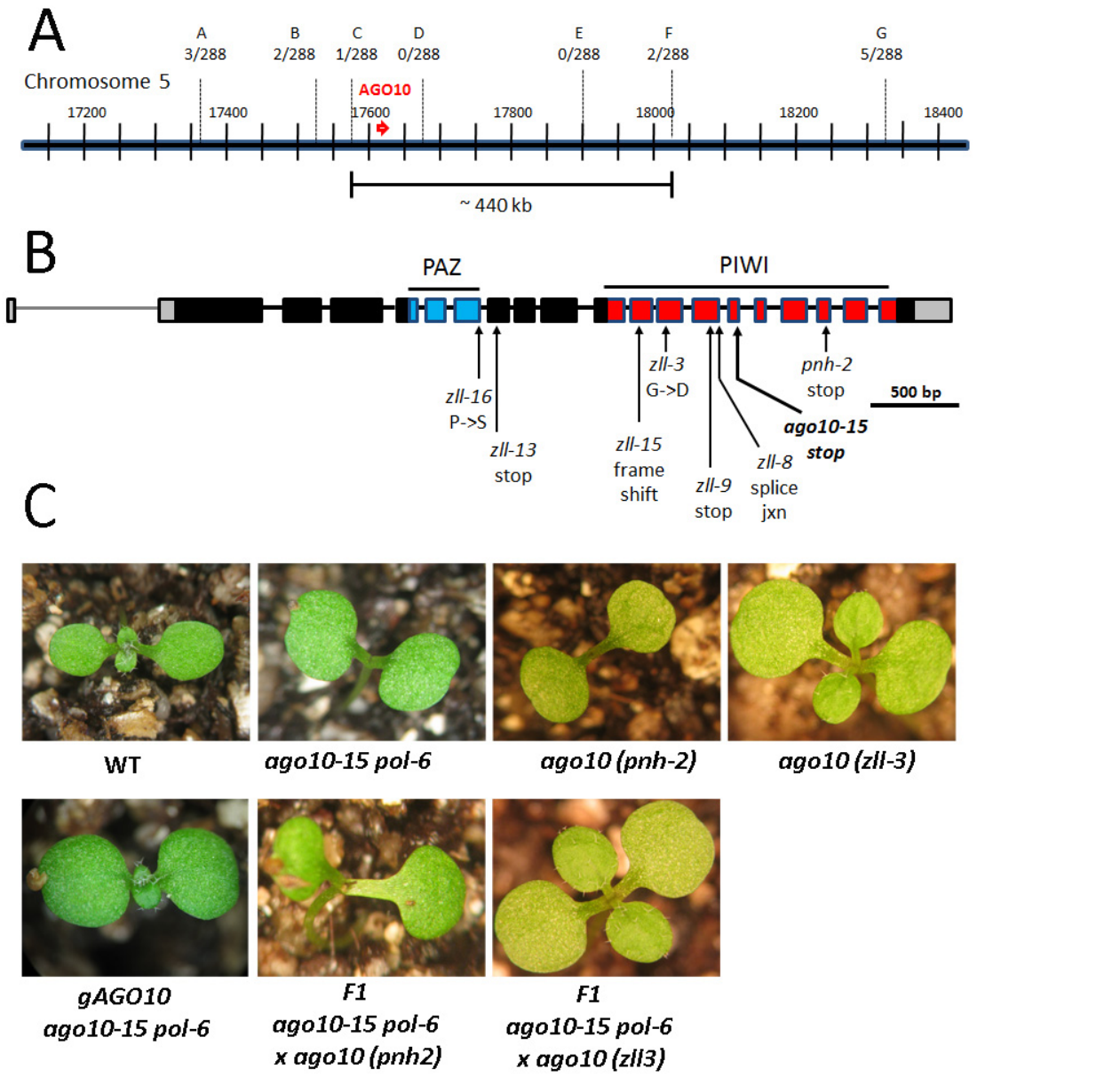

Figure 2.6. Identification of a novel *ago10* allele.
(A) Mapped-based cloning of the isolate #361 *pol* modifier. Letters indicate the location of fine mapping markers (see Table 2.3) and the number of recombinants among 288 mapping chromosomes. The 440 kb region delimited by the closest recombinants is shown.
(B) Diagram of the *AGO10* genomic organization. The location of the coding sequences for the PAZ and PIWI domain as well as the locations and nature of various *ago10* alleles are indicated.
(C) Seedlings phenotypes of wild-type (WT), *ago10-15 pol-6* double mutant, $ago10^{phn-2}$ and $ago10^{zll-3}$ alleles are shown. Complementation of *ago10-15 pol-6* with the *AGO10* genomic DNA as well as allelism tests with the $ago10^{phn-2}$ and $ago10^{zll-3}$ alleles are shown.

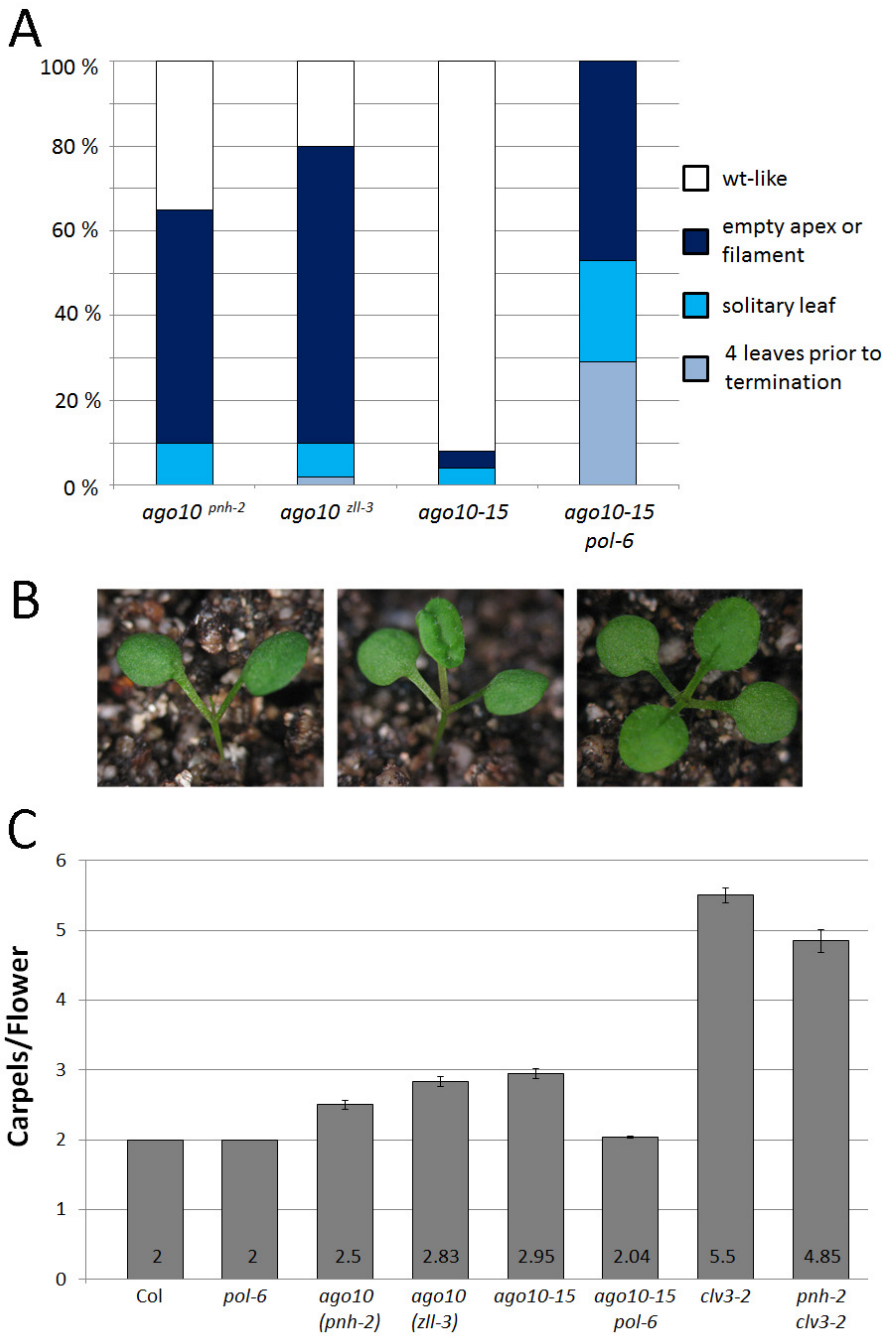

Figure 2.7. The *ago10-15* allele.
(A) Frequency of seedling phenotypes for *ago10* alleles.
(B) Representative phenotypes for *ago10-15 pol* with apex filament, solitary leaf and four leaves prior to termination are shown.
(C) Mean number of carpels per flower with standard error of the mean for wild-type and various mutants are shown.

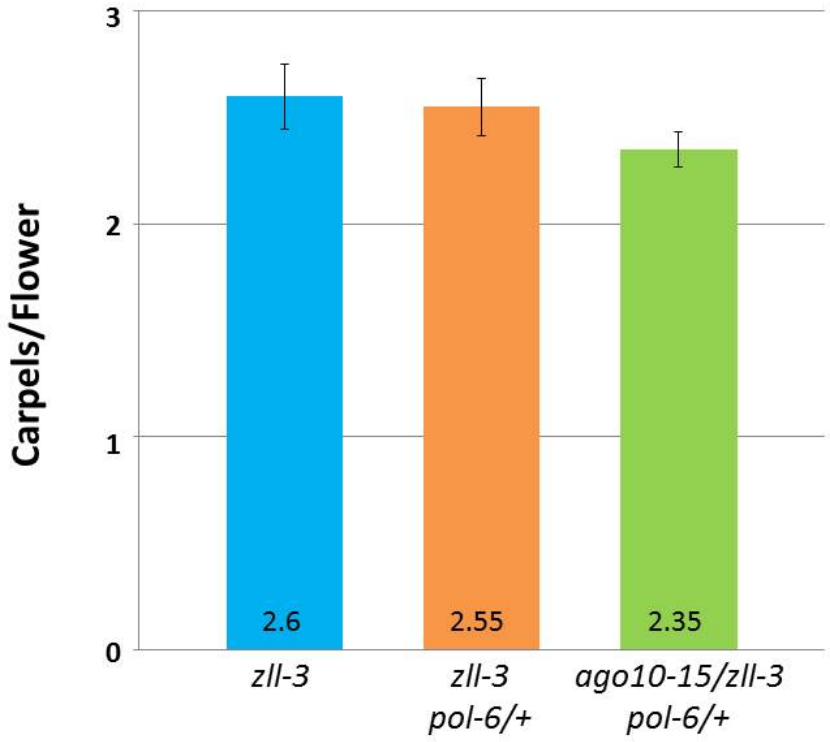

Figure 2.8. *ago10-15* allelism analysis.
Mean number of carpels per flower with standard error of the mean for an allelism test between *ago10*$^{zll-3}$ and *ago10-15*. F1 progeny of *ago10*$^{zll-3}$ x *pol-6* and *ago10*$^{zll-3}$ x *ago10-15 pol-6* were assessed for phenotype.

Table 2.1. Putative *pol* enhancers

Priority	Putative *pol* enhancer lines	Phenotypes	Generation (putative *pol* enhancer x L*er*)	F2 Segregation ration mutant : wild type
1	# 361	Shoot Meristem (SM), Lateral Meristem (LM), Floral Meristem (FM) Termination	F2	19:369
2	# 29	FM termination	F2	12:173
3	# 171	SM, FM termination	F2	14:171
4	# 33	SM, FM termination	F2	Multiple phenotypes
5	# 81	SM, FM termination	F2	Multiple phenotypes
6	# 71	FM termination, Long pedicel	F2	Phyllotaxy defects
7	# 161	FM termination	F1	
8	# 211	FM termination	F2	Multiple phenotypes
9	# 311	PIN	F1	

Table 2.2. Markers used for rough mapping of *ago10-15* from Figure 2.4

Marker	Forward	Reverse	Col	Ler
Chromosome 1				
F3O9	GCCCTTCGTTTTTGTCGAT	TTGAGGAACTTACAATTCTTGTCG	163	130
SO392	TTTGGAGTTAGACACGGATCTG	GTTGATCGCAGCTTGATAAGC	142	156
nF5I14	CTGCCTGAAATTGTCGAAAC	GGCATCACAGTTCTGATTCC	195	290
nga111	GGGTTCGGTTACAATCGTGT	AGTTCCAGATTGAGCTTGAGC	148	154
Chromosome 2				
F17L24	TTGAAAATGCTCAAAACGACAA	ACTGAATGTTTGCTTCCCAGAC	385	340
F26B6	CTCTATCTGCCCACGAACAAG	CAGGCGATAGAGATGGTAGACA	200	220
nga168	TCGTCTACTGCACTGCCG	GAGGACATGTATAGGAGCCTCG	151	135
Chromosome 3				
F2010	AAGAATTGAAATCCCGATGG	GTTGATAAAGCAACGCAGCA	190	215
ciw11	CCCCGAGTTGAGGTATT	GAAGAAATTCCTAAAGCATTC	180	230
ciw4	GTTCATTAAACTTGCGTGTGT	TACGGTCAGATTGAGTGATTC	190	215
nga6	TGGATTTCTTCCTCTCTTCAC	ATGGAGAAGCTTACACTGATC	143	123
Chromosome 4				
F2N1	CAACATGTTTGGGCTCCTCT	TCCCTTCTTGTTTTCACTTTTCA	216	249
ciw6	CTCGTAGTGCACTTTCATCA	CACATGGTTAGGGAAACAATA	150	155
F4B14	TCTTCCACCAGTTCATGCTG	GCGTCTCAGGTGGTTTTAGC	512	357
nga1107b	GCGAAAAAACAAAAAAATCCA	CGACGAATCGACAGAATTAGG	150	140
Chromosome 5				
nga151	GTTTTGGGAAGTTTTGCTGG	CAGTCTAAAAGCGAGAGTATGATG	150	120
MNF13	CGTATTTCATATAAAGTCGTTCTTCGT	ATGTAAATTTGGTATAAGCCGAACA	130	104
MIO24	TGGTGGTGTACGATTTTACCAA	TGCATTTCTCGCCATAGTTG	288	231
K919	CTAATCAACTGCTAAAGTCTGTATTC	GTTTCGACAGCCACAAGAGA	178	166

Table 2.3. Markers used for map-based cloning of *ago10-15* from Figure 2.6A.

	Marker	Enzyme	Forward	Reverse	Col	Ler
A	MNL12- lch1		AGCCGCAAATGGATGTCTCACCA	TGACCCCTCCACATCTCCCAACT	317	303
B	K9D7-lch2		TGTTTGCAGTGATTGGTGGT	TCCCCTTTACCTTCACATGC	407	392
C	MQD19-lch3		GGCCAAATTTTCAACGACAA	TTGGGGAGAGTTTGTGTGGT	663	606
D	MRH10-lch2	DdeI	CGCAAGCCCATTACAAAAAT	AAGGATCTGCTTTGCTTGGA	249	353
E	MFC16-lch1		GGGCGGACTTGTAAAACTT	CGACAATTTTGGGAGCAGAT	332	322
F	K23L20-lch1		GATTTCGCTCTCTGCCAAAA	CGACCGTTTGCTATGCTTCT	156	90
G	K9E15-lch1		CGCGGAATGAAACTACCACT	TCCTCAACTGATGTGCTTGC	187	168

CHAPTER THREE

A WUSCHEL-independent stem cell specification pathway is repressed by PHB, PHV and CNA in Arabidopsis

ABSTRACT

The homeostatic maintenance of stem cells that carry out continuous organogenesis at the shoot meristem is crucial for plant development. Key known factors act to signal between the stem cells and an underlying group of cells thought to act as the stem cell niche. The central player in this regard is the homeodomain transcription factor, WUSCHEL (WUS). WUS is essential for stem cell initiation and maintenance at shoot and flower meristems. Recent data suggest that the WUS protein may move from the niche cells directly into the stem cells to maintain stem cell identity. I have identified a second, previously unknown, pathway for stem cell specification at shoot and flower meristems that bypasses the requirement for WUS. I demonstrate that this novel stem cell specification pathway is normally repressed by the activity of the HD-zip III transcription factors PHABULOSA, PHAVOLUTA and CORONA. When de-repressed, this second stem cell pathway leads to an accumulation of stem cells and an enlargement of the stem cell niche. When de-repressed in a *wus* mutant background, this second stem cell pathway leads to functional meristems with largely normal cell layering and meristem morphology, activation of *WUS* cis regulatory elements, and extensive, but not indeterminate, organogenesis. Thus, WUS is largely dispensable for stem cell specification and meristem function, suggesting a set of key stem cell specification factors remains unidentified.

INTRODUCTION

The shoot meristem is the central source of post-embryonic organogenesis and development, forming all above-ground organs and tissues in plants [1, 2]. In angiosperms, the shoot meristem is formed during embryogenesis in the central cells of the apical region of the embryo [3]. The key to shoot meristem function is the homeostatic maintenance of stem cells, while allowing appropriately-positioned stem cell daughters to begin to differentiate, forming new organs and tissues. The fate of dividing stem cells is determined by position, with central- and apical-positioned daughters remaining stem cells, while lateral and basal daughters switch towards differentiation [4, 5].

A key factor regulating stem cell specification in plants is the WUSCHEL (WUS) homeodomain-containing transcription factor. *WUS* is expressed in the Organizing Center (OC), which comprises the niche cells immediately basal to the shoot and flower stem cells. *WUS* expression in the OC specifies overlying cells as stem cells in a non-cell autonomous manner [6, 7]. *WUS* is necessary and sufficient within the meristem for stem cell specification. In loss-of-function mutants, *wus* seedlings lack a functional shoot meristem, leading to differentiated apex. Later, through an unknown pathway, *wus* mutants form adventitious shoots that establish several leaves and rarely flowers lacking floral organs. Due to the lack of stem cells, these adventitious shoots terminate, followed by a reiteration of this process. *WUS* over-expression leads to ectopic stem cells within the shoot and flower meristem [8-11].

Genetic studies have led to the identification of many other factors affecting stem cell specification and maintenance. Critically those factors when analyzed in detail converge to either regulate the *WUS* expression domain or the organ specification pathway represented by SHOOTMERISTEMLESS (STM) [12-16].

One of the best-studied pathways regulating *WUS* transcription is the CLV signaling pathway. CLV signaling restricts *WUS* expression to the basal daughter of the L3 stem cells, thus limiting the size of the OC and hence, the size of the stem cell population [11, 17]. Many components of CLV signaling have been identified [7, 18-24]. *CLV3*, whose expression appears to mark the stem cell population, codes for the

precursor of the peptide ligand that binds to CLV receptor complexes in the L3 cell layer [20, 21, 25-29]. Mutations in the *CLAVATA* genes result in the opposite phenotype to *wus*, namely enlarged stem cell populations leading to increased organ number and meristem size, all resulting from expanded *WUS* expression [6, 11, 17, 19, 30, 31]. CLV receptor activation represses the activity of the lipid-modified and lipid-binding protein phosphatases POL and PLL1, which are required for *WUS* expression maintenance [22, 23, 32]. While CLV3 signaling limits *WUS* expression, WUS protein promotes *CLV3* transcription, creating a homeostatic feedback loop. Recently, it has been shown that WUS protein may move through plasmodesmata into the overlying stem cells, where it binds directly to *CLV3* cis elements while also activating other unknown stem cell promoting factors [33].

Even with the extensive efforts to identify stem cell regulators that have been carried out by many labs, major gaps in understanding stem cell control remain. In an effort to identify other factors controlling stem cell specification, with a focus on missing CLV signaling components or targets of WUS, I conducted a mutagenesis of *pol-6* single mutants, screening for modifiers that would lead to loss of stem cells specifically in a *pol* mutant background. The *pol* mutant has an almost identical phenotype to wild type, but is an important intermediate in CLV signaling pathway. From the enhanced mutagenesis screening using *pol-6*, I isolated a novel *ago10* allele (*ago10-15*). *ago10-15* plant display early meristem termination in a *pol-6* dependent manner. Because neither *ago10-15* nor *pol-6* exhibit meristem termination, this reflects a synergistic interaction between the mutations.

Additional analyses by myself and several other labs investigating *ago10* genetics and their interaction with other meristem regulators have generated inconclusive findings. The lack of internal consistency of genetic interactions between *ago10* mutants and CLV pathway mutants makes a clear conclusion difficult. This variable genetic response for *ago10* likely reflects the overlapping and antagonistic function of known AGO10 targets. Unlike AGO1, which has a broad ranging function in miRNA action [34-38], AGO10 appears to be fairly specific for the miR165/166 family, where AGO10 paradoxically prevents these miRNAs from repressing their targets [39-42]. Genetic alteration of miR165/166 function results in meristem defects [39, 43]. In

turn, the miR165/166 targets, the homeodomain-leucine zipper class III (HD-zip III) genes. [44-47].

HD-ZIP III members are transcription factors and have overlapping, antagonistic, and distinct roles in Arabidopsis development, including a previously identified but poorly characterized role in stem cells control. The HD-zip III family in Arabidopsis is composed of 5 members: REVOLUTA, PHABULOSA, PHAVOLUTA, CORONA, and ATHB8. REVOLUTA (REV) is necessary for embryonic and lateral shoot meristem initiation, as well as flower meristem intitiation [48]. Triple or quadruple mutants of HD-ZIPIII members display similar mutant phenotypes as loss-of-mutant *ago10* and over-expressed miR166 (*jab-1D*). In addition, transcript levels of HD-ZIPIII are specifically affected by either *ago10* or *jba-1D*.

Post-embyonically, *REVOLUTA* (*REV*) plays an important role in promoting shoot and flower meristem initiation, while *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*) and *CORONA* (*CRN*) play redundant, antagonistic roles in limiting meristem development [46, 47]. *rev* and *phv*/*phb*/*cna* mutants suppress each other's meristem phenotypes post-embryonically [47]. Thus, *ago10* mutants have both a reduction of *REV* activity, leading to meristem loss, and a reduction on *PHB*/*PHV*/*CRN* activity, leading to meristem enlargement.

In an attempt to bypass the functional antagonism inherent in AGO10, miR165/166 and general HD-zip III analysis, I chose to focus specifically on the relationships between PHB/PHV/CNA function and the CLV-WUS pathway. The *phb phv cna* triple mutant has been briefly described as showing effects of stem cell accumulation at shoot and flower meristems (e.g., stem fasciation and additional floral organs) [47]. Thus, I chose to analyze these mutants in detail as well as assess their genetic interactions with the CLV/WUS pathway. My working hypothesis was that PHB/PHV/CNA act either within the CLV/WUS pathway or in a separate pathway controlling WUS transcription and/or activity. This model was based on the essential nature of WUS for stem cell specification described in hundreds of published studies [16]. Surprisingly, I found that WUS is largely dispensable for stem cell specification. Furthermore, a separate stem cell specification implied by this observation is repressed by PHB/PHV/CNA activity.

RESULTS

PHB/PHV/CNA act independently of the CLV pathway

Because of the antagonistic relationships between REV and PHB/PHV/CNA might mask critical meristem functions, I analyzed in detail the *phb phv cna* triple mutant, as well as explored the interactions of *PHB*/*PHV*/*CNA* with the CLV/WUS pathway. *phb phv cna* triple mutants have been only briefly described as exhibiting inflorescence and flower phenotypes reminiscent of *clv* mutants [47], but the detailed defects of these mutants and their relationship with other meristem regulators has remained unexplored.

phb-13 phv-11 cna-2 mutants often displayed a tricotyledon phenotype, stem fasciation, and silique enlargement (Figure 3.1A). Similar to *clv* mutants, *phb phv cna* mutants developed an increase in the number of flower organs in each whorl, with the largest deviation from wild-type found in the central carpels (Figure 3.1B). When analyzed by scanning electron microscopy (SEM), the shoot apical meristems of *phb phv cna* mutants were enlarged and misshapen compared to wild-type, with fasciation (the conversion of the meristem into a line) often observed (Figure 3.2A,B).

To test the effect of *phb phv cna* on expression of the key markers/regulators *CLV3* and *WUS*, I first measured total transcripts by semi-quantitative RT-PCR in wild-type, *phb phv cna* and *clv3-2* seedlings. I observed an up-regulation of both *CLV3* and *WUS* transcript accumulation in *phb phv cna*, consistent with the increase in meristem size in these mutants (Figure 3.1C). I then crossed previously characterized *PWUS:GUS* and *PCLV3:GUS* reporters into the *phb phv cna* mutant background [8, 49]. The expression of both reporters were expanded laterally in 5 days-old *phb phv cna* seedlings compared to wild-type (Figure 3.1D), suggesting that PHB/PHV/CNA may act in the CLV/WUS pathway to limit *WUS* expression.

To test whether PHB/PHV/CNA act within the CLV/WUS pathway, I crossed *phb phv cna* to the strongest *clv* mutant allele *clv3-2*, which is epistatic to other mutations in other *CLV* genes [19, 31]. Surprisingly, the morphology of *clv3 phb phv cna* plants was dramatically altered and enhanced compared to *clv3-2* single mutants. At very early stages of vegetative development, massively enlarged stem cell populations

accumulated, often without any evidence of lateral organ formation (Figure 3.3A-C, F-I). *clv3 phb phv cna* quadruple mutants all eventually initiated organ primordia while the shoot meristem continued to accumulate additional stem cells (Figure 3.3K,L). Inflorescence bolting was impaired in *clv3 phb phv cna* plants, presumably due to the massive size of the meristem (Figure 3.3M).

clv3 phb phv cna flower meristems were also massively enlarged compared to *clv3* single mutants, as apparent from flower morphology, SEM analysis and organs produced (Figure 3.2B,C, Figure 3.3N,O). *clv3 phb phv cna* flowers averaged over 9 carpels per flower and exhibited indeterminate stem cell proliferation leading to massive stem cell populations even very late into flower development. *clv3 phb phv cna* flowers were completely sterile.

Wild-type shoot apical meristems have two very clear clonal cell layers (L1, L2) that undergo strictly anticlinal divisions [4, 50-52]. The underlying L3 layer undergoes divisions in both anticlinal and periclinal orientations. *clv* mutants appear to lose the L3 layer as the OC-factor *WUS* expands into the L3 cells [11]. This affected the morphology of the *clv* meristems, with a lack of a clear L3 layer and a breakdown of all cell layering in the center of the larger *clv* meristems (Figure 3.4D,E). In addition, L1 cells in *clv* meristems often adopted a more columnar shape (Figure 3.4E). *phb phv cna* mutants, despite a similarity to *clv* mutants, retain a clear layering patterning that includes an L3 layer (Figure 3.4F). In addition, I did not observe columnar L1 cells in *phb phv cna* meristems. The *clv3 phb phv cna* quadruple mutants developed columnar L1 cells and lacked a clear L3 layer similar to *clv* single mutants (Figure 3.4G).

PHB/PHV/CNA act independently of WUS

Even though PHB/PHV/CNA act independently of the CLV pathway, they could act in a CLV-parallel pathway controlling *WUS* expression or activity. Indeed, most or all stem cell regulators studied in detail to date appear to act through *WUS* [13-16]. To test this idea, I generated the *wus-1 phb phv cna* quadruple mutant. These plants were assayed as progeny of a *wus/+ phb phv cna* parent. *wus* mutants display a fully penetrant differentiation of the embryonic apex, leading to an absence of leaf primordia that is

clearly visible within 5-7 days after germination [6]. *wus* mutants subsequently form leaves from presumed adventitious shoots at the apical region of the seedling and later in the axil of existing leaves. No meristems have been identified within the developing post-embryonic structures of *wus* mutants. Surprisingly, *wus phb phv cna* quadruple mutants developed 1-8 leaves before the initial shoot apical meristem termination (mean = 2.8 ± 1.5 (standard deviation), n = 26). After the initial termination of the shoot apical meristem, *wus phb phv cna* mutants rapidly formed a single or multiple adventitious meristems (Figure 3.5B-D). These meristems were often capable of developing a full rosette of leaves and an inflorescence with multiple cauline leaves and flowers prior to terminating (Figure 3.5E-L). While no indeterminate structures formed in *wus phb phv cna* plants, the shoots that were present had much of the organogenic capacity typically observed in wild-type shoot meristems. Fasciated stems were occasionally observed suggesting an ectopic accumulation of stem cells despite the lack of WUS function (Figure 3.5I,J,K).

To determine what structures were responsible for this organogenesis, I analyzed *wus phb phv cna* mutants by SEM. While I never observed any meristem-like structures among *wus* seedlings (Figure 3.6A,B), I readily observed meristem-like structures at the apical region of *wus phb phv cna* plants (Figure 3.6C-F). The *wus phb phv cna* meristems exhibited the dome shape typical of wild-type meristems (c.f., Figure 3.6D,F to Figure 3.2A). Histological sectioning of *wus phb phv cna* mutants revealed that normal-sized meristems retained the L1/L2 layering (Figure 3.7C). *wus phb phv cna* meristems larger than wild-type occasionally exhibited a loss of clear layering (Figure 3.7D).

The structures formed in *wus phb phv cna* quadruple mutants had both the appearance and function of normal shoot meristems, although they were not fully indeterminate. To determine if they established a normal Organizing Center comparable to wild-type meristems, I assayed *Pwus:GUS* activity in the quadruple mutant. I readily observed GUS activity in every *Pwus:GUS wus phb phv cna* plant at 12 days old (Figure 3.5O,P). The puncta regions of GUS activity corresponded well in terms of number and position to the meristem structures observed by SEM, suggesting that these meristems are largely normal even in the absence of *WUS* activity. Later

arising adventitious shoot of the quadruple mutant lacked evidence of meristem activity and were indistinguishable from *wus* single mutants (Figure 3.5M,N).
Within the developing flowers, *wus* appeared largely epistatic to *phb phv cna*. The quadruple mutants never formed the central carpels. The mean numbers of flower organs were similar between *wus phb phv cna* and *wus* single mutants (Figure 3.1B). Thus, the phenotypic suppression of *wus by phb phv cna* was limited to vegetative and early reproductive stages.

During the construction of the *wus phb phv cna* quadruple mutant line, I failed to readily observe *wus* suppression until all three HD-zip III genes were homozygous mutant. For example, *wus*/*wus phb*/*phb phv*/+ *cna*/*cna* plants were not clearly different from *wus* single mutants (Figure 3.8).

WOX5 is a *WUS* homologue expressed in the Organizing Center-equivalent Quiescent Center of the root meristem, where WOX5 is important for stem cell maintenance [53, 54]. Interestingly, POL/PLL1 are required for both *WUS* and *WOX5* transcription [55]. To determine if the suppression of *wus* phenotypes by the combined *phb phv cna* mutations was simply the result of the ectopic expression of *WOX5* in the shoot meristem, we tested for changes in WOX5 transcript accumulation in *phb phv cna* mutants. However, no increased WOX5 accumulation was observed (Figure 3.9).

DISCUSSION

A WUS-independent stem cell pathway

Previous research had indicated that WUS is critically required for stem cell initiation and maintenance at shoot and flower meristems [6-11]. Here I provide clear evidence that WUS is dispensable for a functional shoot meristem. *wus phb phv cna* quadruple mutants showed evidence of both embryonic shoot meristem function (in the form of post-embryonic leaf development) and the formation of functional shoot meristems post-embryonically. These post-embryonic meristems in the absence of WUS had many features of normal meristem morphology, histology and activation of the key OC marker

(i.e., *WUS* cis elements). While the post-embryonic vegetative shoot meristems of *wus phb phv cna* plants were not indeterminate, they were capable of extensive organogenesis consisting of a full rosette of vegetative leaves, cauline leaves and over a dozen flower primordia. I even observed fasciated stems and meristems in the quadruple mutant, suggesting ectopic stem cell accumulation in the absence of WUS.

That PHB/PHV/CNA act in a WUS-independent pathway to control stem cell initiation and function is consistent with genetic interactions with *clv3-2*, where I observed a strong enhancement of stem cell accumulation and loss of organogenesis in the *clv3-2 phb phv cna* quadruple mutant. The resulting plants were severely abnormal, often lacked the ability to form lateral organs, and accumulated massive populations of stem cells. *clv3-2 phb phv cna* flowers were similarly enhanced compared to *clv3-2* alone, with significant increases in organ number, stem cell accumulation and indeterminate growth. Taken together, I conclude that PHB/PHV/CNA represent a pathway parallel to CLV/WUS for stem cell initiation and maintenance, and that the role of PHB/PHV/CNA in this pathway is to limit the stem cell population.

While *wus* mutants alone have a completely penetrant loss of stem cells and most studies have focused on the centrality of WUS function, some prior evidence in the literature hinted at a separate WUS-independent stem cell pathway. (1) *WUS* mRNA expression in the inflorescence shoot meristem is low compared to expression at embryonic, seedling or flower meristems [11, 56]. (2) In portions of massively enlarged *clv* shoot apical meristems, *WUS* expression is lost (suggesting stem cell maintenance in the absence of WUS) [56]. (3) In double mutants of *clv3-2* with the dominant-negative *cna-1* allele, the shoot apical meristem defect could be viewed as enhanced compared to *clv3-2* alone; however, the complicated nature of the double mutant phenotype made a clear interpretation difficult [56] (4) Some weak suppression of the *wus* phenotype may have been observed in a combination with the heterozygous *men1* activation-tagged allele of miR166a [43].

The failure to clearly identify AGO10, miR165/166 and the HD-zip III genes as representing a CLV/WUS-independent stem cell pathway despite intense study likely rests on two features of these regulators [39, 42, 43, 47, 57]. First, REV antagonizes PHB/PHV/CNA function in post-embryonic meristem development. Thus, genetic

manipulation of AGO10 or miRNA165/166 function would have a mixed effect by simultaneously promoting and inhibiting stem cell specification. Thus, *wus* was described as epistatic to miR166 over-expression caused by the *jabba1-D* allele [39] and to *ago10-12* [42], while *ago10 clv3* double mutants failed to display any meristem enhancement (Figure 3.2). The second feature likely masking a CLV/WUS-independent pathway is the apparent genetic requirement to completely eliminate PHB/PHV/CNA activity in order to observe *wus* suppression. Even a single wild-type allele of PHB/PHV/CNA appears to prevent *wus* suppression (Figure 3.8). This suggests that even very limited PHB/PHV/CNA activity can block stem cell initiation in a *wus* background.

The PHB/PHV/CNA stem cell pathway

If PHB/PHV/CNA act in a WUS-independent pathway, how do these genes control stem cell specification? From the phenotypes and genetic interactions, it is clear that PHB/PHV/CNA act to limit the stem cell population post-embryonically. This is in contrast to their role in promoting embryonic shoot meristem formation [46, 47]. PHB/PHV/CNA presumably repress a factor(s) that is capable of activating stem cell initiation and maintenance.

This raises the question: what are the genes responsible for stem cell initiation and maintenance? Presumably these genes are the direct or indirect targets of WUS. Perhaps PHB/PHV/CNA repress a related set of target genes that WUS activates and vice versa. While significant steps have been made in transcriptional profiling of the meristem and screens for WUS direct and indirect targets, I still know little about how WUS executes stem cell identity [58-60]. Perhaps the best characterized direct WUS target is *CLV3*, which acts in *WUS* transcriptional control, not in promoting stem cell specification [11, 17, 33]. There is evidence that genes involved in cytokinin signaling are also targeted by WUS – it would be interesting to see if these are targeted by PHB/PHV/CNA as well [59, 61, 62]. Other direct or indirect WUS targets are not yet analyzed in detail, so it remains a mystery which downstream genes carry out stem cell initiation and maintenance in Arabidopsis.

One possibility I considered was that the *WUS* homolog *WOX5* might become active in the shoot meristems of *phb phv cna* plants. *WOX5* is expressed in the stem cell niche of the root meristem (called the Quiescent Center, or QC). *WOX5* expression in the QC acts to maintain stem cell identity in some of the QC-adjacent root stem cells [53, 54]. Interestingly, POL/PLL1 are required for both *WUS* expression maintenance in the OC and *WOX5* expression maintenance in the developing QC [55]. However, I observed no induction of *WOX5* transcript accumulation in either *phb phv cna* nor *wus phb phv cna* plants. This indicates that the stem cell restoration in *wus phb phv cna* is not simply a case of activation of a close functionally similar homolog.

MATERIALS AND METHODS

Plant growth and genetic analysis

Arabidopsis seeds were sown on a 2:1:1 mixture of top soil:perlite:vermiculite supplemented with fertilizer and imbibed for 7 days at 4˚C. Plants were grown under continuous cool-white fluorescent lights at 22˚C. Plants in petri dishes were grown on half-strength Murashige and Skoog salts (Sigma) with 0.8% (w/v) phytoagar. Seeds were imbibed for 4 days at 4°C and grown under continuous cool-white fluorescent lights at 22˚C.

Histological Analysis

Tissue fixation and section were performed as described previously [63]. Eight micrometer sections were prepared using the Leica RM 2065 microtome, stained in 0.025% toluidine blue, and examined with a Nikon OPTIPHOT-2 microscope.

SEM

SEM analysis was performed as described [64]. Briefly, tissue samples were fixed in 4% gluteraldehyde in a sodium phosphate buffer at 4℃ overnight then stained with 0.5% osmium for several days at 4℃. The tissue was then taken through an ethanol dehydration series and critical point dried before mounting with silver paste and gold coating. Images were collected using a Hitachi 3200N SEM.

GUS staining

Pwus:GUS and *PCLV3:GUS* reporter genes [8, 49] were introduced into the *phb phv cna* mutant by crosses. In the F2 population, *phb phv cna*-like plants were isolated and genotyped using *phb*, *phv*, and *cna* primers in [47]. The presence of the reporter gene was tested by PCR and GUS staining. *Pwus:GUS phb phv cna* was crossed to *wus*/+ *phb phv cna* to obtain *Pwus:GUS wus phb phv cna.* GUS staining was performed described [23].

RT-PCR

5 days-old seedlings of Col, *phb phv cna* and *clv3-2* and aerial portions of 14 days-old Col, *wus phb phv cna*, *clv3 phb phv cna* seedlings were used for RNA extraction per manufacturer's instructions (RNeasy Plant Kit, Qiagen, Alameda, CA) and DNaseI treated using the RQ1 RNase-Free DNase (Promega, USA). Two micrograms of RNA was then reverse-transcrbied using Superscript III Reverse Transcriptase using oligo(dT) primers. The set of primers used are listed in Table 3.1.

Image Analysis

Images were collected with a Zeiss stemi sv11 microscope and captured with a Canon digital camera PowerShot S51S. Images were collated in Photoshop with occasional adjustments to brightness and contrast.

ACKNOWLEDGMENTS

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FIGURE LEGENDS

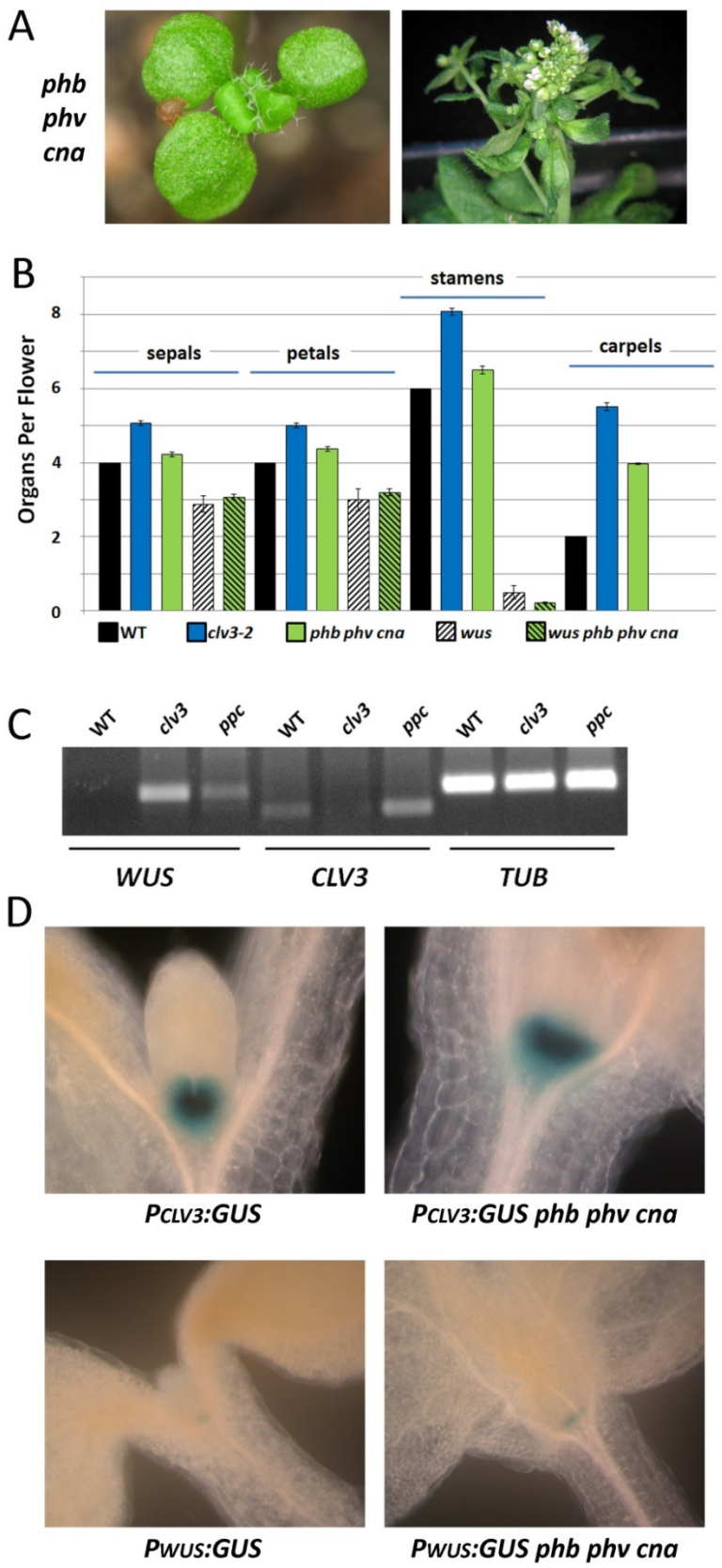

Figure 3.1. PHB PHV CNA restrict meristem size.
(A) *phb phv cna* triple mutants displaying tricotyledon phenotype (left) and inflorescence stem fasciation (right) are shown.
(B) Mean numbers of floral organs per flower with standard error of the mean for wild-type and various mutants are shown.
(C) Semi-quantitative RT-PCR measuring the accumulation of *WUS*, *CLV3* and control *TUBULIN* transcripts in wild-type (WT), *clv3-2* and *phb phv cna* (ppc) 5 days-old seedlings.
(D) A comparison of *PCLV3:GUS* and *PWUS:GUS* reporter line activity of wild-type (left) and *phb phv cna* (right) seedlings.

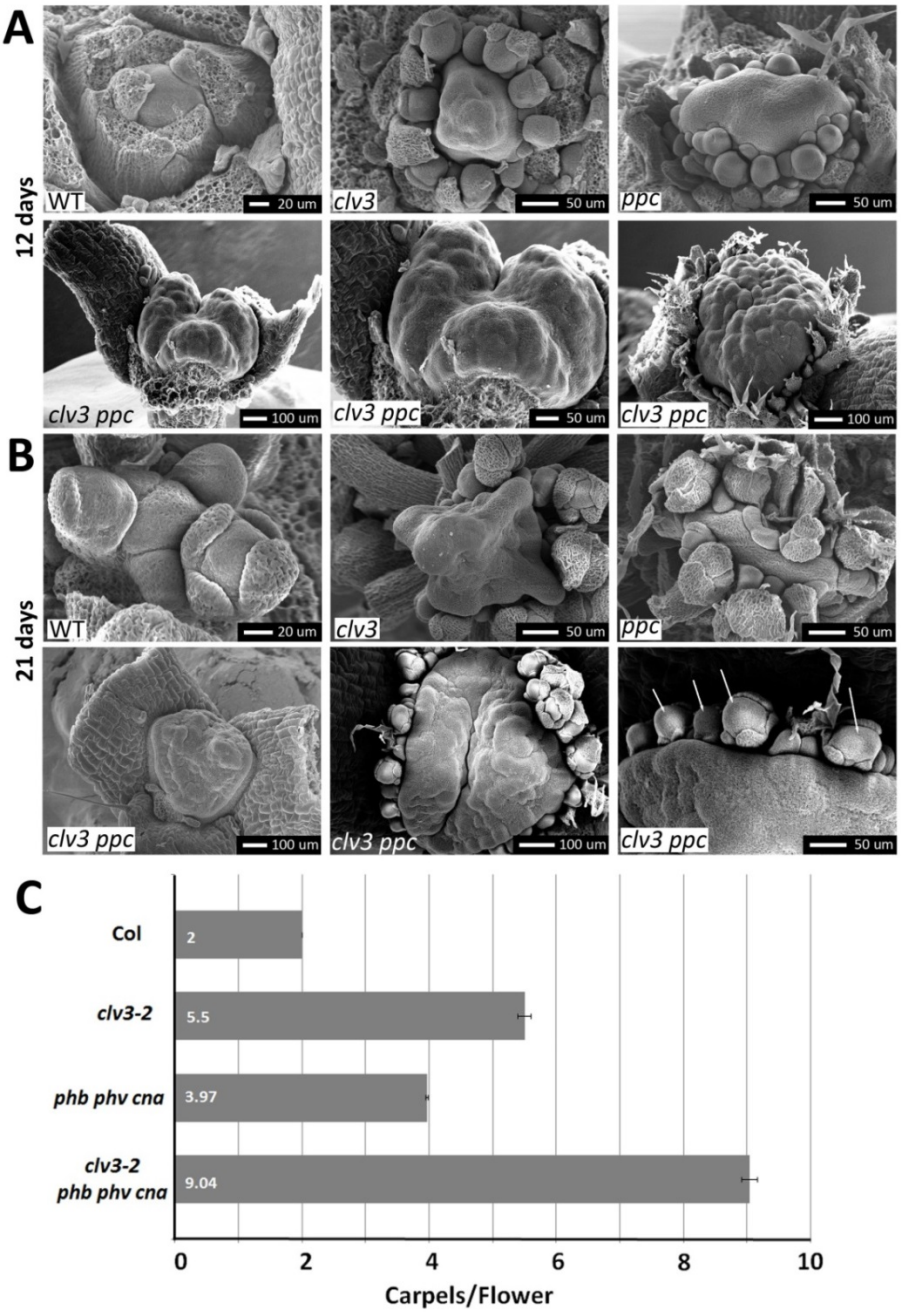

Figure 3.2. *clv3* enhances *phb phv cna* stem cell defects.
(A) Shoot apical meristems of 12 day old wild-type (WT), *clv3-2*, *phb phv cna*, and *clv3 phb phv cna* mutants imaged by scanning electron microscopy. Note the fasciated meristem of *phb phv cna*. *clv3 phb phv cna* mutants occasionally lack all organ primordia as shown the bottom left panel and at higher magnification in bottom center panel. cot, cotyledon.
(B) Shoot apical meristems of 21 days-old plants. Note the continued lack of organ primordia in the bottom left panel. Bottom center panel enlarged on bottom right to show enlarged flower meristems (arrows).
(C) Mean number of carpels per flower with standard error of the mean for various genotypes.

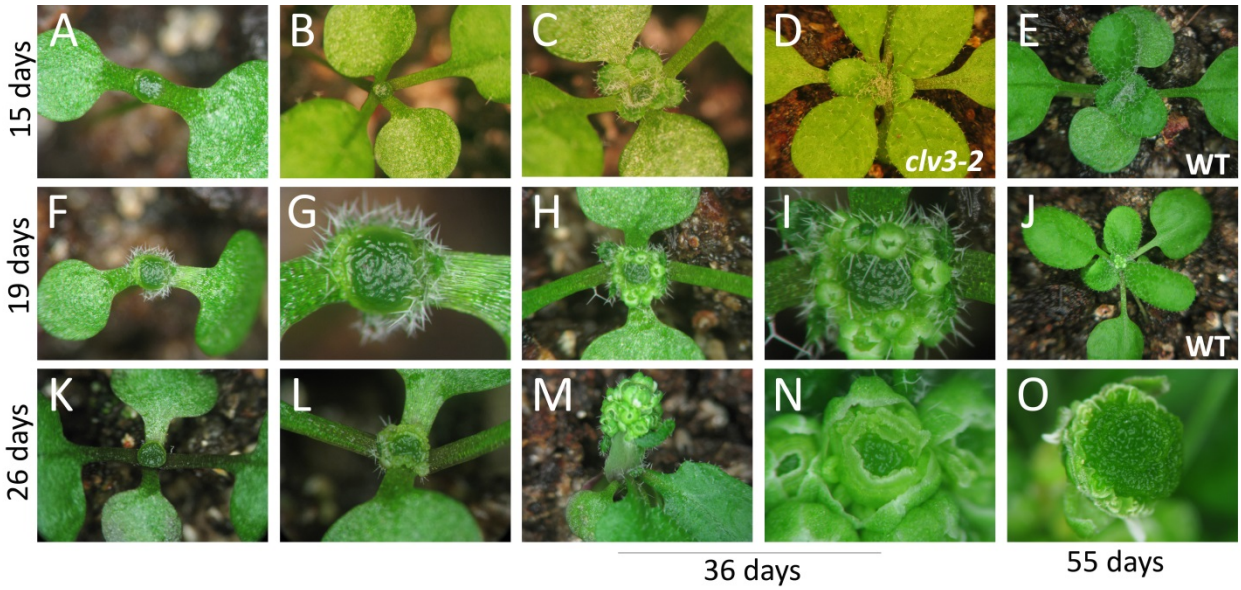

Figure 3.3. Phenotypes of *clv3-2 phb phv cna* plants.
(A)-(L) *clv3-2 phb phv cna* plants (except as noted) showing the shoot apical meristem. (A)-(C) show the range of phenotypes in terms of ability to form organ primordia. Note (G) and (I) are higher magnification views of (F) and (H), respectively.
(M) Side view of typical thick and short inflorescence stem.
(N), (O) *clv3-2 phb phv cna* flowers continue proliferation and accumulation of stem cells.

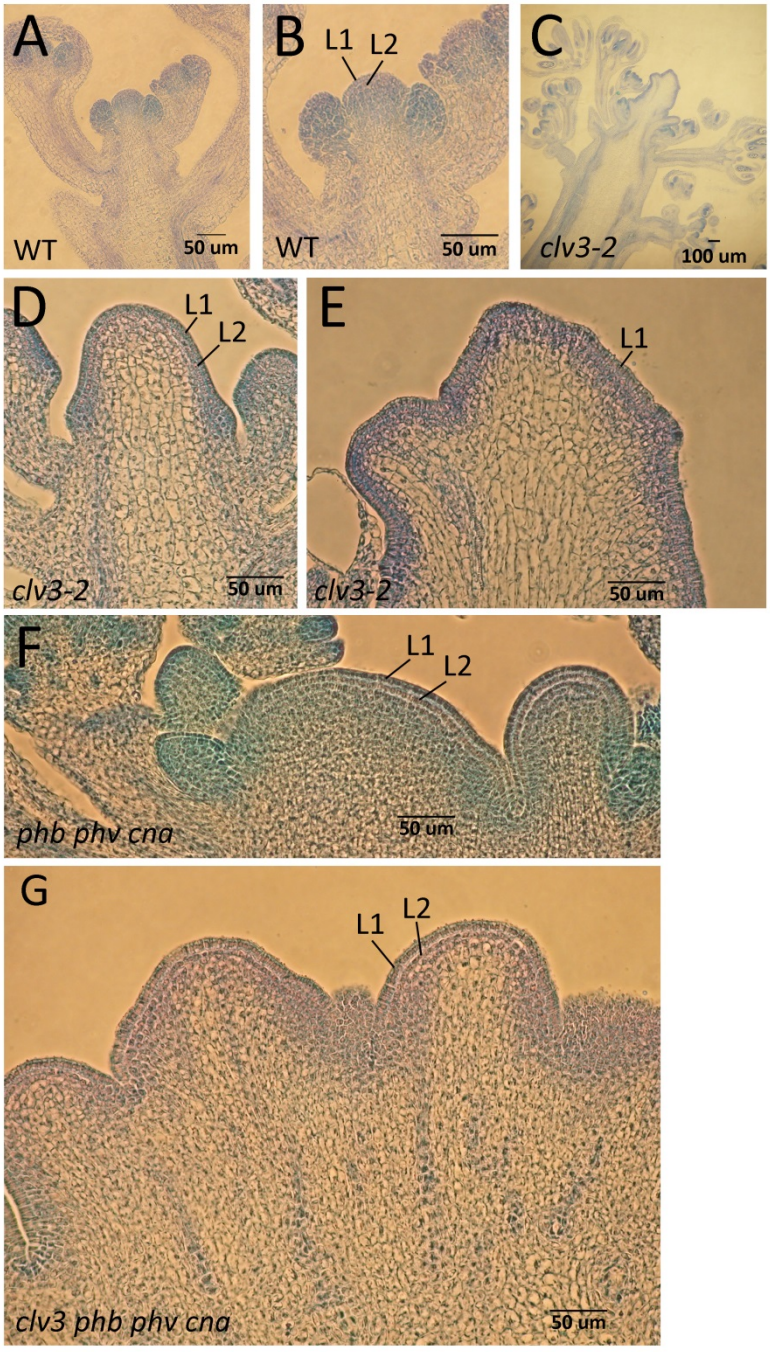

Figure 3.4. Stem cell layering maintained in *phb phv cna*.
Histological sections of wild-type (A, B), *clv3-2* (C-E), *phb phv cna* (F) and *clv3-2 phb phv cna* (G) inflorescence shoot apical meristems. Histologically identifiable L1 and L2 layers are labeled. Note breakdown in clear layering in central apex of *clv3-2* in (D) and the columnar shape of the L1 layer cells in (E) and (G). *phb phv cna* mutants maintain clear layering pattern even in enlarged meristems (F).

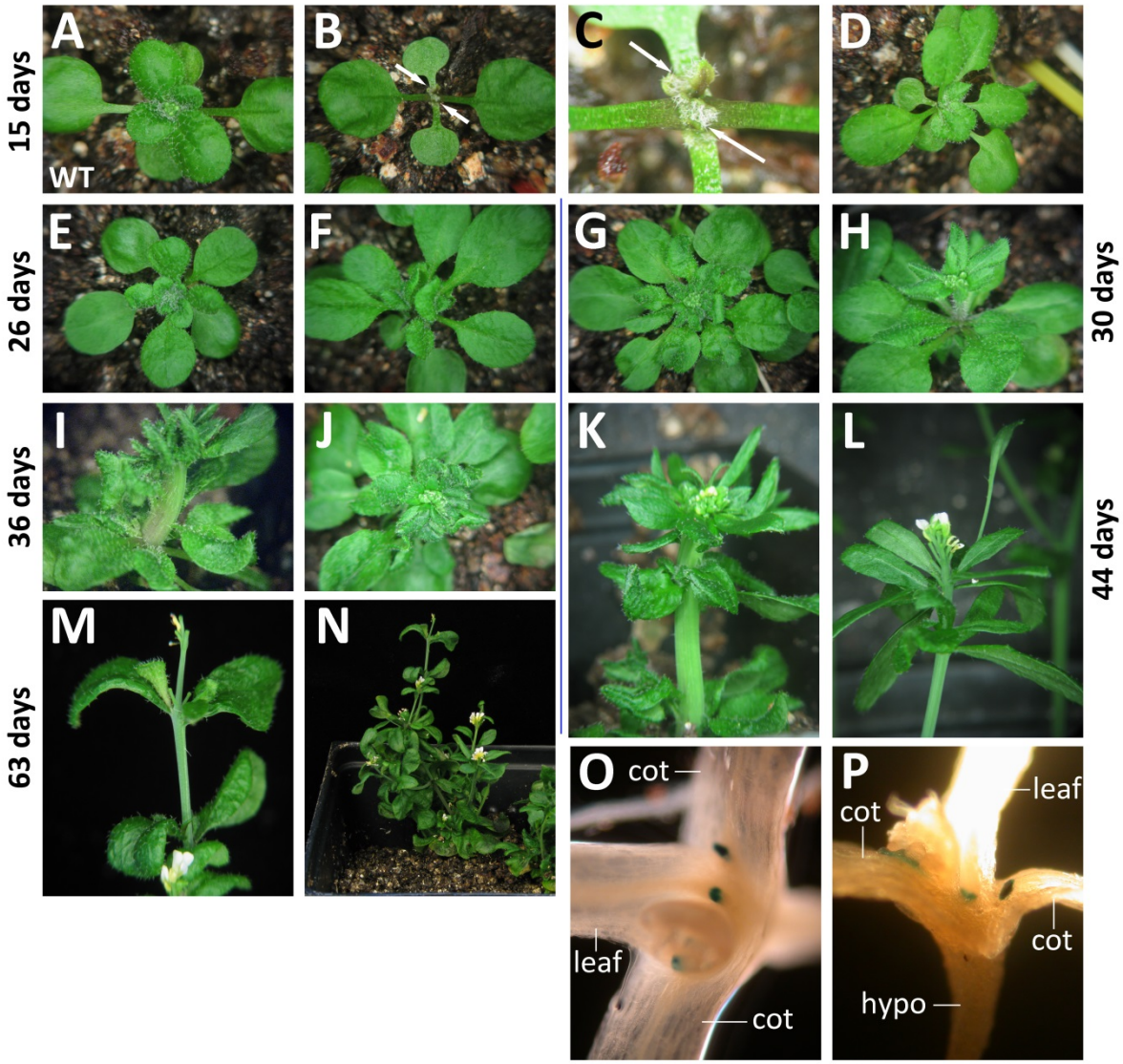

Figure 3.5. *phb phv cna* suppress the *wus* phenotype.
(A) 15 day-old wild-type plant with rosette of vegetative leaves.
(B) *wus phb phv cna* plant with two leaves prior to termination, leading to two adventitious meristems as seen at higher magnification in (C).
(D, E) *wus phb phv cna* plants with a single adventitious meristem leading to a full rosette.
(F) *wus phb phv cna* plant with two adventitious meristems, each forming a vegetative rosette.
(G, H) *wus phb phv cna* plants (top and side views) with emerging inflorescence with many flower primordia.
(I,J) *wus phb phv cna* plants (side and top views) with fasciated inflorescence stems.
(K,L) *wus phb phv cna* early inflorescences form many flowers, but none contain central carpels.
(M,N) Late-arising adventitious shoots on *wus phb phv cna* plants resemble *wus* mutants.
(O,P) Top and side views of *wus phb phv cna* plants carrying *PWUS:GUS*. Note the reporter activity in the axils of leaves and cotyledons. cot, cotyledon; hypo, hypocotyl.

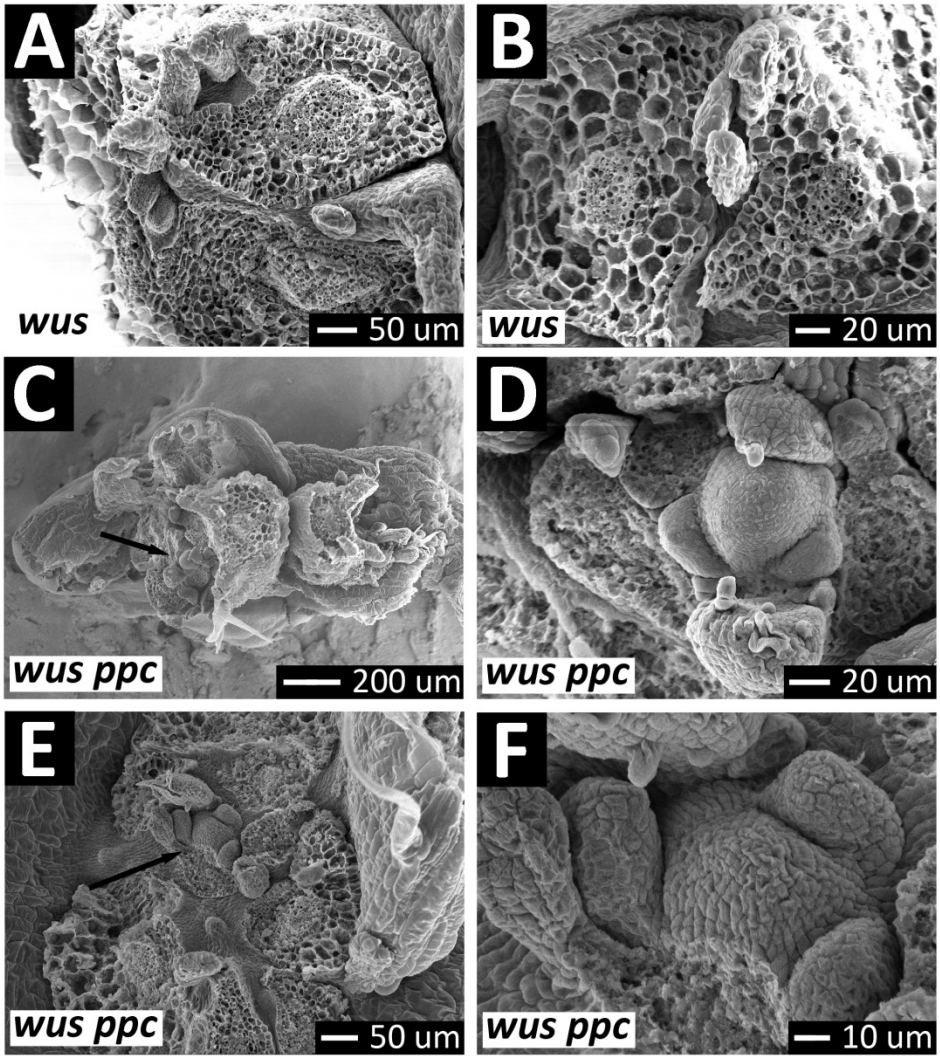

Figure 3.6. Meristems in *wus phb phv cna* plants.
(A, B) Dissected *wus* seedlings imaged by SEM reveal no meristem-like structures.
(C-F) Dissected *wus phb phv cna* seedlings reveal meristem-like structures (C,E – arrows). At higher magnification, meristem structures similar to wild-type are observed (D,F; c.f, Figure 4A).

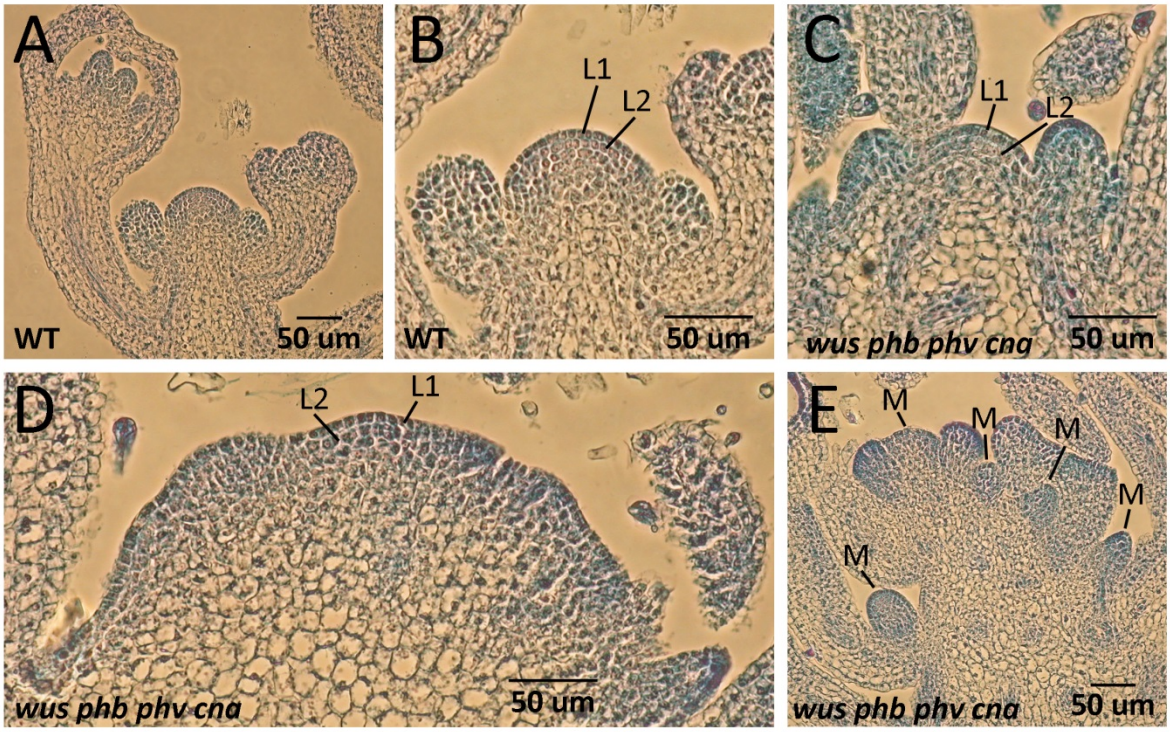

Figure 3.7. Histology of *wus phb phv cna* meristems.
(A, B) Histology of wild-type shoot apical meristems reveals a layering pattern in the central stem cells.
(C, D) Histology of *wus phb phv cna* shoot meristems reveals a layering pattern in normally-sized meristems (C) that is less organized in larger meristems (D).
(E) Extensive development of lateral or adventitious meristems (M) revealed by sections of *wus phb phv cna* plants.

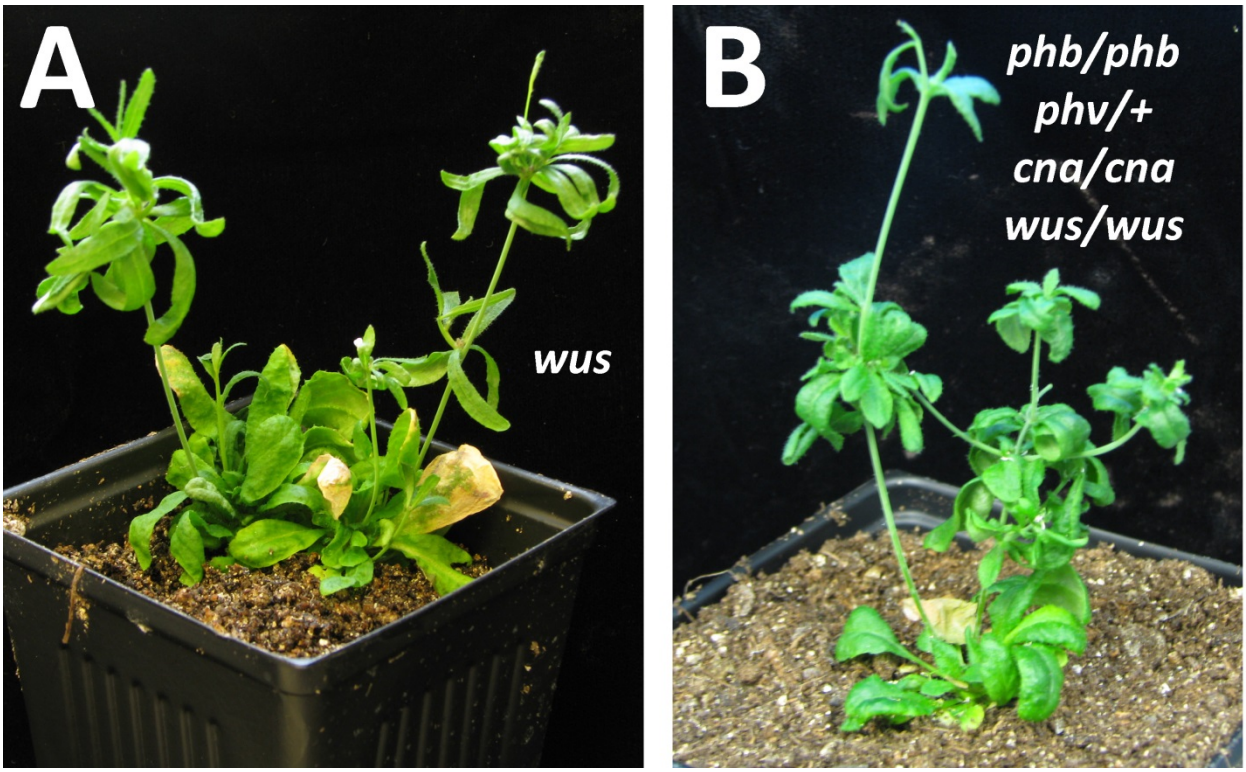

Figure 3.8. *wus* suppression requires *phb*, *phv* and *cna* homozygosity
Mature *wus-1*/*wus-1* and *wus-1*/*wus-1 phb*/*phb phv*/+ *cna*/*cna* plants are shown.

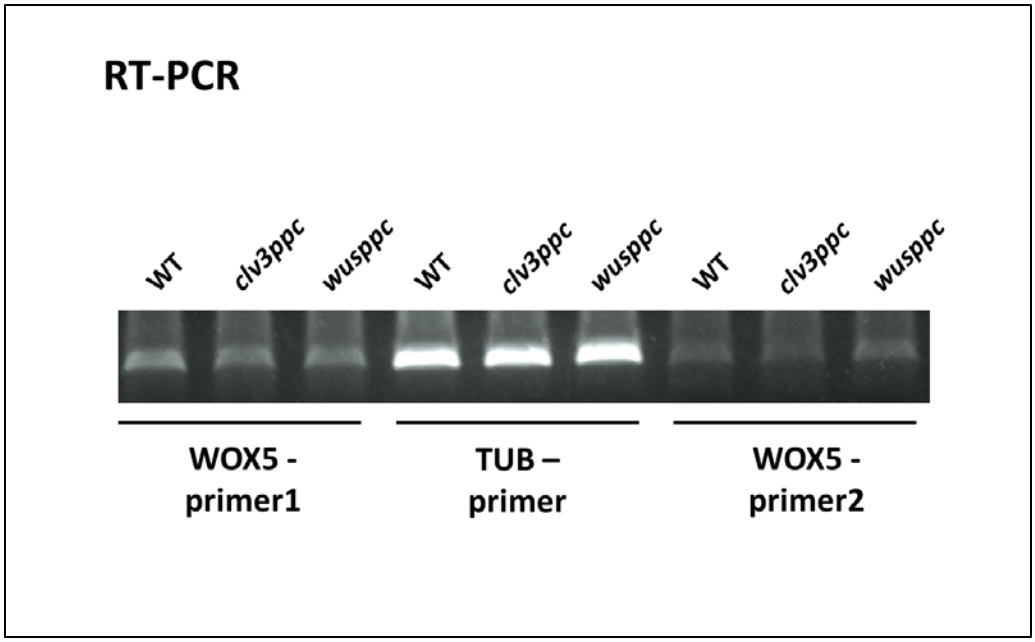

Figure 3.9. Semi-quantitative RT-PCR measuring *WOX5* and control *TUBULIN* transcripts in wild-type (WT), *clv3 phb phv cna,* and *wus phb phv cna* (*ppc*) 14 days-old seedlings.

Table 3.1. Primers used for *WUS* and *CLV3* transcript analysis

RT-PCR Primers	Forward	Reverse
WUS[1]	ACAAGCCATATCCCAGCTTCA	CCACCGTTGATGTGATCTTCA
CLV3[2]	GCTACTACTACTACTCTTCTGCTTCTTGTT	GCTGTCTTGGTGGGTTCACA
WOX-1	CGGCAAGATAGAGAGCAAGAA	GATCTAATGGCGGTGGATGT
WOX-2	CGGTGGAGCAGTTGAAGATA	CACCTTGGAGTTGGAGTCTT
TUB	AGAGGTTGACGAGCAGATGA	CCTCTTCTTCCTCCTCGTAC

WUS[1] : WUS primers were used as described [65].
CLV3[2] : CLV3 primers were used as described [66].

CHAPTER FOUR

Concluding Remarks

Stem cells are fascinating features of plant biology for several reasons. First, plants use stem cells to form the vast majority of their organ and tissues post-embryonically. This feature allows for developmental patterning that is sensitive to environmental conditions and to readily replaced tissue and organs lost to herbivory. Second, the meristem, while homeostatic in overall shape, is a dynamic structure constantly undergoing growth, division, differentiation and organogenesis. The life-long homeostasis of meristems requires a tight balance between stem cell growth/division and differentiation of appropriately positioned daughter cells. Several components regulating this stem cell establishment and maintenance have been identified and characterized.

The central factor regulating stem cell specification is WUSCHEL (WUS). WUS is a homeodomain transcription factor, which is expressed in Organizing Center (OC) [1-4]. The OC provides a signal to the overlying stem cells to maintain them as such. This signal may be the WUS protein itself, which appears to move directly into the overlying stem cells [5]. These recent observations on WUS protein carrying out niche-to-stem-cell signaling on its own matches well extensive genetic analysis showing WUS as both necessary and sufficient for stem cell specification [1-7]. *wus* mutants lack any evidence of shoot or flower stem cell formation, while *WUS* over-expression within the meristem leads to ectopic stem cell accumulation.

Many other factors have been described that alter the meristem and stem cell homeostasis when mutated and/or over-expressed [2, 6-11]. Those subjected to detailed analysis have reinforced the central role for WUS in stem cell specification.

From signal transduction to hormones to chromatin regulators, all appear to act through WUS [12-14].

The best characterized WUS regulatory system is the CLV signaling pathway. CLV signaling limits *WUS* expression to the basal daughter of L3 stem cells. In *clv* mutants, *WUS* expression expands into the apical L3 daughters, presumably enlarging the OC [3, 4]. The result of the enlarged OC in *clv* mutants is the accumulation of stem cells at the expense of differentiation.

POL/PLL1s are signaling intermediates of the CLV pathway that act between the CLV receptors at the plasma membrane and the *WUS* cis elements in the nucleus. The *pol pll1* double mutant is seedling lethal due to loss of asymmetric cell divisions in the basal portion of the early embryo; however, when the apical portion of a *pol pll1* seedling is grafted onto wild-type roots, the *pol pll1* tissue phenocopies *wus* mutants. *pol pll1* tissue lacks the ability to maintain *WUS* expression, indicating that the role of POL/PLL1 is to promote *WUS* expression in a manner that is delimited by CLV signaling [15-17].

Even though many efforts have been undertaken to expand the understanding of the CLV signaling pathway and *WUS* regulation, there remain large gaps in our knowledge. These difficulties may result from a number of issues. First, redundant genes are a common problem in higher plants, with single mutants failing to display mutant phenotypes. This is true for both POL and PLL1, as well as the CLV1-related receptors BAM1, BAM2 and BAM3 [15, 16, 18-20]. *STM* has a large number of homologous, partially redundant genes [21-24]. The HD-zip III transcription factor family is another clear example of the difficulty that genetic redundancy creates in identifying and characterizing gene function [25]. Other factors may remain unidentified if they have essential roles during gamete development and/or embryogenesis.

To bypass these obstacles, I used modifier mutagenesis screening in a *pol-6* background. My reasoning was that the *pol* single mutant has a barely detectable meristem phenotype, hence is a sensitized genetic background in meristem development. In other words, *pol* mutant meristems are just barely able to maintain stem cells. In this scenario, mutations in redundant stem-cell-promoting factors might exhibit a phenotype because of the existing *pol* mutation. Several putative candidates

were isolated and were crossed to L*er* to test if the mutant phenotype was dependent on *pol* and to create a mapping population. In this *pol* enhancer screening, I identified the novel *ago10-15* allele, displaying an early meristem termination phenotype in a *pol* dependent manner. Dr. Lindsey Gish mapped two other putative enhancers and identified *ago10-16* and *tonsoku* alleles.

AGO10 is a central component of the RNA induced silencing complex (RISC) and specifically binds to miR165/166 [26-29]. This is different from other characterized AGO proteins, which have much broader ranges of RNA targets [30-32]. AGO10 appears to use miR165/166-binding to sequester these miRNAs away from AGO1 [33]. Thus, AGO10 acts to protect the mRNAs of the HD-zip III family of genes from repression by miR165/166. While all of these factors (AGO1, AGO10, miR165/166, HD-zip IIIs) have been shown to influence meristem development, the relationships between the AGO10/miR165/166/HD-zip III and the CLV/WUS pathway has remain largely unexplored. Evidence suggesting that AGO10 may regulate shoot meristem in parallel to the CLV pathway comes from the exclusively lateral expansion of *pWUS:GUS* expression by over-expression of miR166g in the activation-tagged *jba-1D* plants, whereas in *clv* mutants, *WUS* expression expands laterally and apically [34]. On the other hand, when I made *ago10^{zll-3}clv3-2* double mutants, they did not show enhancement of shoot or flower meristem enlargement compared to *clv3-2* alone. This is consistent with the previously described *ago10^{zll-3}clv1-4* double which had similar flower meristem defects as *clv1-4* alone [35].

In this study, I demonstrate that PHB/PHV/CNA regulate stem cell specification in parallel with CLV signaling. The first evidence for this conclusion came from creating *clv3 phb phv cna* quadruple mutants. *clv3 phb phv cna* dramatically enhanced shoot and flower meristem accumulation compare to either *clv3-2* or *phb phv cna*. Indeed, the shoot meristems of these plants were often unable to form any differentiated cells for long developmental periods. In addition, the disrupted cell layering pattern of *clv* mutants was observed in that of *clv phb phv cna*, but not in the *phb phv cna*, indicating cell division pattern of shoot meristem are differently affected by *clv* and *phb phv cna* mutations.

Previous studies had indicated that AGO10/miRNA acted through *WUS*. First, *wus* was shown to be epistatic to *jba-1D*, which is an activation-tag allele of *miR166g* [34]. Second, another *ago10* allele, *ago10-12* was crossed to *wus-1* to created *hua1 hua2 ago10-12 wus-1* plants. *hua1 hua2 ago10-12 wus-1* quadruple mutant flowers had identical phenotypes to *wus-1*, suggesting *wus-1* is epistatic to *hua1 hua2 ago10-12* in terms of floral determinacy [36]. Given this background, I expected *wus* to be epistatic to *phb phv cna*. Indeed, *wus* has been shown to be epistatic to all tested meristem-enhancing mutants to date, reinforcing its central role in stem cell specification [12-14].

During the genetic crosses to create the *wus phb phv cna* quadruple mutant, *wus* appeared epistatic as long a single *PHB*, *PHV* or *CNA* allele was retained. However, once the fully homozygous *wus phb phv cna* quadruple was formed, it was clear that these plants were suppressed compared to *wus* single mutants. I found that the plants were capable of extensive organogenesis from structures containing all of the features of shoot meristems: (1) organogeneic capacity; (2) dome-shaped morphology; (3) clear L1/L2 layering pattern; (4) activation of the cis elements for the OC marker *WUS*. This is the first clear evidence showing that WUS is dispensable for stem cell initiation and maintenance.

By characterizing *wus phb phv cna* and *clv3 phb phv cna* quadruple mutant plants, I have provided key insights into understanding stem cell formation. It is clear that major unknown factors are responsible for stem cell specification and many aspects of meristem organization and function. Among these factors are presumably genes targeted for repression by PHB/PHV/CNA.

In conclusion, my findings have changed our current model for meristem development [14] (Figure 4.1). We now propose that AGO10 competes with AGO1 for a limited pool of miR165/166. The role of AGO10 is to limit meristem size by protecting PHB/PHV/CNA from miR165/166. In turn, PHB/PHV/CNA repress a stem cell specification pathway that acts in parallel with the CLV/WUS pathway. The fact that the meristems formed by wild-type WUS activity and those formed in the absence of WUS through the inactivation of PHB/PHV/CNA are so similar in morphology and function suggests that the two pathways act on a common set of factors. A critical question that

we cannot determine at this time is where in the meristem PHB/PHV/CNA are active, and where they are repressed. The level of activity of PHB/PHV/CNA will rest on many factors.

(1) Where are PHB/PHV/CNA expressed? These genes have been assayed by RNA in situ hybridization, where they show very complex patterns within the shoot meristem [37-39]
(2) Where are the miR165/166 genes expressed? As mentioned, there are nine genes in total encoding these miRNAs. The expression for these genes has only been examined at a superficial level [40].
(3) The relative AGO1 and AGO10 activities within or near the meristem. Because the activities of miR165/166 are dependent on the relative activities of AGO1/AGO10 does to their antagonistic function. This information is unknown. One interesting finding is that within the embryonic shoot meristem (where these genes all have the opposite function), AGO10 acts on the meristem from adjacent, underlying tissue [41].
(4) Where are the ZPR proteins active? The ZPR protein family is truncated leucine-zipper proteins that can bind to and thus inactivated HD-zip III proteins [42, 43]. Where this family of redundant proteins act in detail is not understood.
(5) What is the nature of REV antagonism? Whether REV antagonizes PHB/PHV/CNA by directly binding to through another mechanism is unknown. While the transcript accumulation for *REV* in the meristem is known, where the protein is active is unknown [44]. Furthermore, REV activity will also be impact by miR165/166 expression, AGO1/AGO10 activity and ZPR activity.

Answering these questions will be important for unraveling how stem cell specification is controlled in plants. In addition, identifying the unknown factors in stem cell specification that are targeted by PHB/PHV/CNA and carry out actual stem cell maintenance is clearly an obstacle that needs to be overcome. Thus, several strategies for isolating WUS-independent stem cell regulators are suggested:

(1) Mutagenic screening using the *phb phv cna* triple mutant. The goal would be to screen for enhancers and suppressors of the meristem enlargement of *phb phv cna* mutants. For example, a *phb phv cna* enhancer would have an enlarged shoot meristem and flower meristem compare to *phb phv cna*. A *phb phv cna* suppressor mutant would be identified by plants with smaller shoot meristems (reduced fasciation) and/or fewer flower organs. Both of these phenotypes can be readily screened in large populations. Stem-cell promoting factors that are targeted for repression by PHB/PHV/CNA would be expected to fall into this category.

(2) A yeast two-hybrid screen with PHB, PHV, or CNA and a shoot meristem cDNA library may identify HD-zip III-interacting proteins.

(3) RNA-sequencing of collected stem cells from wild type and *wus phb phv cna.* If gene expression level is changed in *wus phb phv cna*, those genes would be candidate genes, which may be expressed for stem cell establishment in WUS-independent manner.

(4) One candidate target gene of PHB/PHV/CNA is *REV*. First, REV and PHB/PHV/CNA have antagonistic roles in shoot and flower meristem development. Critically, *rev* mutants lack lateral shoot and flower meristems, while *phb phv cna* triple mutants have enlarged shoot and flower meristems. Second, when *REV* transcript level was differentially affected by the *jba-1D* and *men1* mutants, the meristems were either enlarged or reduced, respectively. This suggests that *REV* transcript level is important to establish and maintain shoot and flower meristem homeostasis.

To test if *REV* is necessary for stem cell establishment in *wus phb phv cna*, I would cross *men1* mutant, which is an activation-tag allele of *miR166a*, expected to drive reduced *REV* transcripts to the *wus/+ phb phv cna* mutant. If no meristem structures are observed in *wus men1 phb phv cna* quintuple mutants, *REV* is necessary to establish stem cells in *wus phb phv cna*.

To test if *REV* expression could bypass the loss of flower meristem initiation in a *wus* mutant, I would set expression of *REV* under the control of the flower-specific *APETALA (AP1)* cis regulatory elements in a transactivation system, in which

AP1-driven *REV* expression would only occur in the progeny of plants carrying both the *PAP1:LHG4* driver and the *POP6:REV* responder. If the restoration of flower meristem activity by *PAP1:REV* in *wus is observed, REV* is sufficient to initiate flower meristems in *wus* mutant background.

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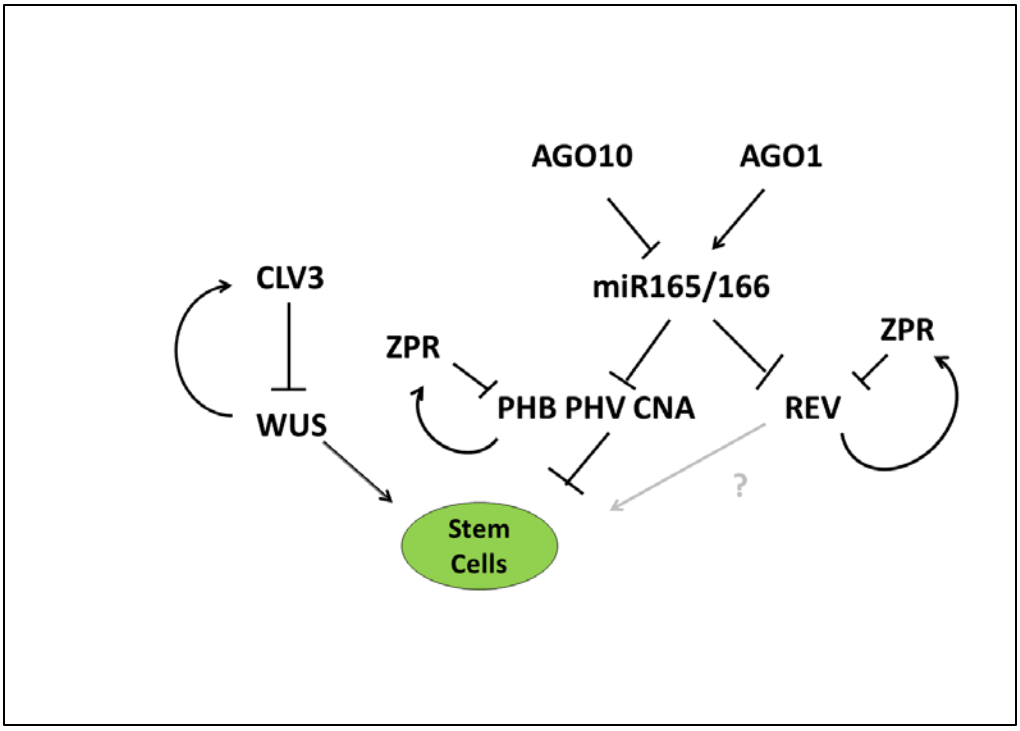

Figure 4.1. a Model of PHB PHV CNA regulate stem cell independently of WUS