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Review

A diverse and intricate signalling network regulates stem cell fate in the shoot apical meristem

Steven Dodsworth *

Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EA, UK

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ABSTRACT

At the shoot apex of plants is a small region known as the shoot apical meristem (SAM) that maintains a population of undifferentiated (stem) cells whilst providing cells for developing lateral organs and the stem. All aerial structures of the plant develop from the SAM post-embryogenesis, enabling plants to grow in a characteristic modular fashion with great phenotypic and developmental plasticity throughout their lifetime. The maintenance of the stem cell population is intimately balanced with cell recruitment into differentiating tissues through intercellular communication involving a complex signalling network. Recent studies have shown that diverse regulators function in SAM maintenance, many of which converge on the *WUSCHEL* (*WUS*) gene. In this review the diverse regulatory modules that function in SAM maintenance are discussed: transcriptional and epigenetic control, hormonal regulation, and the balance with organogenesis. The central role of *WUS* as an integrator of multiple signals is highlighted; in addition, accessory feedback loops emerge as a feature enabling dynamic regulation of the stem cell niche.

Introduction

Stem cells are undifferentiated cells that retain the ability to later differentiate into specific cell types. In plants pluripotent stem cells are confined to specialised regions known as meristems that persist post-embryogenesis. The term 'meristem' was first used by the Swiss botanist Carl Wilhelm von Nägeli in his book "Beiträge zur Wissenschaftlichen Botanik" (1858), derived from the Greek *merizein* ('to divide'). The term meristem therefore pertains to their function of providing cells for all tissues (Lyndon, 1998; Tooke and Battey, 2003). This contrasts with animals, where adults lack a pluripotent stem cell niche and cell lineage plays a large role in embryonic development of animal form. Plants, however, rely on positional cues from neighbouring cells, continuously generating new structures throughout their lifetime and developing in a modular fashion; the action of meristems is therefore crucial to such phenotypic plasticity. The longevity of this stem cell niche is astonishing, and it allows some plants to grow for hundreds or thousands of years such as the living fossil *Welwitschia mirabilis*.

In the meristems, two opposing processes are at work—that of self-renewal to maintain a population of stem cells and that of cell recruitment out of the meristem into developing organs. The number of stem cells in meristems remains remarkably constant despite continual displacement of differentiating cells into organogenesis (Lyndon, 1998; Laufs et al., 1998; Carles and Fletcher, 2003). This indicates that the formation of new stem cells and loss of cells into differentiation is dynamically and almost perfectly balanced (Laufs et al., 1998; Haecker and Laux, 2001). Two apical meristems—the shoot apical meristem (SAM) and root apical meristem (RAM) are responsible for almost all growth of the plant post-embryogenesis, forming the above-ground and below-ground structures respectively. There are also various other types of meristems found in plants, besides the two apical ones: lateral meristems (e.g. axillary in the nodes of leaves, floral meristems), intercalary meristems (e.g. at the base of monocot leaf blades), and meristemoids (precursors of guard cells). Meristems are absolutely essential for plant development and have thus been a topic of intense research for over 100 years.

A deeper understanding of how meristems are formed and maintained has been strongly influenced in recent years by the rise of the model system *Arabidopsis thaliana* and the field of molecular genetics. An exciting discovery was that of a homeobox transcription factor WUSCHEL (WUS), essential for SAM maintenance (Laux et al., 1996). The name of this transcription factor translates as 'fuzzy', so-called in recognition of the disorganised SAMs of *wus* mutants that undergo premature termination. A feedback loop exists between WUS and a glycopeptide signalling pathway encoded by the *CLAVATA* (*CLV*) genes (Schoof et al., 2000). This feedback loop is critical in specifying and maintaining the stem cell niche in a small region of the shoot apex; the niche is a spatial microenvironment in which cells remain in an undifferentiated state in response to positional signals from their

* Fax: +44 1223 333953.
E-mail address: sd426@cam.ac.uk.

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neighbours (Scheres, 2007; Sablowski, 2007; Tucker and Laux, 2007). Cells contained within the niche remain undifferentiated and those which are displaced from the niche undergo differentiation.

This comprehensive review focuses on summarising recent findings and placing them into a contextual framework in order to understand how different pathways and diverse regulators may converge to control the dynamic balance in the SAM. Central themes and key players are discussed, and an integrated model of SAM regulation is proposed.

Dynamic architecture of the SAM

The SAM in *Arabidopsis* consists of approximately 500 cells and can be divided into distinct regions; its anatomy is defined by particular zones and cell layers (Reddy, 2008). Three clonally distinct cell layers (L1–3) are specified: L1 consists of cells at the very tip of the apex that only divide in the anticlinal plane (perpendicular to the surface, thereby spreading laterally) and eventually give rise to the epidermis. Cells in the subepidermal L2 also divide anticlinally (almost exclusively) and give rise to mesophyll cells; those in L3 divide in various random planes (anticlinally and periclinally), and form the central tissues of the leaf and stem (Meyerowitz, 1997; Weigel and Jurgens, 2002; Williams and Fletcher, 2005). In most dicots the surface region, or tunica, consists of the L1 and L2 layers; however in most monocots the tunica consists of just one (L1) layer (Carles and Fletcher, 2003; Kim et al., 2007).

Three functional and cytohistological zones can be juxtaposed onto the cell layers: the central zone (CZ) which contains the pluripotent stem cells themselves, the multipotent peripheral zone (PZ) where differentiation into lateral organs begins, and the rib zone (RZ) which provides multipotent cells for the differentiating stem supporting the SAM (Fig. 1; Lyndon, 1998). Recent work utilising live-imaging of the SAM has provided a more detailed view and better definition of the size and function of different SAM domains. In *Arabidopsis* there are approximately 35 stem cells in the CZ (based on *CLV3* as a molecular marker of stem cells), compared to about nine previously expected from cytological observations alone (Stewart and Dermen, 1970; Reddy and Meyerowitz, 2005; Yadav et al., 2009). The peripheral zone can be further subdivided into the outer peripheral zone (OPZ) and inner peripheral zone (IPZ). Cells located within the IPZ are able to revert back to stem cell fate, but cells located in the OPZ are committed into differentiation pathways; immediate progeny of the stem cells retain the ability to respond to the stem cell-promoting factor, but this ability is lost as cells progress through the IPZ and into the OPZ (Reddy and Meyerowitz, 2005; Reddy, 2008).

Cell division in the CZ is rather slow and the number of CZ cells remains extraordinarily constant. A possible ultimate explanation for this is that it protects the fidelity of the genome by reducing the number of mutations (Irish and Sussex, 1992). A recent study provides support for this idea as enzymes involved in DNA replication and repair are overrepresented in the CZ region (stem cells). The error-free replication of DNA is essential to prevent amplification of mutations and double strand breaks in the daughter cells emerging from the SAM, which would otherwise hinder plant development (Yadav et al., 2009). It has also been recently shown that *BRCA2*-like genes in *Arabidopsis* are required for efficient double strand break repair through homologous recombination and thereby maintain genomic stability; mutants in *BRCA2*-like genes also show altered *WUS* expression and SAM disorganisation (Abe et al., 2009). In the adjacent PZ, however, cells are dividing much more rapidly to provide millions of cells for growth of the developing organ primordia (Clark, 2001; Reddy et al., 2004; Williams and Fletcher, 2005; Reddy, 2008).

A central feedback loop: CLV–WUS signalling is essential for SAM maintenance

WUSCHEL (*WUS*) encodes a homeodomain transcription factor of the WOX (*WUSCHEL-like homeobox*) gene family (Laux et al., 1996), and is expressed in a group of approximately 10 cells directly underlying the stem cells in the L3 and deeper layers, known as the organising centre—OC (Fig. 1). Mutants lacking *WUS* function show premature termination of the SAM, stem cells are not maintained and are consumed by developing organ primordia (Laux et al., 1996; Mayer et al., 1998; Brand et al., 2000; Schoof et al., 2000). Expression of *WUS* ectopically promotes stem cell fate, and therefore *WUS* expression must be tightly confined to the apical region of the SAM. The signal from *WUS*-expressing cells to the overlying stem cells is currently unknown (Lenhard and Laux, 2003).

Regulation of *WUS* expression occurs by a myriad of regulators, but most notably via the *CLAVATA* (*CLV*) pathway. The signal produced by *WUS*-expressing cells of the OC stimulates *CLAVATA3* (*CLV3*) expression. *CLV3* encodes a 96-amino acid protein with a secretory signal peptide sequence in its N-terminal region (Schoof et al., 2000; Miwa et al., 2009). Loss-of-function *clv3* mutants show enlarged SAMs as the expression domain of *WUS* expands. Over-expression of *CLV3* causes premature termination of the SAM (Mayer et al., 1998; Brand et al., 2000; Miwa et al., 2009). CLV3 thus acts as a signal to repress *WUS* expression, and forms a negative feedback loop with *WUS* (Fig. 1; Lenhard and Laux, 1999; Fletcher and Meyerowitz, 2000). The signal from *WUS* to the overlying stem cells could represent a gradient of stem cell-promoting activity that respecifies some PZ cells as CZ ones thereby maintaining the stem cell pool. This respecification/de-differentiation is responsible for the initial enlargement of the CZ in *clv* mutants, as shown by recent live-imaging of the SAM upon transient *CLV3* inactivation (Reddy and Meyerowitz, 2005). The converse has also been shown; inducible upregulation of *CLV3* led to a downregulation of *WUS* and restriction of the CZ domain as it became consumed by developing organ primordia (Muller et al., 2006).

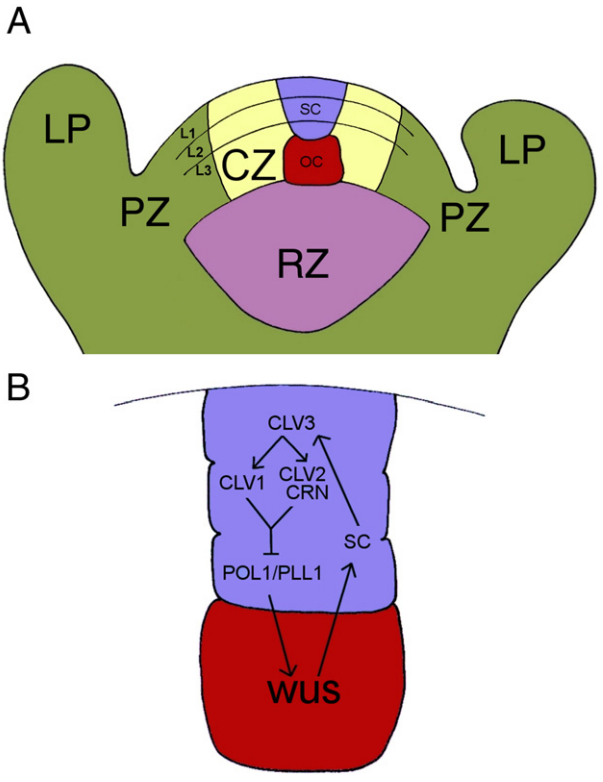

Fig. 1. (A) Basic architecture of the shoot apical meristem. The central zone (CZ), peripheral zone (PZ), rib zone (RZ), stem cells (SC), organising centre (OC), and leaf primordia (LP) are indicated. L1–3 represent the three clonally distinct cell layers. (B) Components of the central feedback loop between *CLAVATA* (*CLV*) and *WUSCHEL* (*WUS*).

There has been much debate over the form of the mature functional CLV3 peptide; however its identity has recently been elucidated, using a combination of MALDI-TOF MS and nano-LC-MS/MS analyses (Kondo et al., 2008; Ohyama et al., 2009). Mature CLV3 is a secreted glycopeptide (13 amino acids) with two hydroxyproline (Hyp) residues. Post-translational arabinosylation of one of the Hyp residues is critical for physiological function and sufficient biological activity. Exogenous application of an artificial CLV3 peptide results in a phenotype similar to the *CLV3* overexpression one, suggesting that this short peptide is the active form of CLV3; similarly exogenous application of CLV3 can rescue the *clv3* phenotype. Very recent results suggest that without the arabinose glycosylation of Hyp residues, the peptide has much reduced activity and a weaker binding affinity to the CLV1 receptor (Ohyama et al., 2009). *CLV3* is expressed in stem cells of all of the three clonal layers (L1–3), with expression occurring down to the fourth layer from the apex (Fletcher et al., 1999; Reddy and Meyerowitz, 2005; Yadav et al., 2009). *CLV3* and *WUS* expression overlap in the L3 layer. CLV3 acts non-cell-autonomously diffusing downwards where it is perceived by several receptors/receptor complexes (Jun et al., 2008; Miwa et al., 2009).

Attention has been drawn to revealing the downstream factors of *CLV3*, and recent results have shed light on the receptors that mediate CLV3 perception. *CLAVATA1* (*CLV1*) encodes a leucine-rich repeat (LRR) receptor-like kinase, and *CLV2* encodes an LRR receptor-like protein lacking a kinase domain (Dievart et al., 2003; Jun et al., 2008; Miwa et al., 2009). Biochemical evidence now exists for a ligand/receptor relationship between the LRR domain of CLV1 and CLV3 (Kondo et al., 2008; Ogawa et al., 2008), but the interaction between CLV2 and CLV3 remains elusive. *CORYNE*/*SUPPRESSOR OF LLP1 2* (*CRN*/*SOL2*) encodes a receptor-like kinase with a short extracellular domain (Miwa et al., 2008; Muller et al., 2008). The *crn*/*sol2* mutant phenotype is similar to that of *clv* mutants, and therefore *CRN*/*SOL2* has been implicated in the *CLV–WUS* pathway. Expression domains of *CLV3* and *WUS* are expanded in the *crn*/*sol2* mutant, as with *clv1*/*2* mutants, suggesting that *crn*/*sol2* similarly negatively regulates *WUS* expression. The *wus* mutation is epistatic to *crn*/*sol2*, indicating that *CRN*/*SOL2* act upstream of *WUS* (Miwa et al., 2008; Muller et al., 2008; Miwa et al., 2009). The *clv1 crn* double mutant has an additive effect on carpel number; however the *clv2 crn* double mutant has a carpel number similar to that of the single mutants. This suggests that CLV2 and CRN might act together but independently of CLV1 (Miwa et al., 2008). It is thought that CRN and CLV2 complement each other as CRN lacks a distinct extracellular domain and CLV2 a kinase domain, but together they establish a functional receptor. CRN/SOL2 and CLV2 therefore act to mediate CLV3 signalling in parallel to CLV1 (Lenhard and Laux, 2003; Miwa et al., 2008). In contrast to the restricted expression domain of *CLV1* in the central zone, *CRN* and *CLV2* are expressed in many plant tissues. The comparable severe mutant phenotypes of *clv1*, *clv2* and *crn* suggest that the two proposed pathways contribute to CLV3 signal transduction at a similar level (Muller et al., 2008; Miwa et al., 2009). Other receptors may be involved, and much less clear is the role of the receptors BAM1 and BAM2—receptors related to CLV1. *bam1*/*2* mutants enhance the *clv1* phenotype, suggesting that they may work redundantly with CLV1 to mediate CLV3 perception (DeYoung and Clark, 2008).

Factors downstream of CLV3 perception have been difficult to reveal, but recent results point to the phosphatases *POLTERGEIST* (*POL1*) and *POL1-LIKE 1* (*PLL1*) (Yu et al., 2000, 2003; Song et al., 2006). All single *pol* mutants are nearly indistinguishable from wild-type, hence the name *POLTERGEIST* ('noisy ghost') as the effect of *pol* is revealed when associated with other mutations. Mutations in *POL1* and *PLL1* suppress the *clv1*, *clv2* and *clv3* phenotypes, placing them downstream of *CLV* genes (Song et al., 2006; Miwa et al., 2009). Overexpression of *POL1*/*PLL1* prevents differentiation and causes stem cell accumulation, exacerbated in a *clv* mutant background. *POL1* and *PLL1* act redundantly to maintain *WUS* transcription, and are negatively regulated by CLV signalling (Yu et al., 2003; Song et al., 2006; Miwa et al., 2009). Interacting partners and downstream targets of *POL1*/*PLL1* are currently unknown.

Bioinformatics database searches have revealed *CLV3*-like genes, which have a conserved 14-amino acid region in the C-terminal region of the protein. This 14 AA domain is now named the CLE domain, after *CLV3* of *Arabidopsis* and *EMBRYO SURROUNDING REGION* (*ESR*) of maize. Thirty-two *CLE* genes are present in the *Arabidopsis* genome and *CLE* genes are expressed in multiple tissues during development, suggesting they may be involved in many aspects of development and morphogenesis (Jun et al., 2008; Sawa et al., 2008; Miwa et al., 2009). Unfortunately no loss-of-function *cle* mutant has been isolated other than *clv3*, to date. However analysis of *CLE* overexpression has provided some clues as to the function of this enlarged family of CLV3-like peptides. Analyses of overexpression and synthetic peptide treatment assays have identified 10 CLE peptides that arrest SAM growth (Strabala et al., 2006; Fiers et al., 2007; Miwa et al., 2009). This may indicate that CLE peptides are at least in part redundant in the SAM, but will require further analysis of multiple loss-of-function mutations and detailed expression patterns.

Conservation of CLV–WUS signalling across the plant kingdom

The discovery of the CLV–WUS loop and its importance in regulating the SAM of *Arabidopsis* raises an obvious question: Do other plants, or perhaps all plants, use a similar genetic mechanism to maintain the SAM? *WUS* homologues have been identified in many plants including the dicots *Antirrhinum majus*, *Petunia hybrida*, *Citrus sinensis*, *Streptocarpus rexii*, and *Solanum lycopersicum* that span several orders of eudicot angiosperms (Fig. 2). In these species, *WUS* appears to be functionally conserved. Mutants in most of these homologues show similar phenotypes to that of the *Arabidopsis wus* mutant, including premature termination of the SAM (Stuurman et al., 2002; Reinhardt et al., 2003; Tan and Swain, 2007; Mantegazza et al., 2009). Expression analyses show that the homologous genes are also expressed in a similar region, underlying the stem cells in the organising centre. A slightly unusual situation is shown by the acaulescent species *S. rexii*, which displays anisocotyly (unequal growth of the cotyledons—seed leaves) and lack of a typical SAM. However, the expression of *WUS* correlates with meristematic activity and stem cell identity, albeit not in the same location as in species with conventional SAMs (Mantegazza et al., 2009).

Homologues of *WUS* have also been identified in monocots, including *Oryza sativa* (rice), *Zea mays* (maize), and *Brachypodium distachyon*. In monocots though, the function of *WUS* seems to have diversified. In rice, *OsWUS* is the orthologue of *WUS*, in maize there are two paralogues *ZmWUS1*/*2*. These genes in both grasses are expressed in the periphery of the meristem and in leaf primordia, and only transiently expressed in the SAM. In contrast, they are expressed in the central region of the SAM (along with CLV1 orthologues) during the reproductive phase (Nardmann and Werr, 2006). Cells that go on to develop the grass leaf are recruited from the entire circumference of the SAM, in contrast to just a few cells in *Arabidopsis* (Nardmann and Werr, 2006; Miwa et al., 2009). The markedly different expression patterns of *OsWUS* and *ZmWUS1*/*2* may therefore be a by-product of the unusual architecture of the grass culm (stem) and developmental mechanism for leaf development, and implies that other mechanisms exist to regulate the vegetative SAM in grasses (Nardmann and Werr, 2006). Very recently, phylogenetic and expression analyses have investigated the ancestry of the *WUS*/*WOX* gene family in basal angiosperms and gymnosperms. It appears that the ancestral function of *WUS* may be in developing lateral organ primordia. However the stem cell-promoting function of *WUS* still appears to be conserved in angiosperms and gymnosperms, although the precise expression pattern found in *Arabidopsis* is not a feature common to all plants (Nardmann et al., 2009).

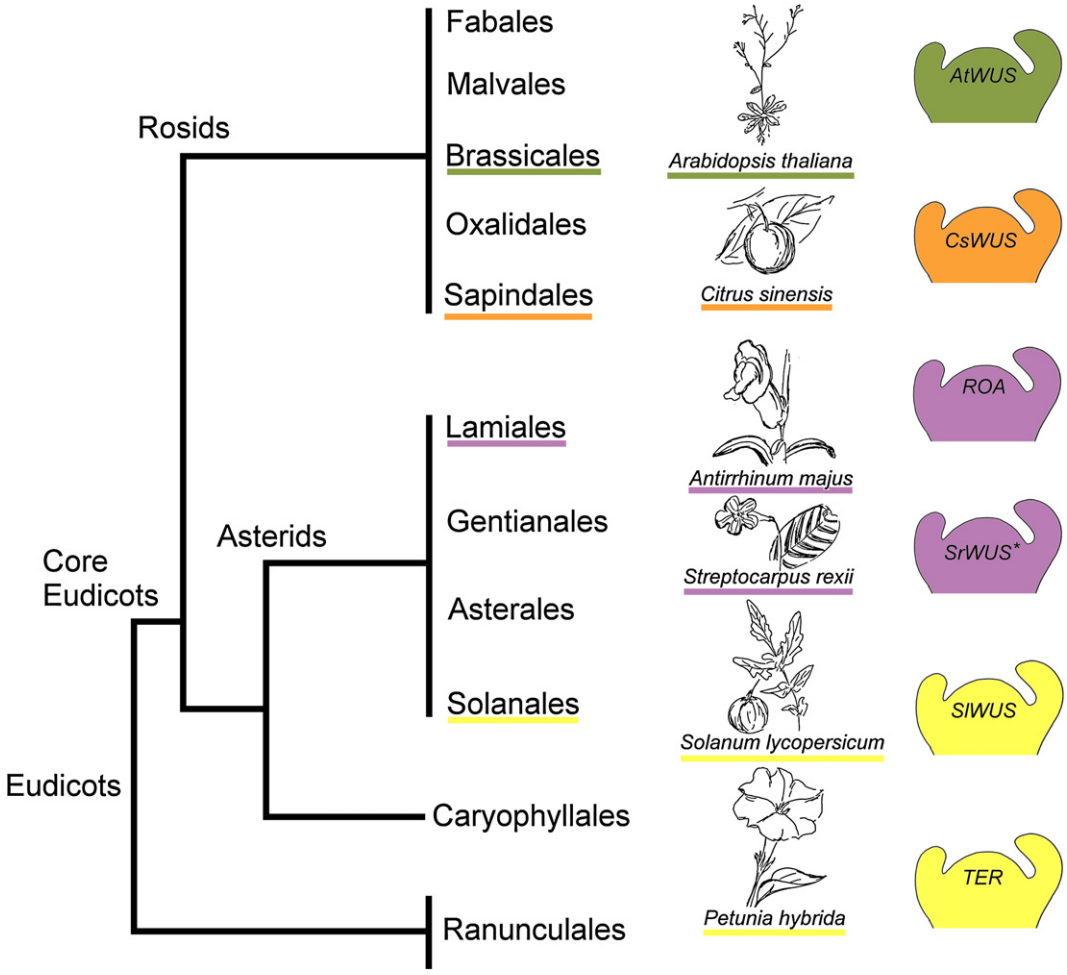

Fig. 2. Evolutionary context: A representative sample of *WUSCHEL* homologues with conserved function are shown mapped onto a simplified phylogeny of the eudicot angiosperms (phylogeny based on Rosin and Kramer, 2009; Burleigh et al., 2009). **SrWUS*—in this acaulescent *Streptocarpus* species the spatiotemporal organisation of the shoot meristem is atypical, however *SrWUS* expression correlates with meristematic activity as would be predicted.

Genes encoding several CLE peptides and their receptors have also been found in a diverse range of plant species (Yamamoto et al., 2000; Oelkers et al., 2008; Sawa et al., 2008), including both monocots (*O. sativa*, *Z. mays*) and dicots (*Glycine max*, *Populus trichocarpa*, *Medicago truncatula*). *CLE* genes are found in the moss *Physcomitrella patens* and even in the alga *Chlamydomonas reinhardtii* (Miwa et al., 2009), and represent one of the largest families of plant polypeptides (Jun et al., 2008). In rice, *FLORAL ORGAN NUMBER 1* (*FON1*) encodes the orthologue of *CLV1*, and loss-of-function mutations in *FON1* result in enlarged meristems and an increased number of floral organs. *FON2* and *FCP1* (*FON2-LIKE CLE PROTEIN1*) encode *CLE* peptides similar to *CLV3*. *FON2* (together with *FON1*) regulate the maintenance of reproductive (inflorescence/floral) meristems, whereas *FCP1* regulates the vegetative SAM and RAM. Thus *FON2* and *FCP1* have diversified in function to regulate the different types of meristem in rice (Suzaki et al., 2008). Orthologues of *CLV1* and *CLV2* have also been isolated in maize—*THICK TASSEL DWARF1* (*TD1*) and *FASCIATED EAR2* (*FEA2*), respectively. However in maize loss-of-function of *TD1*/*FEA2* does not affect vegetative SAM development and maintenance but only floral organ number, in contrast to *CLV1* and *CLV2* in *Arabidopsis*. It has also been shown that *TD1* is not expressed in the vegetative SAM (Bommert et al., 2005; Lunde and Hake, 2009). Whilst *CLV* pathways are present in monocots and CLE peptides play a role in the meristems, the signalling mechanisms and precise function have diversified between monocots and dicots. *CLV1* orthologues in legumes have been shown to play a role in nodule formation rather than SAM maintenance, for example *SUPERNUMERIC NODULES* (*SUNN*) in *M. truncatula* and *HYPERNODULATION ABERRANT ROOT 1* (*HAR1*) from *Lotus japonicus* (Miwa et al., 2009). Mutants in these genes show an increased number of root nodules (with symbiotic nitrogen-fixing bacteria), but no apparent defects in SAM maintenance (Searle et al., 2003; Schnabel et al., 2005). Thus even within the eudicots there has been some diversification of *CLE* gene function.

Transcriptional and epigenetic regulation of WUS: a master regulator of the SAM

The expression of *WUS* is regulated (both positively and negatively) through the activity of various transcription factors and chromatin remodelling factors, in addition to regulation by the glycopeptide CLV3. It is vital that *WUS* expression is maintained and also confined to the organising centre, and many factors act to redundantly regulate this precise expression pattern. The way in which all of these inputs converge to regulate *WUS* expression is currently unknown; however recent work has identified a small region of 57 bp in the promoter of *WUS* that is sufficient in itself to confer the exact expression pattern of *WUS* (Williams and Fletcher, 2005; Baurle and Laux, 2005). This may also imply the action of a single *trans*-activating complex that binds to the two short sequence motifs within the 57 bp region and combines multiple signals to regulate *WUS* expression (Baurle and Laux, 2005). Only two factors to

date have been shown to interact directly with the *WUS* promoter—the chromatin remodelling factors *SPLAYED* (*SYD*) and *BRCA1-ASSOCIATED RING DOMAIN 1* (*BARD1*).

Positive transcriptional regulators: *STIMPY* (*STIP* or *WOX9*) is a positive regulator of *WUS* expression, and encodes a WUS-related homeobox transcription factor (Wu et al., 2005). *stip* mutants lack a functional SAM and the expression of *CLV3* and *WUS*. However the *stip* mutant phenotype can be fully recovered by exogenous addition of sucrose, simulating entry into the cell cycle. Genetic analyses show that loss of *STIP* function suppresses the *clv3* phenotype (enlarged SAM); however overexpression of *STIP* enhances the *clv3* phenotype (Wu et al., 2005). It is therefore postulated that *STIP* acts via maintaining cell division and preventing differentiation, at least partly through positive regulation of *WUS* (Wu et al., 2005; Kim et al., 2007). *STIP* is also subject to negative regulation by the *CLV* genes, forming an auxiliary negative feedback loop (Fig. 4).

APETALA 2 (*AP2*) was originally identified as an A-function floral homeotic gene of the ABC model of floral development. Recently however, *AP2* has been implicated in various other aspects of plant development, including stem cell maintenance in the SAM (Wurschum et al., 2006). The exact mechanism of *AP2* regulation is not yet clear, but it appears it either positively regulates *WUS* (antagonising CLV signalling) or negative regulates signalling downstream of *CLV3*. *ap2* mutants are similar to *wus* mutants and the SAM undergoes premature termination (Wurschum et al., 2006). *OBERON1* (*OBE1*) and *OBE2* encode homeodomain finger proteins that function redundantly (Saiga et al., 2008). The single mutants of these genes show no phenotype, however the double mutant *obe1 obe2* shows termination of the SAM. Levels of both *WUS* and *CLV* gene expression are dramatically reduced in the double mutant. It is suggested that these proteins are required for establishing an appropriate state for SAM regulation via meristem maintenance genes, rather than by specification of the meristem *per se* (Saiga et al., 2008; Miwa et al., 2009). *MERISTEM DEFECTIVE* (*MDF*) encodes an RS domain protein, with a putative role in transcriptional regulation or RNA processing. Several lines of evidence point to a role in meristem maintenance: *mdf* mutants show SAM termination and reduced *WUS* expression, and overexpression of *MDF* causes ectopic meristem activity. These data suggest that *MDF* may positively regulate *WUS* in the vegetative SAM (Casson et al., 2009).

Negative transcriptional regulators: *ULTRAPETALA1* (*ULT1*) encodes a SAND-domain transcription factor that negatively regulates *WUS* expression during later stages of development (Carles et al., 2005). *ULT1* is expressed in developing floral organs and in the embryonic SAM. Mutations in *ULT1* causes enlarged inflorescence and floral meristems suggesting that *ULT1* is important in regulating *WUS* expression post the floral transition (Williams and Fletcher, 2005; Carles et al., 2005). The *ult1 clv* double mutants show that *ULT1* and *CLV* genes act in independent pathways to negatively regulate *WUS* expression, and the *ult1 wus* double mutant shows an additive phenotype—thus implying *ULT1* has *WUS*-independent activity (Carles et al., 2005). *HANABA TARANU* (*HAN*) encodes a GATA-3-like transcription factor and its expression defines a boundary between the central domain of the meristem and organ primordia. *HAN* controls the number and position of *WUS*-expressing cells and its activity is independent of the *CLV* pathway (Zhao et al., 2004; Miwa et al., 2009).

Five class III genes of the homeodomain leucine zipper family (HD-ZIP III) are found in the *Arabidopsis* genome, and have recently discovered roles in plant developmental processes including specification of lateral organ polarity (adaxial-abaxial fates) and meristem functioning: *CORONA* (*CNA*), *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), *REVOLUTA* (*REV*) and *ATHB8*. In wild type plants, *CNA*, *PHB* and *PHV* downregulate *WUS* transcription and thus confine *WUS* (Carles and Fletcher, 2003; Emery et al., 2003; Green et al., 2005; Williams and Fletcher, 2005). Triple mutants in these genes show an enlarged SAM and over-accumulation of stem cells, correlating with expansion of *WUS* expression (Emery et al., 2003).

Regulation of HD-ZIP III genes occurs through RNA silencing and protein dimerisation, adding a novel level of regulation and complexity to the picture. The miR165/166 group of microRNAs (miRNAs) in *Arabidopsis* has been shown to target the five HD-ZIP III genes by causing cleavage of their mRNAs. Recent studies have shown how miRNA regulation of HD-ZIP IIIs is crucial to meristem function (Williams et al., 2005; Jung and Park, 2007). Dominant gain-of-function *jabba-1 D* mutants overexpress the microRNA *miR166g*, which negatively regulates *PHB*, *PHV*, and *CNA*, thus leading to the loss of these transcripts in the SAM (McHale and Koning, 2004; Williams et al., 2005). The result of this is elevated levels of *WUS* and subsequently an enlarged SAM. These three HD-ZIP III genes are involved in embryogenesis and later only *PHB* and *CNA* expression is retained in the SAM, overlapping with *WUS* expression and thus suggesting that they limit meristem size by modulation of *WUS* expression (Jung and Park, 2007).

In addition to miRNAs, LITTLE ZIPPER proteins (ZPR) have a common role in negative regulation of HD-ZIP III genes and exemplify the importance of non-DNA binding proteins as novel regulators (Wenkel et al., 2007; Kim et al., 2008). *LITTLE ZIPPER3* (*ZPR3*) and *ZPR4* encode small ZIP proteins, of 67 and 72 amino acids in size, respectively, with ZIP motifs in their central regions, most similar to HD-ZIP IIIs but they lack DNA-binding domains (Wenkel et al., 2007). The *zpr3* single mutant has no discernible phenotype in the vegetative stages of growth; however the *zpr3 zpr4* double mutant has distinct phenotypic alterations at all stages of growth. The SAM in the *zpr3 zpr4* mutant is very irregular and individual mutant plants exhibited a range of altered SAM-like structures; this shows that ZPR3 and ZPR4 proteins function redundantly as important regulators of SAM maintenance (Kim et al., 2008). Using yeast two-hybrid assays and in vitro pull-down assays it was found that ZPR3 interacts with all five HD-ZIP III proteins via the ZIP motifs (Kim et al., 2008). HD-ZIP IIIs bind DNA as homodimers, and ZPR3 forms non-functional heterodimers with HD-ZIP III proteins, preventing their transcription factor activity (Wenkel et al., 2007; Kim et al., 2008). Thus protein dimerisation and competitive inhibition by ZPR proteins is important in the regulation of HD-ZIP IIIs. Most interestingly, the HD-ZIP III genes positively regulate the transcription of *ZPR* genes, forming a negative feedback loop (Fig. 4). Although both miR165/166 and ZPR proteins have common targets, they have slightly different roles, with miR165/166 suggested to be more prominent in lateral organ patterning, whereas ZPR proteins are more prominent in SAM formation (Kim et al., 2008).

Epigenetic regulation of *WUS*. Remodelling of chromatin organisation is important in developmental processes via the activation and repression of specific sets of genes, particularly key transcription factor genes. Evidence is now emerging that *WUS* is one such target. *FASCIATED1* (*FAS1*) and (*FAS2*) encode subunits of CHROMATIN ASSEMBLY FACTOR-1 (CAF-1). The *fas1* and *fas2* mutants exhibit a range of SAM defects and show varied *WUS* expression, including an expanded expression domain in several cases (Kaya et al., 2001; Guyomarc'h et al., 2005). It is postulated that CAF-1 acts to generally stabilise gene expression in the SAM and thus allowing for stable transcriptional control of *WUS* expression (Guyomarc'h et al., 2005). *BRUSHY1* (*BRU1*) has been associated with regulation of *WUS* and encodes a novel nuclear protein with two predicted protein–protein interaction domains (Takeda et al., 2004). The *bru1* mutant phenotype is similar to that of *fas1* and *fas2*. It is suggested that BRU1 and CAF-1 have similar roles (or function together) in stabilising chromatin structure and gene expression, possibly suppressing the expression of *WUS*. It is currently unknown whether this suppression is direct or indirect (Takeda et al., 2004; Shen and Xu, 2009).

Activation of *WUS* expression occurs through the direct interaction of the chromatin remodelling factor *SPLAYED* (*SYD*). *SYD* encodes an

SNF2 class ATPase, facilitating transcription by creating a DNA template accessible to the transcription apparatus (Kwon et al., 2005; Guyomarc'h et al., 2005; Shen and Xu, 2009). *syd* mutants display a reduced *WUS* expression domain and a smaller SAM. The SYD protein has been shown to specifically interact with the promoter of *WUS* through chromatin immunoprecipitation experiments (Guyomarc'h et al., 2005; Kwon et al., 2005). This was the first evidence of a gene directly regulating *WUS* expression. *BRCA1-associated RING domain 1* (*BARD1*) encodes a protein with two tandem BRCA1 C-terminal (BRCT) domains (Han et al., 2008), which function in phosphorylation-dependent, protein–protein interactions, and a RING domain. *BARD1* has been implicated in DNA repair, but now also in SAM maintenance. Mutations in *BARD1* are shown to cause disruptions of the SAM and *BARD1* functions by confining expression of *WUS* to the OC (Han et al., 2008; Shen and Xu, 2009). The BARD1 protein has been shown to directly interact with the *WUS* promoter. *BARD1* has also been shown to interact with *SYD* using a co-immunoprecipitation (Co-IP) assay. It is therefore thought that BARD1 acts by inhibiting the chromatin remodelling process essential for *WUS* expression, either directly or indirectly through suppression of SYD (Han et al., 2008).

The KNOX pathway: cross-talk with phytohormones and balancing organogenesis

Independent from *WUS* but equally essential for SAM maintenance is the transcription factor *SHOOT MERISTEMLESS* (*STM*), which encodes a TF of the class I *KNOX* (*knotted1-like homeobox*) gene family (Long et al., 1996). *STM* is expressed throughout the meristem and is not confined to a small region like *WUS*. The action of *STM* is independent of *CLV* and *WUS* and it acts by inhibiting differentiation and thus maintaining indeterminate cell fate in the SAM (Endrizzi et al., 1996; Scofield and Murray, 2006). *STM* and *WUS* serve independent but complimentary roles in the SAM; *STM* prevents stem cell differentiation whereas *WUS* specifies a subset of cells as stem cells (Lenhard et al., 2002; Williams and Fletcher, 2005). Both factors are therefore essential in maintaining the balance between organ primordia growth and stem cell maintenance in the SAM; together *STM* and *WUS* can promote the formation of ectopic meristems (Lenhard et al., 2002; Brand et al., 2002; Gallois et al., 2002). Three other *KNOX* genes are expressed at the shoot apex in *Arabidopsis—KNAT1/BP* (*KNOTTED1-LIKE IN ARABIDOPSIS THALIANA* or *BREVIPEDICELLUS*), *KNAT2* and *KNAT6*. These are partially redundant with *STM* and restrict the expression of *ASYMMETRIC LEAVES1* (*AS1*) and *AS2* to leaf primordia, preventing determination into leaf development within the shoot apex (Byrne et al., 2002; Scofield and Murray, 2006). KNOX proteins act non-cell autonomously and have been shown to traffic through plasmodesmata (Kim et al., 2002, 2003). *KNOX* expression is confined to the meristem by epigenetic suppression. Ectopic expression of *STM* was found in mutants of subunits of the polycomb repressive complex 2 (PRC2). PRC2 suppresses transcription of *KNOX* genes by catalysing methylation of histone H3K27. Recent data suggest that the PRC1-like complex acts in conjunction with (and downstream of) PRC2 to silence *KNOX* expression (Shen and Xu, 2009).

Of the phytohormones, auxin and cytokinin play antagonistic roles in division and differentiation at the shoot and root apices. Cytokinin promotes division at the shoot apex, whilst promoting differentiation at the root apex; auxin does the opposite—promoting division at the root apex and differentiation at the shoot apex (Shani et al., 2006; Veit, 2009). High levels of auxin and gibberellin activities are closely associated with the outgrowth of lateral organ primordia at the flanks of the SAM and auxin is implicated in patterning processes at the shoot apex and phyllotaxy (the arrangement of leaves on the stem). In contrast, a high level of cytokinin activity in the central regions of the SAM is linked to the division and maintenance of undifferentiated stem cells (Veit, 2009). Cytokinins stimulate the formation of new SAMs in culture (Skoog and Miller, 1957) and can rescue the *stm* mutant (Long et al., 1996).

Challenges exist in understanding how long-range phytohormonal inputs are integrated with developmental regulators in the SAM; yet recent studies are beginning to reveal such links (Leibfried et al., 2005; Kyozuka, 2007; Veit, 2009). KNOX TFs are important in this regard, with putative roles in stimulating cytokinin (CK) accumulation and suppressing gibberellins (GA) in the SAM, thereby maintaining division and preventing differentiation respectively (Veit, 2009). KNOX exclude gibberellins from the central region of the meristem through two separate processes: inhibition of GA20 oxidase and stimulation of GA2 oxidase. GA20 oxidase is an important enzyme in the biosynthesis of GA and inhibition of this enzyme prevents GA biosynthesis in the apex (Sakamoto et al., 2001; Kyozuka, 2007; Veit, 2009). KNOX proteins also promote transcription of GA2 oxidase at the base of leaf primordia, and this enzyme converts GA into inactive forms such that active forms are confined to leaf primordia. Thus together KNOX proteins prevent accumulation of GA in the central region of the SAM (Fig. 3), consequently preventing differentiation of stem cells (Sakamoto et al., 2001; Jasinski et al., 2005; Veit, 2009).

In the central region of the meristem, KNOX directly activate genes encoding isopentenyl transferases (IPTs), enzymes important in the synthesis of cytokinins. Artificial activation of *IPT* genes and exogenous application of cytokinins have been shown to partially overcome meristem defects in mutants with low KNOX activity e.g. *stm* mutants (Jasinski et al., 2005; Yanai et al., 2005; Scofield and Murray, 2006). Conversely, these *knox* mutant phenotypes are exacerbated by mutations in other aspects of cytokinin signalling. CK signal transduction results in transcription of type A ARRs (*ARABIDOPSIS RESPONSE REGULATORS*), forming a negative feedback loop. It has also been shown that the cytokinin-induced response regulator *ARR7* is directly repressed by WUS, allowing CK to stimulate cell division in a very precise manner in the SAM (Leibfried et al., 2005). Constitutive expression of a mutated *ARR7* allele that mimics the active, phosphorylated form causes a *wus*-like phenotype, which suggests that downregulation of *ARR7* by *WUS* may be biologically relevant (Leibfried et al., 2005; Kyozuka, 2007). The expression of *ABPHYL1*, which encodes a type A response regulator in maize, at the base of leaf primordia may act to dampen cytokinin responses in the developing leaf (Fig. 3). *LONELY GUY* (*LOG*) from rice encodes a novel enzyme with phosphoribohydrolase activity which directly converts inactive forms of CK into active ones (Kurakawa et al., 2007).

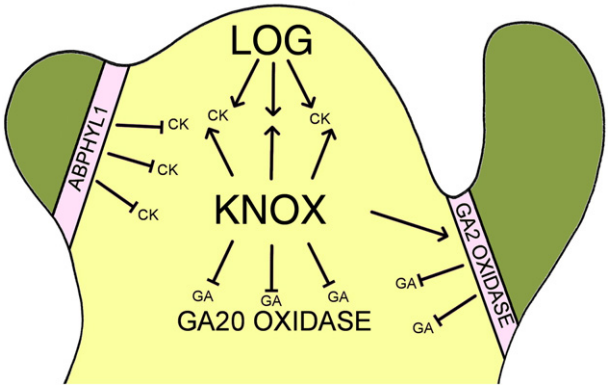

Fig. 3. KNOX interactions and hormonal cross-talk. KNOX proteins inhibit GA20 oxidase expression, an enzyme of gibberellin (GA) biosynthesis, and also stimulate GA2 oxidase at the base of leaf primordia, which converts GA to inactive forms. KNOX also stimulate isopentenyl transferases (IPTs), enzymes of cytokinin (CK) biosynthesis. The LOG enzyme in rice converts cytokinin to active forms specifically at the shoot apex. ABPHYL1 acts to dampen cytokinin at the leaf primordia. Overall these interactions ensure a relatively high concentration of CK and a low level of GA in the central regions of the meristem. Based on Veit (2009).

It is expressed specifically at the very top of the SAM, where stem cells reside (Kurakawa et al., 2007; Veit, 2009). Genes homologous to *LOG* in *Arabidopsis* have been reported to have phosphoribohydrolase activity (Kuroha and Sakakibara, unpublished). Very recently a gene homologous to *LOG* in *Arabidopsis* (*Carboxy-lyase/At5g06300*) has been shown to be expressed in a similar pattern by cell type-specific gene profiling and *in situ* hybridisation (Yadav et al., 2009).

The balance between stem cells and organogenesis in the peripheral zone manifests itself through interactions between KNOX and TFs that promote leaf identity. *ASYMMETRIC LEAVES1* (*AS1*) encodes a MYB transcription factor and *AS2* encodes a member of the plant-specific *LATERAL ORGAN BOUNDARY DOMAIN* (*LBD*) family with a leucine zipper domain (Byrne et al., 2000, 2002; Scofield and Murray, 2006). STM restricts expression of *AS1/2* to the developing leaf primordia and in turn AS1/2 suppress *BP*, *KNAT2* and *KNAT6* expression in the leaf primordia (Fig. 4). Similar interactions have been reported for species other than *Arabidopsis*, including *Z. mays* and *A. majus* (Williams and Fletcher, 2005; Scofield and Murray, 2006). Epigenetic regulation of *AS1* in the leaf primordia occurs through direct targeting by GENERAL TRANSCRIPTION FACTOR GROUP E6 (GTE6), a bromodomain-containing protein that mediates acetylation of histone H3/H4. The histone chaperone protein HIRA binds with AS1 and AS2, and together this chromatin remodelling complex represses *KNOX* expression in the lateral organ primordia (Phelps-Durr et al., 2005; Guo et al., 2008; Shen and Xu, 2009). The absence of *KNOX* expression in developing leaves is essential to their determinate state, as ectopic expression of *KNOX* (e.g. *BP*) such as in the *as1/2* mutants results in leaves with undifferentiated meristematic characters (Byrne et al., 2000, 2002; Scofield and Murray, 2006; Kim et al., 2007).

The situation at the boundary is more complex than this, however. Genes expressed specifically in the boundary between the meristem and organ primordia act to separate organs and ameliorate the interactions between the two; whether they have further roles in morphological development is currently unknown (see Rast and Simon, 2008 for a detailed review). *KNAT6* is expressed in the boundary between the meristem and organ primordia (Belles-Boix et al., 2006). *CUP-SHAPED COTYLEDON* genes (*CUC1-3*) encode NAC-domain TFs and are restricted to the boundary by STM and auxin signalling; *CUC* genes allow activation of *STM* and *KNAT6* (Ragni et al., 2008). The genes *JAGGED LATERAL ORGANS* (*JLO*) and *LATERAL ORGAN BOUNDARIES* (*LOB*) of the LBD family are expressed specifically in the boundary region. *LOB* is activated by *BP/KNAT1*, *AS1/2* and *BLADE ON PETIOLE* (BOP) *1/2*, but its role at the boundary region is not clear. *BOP1/2* activate *AS1/2* in leaf primordia and repress *BP* (Ha et al., 2007). *JLO* represses the *PIN* genes, confining auxin to developing leaf primordia. *JLO* upregulates *KNOX* expression, as misexpression in leaves causes leaf lobing and correlates with ectopic expression of *STM* and *KNAT1/BP* (Borghi et al., 2007).

An integrated network of SAM regulation

As detailed throughout this review, the signalling network that regulates the SAM is complex and consists of numerous regulators and pathways. An integrated model is proposed that aims to present a coherent network of interactions. Several major players implicated in SAM regulation converge on *WUS*, which can be seen as a master regulator of stem cell fate. Similarly the *KNOX* pathway can be seen as a master regulator governing differentiation. Both the *WUS* and *STM/KNOX* pathways are crucial to proper SAM functioning. The *KNOX* and *CLV–WUS* pathways both interact with phytohormones to ensure a high cytokinin and low gibberellin environment in the apex. The involvement of other feedback loops accessory to *CLV–WUS* is apparent, and thus negative feedback loops can be seen as a common element of stem cell homeostasis in the SAM (Fig. 4).

Such a plethora of signals can be understood from two angles: firstly considerable functional redundancy is likely necessary to ensure the SAM continues to function should regulators fail, and

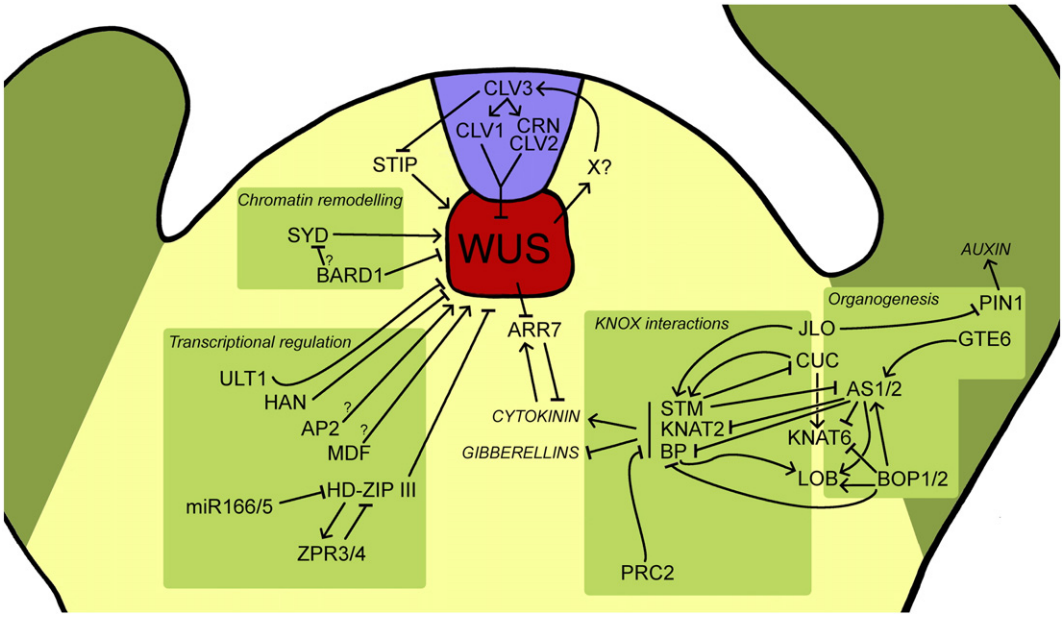

Fig. 4. An integrated network of SAM regulation. The population of stem cells at the apex (blue) are maintained through an unknown signal (X?) that is generated by the underlying *WUS*-expressing cells of the organising centre (red). Stem cells express the signal glycopeptide CLV3, which is perceived by the receptors CLV1 and CRN-SOL2 complex. Transcriptional regulation: ULT1 and HAN restrict *WUS* expression through CLV-independent pathways. The HD-ZIP III proteins also negatively regulate *WUS*, and are themselves subject to regulation by miRNA 166/5 and the competitive inhibitors ZPR3/4. AP2 and MDF interact with the CLV–WUS pathway, probably through positive regulation of *WUS*. STIP positively regulates *WUS* and is subject to negative regulation by the *CLV* genes. Chromatin remodelling: SYD activates *WUS* through direct binding and BARD1 suppresses *WUS* either through direct binding or inhibition of SYD. KNOX interactions: KNOX proteins stimulate cytokinin biosynthesis and inhibit gibberellins, and are subject to polycomb silencing via PRC2. WUS also prevents cytokinin suppression by repressing *ARR7*. Organogenesis: STM inhibits AS1/AS2 to prevent differentiation in the SAM; AS1/AS2 inhibit BP, KNAT2 and KNAT6 expression, and are subject to epigenetic regulation by GTE6. The boundary-specific *LOB* is activated by AS1/2, BOP1/2 and BP. CUC1–3 activate *STM* and *KNAT6*, and STM restricts *CUC* expression. JLO activates *KNOX* expression in the meristem and inhibits *PIN1* expression, accumulating auxin in the developing primordia.

secondly the confinement of stem cells to the niche is crucial for correct morphological development and many signals act to finely tune this system. Future analyses will elucidate in greater detail the spatiotemporal dynamics of gene expression and growth in the SAM, and thereby help transform the static map of interactions into a more dynamic framework.

Concluding remarks

Despite intensive research into the molecular mechanisms underlying SAM regulation, many questions remain unanswered. What is the exact nature of the signal transmitted by WUS to the overlying stem cells? Which genes does WUS directly regulate? What are the components of the CLV signalling cascade and how do these interact to repress *WUS* expression? How do chromatin remodelling factors integrate many developmental signals? This is an especially exciting area of research considering the importance of the SAM in plant development and the great variety of developmental mechanisms involved. Further traditional genetic screens of *Arabidopsis* will undoubtedly provide more information. However the use of advanced techniques (e.g. laser capture microdissection and chromatin immunoprecipitation), and novel experimental approaches (e.g. real-time fluorescent reporter systems, global cell type specific profiling of gene expression and computational modelling) are beginning to provide valuable insights and guide future functional studies. In addition, continued studies in other plant species will reveal exactly how well-conserved the mechanisms that regulate the SAM are within the plant kingdom.

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