STM sustains stem cell function in the Arabidopsis shoot apical meristem and controls KNOX gene expression independently of the transcriptional repressor AS1

Simon Scofield, Walter Dewitte, and James AH Murray*

School of Biosciences; Cardiff University; Cardiff, UK

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Shoot growth in higher plants is dependent on the activity of the shoot apical meristem (SAM). The SAM comprises a small, dome-shaped population of undifferentiated cells used for the formation of new lateral organs such as leaves at the meristem flank and as self-renewing stem cells in the meristem center. The Arabidopsis SHOOT MERISTEMLESS (STM) gene is expressed throughout the SAM, but not in lateral organ primordia, and is required for development of the SAM during embryogenesis and its subsequent maintenance throughout the plant life-cycle.1-3 STM is a member of the class-1 KNOX gene family and encodes a homeodomain transcription factor.4 Other class-1 KNOX genes include KNAT1/ BREVIPEDICELLUS (BP), KNAT2 and KNAT6, and these are expressed in the SAM but excluded from leaf primordia where they are repressed by the MYB-domain transcriptional repressor ASYMMETRIC LEAVES1 (AS1).5,6

Cytokinin cannot replace STM function in the SAM

The stm loss-of-function mutants exhibit defects in SAM formation and maintenance. In strong alleles such as stm-1, the SAM does not form and cotyledons become fused1 (Fig. 1B). Since the SAM is not formed, leaves are not normally produced at the shoot apex. However, in milder alleles such as stm-2, the meristem is formed but is not properly organized.2,3 Organ primordia are initiated inappropriately resulting in irregular phyllostaxy and the eventual consumption of the cells of the SAM into organ primordia. As a consequence, the SAM is not maintained indefinitely, leading to shoot growth arrest. After meristem termination, new shoots are initiated in the axils of leaves before these too cease growth, resulting in an ‘abort-retry’ mode of shoot growth which can also be observed in those stm-1 plants that are occasionally able to produce leaves (Fig. 1C). We find that similar stm-like phenotypes can be recapitulated using STM-specific RNAi (Fig. 1D-F), demonstrating that such defects arise through a post-embryonic requirement for continuous STM function. Overall these observations suggest that STM is required for the initial formation of the SAM and for its continued maintenance through proper organization mediated by restricting the formation of organ primordia.

Previous studies have shown that STM induces cytokinin (CK) synthesis through induction of ISOPENTYL TRANSFERASE (IPT) gene expression, and that application of exogenous CK or targeted expression of IPT genes in the SAM...
partially restores meristem activity to *stm* mutants. However, proper phyllotaxis and sustained meristem function were apparently not restored under these conditions, suggesting that *STM* has additional meristem-organisational roles that cannot be replaced by CK.

We investigated if CK can provide SAM organization and sustained function independently of *STM* by regenerating shoots from *stm* explants under high CK conditions. Shoot formation was stimulated from calli derived from WT and *stm* (*stm-1* or *stm-2*) root explants using various concentrations of CK (BAP or kinetin – see Fig. 1 legend) and auxin, and the morphology of shoots was compared. Shoots regenerated from WT (Ler) root explants showed regular phyllotaxis and sustained function (Fig. 1G, I) and eventually produced flowers. However, regardless of the presence of high concentrations of exogenous CK in the media, shoots regenerated from *stm* root explants consistently recapitulated the *stm* mutant phenotype, and despite forming a few leaves, these terminated in the same abort-retry manner observed in *stm* mutant plants that developed from seed (Fig. 1H, J). The failure of CK to restore normal meristem function was apparent in shoots...
regenerated from root callus of both \textit{stm-1} and \textit{stm-2} alleles, as well as in shoots regenerated from a transgenic STM-RNAi line, which displays a similar phenotype to \textit{stm-2} (Fig. 1D-F; not shown).

We conclude that CK cannot fully replace STM function in the proper formation and maintenance of the SAM, even in tissue culture under a broad range of CK concentrations, supporting the results of Endrizzi et al.\textsuperscript{2} From this perspective, amelioration of the \textit{stm} phenotype by increasing CK levels observed in other studies\textsuperscript{8} is likely due to an increase in the size of the cellular pool available for organ formation, since CK promotes cell proliferation through the CYCD3 pathway,\textsuperscript{10} rather than promoting meristem organization per se. This further supports the hypothesis presented in Scofield et al.\textsuperscript{10} that the role of STM in meristem development is not only to promote synthesis of CK, but also to promote meristem organization in a manner that is not replaceable by CK.

**STM is Required for Maintenance of WUS Expression**

Previous studies have shown that when \textit{STM} activity is lost, organ primordia form within the central zone of stem cells, suggesting a loss of stem cell identity.\textsuperscript{2,10} A key factor in controlling stem cell identity in the SAM is the WUSCHEL (\textit{WUS}) gene, which encodes a WOX homeodomain transcription factor essential for stem cell maintenance.\textsuperscript{11} \textit{WUS} is expressed in the meristem organizing center (OC) and promotes CK responses by repressing A-type \textit{ARR} gene expression.\textsuperscript{12} These are primary CK response genes that act to negatively feedback on CK responses. Hence, while STM promotes CK synthesis in the SAM, WUS promotes CK responses. In agreement with \textit{STM} and \textit{WUS} having differing roles in the SAM, neither gene can replace the function of the other when ectopically expressed.\textsuperscript{13} WUS expression precedes STM in embryonic SAM development, demonstrating that STM is not required for the initial activation of \textit{WUS} expression.\textsuperscript{11} However, induction of de novo meristem formation through ectopic expression of \textit{STM} leads to activation of \textit{WUS} expression and WUS function is required to maintain stem cell identity in these meristems.\textsuperscript{10} We therefore investigated whether \textit{STM} is required to sustain \textit{WUS} expression in the SAM.

We analyzed \textit{WUS} transcript levels by qRT-PCR following short-term downregulation of STM by DEX-inducible RNAi, since the morphological meristem defects and variable re-initiation of shoot growth in \textit{stm} mutants prevent meaningful comparative analysis of \textit{WUS} transcript levels between \textit{stm} and WT. We found \textit{WUS} to be consistently downregulated, with transcript levels ranging from -40\% to -60\% WT level 72h after induction of STM-RNAi (Fig. 1N). The decrease in \textit{WUS} expression was detectable before any morphological changes in shoot development associated with loss of \textit{STM} were apparent, suggesting it is not merely a consequence of loss of meristem cells. \textit{WUS} transcript levels were not significantly affected by short-term upregulation of \textit{STM} (not shown), suggesting the regulation of \textit{WUS} by STM might be indirect or that STM can only regulate \textit{WUS} in certain cell types, consistent with its expression in only a subset of \textit{STM}-expressing cells in the SAM. We therefore suggest that although \textit{STM} is not required for the initial activation of \textit{WUS} expression during embryogenesis, it is required to maintain \textit{WUS} expression in the SAM.

To demonstrate that \textit{STM} is required to maintain the integrity of \textit{WUS}-expression domain (the OC), we examined expression of a p\textit{WUS}:\textit{GUS} reporter in \textit{stm-1} and \textit{stm-2} apices after termination of the SAM. In WT seedlings, GUS activity was detected in the OC in the SAM interior (Fig. 1K). In most \textit{stm} seedlings, GUS activity was not detected after meristem termination, but in a subset we could detect residual GUS activity in terminal leaf primordia and young leaves (Fig. 1L, M). This demonstrates that the \textit{WUS}-expressing cells of the SAM are incorporated into primordia in the absence of \textit{STM}, further demonstrating that \textit{STM} is required to maintain organizing center/stem cell integrity.

**STM Regulates KNOX Genes Independently of AS1**

The class-1 KNOX genes \textit{KNAT1}/\textit{BREVIPEDICELLUS} (\textit{BP}) and \textit{KNAT2} are closely related to \textit{STM} and are repressed in leaf primordia by the MYB-family transcriptional repressor \textit{ASYMMETRIC LEAVES1} (\textit{AS1}), a member of the so-called ARP group of proteins that negatively regulate KNOX gene expression in diverse plant species.\textsuperscript{5,6} Previous studies have suggested that in the SAM, STM represses \textit{AS1} expression, which permits expression of \textit{KNAT1}/\textit{BP} and \textit{KNAT2}. This model is based on 2 observations. First, in the \textit{stm} mutant embryo, \textit{AS1} expression extends between cotyledon primordia into the region that would normally form the SAM, suggesting that STM could act as a repressor of \textit{AS1}.\textsuperscript{3} However, this might also be a consequence of developmental epistasis, where the absence of SAM formation allows the domain of \textit{AS1} expression to become continuous between cotyledons due to the adoption of a more differentiated leaf cell-like morphology. Second, Byrne et al.\textsuperscript{6} showed that in \textit{stm as1} double-mutants, \textit{KNAT1}/\textit{BP} was able to functionally substitute for \textit{STM}, and the authors proposed the regulatory model shown in Figure 2P. However, an alternative model in which STM and \textit{AS1} competitively regulate \textit{KNAT1} has been suggested but not confirmed.\textsuperscript{4}

We analyzed \textit{AS1} expression by qRT-PCR in response to induced upregulation of \textit{STM} (DEX-inducible regulation) and consistently found no significant decrease in its expression.
Figure 2. For figure legend, see previous page.
level at any of the time-points tested, suggesting that STM does not repress ASI (Fig. 2M). Moreover, we detected an increase in ASI transcript levels following long-term induction of STM (up to 2-fold increase 72h after induction and ~3-fold increase in plants constitutively expressing STM from sowing). This increase in ASI levels might be attributable to feedback responses arising from long-term growth with high KNOX gene expression levels, rather than a direct response to STM. Likewise, we did not detect increased ASI transcript levels in STM-RNAi plants (Fig. 2N), again indicating that STM does not repress ASI expression.

We previously showed that STM promotes expression of both KNAT1/2 and KNAT2.10 To establish whether this regulation depends on an STM-dependent repression of ASI not detected in our qRT-PCR experiments, we induced STM upregulation in the asl mutant background and measured the transcript levels of KNAT1/2 and KNAT2. We found that KNAT1/2 and KNAT2 were upregulated following transient STM induction in the asl background (approximately 3-fold change for both genes), and therefore reason that STM-dependent upregulation of these genes cannot be mediated through repression of ASI (Fig. 2O). If STM indeed repressed ASI, we would expect STMoe to repress ASI expression, resulting in little additional phenotypic enhancements in the STMoe/asl background. However, we observed a strongly enhanced phenotype in STMoe/asl compared with STMoe or asl alone suggesting that ASI is indeed normally expressed in the STMoe line (Fig. 2A-L). We found that STMoe/asl seedlings showed enhanced capacity for ectopic meristem formation, especially on cotyledons where such meristems do not normally form in STMoe (Fig. 2F), and leaf outgrowth and differentiation were inhibited more strongly in STMoe/asl compared with STMoe (Figs. 2D, H, J, L). This could be attributable simply to higher levels of KNAT1/2 and KNAT2 expression in STMoe/asl relative to STMoe, or could be due to enhanced KNOX gene sensitivity in the asl background, similar to the enhanced sensitivity to KNOX gene expression conferred by expression of the cell cycle regulator CYCLIND3.10

These data strongly suggest that STM does not repress ASI and that regulation of KNOX genes by STM is ASI-independent. We therefore confirm the alternative model for the regulation of KNOX genes by STM and ASI, initially suggested in Scofield and Murray,4 in which STM and ASI competitively regulate KNAT1/2 expression, though not necessarily directly (Fig. 2Q). This model explains that in the stm mutant, KNAT1/2 is not expressed because ASI represses its transcription and the SAM does not form, while in the stm asl double mutant KNAT1/2 is derepressed and can functionally substitute for STM. In normal development, KNAT1/2 is expressed in the SAM because ASI is absent and is excluded from leaf primordia because STM is absent and ASI is present. In STMoe plants, meanwhile, expression of KNAT1/2 occurs in leaves10 because STM is present in high levels and can alleviate the transcriptional repression by ASI. This model of regulation also applies to KNAT2 but it does not provide meristem functions of STM or KNAT1/2.

This study has addressed some of the key functions of STM and its regulatory interactions with other KNOX genes. We have shown that CK cannot replace STM function in meristem organization, demonstrating that STM has additional roles in the SAM apart from promoting CK synthesis. We also showed that while not essential for initial activation of WUS, STM is required for maintenance of WUS expression and the integrity of the organizing center and stem cell niche. Finally, our data support a refined model which suggests that STM and the MYB-domain transcriptional repressor ASI competitively regulate KNOX gene expression.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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