A terminator of floral stem cells

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Normal flower development requires the termination of stem cell activities in the floral meristem. The floral regulator AGAMOUS (AG) is necessary for this termination and represses the expression of the stem cell determinant WUSCHEL (WUS), but the repression mechanism was not clear. A recent study by Sun and colleagues (pp. 1791–1804) in this issue of Genes & Development has identified a direct target of AG, KNUCKLES (KNU), which encodes a transcriptional repressor of WUS, providing a key missing link in floral meristem determinacy.

Floral homeotic gene AGAMOUS (AG) is required for both floral meristem determinacy and reproductive organ identities (Bowman et al. 1989, 1991; Yanofsky et al. 1990, Coen and Meyerowitz 1991; Mizukami and Ma 1995). AG is a key gene for the C function of the ABC model and a member of the MADS-box gene family (Yanofsky et al. 1990; Bowman et al. 1991; Coen and Meyerowitz 1991). In ag mutants, the floral meristem is active for a much longer period than normal, generating many more floral organs than the wild type (Bowman et al. 1989, 1991; Yanofsky et al. 1990). Similarly, reduction of AG function using sense or antisense transgenes causes extended floral meristem activities (Mizukami and Ma 1995; Mizukami et al. 1996).

Thus, proper stem cell activity in the floral meristem is controlled by the balance between the positive regulator WUS and the negative regulator AG. This is achieved through a positive–negative feedback loop (Fig. 1A). WUS is expressed in the early floral meristem and activates AG expression at floral stage 3 (as defined by Smyth et al. 1990). AG expression is reduced in the wus mutant, whereas ectopic WUS expression prolongs stem cell proliferation and also causes ectopic stamen and carpel development in an AG-dependent way (Lenhard et al. 2001, Lohmann et al. 2001). DNA-binding studies suggest that WUS cooperates with another positive regulator of floral meristem, LEAFY (LFY), to activate AG expression (Lohmann et al. 2001). On the other hand, WUS expression decreases when AG expression is activated and disappears by the time carpel primordia are initiated at stage 6 (Mayer et al. 1998). WUS is required for the floral meristem indeterminacy in the ag-1 mutant because the flowers of the wus-1 ag-1 double mutant appear indistinguishable from those of wus-1 (Laux et al. 1996), supporting the hypothesis that AG negatively regulates WUS expression. Indeed, WUS remains active in the center of the indeterminate floral meristem of the ag-1 mutant through the life of the flower (Lenhard et al. 2001; Lohmann et al. 2001), indicating that AG is required for the cessation of WUS expression at stage 6.

Clearly, the precise duration of stem cell activity in the floral meristem is important for normal development. Terminating the stem cells too early would mean insufficient cells for floral organ formation, whereas overly extended...
stem cell activity would result in abnormally large numbers of floral organs. Furthermore, because of the coupling of floral meristem termination with the formation of the carpels, the female reproductive organs, extended meristem activity is also linked to defects in carpel development. Thus, the above-mentioned positive-negative feedback loop must have a timing component: WUS activates AG expression first at stage 3, and AG represses WUS expression subsequently at stage 6. How is differential timing of the two aspects of the regulatory loop achieved? In particular, what is the mechanism for the delayed repression of WUS by AG?

Sun et al. [2009] have now obtained strong support from a series of genetic and molecular experiments for another gene, KNUCKLES (KNU), that serves to mediate the negative regulation of WUS by AG, and provide evidence for the timing mechanism of the termination of WUS expression. KNU was discovered as a positive regulator of floral meristem determinacy, unlike the normal flower, which produces carpels as the fourth and innermost whorl of organs, knu mutant flowers have extra stamens and carpels that form interior to the fourth-whorl carpels [Payne et al. 2004]. In addition, the KNU protein contains a zinc finger and a putative transcriptional repression motif [Payne et al. 2004], suggesting that it might be a repressor of WUS. Therefore, Sun et al. [2009] hypothesized that KNU might act between AG and WUS to mediate the repression of WUS by AG.

To investigate how AG regulates floral meristem determinacy and the role of KNU in this process, Sun et al. [2009] performed a series of genetic and molecular experiments. First, they ascertained the time at which AG is needed for normal determinacy, using an elegant experiment with inducible AG activity. It was shown previously that AG activity can be controlled by fusing it with a mammalian steroid hormone receptor [glucocorticoid receptor (GR)] that can be activated by an artificial hormone, dexamethasone [dex] [Ito et al. 2004; Gomez-Mena et al. 2005]. By inducing AG activity at different floral stages in transgenic plants with inducible AG activity in an ag mutant background, Sun et al. [2009] showed that AG activity at stage 3, but not stage 4, was able to confer full determinacy, indicating that the normal onset of AG function at stage 3 is required for floral meristem determinacy.

Next, Sun et al. [2009] tested the role of KNU by performing genetic experiments with various combinations of ag, wus, and knu mutations. They found that ag knu double mutants had similar phenotypes to those of ag mutants, consistent with the idea that AG and KNU act in the same genetic pathway and that AG is a positive regulator of KNU. In addition, the knu wus double mutant appeared the same as the wus mutant, indicating that WUS is downstream from KNU. Furthermore, when KNU was expressed using an AG-independent promoter (35S), KNU was able to rescue the indeterminate phenotype of the ag-1 mutant, suggesting that KNU functions downstream from AG. On the other hand, AG-independent KNU expression caused wus-like floral phenotypes, supporting the hypothesis that KNU represses WUS expression. These experiments with mutants and transgenic plants provide key genetic evidence that KNU acts downstream from AG to repress WUS [Fig. 1B].

To examine the relationship between AG, KNU, and WUS further at the level of mRNA expression, Sun et al. [2009] used a transgenic plant system that allows flower development to be induced and found that KNU expression begins ~2 d after the start of AG expression, whereas WUS expression is repressed starting at ~2 d after AG expression. More directly, they showed that induction of AG activity using the AG–GR fusion system is followed by KNU-like floral phenotypes, sup- orting the hypothesis that KNU represses WUS expression. These experiments with mutants and transgenic plants provide key genetic evidence that KNU acts downstream from AG to repress WUS [Fig. 1B].

The delay in KNU expression in comparison with AG expression suggests that the activation of KNU expression might be a key to the timing of stem cell termination in the floral meristem. Further support for the negative regulation of WUS by KNU came from transgenic plants with strong inducible KNU activity, which resulted in premature termination of meristem activity similar to that found in wus mutants. Sun et al. [2009] also determined that the repression motif at the C-terminal region of the KNU protein was required for termination of WUS expression, suggesting that KNU acts as a transcriptional repressor.

The delay in KNU expression in comparison with AG expression suggests that the activation of KNU expression might be a key to the timing of stem cell termination in the floral meristem, thereby allowing the needed amount of stem activity for floral organ generation. To examine this temporal relationship between AG and KNU further, Sun et al. [2009] combined the inducible AG (using the AG–GR fusion) with a KNU–GUS reporter gene and found that 1 d after AG induction, GUS expression was...
not detected, but 2 d after AG induction, strong GUS expression was detected. As a positive control, another AG target gene, SPL, was activated immediately after AG induction. Although it is not known how KNU expression is delayed compared with AG induction, the delayed KNU expression is likely a key to allowing WUS to be expressed long enough for sufficient floral stem cell activity to generate floral organs before the termination of the floral meristem.

The next question Sun et al. (2009) addressed was whether KNU is a direct target of transcriptional regulation by AG. Sun et al. (2009) noted that the putative KNU promoter region does not contain a perfect AG target site, as defined by earlier in vitro studies (Huang et al. 1993; Shiraishi et al. 1993), nevertheless, there are three partial sites of AG-binding consensus sequences that were found previously to bind to AG weakly (Ito et al. 1997). To test whether AG binds to the KNU promoter region in vivo, Sun et al. (2009) performed chromatin immunoprecipitation (ChIP) experiments. They found that the KNU promoter region containing the partial AG-binding sites was enriched after 1 d of the induction of AG activity, and this enrichment was further strengthened 2 and 3 d after induction. Furthermore, when the partial AG-binding sites were mutated in a fusion of the KNU promoter to the GUS reporter gene, GUS expression was not detected in 88% of the transgenic plants, in contrast to GUS expression in 80% of the transgenic plants carrying a fusion with the wild-type KNU promoter. These results support the hypotheses that AG binds directly to the KNU promoter and that AG binding activates KNU transcription.

At the same time, it was puzzling why AG binding was observed by ChIP 1 d after the induction of AG function, but KNU expression was not detectable until 2 d after the induction. To probe further into the mechanism of the activation of KNU expression, Sun et al. (2009) examined the status of transcription-repressive marker histone H3 Lys 27 trimethylation (H3K27me3). They found that the level of H3K27me3 was high near the KNU transcriptional start and in the coding region; moreover, the deleted KNU–GUS reporter gene. GUS expression was not detected in 88% of the transgenic plants, in contrast to GUS expression in 80% of the transgenic plants carrying a fusion with the wild-type KNU promoter. These results support the hypotheses that AG binds directly to the KNU promoter and that AG binding activates KNU transcription.

Is the H3K27me3 level, or some other cis elements in the deleted KNU region, relevant to KNU regulation? Sun et al. (2009) addressed this question by testing for KNU expression in mutants that are defective in genes encoding polycomb group proteins, which maintain H3K27me3 levels (Katz et al. 2004; Schubert et al. 2006). Indeed, KNU was expressed ectopically in leaves of these mutants. Another test was to see whether another factor associated with H3K27me3, the epigenetic repressor TFL2/LHP1, was involved in repressing KNU expression. In the tfl2 mutant, KNU was overexpressed in the flower and ectopically expressed in the inflorescence stem. Therefore, defects in maintaining H3K27me3 levels could de-repress KNU expression, suggesting that relatively high levels of H3K27me3 at the KNU locus might be responsible for the repression of KNU expression, although the formal possibility that the epigenetic regulators polycomb group proteins and TFL2 might affect KNU expression indirectly could not be ruled out.

Whereas Sun et al. (2009) convincingly showed that AG likely activates KNU transcription by binding directly to the KNU promoter and that KNU expression is delayed relative to the onset of AG function, probably by repressive chromatin as marked by H3K27me3, several questions remain. First, it is not known whether and how AG acts to counter the repressive chromatin. Second, the difference for AG function between 1 d and 2 d after AG is induced is not known. In other words, why was AG able to counter the effect of repressive chromatin 2 d, but not 1 d, after AG induction? Sun et al. (2009) proposed that the repressive marker is removed in an AG-dependent manner and suggested that the period of 2 d might be required because of a need for active cell division. However, AG is not a histone demethylase. Could AG recruit a histone demethylase to the KNU promoter region? Is the removal of histone methylation achieved passively by preventing methylation following DNA replication during the cell cycle?

A second area that requires further investigation concerns the mechanism by which KNU represses WUS expression. Although Sun et al. (2009) showed that the KNU repression motif is required for the termination of WUS expression, it is possible that KNU represses an activator of WUS expression, thereby negatively regulating WUS expression in an indirect fashion. Sun et al. (2009) indicated that they performed a ChIP experiment for KNU binding to the WUS promoter, but the result was not conclusive. Perhaps more importantly, KNU has not been shown to be, or to associate with, a DNA-binding protein, although it has a C2H2-type zinc finger (Payne et al. 2004). Nevertheless, the observation by Sun et al. (2009) that the activity of a KNU–GR fusion protein can be induced by the artificial hormone dexam suggests that KNU is a transcriptional regulator. Future experiments are needed to determine whether KNU is a DNA-binding protein and, if so, what the binding sequence for KNU is.

Yet another puzzle is the relationship between AG, KNU, and other genes that also play a role in regulating floral meristem determinacy [Fig. 1C]. One such additional regulator is the C2H2 zinc finger protein SUPERMAN (SUP), which has been shown to repress stem cell proliferation at floral stages 3 and 4 [Bowman et al. 1992; Sakai et al. 1995]. Genetic studies indicated that AG and SUP control floral meristem determinacy through independent pathways [Schultz et al. 1991; Bowman et al. 1992]. SUP is also involved in the termination of WUS expression [Prunet et al. 2008]. What is the relationship between the two C2H2 zinc finger proteins KNU and SUP? Another contributor to the floral determinacy is CRABS CLAW [CRC], which also negatively regulates stem cell proliferation [Bowman and Smyth 1999] and might act downstream from AG and two other floral organ identity genes—APETALA3 and PISTILLATA [Lee et al. 2005].
However, the relationship between CRC and KNU is unclear, and the interactions between AG, SUP, and CRC at the molecular level are yet to be understood. Recent studies have uncovered other positive regulators of floral meristem determinacy—REBELOTE (RBL), SQUINT (SQN), and ULTRAPETALAL1 (ULT)—that function upstream of AG and SUP [Fig. 1C; Carles et al. 2004; Prunet et al. 2008]. The proteins encoded by these genes have various predicted activities, suggesting that they act with different mechanisms. With rapid progress fueled by new technologies, future investigations will likely yield new insights regarding the control of stem cell activities in the floral meristem, ever enriching us in this fertile ground of plant development.

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