Requirement of Homeobox Gene *STIMPY/WOX9* for *Arabidopsis* Meristem Growth and Maintenance

Xuelin Wu,[1] Tsegaye Dabi,[1] and Detlef Weigel[1,2,*]
[1]Plant Biology Laboratory
The Salk Institute for Biological Studies
La Jolla, California 92037
[2]Department of Molecular Biology
Max Planck Institute for Developmental Biology
D-72076 Tübingen
Germany

Summary

Most organs of flowering plants develop postembryonically from groups of pluripotent cells called meristems [1]. The shoot apical meristem (SAM) is specified by two complementary pathways [2–4]. *SHOOT MERISTEMLESS* (*STM*; [5]) defines the entire SAM region [6]. *WUSCHEL* (*WUS*), on the other hand, functions in a more restricted set of cells to promote stem-cell fate and is regulated by the *CLAVATA* genes in a negative feedback loop [7–10]. In contrast, little is known about how the growth of the SAM, which increases in size during vegetative development [11], is regulated. We have characterized *STIMPY* (*STIP*; also called *WOX9* [12]), a homeobox gene required for the growth of the vegetative SAM, in part by positively regulating *WUS* expression. In addition, *STIP* is required in several other aerial organs and the root. What sets *STIP* apart from *STM* and *WUS* is that *stip* mutants can be fully rescued by stimulating the entry into the cell cycle with sucrose. Therefore, *STIP* is likely to act in all these tissues by maintaining cell division and preventing premature differentiation. Taken together, our findings suggest that *STIP* identifies a new genetic pathway integrating developmental signals with cell-cycle control.

Results and Discussion

Identification of *STIP*
STIMPY (*STIP*) was first identified in an activation-tagging screen [13] through a dominant gain-of-function allele, *stip*-D. Compared to the wild-type, *stip*-D plants develop wavy leaf margins (Figure 1A), which are a general indication of misregulated cell division in leaves [14–18]. These plants also develop an increased number of axillary shoots, resulting in a mildly bushy phenotype (not shown). Flower and ovule development are also affected in *stip*-D plants, leading to reduced fertility (not shown). Recapitulation experiments show that these phenotypes result from overexpression of the homeobox-containing gene At2g33880, which has recently been called *WOX9* on the basis of its distant homology to *WUS* [12]. Because there is a tradition of naming mutants after phenotypes, even when they were initially annotated on the basis of sequence similarity (e.g., [19]), we will refer to this gene as *STIMPY* (*STIP*) in this study.

*Correspondence: weigel@weigelworld.org

Previously, it has been shown that *STIP* is expressed in developing embryos [12]. With in situ hybridization and analysis of promoter fusions to the *GUS* reporter, we found that *STIP* is also expressed at low levels in proliferating tissues postembryonically. Soon after germination, *STIP* can be detected in the vegetative SAM and leaf primordia (Figures 1E and 1F). After the floral transition, it is expressed in floral meristems and transiently in emerging floral organs during their initial growth period (Figure 1B). Little, if any, expression is detected in the inflorescence meristem (Figure 1G). It is also expressed in the epidermal layer of the placenta and growing septum (Figure 1C; [12]). In addition, *STIP* is expressed in the upper portion of the root meristematic zone (Figure 1D).

STIP Is Required for Early Development
We isolated two strong hypomorphic alleles of *STIP* (see the Supplemental Experimental Procedures in the Supplemental Data available with this article online). *stip* loss-of-function mutants have smaller, hyponastic cotyledons (compare Figures 1J and 1K to the wild-type in 1H), and some lack the primary root (Figure 1K). This is in contrast to the epinastic cotyledons found in *stip*-D seedlings (Figure 1I). Growth arrests soon after germination in more than 80% of *stip* seedlings, resulting in early seedling lethality. The remaining plants are delayed in development in comparison to the wild-type, and about half of them die during later vegetative development. The surviving plants can develop into largely normal adults that have defects only in the fruits (not shown).

The early *stip* seedling phenotypes indicate possible defects in embryogenesis. When we examined the progeny of a *stip*/+ plant, approximately 25% of the embryos, presumed to be *stip* homozygotes, showed a variable reduction in the size of the embryonic hypocotyl and cotyledons (compare Figures 1M and 1N to the wild-type in 1L). The name *STIMPY* refers to this "stumped" phenotype. Closer examination revealed that the basal portion of *stip* embryos have fewer cells, possibly owing to reduced apical-basal division (Figure S1; data not shown). Severely affected embryos arrest at the globular stage (Figure 1O).

STIP Promotes Growth of the Vegetative SAM
At the structural level, the SAM region of *stip* seedlings immediately after germination appears identical to the wild-type counterpart (compare Figure 2E to 2A). The intense red staining by Safaranin O [20] suggests that this region is undifferentiated, like the wild-type SAM (Figures 2A and 2E). CLV3::GUS, which marks the stem cells in the SAM [16], is expressed at lower levels than in the wild-type (compare Figure 2F to 2B), indicating a functional SAM with a reduced stem-cell population. A similar reduction can also be seen in mature *stip* embryos (Figure S2), suggesting that *STIP* is also involved in SAM formation during embryogenesis. Six days after germination, the wild-type SAM has grown into the dome-shaped structure (Figure 2C). In contrast, the SAM
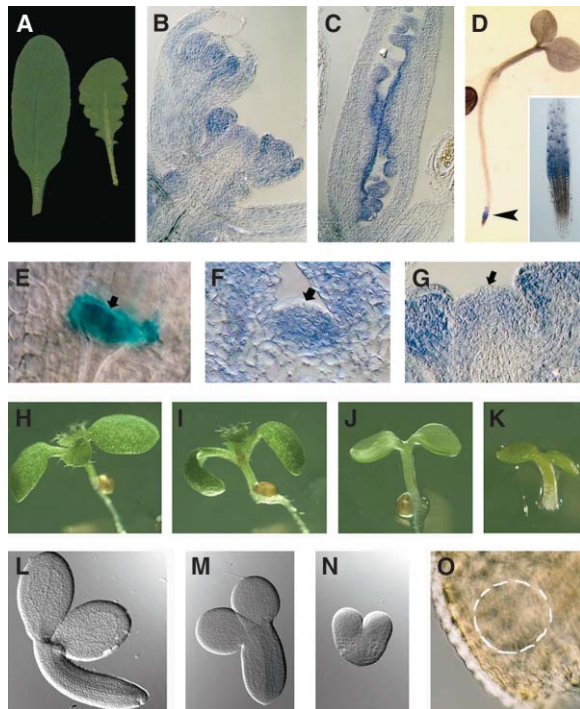
Figure 1. Changes in *STIP* Activity Affect Plant Growth

(A) Comparison between a wild-type (left) and a *stip*-D (right) rosette leaf.

(B–D, F, and G) In situ hybridization with *STIP* probe to inflorescence apex (B), developing carpels (C), 1-day-old seedling (D), and vegetative apex of a 6-day-old seedling (F). The arrowhead in (D) points to the region where *STIP* expression is detected. A higher magnification of the root tip is shown in the inset. (G) The inflorescence meristem in (B) is shown in the same magnification as (F) for comparison.

(E) STIP::GUS expression in whole-mount 1-day-old seedling. The SAM in (E)–(G) are marked with arrows.

(H–K) Five-day-old wild-type (H), *stip*-D (I), and *stip* knockout mutant (J and K) seedlings. Some *stip* mutants germinate but lack the primary root (K).

(L) Mature wild-type embryo, dissected and viewed with differential interference contrast optics.

(M–O) Mature *stip* embryos with different degrees of growth reduction. The embryo in (O) has not been dissected from the seed coat and is shown at higher magnification than those in (L)–(N); its outline is marked by dotted lines.

of *stip* mutants remains flat and becomes fully differentiated (Figure 2G). The flat SAM of *stip* mutants resembles that of a *wus-1* mutant [21]. The shoot apex of 6-day-old *stip* mutants lacks both *CLV3* (compare Figure 2H to 2D) and *WUS* expression (Figure S2). *STM* expression is present in these mutant seedlings, but in fewer cells than in the wild-type because of the flatness of the SAM (Figure S2). These observations suggest that *STIP* is required for the maintenance of *WUS* and *CLV3* expression and that it acts largely independently of *STM* after germination.

stip-D seedlings have slightly increased *CLV3* expression (compare Figure 2L to 2D), consistent with these observations. However, no obvious increase in the size of the SAM can be detected in *stip*-D plants, indicating complex interactions between *STIP* and *CLV3*. To further understand the relationship between *STIP* and the *WUS/CLV3* pathway, we introduced both *stip* loss-of-
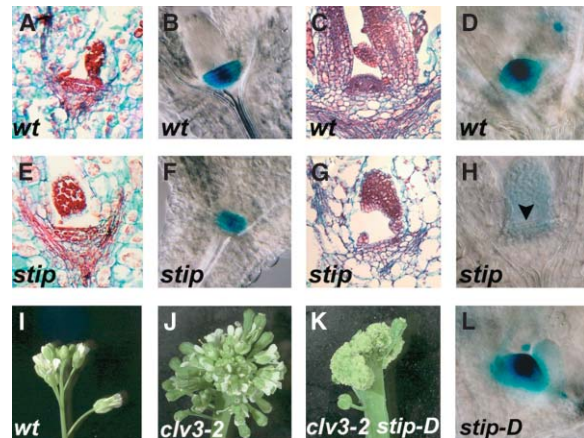

Figure 2. *STIP* Promotes Meristem Growth

(A and E) Longitudinal sections through shoot apices of 1-day-old seedlings, stained with Alcian blue and Safranin O.

(B and F) CLV3::GUS expression in whole-mount 1-day-old seedlings.

(C and G) Longitudinal sections through shoot apices of 6-day-old seedlings, stained with Alcian blue and Safranin O.

(D, H, and L) CLV3::GUS expression in whole-mount 6-day-old seedlings. In (H), a leaf that is arrested in growth can be seen behind the flat SAM (marked by arrowhead), which lacks the typical intense blue staining of the wild-type SAM (D).

(I–K) Inflorescences of 5-week-old plants.

function mutations and *stip*-D into the *clv3* background. *clv3* mutants show enlarged SAMs and increased organ number owing to stem-cell overproliferation [9]. Loss of *STIP* function completely suppresses the *clv3* phenotype, and *stip clv3-2* double mutants show the growth-arrest phenotype identical to that of *stip* single mutants (not shown). This suggests that *STIP* is epistatic to *CLV3* in SAM regulation. *stip*-D enhances the *clv3* phenotype, with *stip*-D *clv3-2* plants having larger SAMs than *clv3-2* alone. The size increase can be seen with the unaided eye by the late vegetative stage (not shown). It becomes more dramatic in the inflorescence meristem, when the entire *stip*-D *clv3-2* inflorescence apex is turned into a mass of meristematic tissue with very few flowers (compare Figure 2K to *clv3-2* in 2J). *WUS* expression can be detected in the second and third cell layers throughout the greatly overproliferated inflorescence meristem (Figure S2), confirming its meristematic identity. Moreover, *stip*-D fails to rescue the SAM defects of *wus-1* (not shown), suggesting that *STIP* functions upstream of *WUS*.

In contrast, only additive effects are observed between *stip* loss-of-function mutations and *stm-1* [5] (not shown). Similarly, *stip*-D does not enhance or suppress the SAM defects of strong or intermediate *stm* alleles, *stm-1* [5] or *stm-2* [22] (not shown), supporting the notion that *STIP* acts independently of *STM* during vegetative development.

STIP Maintains Growth and Cell Division in the Shoot and Root Apex

We have so far presented evidence that *STIP* is required for *WUS* maintenance in the SAM. However, leaves or secondary shoots are never formed after the vegetative SAM fails in *stip* mutants. This phenotype is far more
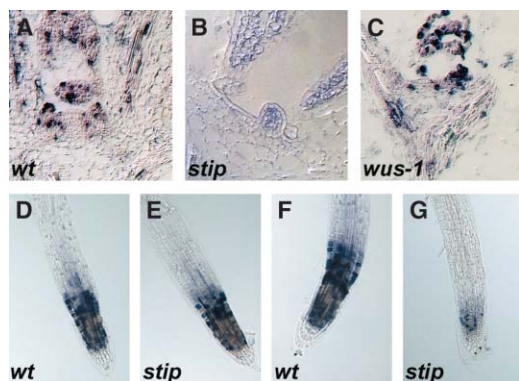
Figure 3. *STIP* Maintains Cell Division in the Vegetative Shoot Apex and the Root

(A–C) In situ hybridization of histone H4 probe to vegetative apices of 6-day-old seedlings. The sample in (B) has been intentionally overdeveloped to detect any residual histone H4 expression, and the faint color is background staining.

(D–G) In situ hybridization of histone H4 probe to root tip of 1-day-old (D and E) and 5-day-old (F and G) seedlings.
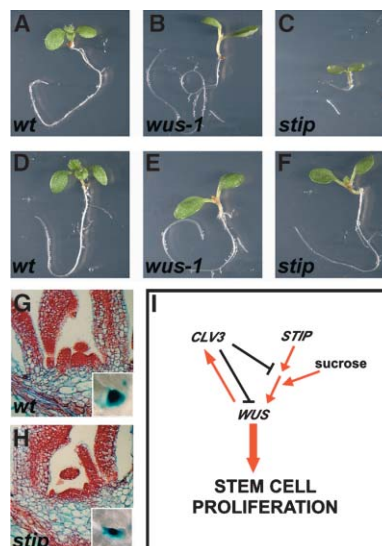

Figure 4. Sucrose Rescues Loss of *STIP* Function during Seedling Development

(A–F) Six-day-old seedlings grown on medium without (A–C) and with (D–F) sucrose.

(G and H) Longitudinal sections through the SAM of 6-day-old wild-type (G) and *stip* (H) seedlings grown with the presence of sucrose and stained with Alcian blue and Safranin O. Insets show CLV3::GUS expression in whole-mount samples of the same genotype.

(I) A genetic model of *STIP* function in stem-cell maintenance in the vegetative SAM. *STIP* positively regulates *WUS* expression in the vegetative SAM, and it is subject to negative feedback back regulation via the *CLAVATA* genes. Other exogenous factors, such as sucrose, can also promote *WUS* expression in the vegetative SAM by stimulating cell division.

severe than that of strong *wus* mutants, which despite their disordered growth eventually produce abnormal flowers [21]. This suggests that *STIP* has a broader role in maintaining growth in the vegetative shoot apex, which may be the cause of the complete growth arrest of most *stip* seedlings.

To further investigate the differentiation status in the *stip* vegetative apex, we compared cell-division activity in wild-type, *stip,* and *wus* seedlings with expression of histone H4, an S-phase-specific marker [23]. In a 6-day-old wild-type shoot apex, most of the histone H4 expression is detected in the SAM peripheral zone, where leaf primordia emerge, and in the growing leaves (Figure 3A). In a *wus*-1 seedling, histone H4 is expressed sporadically in the shoot apex (Figure 3C), which is consistent with its ability to continue to grow, albeit in a limited manner. Histone H4 expression is not detected in the apical region of *stip* seedlings, including the first pair of leaf primordia (Figure 3B), indicating that *STIP* maintains the ability of cells to divide in the vegetative SAM and leaf primordia.

Because root growth also arrests in *stip* seedlings, and no lateral root is initiated (Figure 4C), we compared histone H4 expression in wild-type and *stip* roots. In wild-type roots, most of the cell division occurs in the meristematic zone above the quiescent center (Figure 3D; [1]). The roots of 1-day-old *stip* seedlings are morphologically identical to wild-type roots, with slightly less histone H4 expression (compare Figure 3E to 3D). By 5 days after germination, very little cell division can be detected in the roots of the *stip* mutants, and the cytoplasmic dense meristematic zone has mostly disappeared (compare Figure 3G to 3F). Thus, *STIP* maintains cell division and growth in the root, which is similar to its role in the aerial part of the plant.

Exogenous Sucrose Bypasses the Requirement for *STIP* Activity

If cell-cycle arrest is the main cause of the *stip* phenotype, we reasoned that it might be possible to rescue

stip mutants by stimulating cell division through an alternative route. Previous reports have shown that the presence of sucrose in the growth medium can induce the expression of CycD, which can then lead to increased cell division [24–26]. Unlike plant hormones such as cytokinin, low levels of sucrose do not disturb the overall development of wild-type seedlings. We germinated wild-type, *wus*, *clv3*, and *stip* seeds on medium with and without sucrose. As expected, sucrose does not affect the growth of wild-type (Figures 4A and 4D), *wus*-1 (Figure 4B and 4E), or *clv3*-2 (not shown) plants. In contrast, *stip* mutants show a dramatic response to sucrose. Without sucrose, the growth arrest of most *stip* seedlings is apparent 2 days after germination, and they do not undergo further morphological changes (Figure 4C). In contrast, *stip* seedlings on sucrose-containing medium resume near-normal growth by the second day. All mutant seedlings reach near wild-type size 6 days after germination (Figure 4F). Longitudinal sections of the rescued seedlings show that the SAM has partially grown (compare Figure 4H to 4G). The presence of a reduced stem-cell population is confirmed by CLV3::GUS expression (Figure 4H, inset). Remarkably, germinated *stip* seedlings that have survived for up to 8 days without exogenous sucrose can also be fully rescued by sucrose-containing medium. Because 5-day-old *stip* seedlings have already lost all signs of growth (Figures 2G and 2H; Figures 3B and 3G), this suggests that some

cells in the *stip* shoot and root apex retain meristematic potential after arrest of growth.

The rescued *stip* seedlings can grow into phenotypically normal plants when transferred to soil after being grown on sucrose medium for 10–12 days, at which point the SAM has already entered the reproductive phase. The only remaining defects are seen in the developing carpels. This is consistent with the absence of *STIP* expression in the inflorescence SAM and supports the hypothesis that *STIP* is not required in the inflorescence SAM.

Role of *STIP* in Stem-Cell Maintenance and Meristem Growth

The growth of the vegetative SAM is mainly achieved by an increase in cell number [11]. We propose that in the central zone, *STIP* maintains the stem-cell population by positively regulating the expression of *WUS* in the organizing center, which promotes stem-cell fate. Overexpression of *STIP* can only lead to overproliferation of the SAM when *CLV3* is removed from the *WUS/CLV* feedback loop, suggesting that this activity is subject to *CLV3* repression. Therefore, it is possible that the negative feedback regulation of *WUS* by the *CLV* pathway [16, 27] is partially carried out via *STIP* in the vegetative SAM. However, the influence of *STIP* on *WUS* expression may be indirect because sucrose is able to rescue *stip* seedlings but not *wus* seedlings. In addition, *stip wus*-1 double mutants germinated on sucrose-containing medium grow up as *wus*-1 does. This suggests that exogenous sucrose affects genes upstream of *WUS* (Figure 4I). Although *STIP* is required for maintaining cell division in the peripheral zone, *stip*-D mutants do not show an overproliferation of the peripheral zone or increased growth rate, suggesting that like *CLV3* in the central zone, other factors may prevent overgrowth in the peripheral zone by negatively regulating STIP activity.

STIP Promotes Cell-Cycle Entry

Not only does *STIP* promote the growth of the vegetative SAM, it also affects the growth of many other tissues. Loss of STIP activity leads to a reduction in embryonic growth, failure in axillary SAM and leaf-primordia initiation and growth, and failure in primary root growth and lateral root initiation. These phenotypes suggest that *STIP* functions as a general positive regulator of undifferentiated growth in *Arabidopsis*.

HOBBIT (*HBT*; [28, 29]), which encodes a CDC27 homolog, provides clear evidence for the requirement of cell-cycle control in meristem and primordia maintenance [28, 29]. Because sucrose stimulates cell-cycle entry by upregulating CycD expression [24], *STIP* may also act upstream of the Cyclins to prevent differentiation and to promote cell division. The molecular nature of STIP as a homeodomain protein makes it unique in connecting transcriptional regulation of primordia growth to cell-cycle entry. The effects of *STIP* also share common aspects with cytokinin, which upregulates CycD3 expression, promotes undifferentiated growth, and regulates organ initiation in the shoot ([23, 30]; reviewed in [31]). However, different from STIP, cytokinin has oppo-

site roles in shoot and root development [30, 32–34]. Whether *STIP* interacts with the cytokinin pathways, and how its function is related to cell-cycle control, remains to be seen.

References

1. Steeves, T.A., and Sussex, I.M. (1989). Patterns in Plant Development, Second Edition (Cambridge: Cambridge University Press).
2. Carles, C.C., and Fletcher, J.C. (2003). Shoot apical meristem maintenance: The art of a dynamic balance. Trends Plant Sci. *8*, 394–401.
3. Baurle, I., and Laux, T. (2003). Apical meristems: The plant's fountain of youth. Bioessays *25*, 961–970.
4. Lenhard, M., and Laux, T. (1999). Shoot meristem formation and maintenance. Curr. Opin. Plant Biol. *2*, 44–50.
5. Barton, M.K., and Poethig, R.S. (1993). The formation of the shoot apical meristem in *Arabidopsis thaliana*: An analysis of development in the wild type and in the *shoot meristemless* mutant. Development *119*, 823–831.
6. Long, J.A., Moan, E.I., Medford, J.I., and Barton, M.K. (1996). A member of the KNOTTED class of homeodomain proteins encoded by the *STM* gene *Arabidopsis*. Nature *379*, 66–69.
7. Mayer, K.F.X., Schoof, H., Haecker, A., Lenhard, M., Jürgens, G., and Laux, T. (1998). Role of *WUSCHEL* in regulating stem cell fate in the *Arabidopsis* shoot meristem. Cell *95*, 805–815.
8. Clark, S.E., Running, M.P., and Meyerowitz, E.M. (1993). *CLAVATA1*, a regulator of meristem and flower development in *Arabidopsis*. Development *119*, 397–418.
9. Fletcher, J.C., Brand, U., Running, M.P., Simon, R., and Meyerowitz, E.M. (1999). Signaling of cell fate decisions by *CLAVATA3* in *Arabidopsis* shoot meristems. Science *283*, 1911–1914.
10. Jeong, S., Trotochaud, A.E., and Clark, S.E. (1999). The *Arabidopsis CLAVATA2* gene encodes a receptor-like protein required for the stability of the CLAVATA1 receptor-like kinase. Plant Cell *11*, 1925–1934.
11. Medford, J.I., Behringer, F.J., Callos, J.D., and Feldmann, K.A. (1992). Normal and abnormal development in the *Arabidopsis* vegetative shoot apex. Plant Cell *4*, 631–643.
12. Haecker, A., Gross-Hardt, R., Geiges, B., Sarkar, A., Breuninger, H., Herrmann, M., and Laux, T. (2004). Expression dynamics of *WOX* genes mark cell fate decisions during early embryonic patterning in *Arabidopsis thaliana*. Development *131*, 657–668.
13. Weigel, D., Ahn, J.H., Blázquez, M.A., Borevitz, J., Christensen, S.K., Fankhauser, C., Ferrándiz, C., Kardailsky, I., Malancharuvil, E.J., Neff, M.M., et al. (2000). Activation tagging in *Arabidopsis*. Plant Physiol. *122*, 1003–1013.
14. Gallois, J.L., Woodward, C., Reddy, G.V., and Sablowski, R. (2002). Combined *SHOOT MERISTEMLESS* and *WUSCHEL* trig-

ger ectopic organogenesis in *Arabidopsis*. Development *129*, 3207–3217.

15. Lenhard, M., Jurgens, G., and Laux, T. (2002). The *WUSCHEL* and *SHOOTMERISTEMLESS* genes fulfil complementary roles in *Arabidopsis* shoot meristem regulation. Development *129*, 3195–3206.

16. Brand, U., Grunewald, M., Hobe, M., and Simon, R. (2002). Regulation of *CLV3* expression by two homeobox genes in *Arabidopsis*. Plant Physiol. *129*, 565–575.

17. Nath, U., Crawford, B.C., Carpenter, R., and Coen, E. (2003). Genetic control of surface curvature. Science *299*, 1404–1407.

18. Palatnik, J.F., Allen, E., Wu, X., Schommer, C., Schwab, R., Carrington, J.C., and Weigel, D. (2003). Control of leaf morphogenesis by microRNAs. Nature *425*, 257–263.

19. Ferrándiz, C., Liljegren, S.J., and Yanofsky, M.F. (2000). Negative regulation of the *SHATTERPROOF* genes by *FRUITFULL* during *Arabidopsis* fruit development. Science *289*, 436–438.

20. Graham, E.T., and Trentham, W.R. (1998). Staining paraffin extracted, alcohol rinsed and air dried plant tissue with an aqueous mixture of three dyes. Biotech. Histochem. *73*, 178–185.

21. Laux, T., Mayer, K.F.X., Berger, J., and Jürgens, G. (1996). The *WUSCHEL* gene is required for shoot and floral meristem integrity in *Arabidopsis*. Development *122*, 87–96.

22. Clark, S.E., Jacobsen, S.E., Levin, J.Z., and Meyerowitz, E.M. (1996). The *CLAVATA* and *SHOOT MERISTEMLESS* loci competitively regulate meristem activity in *Arabidopsis*. Development *122*, 1567–1575.

23. Riou-Khamlichi, C., Huntley, R., Jacqmard, A., and Murray, J.A. (1999). Cytokinin activation of *Arabidopsis* cell division through a D-type cyclin. Science *283*, 1541–1544.

24. Riou-Khamlichi, C., Menges, M., Healy, J.M., and Murray, J.A. (2000). Sugar control of the plant cell cycle: Differential regulation of *Arabidopsis* D-type cyclin gene expression. Mol. Cell. Biol. *20*, 4513–4521.

25. Cockcroft, C.E., den Boer, B.G., Healy, J.M., and Murray, J.A. (2000). Cyclin D control of growth rate in plants. Nature *405*, 575–579.

26. Dewitte, W., and Murray, J.A. (2003). The plant cell cycle. Annu. Rev. Plant Biol. *54*, 235–264.

27. Schoof, H., Lenhard, M., Haecker, A., Mayer, K.F.X., Jürgens, G., and Laux, T. (2000). The stem cell population of *Arabidopsis* shoot meristems in maintained by a regulatory loop between the *CLAVATA* and *WUSCHEL* genes. Cell *100*, 635–644.

28. Willemsen, V., Wolkenfelt, H., de Vrieze, G., Weisbeek, P., and Scheres, B. (1998). The *HOBBIT* gene is required for formation of the root meristem in the *Arabidopsis* embryo. Development *125*, 521–531.

29. Blilou, I., Frugier, F., Folmer, S., Serralbo, O., Willemsen, V., Wolkenfelt, H., Eloy, N.B., Ferreira, P.C., Weisbeek, P., and Scheres, B. (2002). The *Arabidopsis HOBBIT* gene encodes a CDC27 homolog that links the plant cell cycle to progression of cell differentiation. Genes Dev. *16*, 2566–2575.

30. Giulini, A., Wang, J., and Jackson, D. (2004). Control of phyllotaxy by the cytokinin-inducible response regulator homologue ABPHYL1. Nature *430*, 1031–1034.

31. Mok, D.W., and Mok, M.C. (2001). Cytokinin metabolism and action. Annu. Rev. Plant Physiol. Plant Mol. Biol. *52*, 89–118.

32. Werner, T., Motyka, V., Strnad, M., and Schmulling, T. (2001). Regulation of plant growth by cytokinin. Proc. Natl. Acad. Sci. USA *98*, 10487–10492.

33. Werner, T., Motyka, V., Laucou, V., Smets, R., Van Onckelen, H., and Schmulling, T. (2003). Cytokinin-deficient transgenic *Arabidopsis* plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell *15*, 2532–2550.

34. Chaudhury, A.M., Letham, S., Craig, S., and Dennis, E.S. (1993). *amp1*—a mutant with high cytokinin levels and altered embryonic pattern, faster vegetative growth, constitutive photomorphogenesis and precocious flowering. Plant J. *4*, 907–916.