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WUSCHEL regulates cell differentiation during anther development

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Abstract

During anther development a series of cell specification events establishes the male gametophyte and the surrounding sporophytic structure. Here we show that the homeobox gene *WUSCHEL*, originally identified as a central regulator of stem cell maintenance, plays an important role in cell type specification during male organogenesis. *WUS* expression is initiated very early during anther development in the precursor cells of the stomium and terminates just before the stomium cells enter terminal differentiation. At this stage the stomium cells and the neighboring septum cells that separate the pollen sacs undergo typical cell wall thickening and degenerate which leads to rupture of the anther and pollen release. In *wus* mutants, neither stomium cells nor septum cells differentiate or undergo cell death and degenerate. As a consequence, the anther stays intact and pollen is not released. CLAVATA3 which is activated by *WUS* in stem cell maintenance, is not activated in anthers indicating a novel pathway regulated by *WUS*. Comparing *WUS* function in stem cell maintenance and sexual organ development suggests that *WUS* expressing cells represent a conserved signaling module that regulates behavior and communication of undifferentiated cells.
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Keywords: Anther development; Dehiscence; Stomium; Plant development; *WUSCHEL*

Introduction

Stamens are the male reproductive organs of plants consisting of a basal filament and the four-lobed sporogenic structure, the anther. During *Arabidopsis* anther development, a series of processes generates a set of cells that serve microspore production and dispersal (Sanders et al., 1999). First, four subepidermal archesporial cells give rise to sporogenous cells and contribute to the formation of the three surrounding cell layers, tapetum, middle layer and endothecium (Fig. 1A). Soon thereafter the anther primordium assumes a butterfly shape in cross-sections with four locules, each of which contains one developing pollen sac, at maturity (Fig. 1B). The two locules on each side of the anther become gradually separated by a furrow that contains a group of specialized cells, the stomium. After meiosis, tetrads of haploid microspores are formed that subsequently become separated as individual pollen grains. At the same time, the stomium differentiates and eventually, together with the endothecium and the septum, degenerates to release the pollen. It is unknown how this process is regulated but increased expression of a thiol-endopeptidase gene in the stomium and the connective tissue shortly before anther degeneration is consistent with these tissues undergoing programmed cell death (Koltunow et al., 1990). Nevertheless, elegant experiments demonstrate that genetic ablation of stomium cells itself is not sufficient for anther opening, indicating that the stomium has an active role in anther differentiation (Beals and Goldberg, 1997).

Genetic screens in *Arabidopsis* have identified a number of mutants defective in anther formation, microsporangial development, pollen formation, or dehiscence (Ito et al., 2004; Sanders et al., 1999, 2000; Sorensen et al., 2002, 2003; Wilson et al., 2001; Yang et al., 2003). Since such mutants are usually isolated in screens for male sterile plants, mutations in regulatory genes that result in phenotypes before anther development takes place might be missed.

The homeobox gene *WUS* was originally identified as a central regulator of shoot and floral meristems in *Arabidopsis* (Mayer et al., 1998) where it is expressed in a small group of

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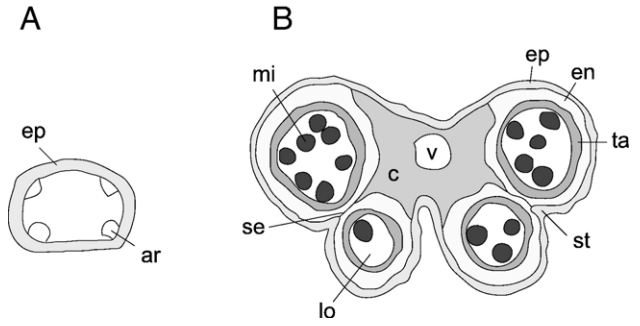

Fig. 1. Anther development. Schematic overview of the tissue types in anther development at stage 2 (A) and stage 10 (B). ar, Archesporial cell; ep, epidermis; en, endothecium; lo, locule; mi, microspore; se, septum; st, stomium; ta, tapetum.

cells, termed the organizing center, and is required to maintain the overlying stem cells undifferentiated. In several experimental settings *WUS* has been found to be sufficient to ectopically induce stem cells as judged from the expression of the stem cell marker gene *CLV3* (Gallois et al., 2004; Lenhard and Laux, 2003). However, the expression of *WUS* in developing female organs that lack stem cells indicates that *WUS* function is more complex (Gross-Hardt et al., 2002). In ovule development, *WUS* is expressed in the apical nucellus of the ovule primordium and is required to define the boundary between nucellus and underlying chalaza and to induce organ formation at that boundary (Gross-Hardt et al., 2002; Sieber et al., 2004). Recent studies indicate that *WUS* can act both as a transcriptional activator of the floral gene *AGAMOUS* and as a transcriptional repressor of cytokinin response genes (Kieffer et al., 2006; Leibfried et al., 2005; Lohmann et al., 2001), suggesting that its molecular function is modified by the developmental context. Here we report a novel function of the *WUS* gene during anther development.

Material and methods

Plant work

Plants were grown at 18°C with 16 h of light and 8 h of darkness. Landsberg *erecta* (L*er*) ecotype was used in all the experiments and here is referred to as wild type. *wus-1* and the *wus-3* mutants have been described previously (Laux et al., 1996; Mayer et al., 1998). The *wus-6* allele was kindly provided by Dr. Machida (Nagoya University) (Hamada et al., 2000). PCR-based genotyping of *wus-1* plants and transgenic plants carrying the *CLV1:WUS* transgene, and *WUS*::*NLSGUS* or *CLV3*::*NLSGUS* reporter genes have been described previously (Gross-Hardt et al., 2002).

In situ hybridization

In situ hybridization using a *WUS* antisense probe was essentially done as described previously (Mayer et al., 1998) with the exception that hybridization and washing temperatures were 56°C. For the anti-*WUS* probe, a cDNA fragment without the homeobox was used that does not cross-react with mRNA of related homeobox genes (Haecker et al., 2004). In addition, expression was verified by a *WUS:GUS* reporter gene (Gross-Hardt et al., 2002).

Histology

GUS staining was performed as previously described (Schoof et al., 2000). For histological sections of anthers, different stages of flowers were embedded either in LR-White (Polysciences, Warrington, PA) or Spurr's epoxy resin (Spurr, 1969), sectioned, and stained with toluidine blue as described previously (Endrizzi et al., 1996; Laux et al., 1996). 2- to 5-μm sections were analyzed under the bright field microscope. Scanning electron microscopy was done as previously described (Laux et al., 1996) with the exception that post-fixation with OsO_4 was omitted.

Results

WUS is expressed in immature stomium cells

To uncover unknown functions of *WUS* in plant development, we scrutinized the complete plant for expression of a *WUS*::*GUS* reporter gene (Baurle and Laux, 2005). We detected expression in developing anthers (see below), in addition to ovules and shoot and floral meristems consistent with previous studies (Green et al., 2005; Gross-Hardt et al., 2002; Wellmer et al., 2004), but nowhere else in the plant.

For a detailed developmental expression study we analyzed the spatial and temporal *WUS* mRNA pattern by situ hybridization studies throughout anther development. At stage 1 the circular anther primordium does not express detectable levels of *WUS* (Fig. 2A, Stages according to Sanders et al., 1999). At stage 2, the anther primordium has assumed an oval shape in cross-sections (Fig. 2B). At this stage, *WUS* expression is detected in 2–3 cells of the stomium region. In longitudinal sections through anther primordia, *WUS* is expressed in the stomium furrow throughout the length of the anthers (Fig. 2L). *WUS* expression continues until stage 11 when the stomium cells start to display specific differentiation, such as cell wall thickening (see below, Figs. 2I, K). *WUS* expression appears generally restricted to the epidermis and one or two subepidermal cells (Figs. 2C–E insets). In some cases isolated spots of signal are also detected one or two cell distances underneath the epidermis (Fig. 2G, inset). No *WUS* mRNA is detected in anthers after stage 11 (Fig. 2J).

WUS is essential for anther development

WUS expression in the anther primordia does not result in any detectable localized *CLV3*::*GUS* expression (not shown), suggesting a different function of *WUS* in anthers compared to its role in shoot and floral meristems. To investigate the function of *WUS* in anthers, we compared anther development in wild type and *wus* plants. We obtained identical results for the putative null allele *wus-1* and for the weak allele *wus-3*. For brevity, we will focus on *wus-1*.

Wild type anthers contain four locules with one pollen sac each that open at the end of anther development to release the pollen grains (Fig. 3A). By contrast, *wus* anthers often have less, smaller or malformed lobes in comparison to wild type and do not open (Figs. 3B–D). This indicates that *WUS* is required for normal anther development. Since *WUS* is required for floral meristem activity (Laux et al., 1996), defective anther development could reflect missing *WUS* function in the floral meristem leading to aberrant initiation of anther primordia due to, for example, a shortage of cells or misplacement of the primordia. Alternatively it could result from the lack of *WUS*

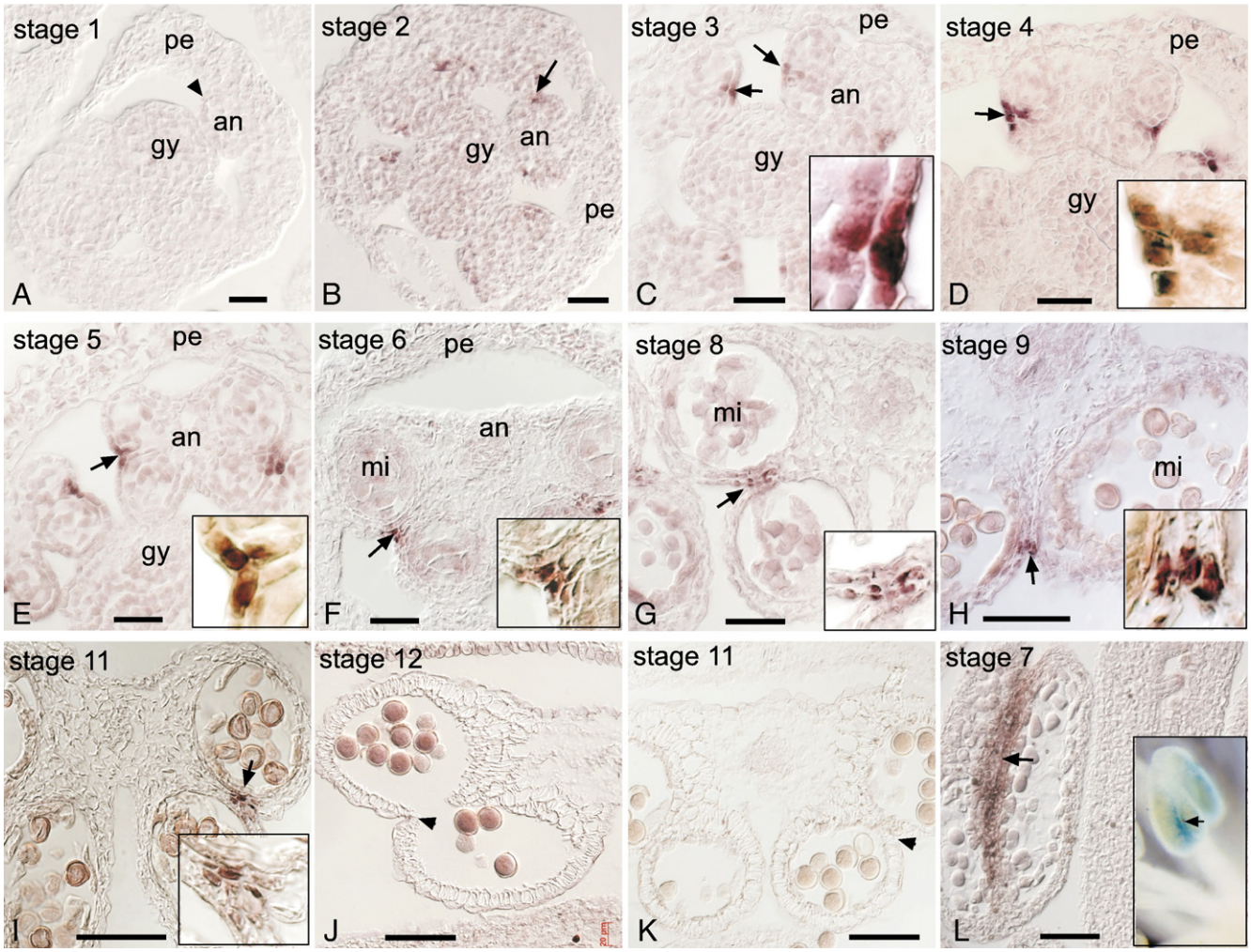

Fig. 2. *WUS* expression pattern during wild type anther development. In situ hybridization using *WUS* antisense mRNA (A–J, L), and sense mRNA as a control (K). Cross-sections (A–K) and a longitudinal section (L) of wild type stamens. Stages (according to Sanders et al., 1999) are indicated. *WUS* mRNA is undetectable at stage 1 (A). Arrowhead indicates the stomium region. Between stage 2 (B) and stage 11 (I), *WUS* mRNA is specifically detected in the stomium region (arrows). Inlays in panels B–I show the stomium region at higher magnification. Inlay in panel L shows *WUS*: :*GUS* expression in the stomium of a wild type anther. Subsequently no *WUS* expression is detectable (J). Hybridization signal is detected as a brown color. Arrows indicate *WUS* expression in stomium. Arrowhead indicates the stomium region. gy, gynoecium; mi, microspore; pe, petal; an, anther. Scale bars: 25 μm.

function in the stomium itself. To distinguish between these possibilities, we analyzed flowers of *wus*-plants carrying a *CLV1*: :*WUS* transgene that rescues stem cell maintenance in shoot and floral meristems (Gross-Hardt et al., 2002). The rescued flowers display variable organ numbers in the central whorls ranging from a single stamen, similar to flowers of the *wus-1* mutant, to flowers with six stamens and a gynoecium indistinguishable from wild type (Table 1). Interestingly, we found malformed anthers with abnormal locule numbers only in flowers with less than four stamens, whereas flowers with four or more stamens always have anthers with wild type locule numbers (Table 1). Thus, malformation of anther morphology correlates with stamen number and thus appears to be the consequence of reduced floral meristem activity.

WUS specifically affects stomium development

The vast majority (96%; n-340) of anthers in *CLV1:WUS wus* flowers do not open and release pollen similar to anthers of the *wus* mutant alone. In contrast to anther morphology this defect is independent of floral organ numbers and is thus not affected by floral meristem activity, indicating that anther opening requires *WUS* expression in the stomium.

To address the role of *WUS* in dehiscence at the cellular level, we analyzed histological sections of developing anther primordia of *wus* mutants and wild type. In wild type anthers, the four lobes containing one locule each can readily be recognized at stage 4 (Fig. 4A). The microspore mother cells start to undergo meiosis at stage 5 (Fig. 4B). The stomium cells (arrows) at the junctions of the two lobes of each theca and the interlocular septum cells remain relatively small and unvacuolated until stage 10 of anther development, whereas surrounding cells appear to differentiate, as recognized by their enlargement and, for example, the development of fibrous bands (Figs. 4A–F). At stage 11, however, the stomium cells become flat and their cell walls appear thickened (Fig. 4G). Subsequently, the interlocular septum adjacent to the stomium disintegrates resulting in the fusion of both locules of one theca. Eventually, degeneration of

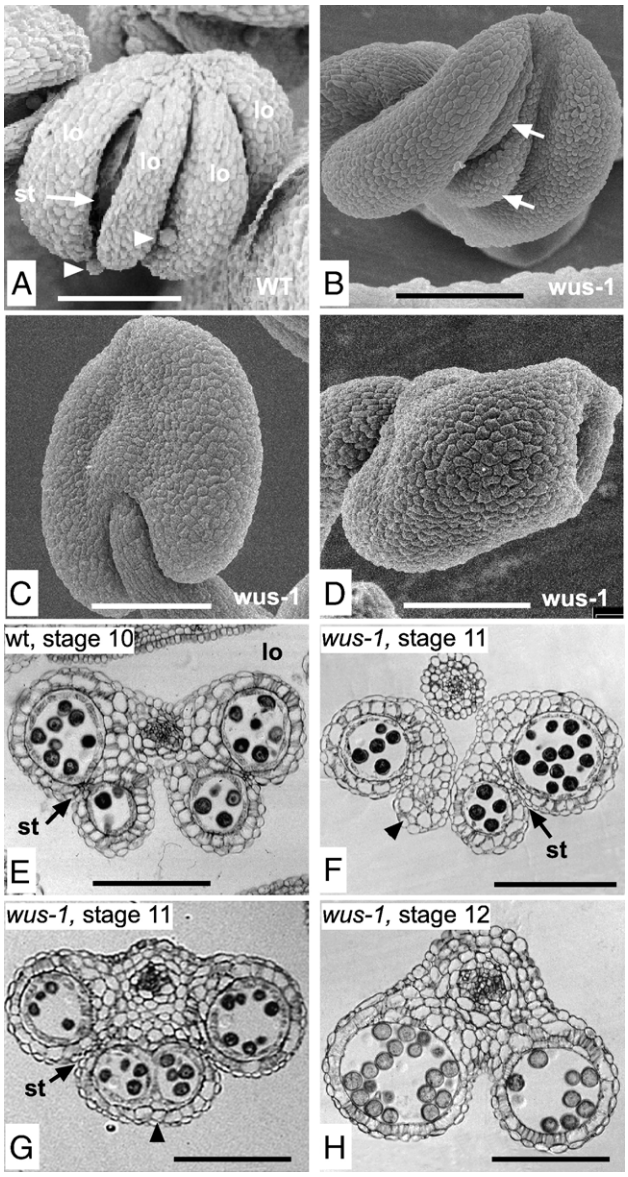

Fig. 3. *wus-1* anther morphology is altered. (A–D) Scanning electron microscopy images of anthers. (A) A wild type anther with four locules: the 2 outer and 2 inner locules are of similar size each. The stomium has opened (arrow) and pollen grains are visible (arrowheads). (B–D) *wus* anthers display a reduction in the size of central locules (arrow in panel B), a reduced number of locules (C), or a single fused structure (D). (E) Histological cross-sections of a wild type anther displaying 4 locules. (F–H) Cross-sections of *wus-1* anthers with abnormal morphology. Scale bars: 100 μm. lo, locule; st, stomium.

the stomium cells opens the anther which leads to the release of the pollen grains (Fig. 4H).

We did not observe any abnormal phenotypes in the gametogenic tissue of *wus-1* anthers and scarcely released *wus* pollen is fertile when hand pollinated onto a wild type pistil (not shown). Furthermore, except for the aberrant number of locules, anthers of *wus-1* mutants are histologically indistinguishable from wild type until stage 10 in antherogenesis (Figs. 4I, J). Notably, there are no abnormalities in *wus-1* anthers in the stomium region (arrows) at the developmental stages when *WUS* is expressed in wild type. At stage 11, however, when the cells in the stomium and the neighboring septum cells undergo cell wall thickening and eventually degenerate in wild type, they remain unchanged in the *wus-1* mutant (Figs. 4K, L). As a consequence, *wus-1* anthers do not open and release pollen grains. Taken together, expression of *WUS* is essential for proper development of the stomium region and the neighboring septum cells.

Discussion

Here we have reported a specific role of the *WUS* gene in cell differentiation during anther organogenesis. In analogy to its role in stem cell maintenance, a potential function of *WUS* in anther organogenesis could be to maintain anther primordium cells in an undifferentiated state. Indeed, as long as the stomium cells express *WUS*, they appear undifferentiated in that they are small and cytoplasmic. Only when *WUS* expression has ceased, the cells differentiate and degenerate, consistent with a model in which *WUS* blocks differentiation.

However, our data argue against this possibility and rather suggest that *WUS* has a novel function in anther organ development: firstly, we have not found any evidence for premature or ectopic differentiation of anther cells as observed in the stem cell niche in loss of function *wus* mutants. Furthermore, during male organogenesis *WUS* does not induce *CLV3* expression, as it does in the stem cell niche, nor does it induce expression of the *WUSCHEL TARGET* 1 gene, as it does in ovule development (Stern, D and T.L., unpublished).

Surprisingly, only the developmental processes that in wild type anthers take place after *WUS* expression has become undetectable, differentiation and degeneration of stomium and septum cells, are detectably affected in the *wus* anther. Therefore, we propose that in anther development *WUS* function is required to provide the competence of stomium and septum cells to properly differentiate later. Based on the expression of the *WUS* homolog in *Antirrhinum* (Kieffer et al., 2006) this function appears to be conserved between plant species.

Besides the differences, are there also commonalities of *WUS* action in sexual organogenesis and stem cell maintenance? Notably, in all cases *WUS* is expressed in small groups of relatively undifferentiated cells and affects the fate of both the expressing cells and the neighboring cells. A plausible hypothesis is therefore that *WUS* expressing cells function as conserved signaling modules in cell specification. As a primary consequence, these cells appear to be maintained in

Table 1
Anther morphology in *wus-1* correlates with floral meristem activity

Locule number	Wild type	CLV1: *WUS*, *wus-1*				
	Anthers per flower					
	6	1	2	3	4	5	6
1	0						
2	1	10	13	6		1	
3	0	13	13	11		1	
4	99	77	74	84	100	98	100
n	238	30	69	18	41	125	60

The fraction of anthers with a given locules number is given in percentages. *n*, number of anthers analyzed.

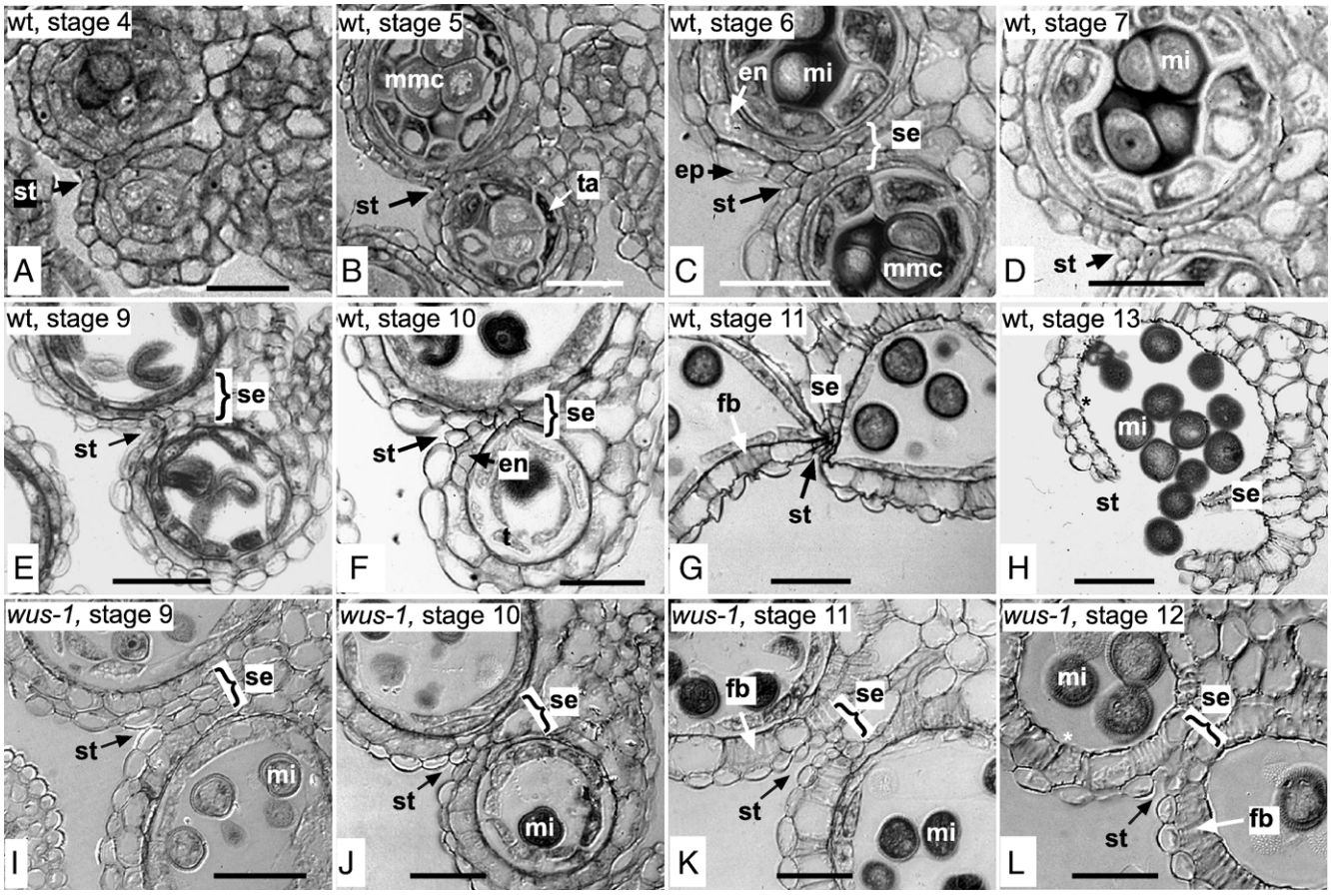

Fig. 4. Defective stomium and septum development in *wus-1* anthers. (A–H) Histological cross-sections of developing wild type anthers. The developmental stages (according to Sanders et al., 1999) are indicated. (A–E) The stomium cells remain relatively small and cytoplasmic in comparison to, e.g., the connective cells until stage 9. At stage 10 (F) septum cells become flattened. At stage 11 (G) stomium and septum cells display localized cell wall thickening. At stage 13 (H), the stomium cells and the septum have been disrupted and the pollen grains are released. (I–L) Cross-section of developing *wus-1* anthers. Until stage 9 (panel I, compare to panel E), *wus-1* anthers are indistinguishable from wild type. At stage 10 (J), septum cells remain unchanged and are not flattened. At stages 11 (K) and 12 (L) stomium and septum cells stay intact and have partially increased in size. Scale bars: 25 μm. ep, Epidermis; en, endothecium; fb, fibrous bands; mmc, microspore mother cell; m, microspore; se, septum; st, stomium; ta, tapetum.

an uncommitted state and in turn affect the fate of their neighbors. This model might also apply to several *WUS RELATED HOMEOBOX* genes that are transiently expressed in small cell groups during embryogenesis and appear to affect specification of expressing and neighboring cells (Haecker et al., 2004; Breuninger, H., Rikirsch, E., Hermann, M., and T.L. unpublished).

The restriction of the *WUS* signaling module to the shoot apex and to both sexual organs raises the question whether these structures have a common evolutionary origin or whether *WUS* signaling has been recruited independently. Indeed, expression of *WUS* in the shoot meristem and the nucellus provides molecular support for the hypothesis that the nucellus is homologous to a shoot meristem which was derived from paleobotanical studies (Gross-Hardt et al., 2002; Kenrick and Crane, 1997). The origin of the angiosperm anther, however, is not clear. In one view, based on comparison of anthers to synangia of seed ferns, stamens may be derived from sporophylls bearing two synangia, each of which is comprised of two fused sporangia (Doyle, 1994). In this view, the stomium would represent the site of fusion between two adjacent sporangia that has recruited some aspect of apical meristem function.

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References

Baurle, I., Laux, T., 2005. Regulation of WUSCHEL transcription in the stem cell niche of the *Arabidopsis* shoot meristem. Plant Cell 17, 2271–2280.

Beals, T.P., Goldberg, R.B., 1997. A novel cell ablation strategy blocks tobacco anther dehiscence. Plant Cell 9, 1527–1545.

Doyle, J., 1994. Origin of the angiosperm flower: a phylogenetic perspective. Plant Syst. Evol., Suppl. 8, 7–29.

Endrizzi, K., Moussian, B., Haecker, A., Levin, J., Laux, T., 1996. The SHOOTMERISTEMLESS gene is required for maintenance of undifferentiated cells in *Arabidopsis* shoot and floral meristems and acts at a different regulatory level than the meristem genes WUSCHEL and ZWILLE. Plant J. 10, 967–979.

Gallois, J.L., Nora, F.R., Mizukami, Y., Sablowski, R., 2004. WUSCHEL induces shoot stem cell activity and developmental plasticity in the root meristem. Genes Dev. 18, 375–380.

Green, K.A., Prigge, M.J., Katzman, R.B., Clark, S.E., 2005. CORONA, a member of the class III homeodomain leucine zipper gene family in *Arabidopsis*, regulates stem cell specification and organogenesis. Plant Cell 17, 691–704.

Gross-Hardt, R., Lenhard, M., Laux, T., 2002. WUSCHEL signaling functions in interregional communication during *Arabidopsis* ovule development. Genes Dev. 16, 1129–1138.

Haecker, A., Gross-Hardt, R., Geiges, B., Sarkar, A.K., Breuninger, H., Herrmann, M., Laux, T., 2004. Expression dynamics of WOX genes mark cell fate decisions during early embryonic patterning in *Arabidopsis thaliana*. Development 131, 657–668.

Hamada, S., Onouchi, H., Tanaka, H., Kudo, M., Liu, Y.G., Shibata, D., MacHida, C., Machida, Y., 2000. Mutations in the WUSCHEL gene of *Arabidopsis thaliana* result in the development of shoots without juvenile leaves. Plant J. 24, 91–101.

Ito, T., Wellmer, F., Yu, H., Das, P., Ito, N., Alves-Ferreira, M., Riechmann, J.L., Meyerowitz, E.M., 2004. The homeotic protein AGAMOUS controls microsporogenesis by regulation of SPOROCYTELESS. Nature 430, 356–360.

Kenrick, P., Crane, P., 1997. The Origin and Early Diversification of Land Plants: A Cladistic Study. Smithsonian Institution Press, Washington.

Kieffer, M., Stern, Y., Cook, H., Clerici, E., Maulbetsch, C., Laux, T., Davies, B., 2006. Analysis of the transcription factor WUSCHEL and its functional homologue in *Antirrhinum* reveals a potential mechanism for their roles in meristem maintenance. Plant Cell 18, 560–573.

Koltunow, A.M., Truettner, J., Cox, K.H., Wallroth, M., Goldberg, R.B., 1990. Different temporal and spatial gene expression patterns occur during anther development. Plant Cell 2, 1201–1224.

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

Leibfried, A., To, J.P., Busch, W., Stehling, S., Kehle, A., Demar, M., Kieber, J.J., Lohmann, J.U., 2005. WUSCHEL controls meristem function by direct regulation of cytokinin-inducible response regulators. Nature 438, 1172–1175.

Lenhard, M., Laux, T., 2003. Stem cell homeostasis in the Arabidopsis shoot meristem is regulated by intercellular movement of CLAVATA3 and its sequestration by CLAVATA1. Development 130, 3163–3173.

Lohmann, J., Huong, R., Hobe, M., Busch, M., Parcy, F., Simon, R., Weigel, D., 2001. A molecular link between stem cell regulation and floral patterning in *Arabidopsis*. Cell 105, 793–803.

Mayer, K.F.X., Schoof, H., Haecker, A., Lenhard, M., Jürgens, G., Laux, T., 1998. Role of WUSCHEL in regulating stem cell fate in the *Arabidopsis* shoot meristem. Cell 95, 805–815.

Sanders, P., Bui, A., Wetering, K., McIntire, K., Hsu, Y., Lee, P., Truong, M., Beals, T., Goldberg, R., 1999. Anther developmental defects in *Arabidopsis thaliana* male sterile mutants. Sex. Plant Reprod. 11, 297–322.

Sanders, P.M., Lee, P.Y., Biesgen, C., Boone, J.D., Beals, T.P., Weiler, E.W., Goldberg, R.B., 2000. The *Arabidopsis* DELAYED DEHISCENCE1 gene encodes an enzyme in the jasmonic acid synthesis pathway. Plant Cell 12, 1041–1061.

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

Sieber, P., Gheyselinck, J., Gross-Hardt, R., Laux, T., Grossniklaus, U., Schneitz, K., 2004. Pattern formation during early ovule development in *Arabidopsis thaliana*. Dev. Biol. 273, 321–334.

Sorensen, A., Guerineau, F., Canales-Holzeis, C., Dickinson, H.G., Scott, R.J., 2002. A novel extinction screen in *Arabidopsis thaliana* identifies mutant plants defective in early microsporangial development. Plant J. 29, 581–594.

Sorensen, A.M., Krober, S., Unte, U.S., Huijser, P., Dekker, K., Saedler, H., 2003. The Arabidopsis ABORTED MICROSPORES (AMS) gene encodes a MYC class transcription factor. Plant J. 33, 413–423.

Spurr, A.P., 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26, 31–43.

Wellmer, F., Riechmann, J.L., Alves-Ferreira, M., Meyerowitz, E.M., 2004. Genome-wide analysis of spatial gene expression in *Arabidopsis* flowers. Plant Cell 16, 1314–1326.

Wilson, Z.A., Morroll, S.M., Dawson, J., Swarup, R., Tighe, P.J., 2001. The Arabidopsis MALE STERILITY1 (MS1) gene is a transcriptional regulator of male gametogenesis, with homology to the PHD-finger family of transcription factors. Plant J. 28, 27–39.

Yang, S.L., Xie, L.F., Mao, H.Z., Puah, C.S., Yang, W.C., Jiang, L., Sundaresan, V., Ye, D., 2003. Tapetum determinant1 is required for cell specialization in the *Arabidopsis* anther. Plant Cell 15, 2792–2804.