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Stéphanie Breuil Broyer, Patrice Morel, Janice de Almeida-Engler, Vincent Coustham, Ioan Negrutiu, et al.. High-resolution boundary analysis during *Arabidopsis thaliana* flower development.. *Plant Journal*, 2004, 38 (1), pp.182-192. 10.1111/j.1365-313X.2004.02026.x . hal-02677983

HAL Id: hal-02677983

<https://hal.inrae.fr/hal-02677983>

Submitted on 31 May 2020

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TECHNICAL ADVANCE

High-resolution boundary analysis during *Arabidopsis thaliana* flower development

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Received 17 October 2003; revised 16 December 2003; accepted 23 December 2003.

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Summary

We report a comparative analysis of cell proliferation patterns during *Arabidopsis* flower development. Cell division was evaluated by a direct method, i.e. the 5-bromo-2'-deoxyuridine (BrdU) incorporation/immunodetection procedure. BrdU patterns in wild-type plants were correlated with the expression profiles of both several cell cycle genes involved in the control of the G₁/S transition and cell cycle-related repressor genes, *MSI4* and *MSI5*, encoding WD-repeat proteins. To evaluate how proliferation patterns arise with respect to boundaries and vice versa, the expression of a boundary gene, *CUP SHAPED COTYLEDON (CUC)2*, was determined. Combining these approaches, we demonstrate that boundaries between inflorescence and floral meristems and between floral whorls are narrow bands of non-dividing cells. In addition, we show that negative and positive regulators of cell proliferation are simultaneously and continuously expressed in dividing meristematic domains, being excluded from boundary cells. Finally, BrdU incorporation and *CUC2* *in situ* hybridisation patterns were analysed in two mutant backgrounds, *agamous (ag)-1* and *superman (sup)-1*, in order to assess changes in boundary establishment and different levels of indeterminacy under conditions of altered proliferation at the floral meristem centre.

Keywords: BrdU, flower development, boundary, *Arabidopsis*, *SUPERMAN*, *AGAMOUS*.

Introduction

Proper development of multicellular organisms requires the establishment of compartments with distinct identities (insect parasegments, worm recurrent cell lineage, mammalian rhombomeres, flower whorls) separated by boundaries that prevent the movement of cells and their descendants from one compartment to another (Cooke and Moens, 2002; Dahmann and Basler, 1999). Boundary setting is particularly important during animal development – where cell identity is established during embryogenesis and where cell migration is possible – and usually relies on differential cell affinity (Dahmann and Basler, 1999). Such mechanisms are probably different in plants where cell migration is

prevented by the rigid cell wall and where cell identity is determined through its position. However, as plants develop following strict and reiterative patterns and rarely develop chimaeric organs, compartment and boundary clearly do exist (Callos and Medford, 1994; Doonan, 2000; Irish and Jenik, 2001). An example of a biological process displaying such boundaries is flower development.

Floral patterning is tightly controlled in eudicots as a given species develops the same number of organs, free or fused, at defined positions within the flower meristem (FM). In *Arabidopsis*, the flower consists of four free sepals, four free petals, six free stamens and two congenitally

fused carpels (Irish, 1999). Much progress has been made during the past 15 years in our understanding of flower development (Irish, 1999; Lohmann and Weigel, 2002) through the identification of genes responsible for shoot apical meristem identity, floral meristem identity, floral organ identity (ABC genes) and of cadastral genes. The latter delimit whorls and organ primordia territories and prevent organ fusion. These genes include *NO APICAL MERISTEM* (*NAM*; Souer *et al.*, 1996), *CUP SHAPED COTYLEDON* (*CUC*; Ishida *et al.*, 2000), *UNUSUAL FLOWER ORGANS* (*UFO*; Ingram *et al.*, 1995; Laufs *et al.*, 2003) or *SUPERMAN* (*SUP*; Bowman *et al.*, 1992; Schultz *et al.*, 1991). It is assumed that such cadastral genes act by inhibiting cell proliferation in regions delimiting organogenic territories within the FM (Bereterbide *et al.*, 2001; Doonan, 2000; Hiratsu *et al.*, 2002; Vroemen *et al.*, 2003).

The underlying cellular patterning during the early steps of floral development has so far been explored through physical and phyllotactic models (Callos and Medford, 1994; Green, 1999) or by indirect methods such as sector boundary (Bossinger and Smyth, 1996; Furner, 1996; Jenik and Irish, 2000; Vincent *et al.*, 1995) and cytological (Barton and Poethig, 1993) analyses. Sector boundary analyses in *Antirrhinum* and *Arabidopsis* have shown that lineage restrictions arise between whorls at about the florotypic stage correlating with the onset of the ABC gene expressions (Vincent *et al.*, 1995), and that floral and floral organ primordia are initiated from a constant number of cells (Bossinger and Smyth, 1996). Mutations in floral homeotic genes do not affect this patterning (Bossinger and Smyth, 1996) but rather the rate and orientation of cell division in the three cell layers (L1–L3) from stage 6 onwards (Jenik and Irish, 2000). Cell proliferation patterns during floral development have also been analysed by *in situ* hybridisation with cell cycle genes (Doonan, 2000). The observed patterns are usually patchy, presumably because of the phase-specific expression of cell cycle genes. The only apparent exceptions are the D-type cyclins, with no or weak expression at the base of flower organ primordia (Gaudin *et al.*, 2000; Towers *et al.*, 2003). Taken together, these data support the view that organ primordia arise as bulges of cells separated by boundary regions with low rates of cellular proliferation. However, despite the various reported approaches, we still need a more direct demonstration linking developmental dynamics, and in particular boundary formation, to the proliferative status of the FM.

Direct labelling of dividing cells can be achieved not only by ^3H -thymidine incorporation into DNA (Brown *et al.*, 1964) but also by using the non-radioactive thymidine analogue, 5-bromo-2'-deoxyuridine (BrdU), shown to be as valid as ^3H -thymidine autoradiography (Hervas *et al.*, 2002). BrdU labelling and immunodetection are widely used in animal systems and especially in *Drosophila* to study cell cycle patterning during development (Duman-

Scheel *et al.*, 2002; Johnston and Edgar, 1998; Johnston *et al.*, 1999; Weigmann *et al.*, 1997). In plants, BrdU incorporation and immunodetection experiments have been carried out to investigate DNA structure and replication, DNA methylation, cell division rates and synchronisation of cell cycle events (Armstrong *et al.*, 2001; de-Castro *et al.*, 2000; Nagar *et al.*, 2002; Zluvova *et al.*, 2001), but only rarely to evaluate proliferation patterns (Sato-Nara and Fukuda, 2000).

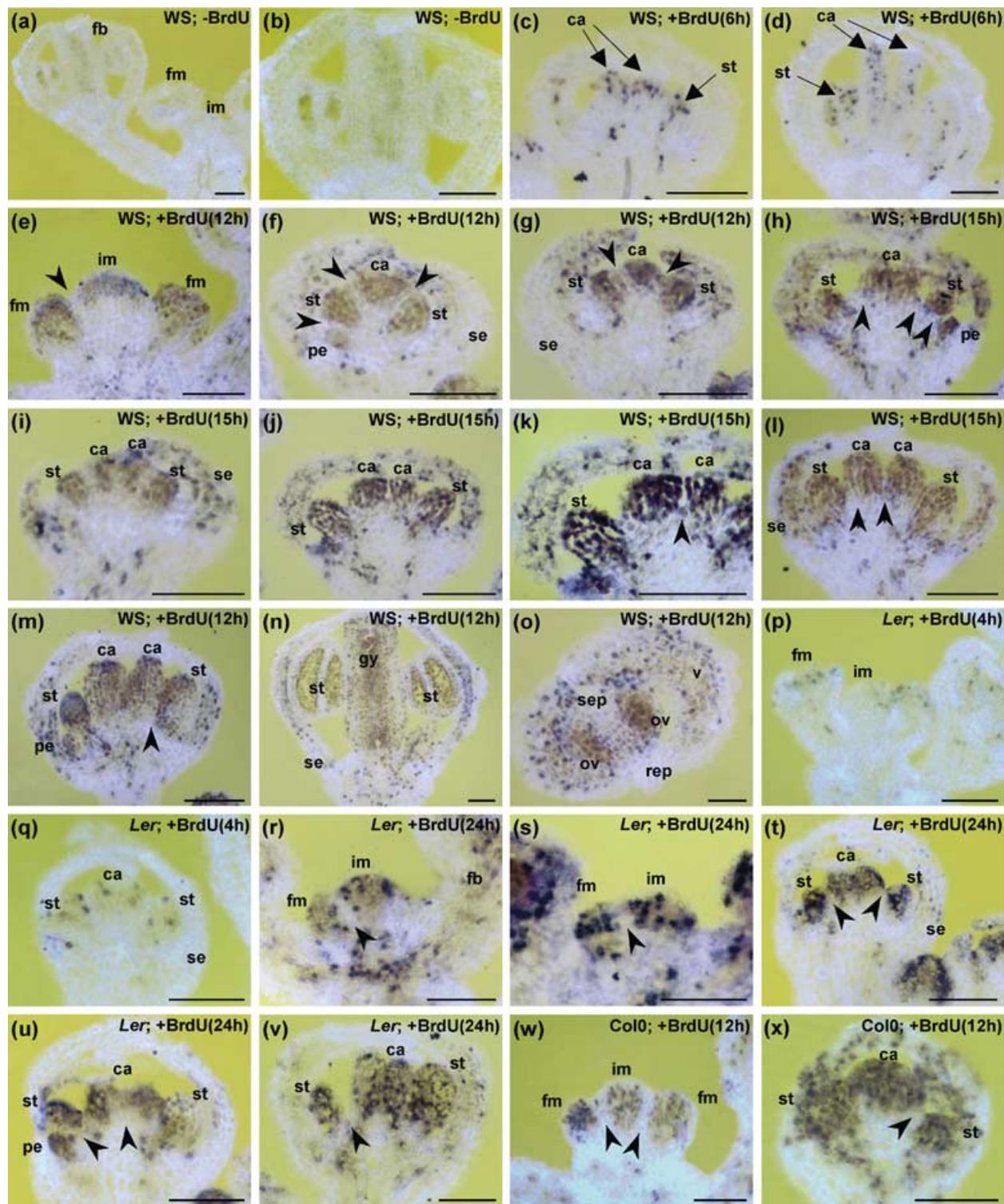
We report here the analysis of cell proliferation patterns during *Arabidopsis* flower development, by applying the BrdU incorporation/immunodetection method. BrdU labelling was first performed on wild-type flowers from various ecotypes and then correlated with the expression profiles of several cell cycle genes involved in the control of the G₁/S transition, generally accepted as the principal event in the commitment to cell division (Stals and Inze, 2001). BrdU patterns were also correlated with the expression profiles of the cell cycle-related repressor genes, *Multicopy suppressor CFIRAI*, *MSI4* and *MSI5*, encoding WD-repeat proteins (Delichère *et al.*, 1999; Hennig *et al.*, 2003; Morel P., manuscript in preparation). Furthermore, in order to evaluate the relationship between proliferation patterns and organ boundaries, we have also determined the expression patterns of a boundary gene, *CUC2* (Aida *et al.*, 1997). Subsequently, the BrdU and *in situ* hybridisation patterns were assessed in the *Arabidopsis* mutants *agamous (ag)-1* (Bowman *et al.*, 1989) and *superman (sup)-1* (Bowman *et al.*, 1992; Schultz *et al.*, 1991). These genes control FM determinacy (*AG* and *SUP*), floral organ identity (*AG*) and boundary formation between male and female domains at the flower meristem centre (*SUP*).

Results

BrdU incorporation patterns in the floral meristem

Cell proliferation patterns during floral development were first examined in wild-type inflorescences. Excised inflorescences were cultured *in vitro*, as described by Magnard *et al.* (2001), in medium supplemented with BrdU, and for each time point, three independent inflorescences were processed and monitored for BrdU by immunostaining. Preliminary tests had shown that 36-h culture in the presence of BrdU does not perceptibly alter flower development (data not shown).

BrdU incorporation was first assayed in the Wassilewskija (WS) ecotype (Figure 1a–o). No labelled nuclei could be detected in control inflorescences (24-h culture without BrdU; Figure 1a,b). A 6-h BrdU pulse yielded small numbers of labelled cells appearing as patchy patterns in floral organ primordia (for example stamens and carpels; Figure 1c,d). A 12–15-h BrdU pulse (both conditions gave similar results)



increased the number of labelled cells, by detecting all the dividing cells that had entered S phase at least once during the pulse. Such a labelling made it possible to visualise proliferation domains corresponding to inflorescence meristems (Figure 1e), young floral meristems (Figure 1e) and floral organ primordia (Figure 1f–m). Remarkably, narrow bands of cells that do not incorporate BrdU clearly separate the proliferating domains. Such non-dividing cells, usually two or three rows wide (Figure 1e,k), are formed during various stages of flower meristem progression and could be considered as presumptive boundaries.

Thus, BrdU labelling dynamics has been studied at various developmental stages. At stage 2, the FM appears as a uniform group of dividing cells separated from the inflorescence meristem by non-dividing cells (Figure 1e). At stage 4, the floral meristem is composed of islets of proliferating cells corresponding to presumptive organ primordia, with each islet separated from the others by non-proliferating cells (Figure 1f). At stage 5, petal and stamen primordia arise from the meristem dome as individual groups of dividing cells (Figure 1g,h). At this stage, the male–female border is clearly visible. At stages 6 and 7 (Figure 1i–k), the two carpel primordia emerge from the central dome of the meristem. Interestingly, the separation between the two carpel primordia corresponds to a zone of non-proliferating cells at the very centre of the meristem (Figure 1k), which do not take part in placentation. From stage 10 onwards, BrdU incorporation occurs mainly within the gynoecium (Figure 1n). A transverse section through the gynoecium shows that the labelling is because of cell proliferation in the ovules (Figure 1o). At this stage, the dehiscence zone in the anther (Figure 1n), the replum and the septum in the pistil (Figure 1o) show little or no BrdU incorporation.

To check whether similar proliferation patterns occur in other *Arabidopsis* ecotypes, the BrdU incorporation experiment was repeated with the Landsberg *erecta* (*Ler*; Figure 1p–v) and Columbia (*Col-0*; Figure 1w,x) ecotypes. Similar to *WS*, short 4-h BrdU pulses yielded patchy signals (Figure 1p,q). On the contrary, long BrdU pulses (24 h for *Ler* and 12 h for *Col-0*) allowed the visualisation of distinct zones of proliferating cells corresponding to inflorescence and floral meristems (Figure 1r,s,w) or to floral organ

primordia (Figure 1t–v,x). In all examples, proliferating zones were delimited by layers of non-dividing cells that do not incorporate BrdU (Figure 1r–x). Thus, proliferation patterns in inflorescences appear to be identical in all *Arabidopsis* ecotypes. Islets of dividing cells, which correspond to floral organ primordia, are defined very early in development and are strictly delimited by layers of non-dividing cells, i.e. potential boundaries.

Expression patterns of genes related to cell cycle control and of the boundary gene CUC2

The BrdU patterns were correlated with the expression profiles of cell cycle, cell cycle-related and boundary genes. The spatial and temporal expression patterns of the following genes were analysed during flower development by mRNA *in situ* hybridisation (Figure 2). The cell cycle-related genes selected for analysis were the cyclin-dependent kinase (CDK) subunit *Arath;CKS1* (Figure 2a,b; De Veylder *et al.*, 2001b), the retinoblastoma tumour suppressor protein *Arath;Rb* (Figure 2c,d; Kong *et al.*, 2000), D-type cyclins *Arath;CycD2;1* and *Arath;CycD3;1* (Figure 2e,f,g,h, respectively; Soni *et al.*, 1995), transcription factors *Arath;E2Fa* and *Arath;E2Fb* (Figure 2i,j,k,l, respectively; Vandepoele *et al.*, 2002), CDK inhibitors Kip-Related Protein *Arath;KRP2* and *Arath;KRP3* (Figure 2m,n,o,p, respectively; De Veylder *et al.*, 2001a) and the *MSI4* and *MSI5*, encoding WD-repeat proteins (Figure 2q,r,s,t, respectively; Delichère *et al.*, 1999; Hennig *et al.*, 2003). In addition, the organ boundary gene *CUC2* (Aida *et al.*, 1997) was also analysed (Figure 2u–x). For each gene analysed, gene-specific probes were used. Hybridisation with sense probes resulted in no significant background except for a weak stain seen for *Arath;Rb* and *Arath;CycD3;1* sense probes (data not shown).

In situ hybridisation results (Figure 2) yielded two primary conclusions. First, the expression profiles of all cell cycle-related genes are similar to each other during floral development, being expressed in dividing cells, such as floral organ primordia (Figure 2a–t) or vascular tissues (precambium, Figure 2g,h, for example). In addition, none of these genes show tissue, layer (L1–L3) or polarity (abaxial/adaxial) specificities. Second, the expression patterns of these genes parallel the BrdU profiles, displaying groups

Figure 1. Patterns of BrdU incorporation during wild-type floral development.

(a–o) Immunostaining performed on *WS*: (a,b) controls, inflorescences and floral buds from plants grown for 24 h without BrdU; (c,d) flower buds from inflorescences incubated for 6 h with BrdU; (e–o) successive floral developmental stages (see text) from inflorescences incubated 12–15 h with BrdU.

(p–v) Immunostaining performed on *Ler*: (p,q) inflorescences and floral buds from plants incubated for 4 h with BrdU; (r–v) inflorescences meristem and floral buds incubated for 24 h with BrdU.

(w, x) Immunostaining performed on *Col-0* inflorescence meristems and floral buds incubated for 12 h with BrdU.

Arrowheads point to non-dividing cells that did not incorporate BrdU. Immunostaining was performed on longitudinal sections, except for (o) performed on transversal section. All flowers and meristems are oriented with the apex of the flower towards the top. Key: im, inflorescence meristem; fm, floral meristem; fb, floral bud; se, sepal; pe, petal; st, stamen; ca, carpel; gy, gynoecium; ov, ovules; rep, replum; sep, septum. Scale bars = 60 µm.

Incorporated BrdU is visualised with immunostaining. Cell walls were stained with calcofluor, and observations were made under UV. Details of ecotypes and BrdU labelling methods used are given in the top right corner of each picture.

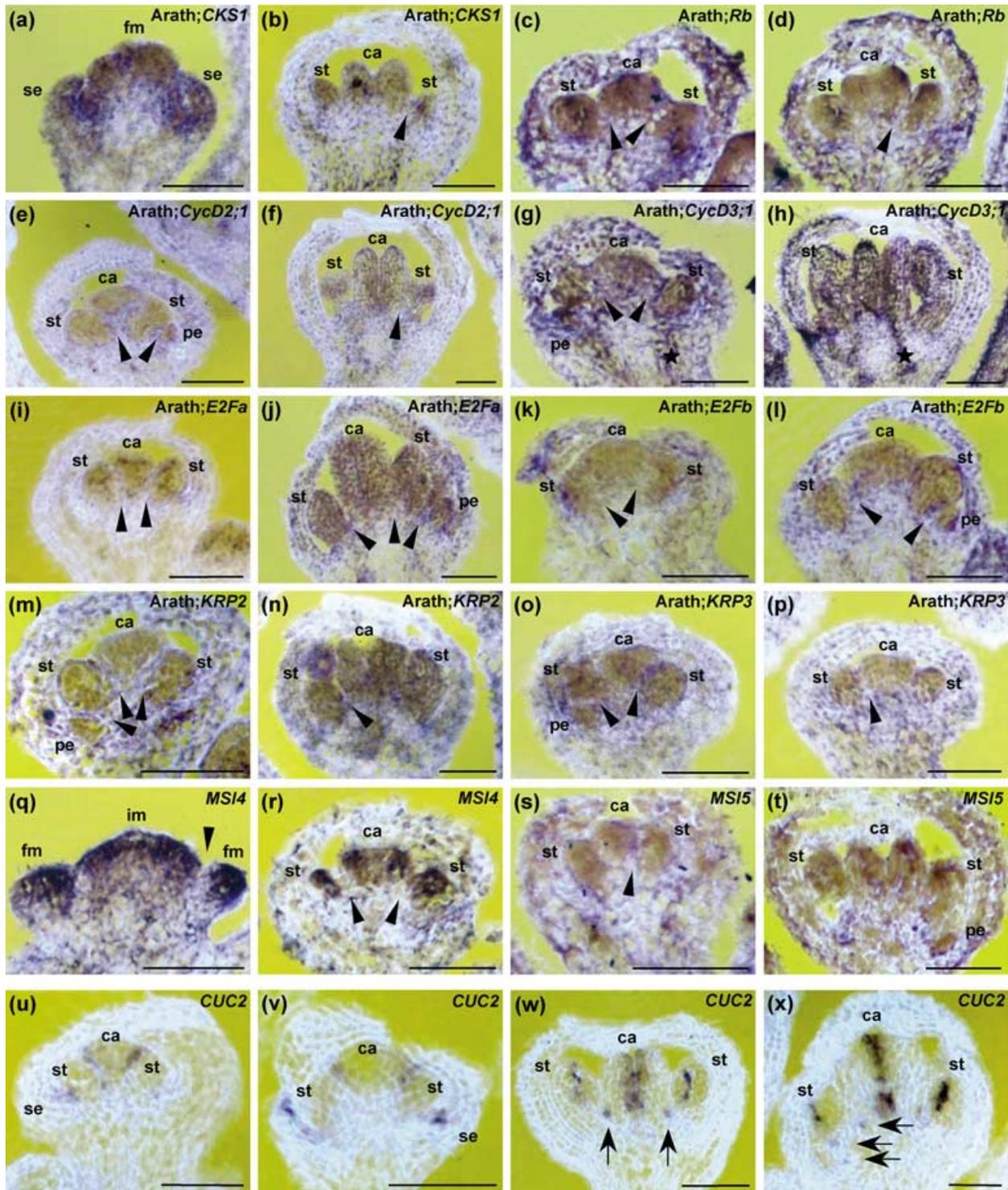


Figure 2. Expression of cell cycle, cell cycle-related genes and of *CUC2* in wild-type developing flowers. Expression patterns were obtained by non-radioactive *in situ* hybridisation. Cell walls were stained with calcofluor, and observations were made under UV. The analysed genes (the names are also written in the top right corner of each picture) were: (a,b) *Arath;CKS1*; (c,d) *Arath;Rb*; (e,f) *Arath;CycD2;1*; (g,h) *Arath;CycD3;1*; (i,j) *Arath;E2Fa*; (k,l) *Arath;E2Fb*; (m,n) *Arath;KRP2*; (o,p) *Arath;KRP3*; (q,r) *MSI4*; (s,t) *MSI5*; and (u-x) *CUC2*. Stars indicate vascular tissues (pre-cambium), arrowheads cells where cell cycle or cell cycle-related genes are not expressed and arrows small groups of cells that express *CUC2*. Hybridisations were performed on longitudinal sections, and all flowers are oriented with the apex of the flower towards the top. Key: im, inflorescence meristem; fm, floral meristem; se, sepal; pe, petal; st, stamen; ca, carpel. Scale bars = 60 μ m.

of cells expressing and other groups of cells not expressing the tested genes. For example, an absence of *MSI4* expression is visible in cells between the inflorescence and the floral meristems (Figure 2q). Similarly, *Arath;Rb* (Figure 2c), *Arath;CycD2;1* (Figure 2e), *Arath;CycD3;1* (Figure 2g), *Arath;E2Fa* (Figure 2i), *Arath;KRP2* (Figure 2m), *Arath;KRP3* (Figure 2o) and *MSI5* (Figure 2s) expression patterns define islets corresponding to the floral organ primordia delimited by narrow boundaries with cells not expressing the tested genes (arrowheads). Comparably to patterns observed with BrdU, such boundary cells can be detected as early as stage 4 during FM development. At later stages, cell cycle gene transcripts are not present in the cells between whorls or immediately adjacent to the base of floral organ primordia, therefore defining interwhorl boundaries (Figure 2b,d,f,j,n,r,t).

To get further insight into the nature of the non-dividing cells domains, we have examined the expression pattern of the *CUC2* gene, a specific boundary marker, during reproductive developmental stages (Figure 2u–x). At the early stages of flower development, *CUC2* expression is strictly the opposite of cell cycle or cell cycle-related gene expression profiles. Hybridisation signals, one or two cell layers wide and four or five cell layers deep, delimit domains corresponding to sepal, anther and carpel whorls. Petal primordia remain difficult to observe because of their reduced size. The results show that such boundaries are settled before each primordium is initiated (Figure 2u). Later, in addition to expression in anthers and gynoecium, *CUC2* continues to be expressed in very small groups of cells immediately adjacent to the base of each floral organ, i.e. the peri-organ boundaries (Figure 2w,x; Ishida *et al.*, 2000).

Cell proliferation patterns in *ag-1*

Mutations in the C-class gene *AG* result in indeterminate flower development with a reiteration of the sepal/petal/petal motif (Bowman *et al.*, 1989). In this scheme, BrdU incorporation and *Arath;CycD3;1* and *CUC2* expression patterns in *ag* would be expected to mark discontinuous proliferating areas separated by non-proliferating cell regions. This is indeed the case: Figure 3(a) (24-h BrdU labelling) and Figure 3(b) (*Arath;CycD3;1* expression) show that dividing cells only occur at the central dome of the FM and in organ primordia and young organs. In contrast, Figure 3(c) shows that *CUC2* is only expressed in cells surrounding the centre of the meristem, which correspond to whorl boundaries (compare with Figure 3a,b).

Cell proliferation patterns in *sup-1*

Mutations in the *SUP* gene affect the boundary between whorls 3 and 4. In *sup-1*, the stamen/carpel boundary is shifted towards the centre of the FM, leading to the absence

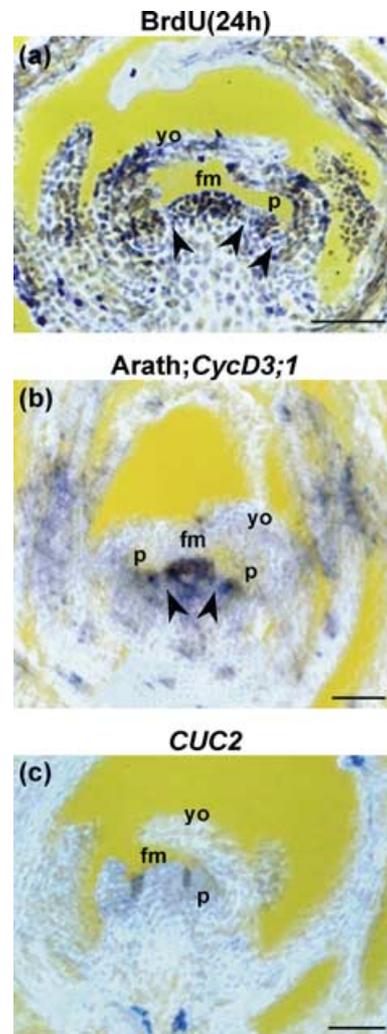


Figure 3. Cell division patterns in *ag-1* developing flowers. Cell proliferation has been analysed by (a) BrdU incorporation patterns (24-h labelling) and *in situ* localisation of (b) *Arath;CycD3;1* and (c) *CUC2*. Arrowheads point to non-dividing cells that did not incorporate BrdU and that do not express the D-type cyclin *Arath;CycD3;1*. All flowers are oriented with the apex of the flower towards the top. Key: fm, floral meristem; p, primordium; yo, young organ. Scale bars = 60 μ m.

of or a very reduced whorl 4, as shown by scanning electron microscopy and *AP3* expression pattern analysis (Bowman *et al.*, 1992; Schultz *et al.*, 1991). This results in the formation of extra whorl(s) of stamens at the expense of carpels. In addition, mosaic carpel–stamen structures can frequently be observed at the floral meristem centre (FMC) and are assumed to correspond to congenital stamen–carpel fusions (Figure 4c,f,g and also see Bowman *et al.*, 1992).

We describe below the concerted effects of a mutation in *SUP* on stamen whorl reiteration and malfunction of the stamen/carpel boundary. These effects were assessed by examining the distribution of BrdU-labelled and *CUC2*-expressing cells in *sup-1* flowers.

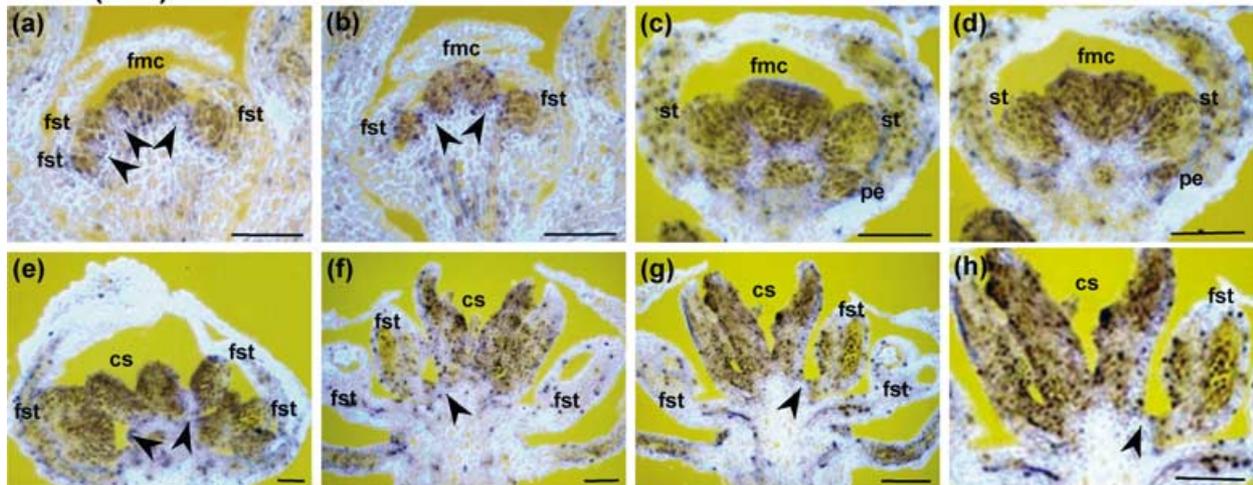
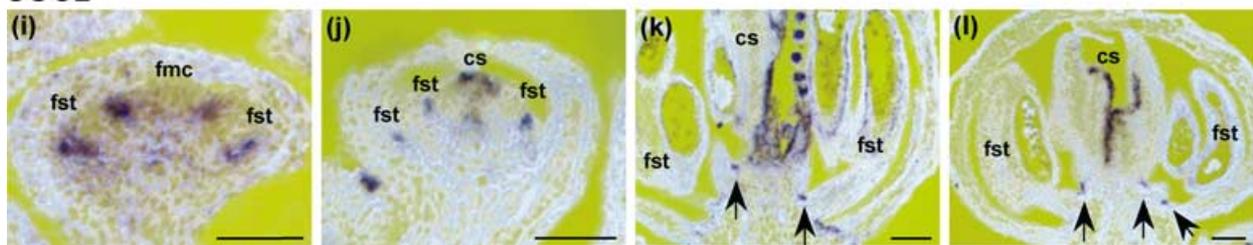
BrdU (12h)**CUC2**

Figure 4. Cell division patterns in *sup-1* developing flowers.

Cell proliferation has been investigated through (a–h) BrdU incorporation patterns (12-h labelling) and (i–l) *in situ* localisation of *CUC2*. Patterns were analysed in (a–d, i, j) young floral meristems (up to stage 6) and (e–h, k, l) older flower buds (up to stage 12). (h) Close-up view of (g). Arrowheads point to non-dividing cells that did not incorporate BrdU, and arrows indicate small groups of cells at the base of stamens that express *CUC2*. All flowers are oriented with the apex of the flower towards the top. Key: fmc, flower meristem center; pe, petal; st, stamen; fst, free stamen; cs, chimaeric carpel/stamen structure. Scale bars = 60 μ m.

In the early stages (4–6) of flower development, BrdU labelling profiles reveal domains of actively proliferating cells alternating with regions of non-proliferating cells (Figure 4a,b). In agreement with previous description of *sup-1* phenotype (Bowman *et al.*, 1992), the domains of dividing cells correspond to successive whorls of stamen primordia (flanks of the meristem; Figure 4a,b) and to the as yet undetermined region at the FMC. These two same consecutive sections make it possible to observe an emerging extra-stamen (Figure 4b). At stage 8, the cells of most primordia were still actively dividing (Figure 4e). Primordia formed at the FMC are usually separated from the innermost stamen (sub)whorl by proper boundaries. However, at the FMC, we have been unable to observe the zone of non-proliferating cells equivalent to that observed in the wild type between carpel primordia (compare Figure 4e to Figure 1k). Taken together, these results suggest prolonged meristematic activity producing multiple whorls of free stamens with relatively clear-cut organ separation.

However, the boundaries appear sometimes less broad and therefore the primordia domains are less distinctly

separated than in the wild type (compare the two consecutive sections in Figure 4c,d to Figure 1t,u; also see Figure 1f–k). As a matter of fact, this is particularly clear at the level of the epidermal or upper cell layers (L1, and possibly L2), which show erratic labelling, as compared to the subepidermal layers (mainly L3), which remain unlabelled. At stage 12, BrdU labelling shows that proliferating zones were, like in the wild type, restricted to the youngest organs located at the FMC (Figure 4f–h). As for early stages, BrdU-unlabelled cells, i.e. boundaries between organs, are only visible at the base of well-developed and unfused stamens (Figure 4f–h) and not at the FMC where chimaeric stamen/carpel structures develop.

CUC2 expression patterns were analysed to show that during the first stages of flower development, the signal is detected in cells between whorls (Figure 4i,j). They clearly delimit stamen primordia from the FMC. Surprisingly, the transcripts were sometimes observed at the summit of the FMC (Figure 4j). At stage 12, *CUC2* expression was still detected not only at the base of the stamen filaments (Figure 4k,l) but also in ovules (Figure 4k), in the lower (Figure 4k) and the inner (Figure 4l) part of the carpel-like

structures, which corresponds to the presumptive placenta and/or septal primordia according to Ishida *et al.* (2000).

Discussion

This study reports on cell proliferation patterns during *Arabidopsis* flower development. To this end, a BrdU labelling method in a semi-*vivo* culture system of cut flowers has been established. This method has been evaluated in three *Arabidopsis* ecotypes, in two mutant backgrounds (*ag* and *sup*) and has been compared with *in situ* hybridisation results obtained using cell cycle and boundary genes.

BrdU labelling as a highly reliable procedure to study proliferation patterns during flower development

Based on our results and those already published (de-Castro *et al.*, 2000; Sato-Nara and Fukuda, 2000), the BrdU incorporation protocol becomes a method of choice in plant developmental studies, with comparable benefits to those routinely employed in animal systems (Duman-Scheel *et al.*, 2002; Johnston and Edgar, 1998; Johnston *et al.*, 1999; Weigmann *et al.*, 1997). The cut flower/BrdU procedure developed is simple, non-radioactive and reliable. The duration of the pulse can be modulated to generate images of a high resolution. The method can therefore be calibrated to accurately measure division rates. For example, all cells in inflorescence meristem (IM), FMs and organ primordia (stages 1–8) were shown to divide at least once every 12–15 h, which is slightly higher than average division rates observed in *Antirrhinum* during flower development (Vincent *et al.*, 1995).

Cell proliferation domain and boundary region topology during floral development

Following long labelling pulses, the reported BrdU patterns reveal the topology of cell proliferation domains and allow monitoring the progressive organisation of the FM into regions of actively proliferating cells and regions showing no cell division (Figure 1). At stages 1 and 2, the FM appears as a highly homogenous population of dividing cells. As early as stage 4, the FM becomes divided into sectors of proliferating cells giving rise to floral organ primordia. The dividing sectors are separated by two to three cell layers that do not incorporate BrdU and that express *CUC2* (Figure 2), a gene involved in organ separation (Aida *et al.*, 1997; Vroemen *et al.*, 2003). Our studies indicate that boundary cells remain BrdU negative even after 24-h labelling and do not express any of the cell cycle genes tested. These results strongly suggest that cells in boundary zones between inflorescence and floral meristems and between whorls are comprised of non-dividing cells rather than slow-dividing. This assumption is also supported by the

fact that boundary cell–cell walls appear thicker than flower meristem and organ primordia cell–cell walls when observed under UV after calcofluor staining (data not shown). We therefore confirm, through a direct method, hypotheses on boundary control processes supported by mutant analyses (Barton and Poethig, 1993; Vroemen *et al.*, 2003), expression patterns of cell cycle genes (Doonan, 2000; Gaudin *et al.*, 2000) or physical models (Green, 1999). In addition, our results indicate that each flower organ, including the two carpels ultimately giving rise to the single fused gynoecium (Figure 1k), develops as an autonomous group of dividing cells that are delimited before primordia arise. The data are consistent with boundary sector analysis, showing that flower organs develop from a fixed number of founder cells (Bossinger and Smyth, 1996; Furner, 1996) and that lineage restrictions between whorls are set at about the florotypic stage, before the morphological manifestation of whorls 2–4 (Vincent *et al.*, 1995).

Data also show (Figure 2) that positive and negative regulators of the cell cycle are simultaneously expressed in meristematic domains and organ primordia, resulting in a constant fine-tuning of meristem homeostasis in organ patterning and morphogenesis. Interestingly, all tested genes are excluded during both early and late stages of development from boundary cell layers.

Boundary region analysis in AG and SUP

The analysis of developmental mutants through a combination of BrdU labelling and the expression pattern of the boundary marker gene *CUC2* illustrates at cellular level that the *ag-1* and *sup-1* mutants have gene-specific effects on floral determinacy control and that the formation of boundaries (composed of non-dividing cells expressing *CUC2*) in the flower meristem is not affected in *ag-1*. This result together with those of Vincent *et al.* (1995), Bossinger and Smyth (1996) and Jenik and Irish (2000) suggest that AG *per se* does not play a boundary role during the initial stages of flower development even if its expression domains approximately coincide with boundary setting at the florotypic stage (Figure 2; Irish, 1999).

Concerning *sup-1*, BrdU labelling together with *CUC2* expression patterns reveal the existence of well-defined boundaries between the successive stamen (sub)whorls, which is in agreement with the work of Baum *et al.* (2001) who showed that the reiterated whorls in *sup-1* correspond to proper whorl 3. However, *sup-1* fails in establishing boundaries (as non-dividing cells) between the innermost stamen and the carpeloid structures or between the carpeloid structures at the FMC, resulting in mosaic structure development. *CUC2* expression at the summit of the FMC in the mutant possibly indicates the almost coincident position of the last stamen (sub)whorl with the FMC. This is in agreement with the observed variability of the *sup-1*

phenotype at the FMC, ranging from stamens only, filamentous or carpeloid stamens to occasional and reduced carpels. Finally, the results suggest that boundary function(s) are primarily controlled at L1 (L2) cell level (Figure 4c,d). Consistent with this observation, it was recently reported that *CUC3* (also involved in boundary setting) expression is strongest in the epidermal cell layer, as compared to *CUC1* and *CUC2* (Vroemen *et al.*, 2003).

In conclusion, using our efficient BrdU protocol in combination with the expression pattern of the boundary marker gene *CUC2*, we show that boundaries between inflorescence and floral meristems or between whorls are confined to non-dividing cells, thus preventing the development of chimaeric structures. The reported BrdU method can now be used to analyse a variety of mutants affected in boundary setting and to assess whether the chimaeric organs formed in such mutants originate from congenital or post-genital fusions.

Experimental procedures

Plant material and growth conditions

All the experiments were performed on the *Arabidopsis thaliana* ecotypes *Ler*, Col-0 and WS. *ag-1* (*Ler* background) and *sup-1* (*Ler* background) were provided by E. Meyerowitz (California Institute of Technology, Pasadena, CA, USA). Wild-type and mutant plants were grown in soil under short-day conditions (10 h light; 10 000 lux luminosity; 55–80% of humidity; 22°C (day)–17°C (night)) and then transferred to the greenhouse (14 h light; 10 000–20 000 lux luminosity; 60–66% of humidity; 21–26°C (day)–16°C (night)).

Inflorescence (cut flower) culture and BrdU incorporation

Inflorescences were grown using a culture system developed by Magnard *et al.* (2001). The primary inflorescences were excised 3 cm from the top and immediately placed in a 6-well cell culture cluster (Costar 3516, Corning Incorporated, NY, USA) through holes made in the lid of the plate. Surface sterilisation of the peduncle was not necessary for cultures maintained for less than 48 h. Each well of the tissue culture plate was filled with approximately 8 ml of culture medium supplemented with BrdU (2 mg ml⁻¹, Sigma, St Louis, MO, USA). The plates were placed under vacuum for 15 min and incubated in the growth chamber (12 h light; 22°C). These conditions allow the inflorescence culture system to mimic development in the intact plant and a vital dye to reach the flowers. The culture medium was 1× Murashige and Skoog basal salt mixture (Duchefa M0221, Haarlem, the Netherlands), 3% (w/v) sucrose, and 1× Gamborg B5 vitamin mixture (Duchefa G0415, Haarlem, the Netherlands), pH 5.8. Stock of BrdU (50 mg ml⁻¹) was prepared in NaOH (0.1 mM).

Preparation of tissue sections

Tissue preparation, for both *in situ* hybridisation and immunohistochemical detection of BrdU, was as described by Bradley *et al.* (1993). Tissue sections were 10 µm thick.

In situ hybridisation

In situ detection of mRNA on paraffin-embedded inflorescences was performed as described by Bradley *et al.* (1993). cDNAs from all genes tested were amplified by PCR with specific primers and cloned in the plasmid pGEMTeasy (Promega, Madison, WI, USA). The resulting pGEMT vectors were used as a template to generate the sense and the antisense probes by using the SP6 and T7 promoters, respectively. Fluorescence was visualised using an epifluorescence microscope (Zeiss, Jena, Germany).

Immunohistochemical detection of BrdU incorporation

Basically, the immunodetection of BrdU was performed as described by Coen *et al.* (1990). Paraffin was removed from sectioned inflorescences and subsequently hydrated. Slides containing samples were then washed for 2 × 10 min in PBS and incubated for 20 min in HCl (2 mol l⁻¹). Slides were then neutralised for 3 × 5 min in PBST and treated with 10 µg ml⁻¹ Proteinase K in the enzyme buffer at 37°C for 30 min. The enzymatic reaction was stopped by 5 min in glycine (2 mg ml⁻¹ in PBS), and slides were washed for 5 min in PBS. After two blocking reactions, the monoclonal primary antibody raised against BrdU (Beckton Dickinson, San Jose, CA, USA) – diluted (1 : 500) in the Buffer A (1% BSA, 0.5% Triton X-100, 100 mM Tris-HCl, 150 mM NaCl, pH 7.5) – was applied and the slides were incubated at room temperature in a wet chamber for 1.5 h. After four washes of 15 min each in Buffer A, the secondary AP-labelled goat antimouse IgG antibody (Sigma; 1 : 1000 in Buffer A) was applied and slides were incubated at room temperature in a wet chamber for 1.5 h. Signal detections and cell wall labelling were performed as described by Bradley *et al.* (1993). Fluorescence was visualised using an epifluorescence microscope (Zeiss).

Acknowledgements

Authors are thankful to Prof. Christian Dumas (Laboratory RDP, ENS-Lyon) for supporting this project. We also thank Isabelle Courseau for her excellent technical help, Pradeep Das, Toshiro Ito, Venugopala Gonehal (Cal. Institute of Tech.), John Bowman (U.C. Davis), Charlie Scutt (ENS-Lyon) for their encouraging discussions and comments and Lieven De Veylder and Dirk Inzé (University of Ghent) for providing cell-cycle gene sequences before publication.

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