

Determination of floral initiation in *Malus domestica*: a novel morphogenetic approach

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Abstract

Floral initiation in apple (*Malus domestica* Borkh) was studied by a novel morphogenetic approach. Developmental stages of apices were evaluated based on the morphology of shoot apical meristem (SAM) from various collection dates. Besides, the frequency of each stage was calculated within apices populations after full blooming (DAFB). Prior to doming of apex, three marked phases were found based on SAM morphology: 1) narrow appearance (vegetative phase), 2) broadened form (transition phase), and 3) prominent shape (commitment phase). A furrow region was formed at the base of leaf primordium during the bract initiation, while significant broadening of SAM was observed. Cell division patterns manifested in modification of anisotropic clusters from isotropic cellular packets, as a result of which profound morphological changes of apices occurred. Based on these findings, we propose that the structural alterations prior to doming may be taken into account for determination of the initial development and reproduction signs in apple trees.

Additional key words: apple, cell division pattern, epi-illumination light microscopy, fluorescence microscopy, shoot apical meristem.

Introduction

Flower formation involves a long multi-step process, in which floral initiation is defined by changes of shoot apical meristem (SAM) from vegetative to reproductive status, which can be recognised on the basis of histological and morphological differences of apex (Bernier 1997, Clark 1997, Grandjean *et al.* 2004, Kwiatkowska 2008). So far, numerous investigations have been carried out to describe flowering and fruit development in *Malus domestica*, (Dencker and Hansen 1994, Hirst and Ferree 1996, Foster *et al.* 2003, Oukabli *et al.* 2003, Evans and Dickinson 2005) but only few studies have been conducted on the time of floral initiation as well as its morphological signs (Buban and Faust 1982, Foster *et al.* 2003). Characterisation of floral initiation seems to be ambiguous because of some uncertainties on description of flowering stages. Dencker and Hansen (1994) reported that the doming of apex in apple basically coincides with the cessation of shoot growth, which has also been considered as the first sign for floral initiation (Hirst and Ferree 1995). Foster *et al.* (2003) quantitatively analysed the diameter of SAM over its developmental course and showed that the apex

broadening happens prior to its doming. However, the broadening of apex itself may not be considered as the first sign of floral initiation, and it should be evidently clarified upon morphological changes showing cellular division patterns. In fact, little is known about architectural aspects of SAM prior to broadening, although some insights have been emphasised for the floral ontogeny of the *Maloideae* from three-dimensional or cross sectional studies (Pratt *et al.* 1988, Evans and Dickinson 1999, 2005).

Further, cells in central and peripheral zones of the vegetative and reproductive apices have been shown to play a major role on organ formation and morphogenesis (Gross-Hardt and Laux 2003, Veit 2004, Reddy and Meyerowitz 2005). The centrally located cells remain undifferentiated on the SAM and show non-polar and isotropic appearance, while cells in peripheral zones display multidirectional division and anisotropic characteristics implying high capacity of differentiation, and consequently forming of organs (Gross-Hardt and Laux 2003, Tooke and Battey 2003, Kwiatkowska 2004).

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Abbreviations: ELM - epi-illumination; DAFB - days after full bloom; SAM - shoot apical meristem.

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In addition, the groups of peripheral cells are rapidly dividing lineage with anisotropic and polar appearance in contradiction of initial cells (Rolinson 1976, Korn 1980). What is more, cell orientation patterns in SAM may be changed during its transition to reproductive phase (Dumais and Kwiatkowska 2002, Grandjean *et al.* 2004, Fleming 2006, Kwiatkowska 2006). Recently some

important genetic changes during transition to flowering in *Arabidopsis* have also been reported (Wang and Wang 2009).

In the current study, we aimed to determine the initial development and reproduction signs through microscopic examinations of the architectural characteristics and the cellular pattern of SAM.

Materials and methods

The changes of SAM morphology were studied during transition from vegetative to reproductive status using eight-year-old Golden Delicious/MM106 trees cultivated at the private orchard in Tabriz, Iran. Uniform limbs and two-year-old spurs were tagged on 50 trees for subsequent sampling. Collection of samples was precisely performed 10 d after full bloom until the end of dormancy with an interval of 10 d. In each sampling, a number of 100 well-developed buds on the southern side of trees were randomly selected and immediately fixed in formalin : 100 % acetic acid: ethanol, 5:5:90; (FAA; Posluszny *et al.* 1980). After 24 h fixation in FAA, samples were rinsed, dehydrated in series of ethanol and stored in 95 % ethanol for microscopic examinations.

To evaluate the morphology of SAM during its transition from vegetative to reproductive stages, 3D microscopic techniques were utilised. The prepared samples were dissected under a SZX 9 stereomicroscope (Olympus, Tokyo, Japan) and stained with alcohol soluble nigrosin (Merck, Darmstadt, Germany) according to a previous report (Charlton *et al.* 1989). Further washing of the samples was carried out in 95 % ethanol for 2 d. Nigrosin stained apices were examined using an Olympus BX60 microscope equipped with U-URBL illuminator and U-RLBC mirror cassette. Epi-photographs of apices in absolute ethanol were taken according to Posluszny *et al.* (1980) by means of an Olympus DP70 high resolution digital camera, for which a reflected dark field mode and yellow contrasting filters were employed. Digital images were then saved using RGB mode with the resolution of 2040 × 1536 pixels and assembled into plates using AnalySIS Pro 5.0 software (Soft Imaging System, Münster, Germany).

To investigate the arrangement of stem and periphery cells and their division pattern, we exploited a novel fluorescence microscopy technique. First, dehydrated materials were immersed in glacial acetic acid for 15 min.

They were then stained by means of saturated aniline blue solved in methyl cellosolve (2-methoxy-1-ethanol) for 24 h for minimising the background noises. Further complementary staining was performed using 0.2 % (v/v) acid fuchsin for 24 h. Compared to previously reported method (Lemon and Posluszny 1998), we have used the mentioned counter-staining approach to maximise the contrast of the micrographs. The prepared specimens were examined utilising an Olympus BX51 fluorescence microscope equipped with a BX-RFA fluorescence illuminator. To optimise fluorescence excitation, U-MWG3 mirror cube unit at 510 - 550 nm was used. Serial images were taken from top to bottom of apices (5 µm increment per focal step) using Hamamatsu C7780-10 cooled CCD (Hamamatsu Photonic, Tokyo, Japan) and images (1344 × 1024 pixels resolution) were then imported to ImageJ 1.37 software (<http://rsbweb.nih.gov/ij/>) to produce the superimposed images. Fluorescence images were then extracted manually by positioning of cell vertices. The thin and thick vertices were grouped by different colours for determination of the cellular division pattern.

Time of floral inception was determined based on the transition phase from vegetative to reproductive stages. The morphological assessments of apices were categorised by comparing the shape of apex as well as the orientation and order of formed organs. The frequency of each observed stage at sampling time points was calculated as a percentage of total observations. The time of floral inception was determined based on the first sign of marked alterations of meristem which can be observed 20 d after full bloom. At the end of dormancy period, the percentage of vegetative and floral buds was also calculated. A paired *t*-test was used to analyse the frequency of the morphological changes at sampling time points using SPSS 11.5 software.

Results

Morphological changes and cellular division patterns of SAM during the transition from vegetative to reproductive phase were obtained by reflected light and fluorescence microscopy, respectively. As seen for the developmental stages of SAM at the beginning of growing season, the apex (AP) appeared to be very narrow and enclosed by the latest leaf primordium (LP) at

the beginning of bud development at stage 1 (Fig. 1A). By formation of new leaf initial at the flank of apex (stage 2), slight broadening and escalation of the apex toward the new incepted leaf primordium were observed (Fig. 1B). These changes in the morphology of apex appear to be repeated upon initiation of new leaf primordia during growing season. The rate of

organogenesis of SAM was gradually decreased as a result of the bud aging during the growth and development. The latter event was also followed by cessation of leaf inception activity of meristem. At stage 3, the apex became more broadened than that of the first stage, where the broadening appeared to be in synchronisation with initiation of the first bract (Fig. 1C). Consequently, the apex experienced new developmental pattern (stage 4),

which was characterised by formation of furrow (FU) between the latest leaf primordium and meristem mantel. Consequently, the leaf primordium began to separate from apex, which in return became markedly protuberant (Fig. 1D).

After stage 4, another distinct stage (stage 5) could be clarified by initiation of the next bract at the flank of SAM (Fig. 1E). Then, apex began to expand (stage 6)

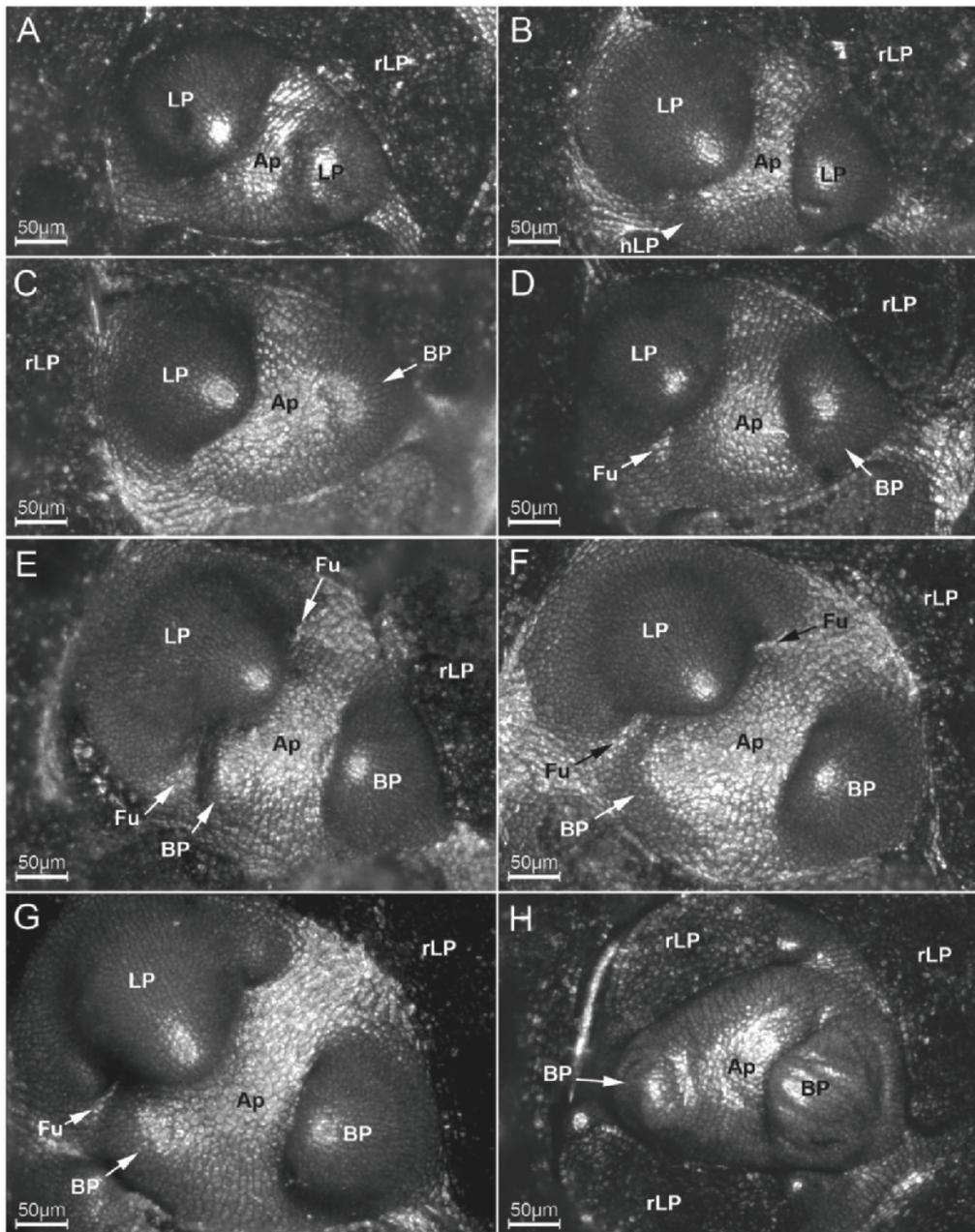


Fig. 1. Developmental stages of apex at the beginning of growing season. *A* - stage 1, apex with narrow appearance enclosed with the latest leaf primordium; *B* - stage 2, slight broadening of apex due to initiation of the new leaf primordium; *C* - stage 3, extra broadening of apex with initiation of the first bract; *D* - stage 4, formation of furrow at the base of the latest leaf primordium; *E* - stage 5, increased broadening of apex (at this stage, the other bract initiates at the flank of apex); *F* - stage 6, extra expansion of apex and depressed furrow; *G* - stage 7, maximum expansion of apex prior to doming; *H* - stage 8, doming of apex. Ap - apex, LP - leaf primordium, nLP - new leaf primordium, Fu - furrow, rLP - removed leaf primordium, BP - bract primordium.

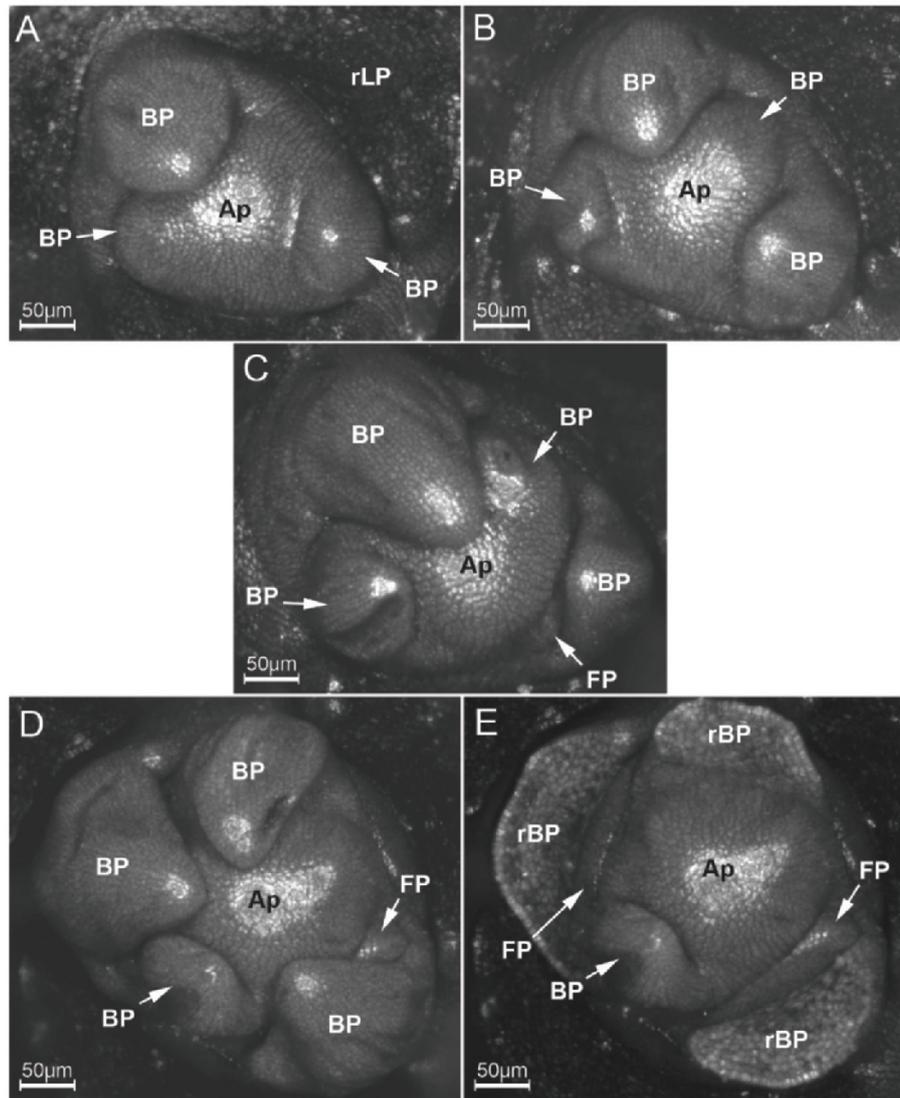


Fig. 2. Inflorescence developmental stages of apex. *A* to *D* - formation of new bracts at the axis of inflorescence after doming during various progressive stages; *E* - the removed bracts from sample shown in panel *D* displaying initiated lateral flowers. Ap - apex, rLP - removed leaf primordium, BP - bract primordium, rBP - removed bract primordium, FP - flower primordium.

simultaneously with development of the formed bracts (Fig. 1*F*). Further development of SAM appeared to be continued until achieving its maximum size (Fig. 1*G*). Development of the latest leaves displayed the furrow lying beneath the latter bract, concurrent with greater expansion of apex (stage 7). A new stage of apex development (stage 8) can be detected as a dome-shaped SAM mantel and deeper furrow (Fig. 1*H*). Based on this pattern, apex began to arise from subtending appendages. Thus, stage 8 could be considered as the developmental step, at which the axis of inflorescence is formed.

Additional development of incepted inflorescence can also happen by initiation of new bracts (Fig. 2*A,B*). In fact, the first flower seems to be initiated upon the manifestation of the first lateral flower primordium at the axil of bract (Fig. 2*C*). By this phenomenon, the volume

of inflorescence was increased and covered by rapidly developing bracts as well as leaves (Fig. 2*D*). The bracts were removed to ensure about the possible formation of underneath lateral flower primordia, in which lateral protuberances were seen after doming of apex in progressive stage of inflorescence development (Fig. 2*E*).

Frequency analyses of apices at different developmental stages revealed that, at the beginning of growing season, stages 1 and 2 appeared to be predominant phases (up to 70 DAFB). A profound descending trend can be explicitly seen for the frequency of SAM organogenesis during stages 1 and 2. Such trend occurs due to possible transitional changes from proliferation to differentiation (*i.e.*, the steady state phase from 80 to 120 DAFB). The apices growth profiles at stages 1 and 2 showed an intriguing similarity implying

insignificant differences within these two stages ($P > 0.05$). The frequency of stages 3 and 4 showed a

slight increase of up to 60 and 80 DAFB, respectively (Table 1). The lowest rate of apices development was

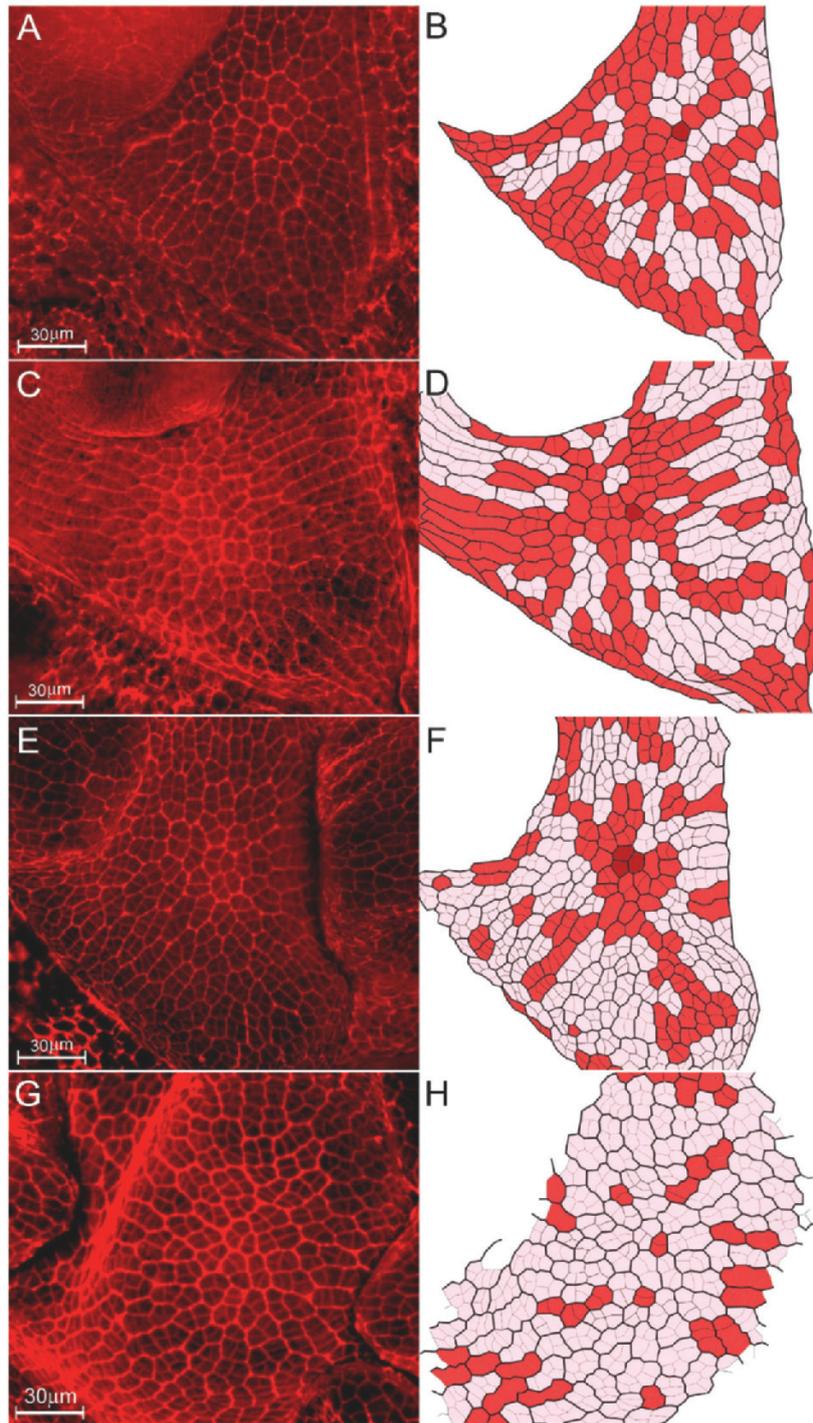


Fig. 3. Fluorescence and extracted images of apex at different developmental stages. *A,B* - isotropic and anisotropic groups of cells in central and peripheral zones represent cell division patterns at stage 1 (vegetative phase); *C,D* - broadened apex at stage 3 (transition phase) demonstrates increment in anisotropic cellular divisions at the periphery zone and synchronised expansion of meristem with multidirectional cell division compared to unidirectional cellular division; *E,F* - apex at stage 4 (commitment phase) shows the high range of multidirectional cell division, especially at the peripheral zone; *G,H* - apex of the inflorescence indicates highly manifested cellular division pattern within entire meristem with abundant cell lineage at the central zone. Cellular division changes occur from uni- to multi-directional patterns during transition process from vegetative to reproductive phases. Deep red, red and pink colours represent the non-divided, unidirectional and multidirectional divided cells, respectively.

Table 1. Frequency [%] of apices at different stages during sampling dates. DAFB - days after full bloom.

| DAFB | Stage 1 | Stage 2 | Stage 3 | Stage 4 | Stage 5 | Stage 6 | Stage 7 | Stage 8 |
|------|---------|---------|---------|---------|---------|---------|---------|---------|
| 10 | 50 | 48 | 2 | 0 | 0 | 0 | 0 | 0 |
| 20 | 47 | 49 | 4 | 0 | 0 | 0 | 0 | 0 |
| 30 | 44 | 46 | 8 | 2 | 0 | 0 | 0 | 0 |
| 40 | 44 | 43 | 8 | 4 | 1 | 0 | 0 | 0 |
| 50 | 38 | 39 | 13 | 8 | 1 | 1 | 0 | 0 |
| 60 | 34 | 33 | 15 | 10 | 4 | 3 | 1 | 0 |
| 70 | 29 | 30 | 13 | 17 | 5 | 4 | 2 | 0 |
| 80 | 18 | 17 | 11 | 21 | 13 | 11 | 9 | 0 |
| 90 | 14 | 16 | 7 | 18 | 17 | 15 | 11 | 2 |
| 100 | 13 | 15 | 6 | 11 | 19 | 17 | 16 | 3 |
| 110 | 15 | 13 | 5 | 8 | 17 | 20 | 15 | 7 |
| 120 | 13 | 15 | 4 | 5 | 14 | 16 | 14 | 19 |

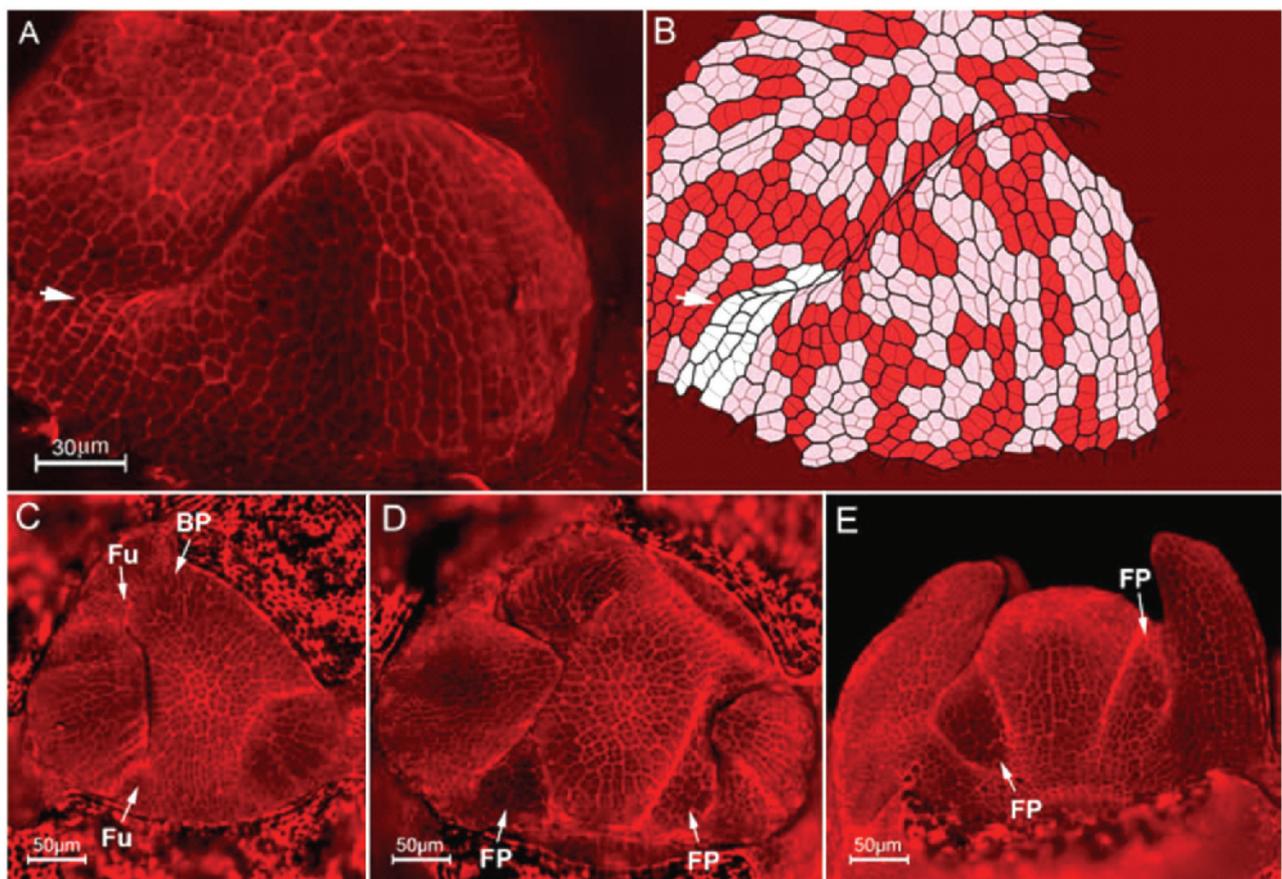


Fig. 4. Fluorescence and extracted images of apices during furrow formation and inflorescence development. *A, B* - the furrow formation between apex and leaf primordium, the depressed strip of small cells with unidirectional cellular division is shown with *white arrows* at the furrow region; *C* - formation of furrow as a depressed region, a new bract primordium; *D* - initiation of protuberant lateral flower primordium at the furrow region; *E* - side view of the formation of new bracts and flower primordia at the furrow region. Fu - furrow, BP - bract primordium, FP - flower primordium.

achieved for stage 3 in comparison with the other stages. This stage can be considered as the critically transitional phase in terms of apices development, in which apex began to differentiate. Such differentiation appeared to be highlighted at stage 4 (Fig. 1D) showing greater rate of apices morphogenesis (up to 80 DAFB). Stages 5 to 8

revealed similar patterns for organogenesis with lag phases (Table 1). Stages 7 and 8 showed somewhat apex development toward formation of the inflorescence axis.

To substantiate the development of SAM in apple trees, the cellular patterns of apex at stages 1, 3 and 4 (prior to doming) were investigated. In practice, at the

progressive stages after doming, the cellular division patterns were compared with that of previous critical stages using fluorescence microscopy. The most profound stages of apex development were further examined with highly magnified fluorescence micrographs and the related extracted images (Figs. 3, 4). Slim structures were observed for apex at stage 1, where isotropic cluster of initial cells on the central zone (Fig. 3*A,B*) displays somewhat different morphology compared to anisotropic groups of peripheral cells. Bold high dense and faint less dense cell walls can be recognised between and within partially grouped cells, respectively (Fig. 3). These grouped cells tend to undergo cellular divisions with specialised orientations for subsequent developments. We observed more broadening of SAM as a result of cellular expansion within the peripheral zone at stage 3 (Fig. 3*C,D*), even though these cells appeared to be grouped as a packet beginning to enhance the surface of meristem. The formation of furrow at the base of the leaf primordium at stage 4 is perhaps an indication for beginning of the appendage rising from the apex (Fig. 3*E,F*). The cellular distribution pattern of the inflorescence after apex doming indicated the formation of multidirectional cell division both at the central and peripheral zone (Fig. 3*G,H*).

Altogether, cellular division pattern and distribution

of isotropic and anisotropic groups of cells showed transitional pattern from vegetative to reproductive states. So that, at stage 3, the apex can be expanded leading to a uniform distribution of anisotropic cluster of cells that appear to endure more specialised pattern of distribution at stage 4.

The side view of furrow, shown as white strip in extracted image, demonstrates anisotropic group of cells which appeared to be oriented as unidirectional ribbons (Fig. 4*A,B*). In fact, the cellular division pattern above the furrow displayed an irregular orientation (Fig. 4*B*). The inflorescence development is shown as formation and differentiation of meristem (Fig. 4*C,D*). The side view of the differentiated meristem can also be seen as mounted masses at the furrow region (Fig. 4*E*).

Profound emission of fluorescence was seen as thick and thin cell walls (Fig. 5*A,C*). The red bold and yellow thin lines in extracted images show the cell walls of the primary and secondary cells, respectively (Fig. 5*B,D*). The bold green and white circles represent the vertices of these primary and secondary cells, respectively (Fig. 5*B,D*). These explicitly demonstrate detection of two main groups of cells, which display different fate at the central and peripheral zones even though they are segregated from the primary cells.

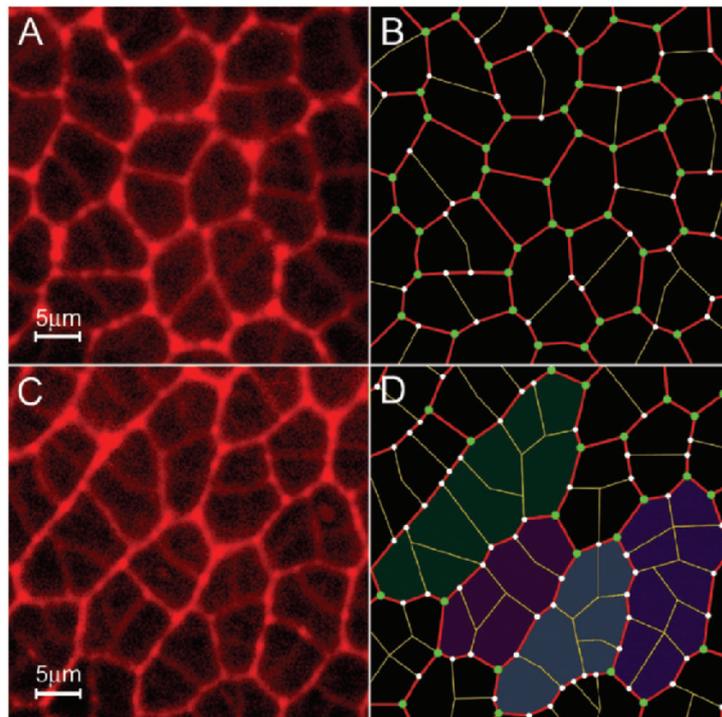


Fig. 5. Highly magnified fluorescence and extracted images of apex at stage 4. *A,B* - apex at the central zone shows the isotropic cells undergoing unidirectional divisions; *C,D* - multidirectional cellular division at the peripheral zone with expansion in apex. Red and yellow lines (panels *B* and *D*) represent the high and low dense cell walls, respectively. The green and white circles on the cell walls represent cellular division patterns as high and low dense vertices. Examples of the anisotropic cell clamps at the periphery zone are shown with different colours.

Discussion

Flowering of apple is a complex process with several stages including floral induction, initiation and organogenesis as well as anthesis (Pratt *et al.* 1988). However, it is difficult to distinguish the precise time for floral induction/initiation, and often these two terms are constitutionally used in the literature (Tromp 1976, Ryugo 1986, Foster *et al.* 2003). Morphologically, the doming of SAM is believed to be the first sign for floral initiation (Luckwill and Silva 1979, Huang *et al.* 1986). Likewise, the doming of SAM was also shown to be regarded as the first sign of floral initiation in some other plants including sour cherry (Diaz *et al.* 1981), Japanese pear (Banno *et al.* 1985), and sweet cherry (Guimond *et al.* 1998). Besides, it has been reported that the broadening of SAM is the first sign for floral inception in apple (Foster *et al.* 2003) and almond (Lamp *et al.* 2001), which is believed to happen prior to the doming of SAM. To pursue these intriguing issues, in this investigation, we undertook microscopic analyses approaches on SAM development in apple tree.

Based on the morphological analysis of shoot apices during their development, we detected some distinct morphological changes for SAM prior to the doming (Fig. 1). After the formation of two consecutive leaf primordia, the vegetative SAM displayed a flat and narrow structure (Fig. 1A). Once the new leaf was initiated at the flank of SAM, it becomes partially broaden (Fig. 1B). In consensus with our findings, Foster *et al.* (2003) classified two types of the vegetative meristems including, "flat and narrow meristems" and "broad meristems". Similarly, distinct morphological manifestations of SAM indicate two types of broadening before and after furrow formation (Fig. 1C,E,G). This clearly shows existence of profound morphological expression and depression in surface architecture of the SAM showing significant growth after furrow formation as expansion of meristem (Fig. 1C,H). After doming, SAM seems to reach almost the maximum expansion, at which apex begins to form bracts and inflorescence axis (Fig. 2A,E). To pursue these of note developmental events, we statistically analysed the frequency profile of SAM structure within various stages. We found profound consistency for the frequency summation of stages 1 and 2 (Fig. 3). A lag phase can be seen from stage 4 onward, perhaps because of deceleration in morphogenesis.

The broadening of SAM is recognised as the first sign of floral initiation (Pratt *et al.* 1959, Foster *et al.* 2003). However, Hirst and Ferree (1995) reported that the apical doming, as the indicator trait for floral initiation, generally occurs between 85 and 109 DAFB. Hoover *et al.* (2004) conducted a similar study to determine the effect of cultivar on the course of organogenesis on apple buds and also showed that doming occurs at 86 to 112 DAFB. These researchers showed that the probability of doming stage can rarely exceed 0.13, upon which it can be literally deduced that the flower morphogenesis is a rapid process. Based on our results, the doming of SAM

at stage 8 took place at 90 DAFB and can reach its maximum rate at 120 DAFB. Furthermore, the frequency of doming stage showed fluctuation between 0.03 to 0.17 at 90 and 120 DAFB, respectively. Our results were in agreement with the previous findings (Hirst and Ferree 1995, Hoover *et al.* 2004) from the time and probability of apical doming viewpoints. We observed a new stage for floral initiation prior to the doming (Fig. 1D), thus it should be considered for definition of the first sign of floral initiation.

Since the differences between groups of cells at the apical meristem were not very pronounced in the epillumination images, we developed a new fluorescence microscopy method for further examination of these findings. This new method provides colourful high contrast images with pronounced details of cellular architectures, where the optical properties of cell walls perfectly reflect cellular division patterns. These fluorescence micrographs revealed that cell division increases at the peripheral zone of SAM when new leaf primordium begins to form (Fig. 3A). Besides, the cell cluster formation may cause a slight expansion between the two successive organs (Fig. 3B). These results clearly imply that the organ formation may be correlated with the high rates of cell division at the periphery region of SAM (Dumais and Kwiatkowska 2002, Kwiatkowska and Dumais 2003). Presumably, such alterations in SAM during the formation of new leaf primordium can be considered as an expected regular process of organogenesis. Acquired fluorescence images from apices obtained at stage 3 (Fig. 3E,F), clearly shows a marked increase in the periphery cells mass in comparison with that of stage 1 (Fig. 3A,B). It can be figured out that the outspread periphery cells at stage 4 associated with apex expansion is likely the main sign for meristem commitment to flower at the cellular levels. Likewise, it has been shown that the periphery anisotropic groups of cells neighbouring to bract tend to present a "Lagrangian growth" (Coen *et al.* 2004), where cells reveal size-oriented growth (Figs. 3E,F and 4A,B). In fact, apex expansion is a prerequisite for formation of the first bract as the reproductive organ (Fig. 3C,D). After this transitional stage, distinct morphological alterations can be observed (Figs. 3E,F and 4A,B), upon which some reflective changes in topography of SAM may occur. Nevertheless, despite high mitotic activity of the shoot apical meristem, its size could not exceed from distinct range. It has been shown that a subtle depression at the summit of the apical dome became rounded during the early phase of flower induction, by which an increase in the ratio of height to diameter of the dome occurs (Albrechtova *et al.* 2004). Our results support the latter findings that are in consensus with the constant cellular context of central zone due to feedback signalling loop between the organising periphery cells and stem cells (Laux *et al.* 1996, Sharma and Fletcher 2002, Sharma *et al.* 2003, Muller *et al.* 2006). Interestingly, the furrow at

the base of SAM can also be seen as depressed cell striped lineages (Fig. 4A,B).

By using counterstaining of apex with two types of dyes with different excitation properties, more details of cellular arrangement are detectable. Acid fuchsin shows high tendency to cell wall, thus the thicker the wall, the higher the fluorescence emission can be attained (Fig. 5A,C). Upon the optical differences in cell walls at the surface of SAM, the thick and thin cell walls display some reorganisation indicating existence of groups of cells. After floral initiation, it was found that the rate of cell division appeared to progressively increase at the central zone. In fact, as a result, cellular clusters can be determined by distinct cell wall properties (Fig. 5D). Changes in cell division pattern from centrally located isotropic cellular clusters to the anisotropic periphery cellular packets (Fig. 3E,F) clearly indicate profound

tendency towards differentiation during floral initiation. These findings add some new aspects to the previous reports (Albrechtova *et al.* 2004, Kwiatkowska 2004), whose works showed that expansion of the central zone of SAM seems to be isotropic and slower than in the peripheral zone.

Upon our findings obtained using ELM and the new fluorescence imaging methodology, we suggest that the following three critical forms of SAM during developmental process could be classified as: 1) narrow appearance (vegetative phase), 2) broadened form (transition phase), and 3) prominent shape (commitment phase). Taken all these findings together, we propose that the "broaden SAM with furrow" at stage 4 should be considered as the first signs for the floral initiation in apple trees.

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