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Cloning and expression analysis of a *Petunia hybrida* flower specific mitotic-like cyclin

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Abstract **A cyclin cDNA clone (*Pethy;CycB1;1*) was isolated from a *Petunia hybrida* ovary specific cDNA library. Sequence comparison revealed that Pethy;CYCB1;1 protein is highly homologous to mitotic B1 cyclins. Northern analysis and in situ hybridisation experiments showed that its expression is developmentally regulated and restricted to flower organs. We have attempted to define some of the cell division patterns which contribute to shaping each floral organ by analysing *Pethy;CycB1;1* expression on *Petunia* flower sections. While in sepals, epidermis and parenchyma cell division patterns were comparable, there were two distinct cell division patterns in petals. In the epidermis, *Pethy;CYCB1;1* expression was found both at the petal tip and along epidermis, whereas in the parenchyma only at the petal tips. In reproductive organs cell divisions were detected only in sporophytic tissues. No signals were detected inside meiotic cells.**

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Key words: Cell-cycle; Mitotic cyclin; Flower development; *Petunia hybrida*

1. Introduction

Cell cycle progression in eukaryotes is regulated by major controls operating at the G1/S and G2/M phase boundaries. Passage through these controls requires the activation of cyclin dependent kinases (CDK) whose catalytic activity and substrate specificity are modulated by (1) the association with specific regulatory subunits known as cyclins, (2) the phosphorylation state of the complex and (3) the binding to protein CDK inhibitors. Originally described as proteins which accumulate periodically during early cleavage of sea urchin eggs [1], cyclins were subsequently identified in many eukaryotic species that are evolutionary as distant as yeast, animals and plants [2].

Three types of cyclins have been identified in plants: A, B and D [3]. A and B types, usually referred to as mitotic cyclins, are expressed predominantly during late S, G2 and M phase with cyclins A slightly earlier than cyclins B. Based on sequence similarity cyclins A are further subdivided in three classes: *CycA1*, *CycA2* and *CycA3*. B type cyclins are divided into *CycB1* and *CycB2*. D type cyclins, originally identified by their ability to rescue yeast mutants lacking endogenous G1 cyclin activity, are divided into *CycD1*, *CycD2* and *CycD3*.

Plants display unique developmental features found in neither animals nor fungi. For example plant embryogenesis, unlike embryogenesis in animals, defines only the basic morphology of the organism. Growth is achieved as a result of repetitive cell divisions in specialised regions known as meristems and subsequent differentiation. New organs are differentiated after germination according to internal developmental programs or in response to environmental stimuli. Thus, throughout plant life there must be a perfect integration between developmental controls and the mechanisms regulating meristem activity and differentiation processes. Transcriptional regulation of a rate-determining factor could be one of the mechanisms that control cell division rate [4].

Cdk expression in plants not only correlates with mitotic activity, but also with competence for cell division [5,6], whereas expression of mitotic cyclins is restricted to dividing cells [7,8]; therefore it has been proposed that differential expression of mitotic cyclins is a critical factor for the proper regulation of mitotic timing in at least some developmental programs [9].

Flowers are complex structures composed of different organs arranged in whorls. After floral induction the meristem changes its activity from the production of leaf primordia to the production of floral primordia. With the proceeding of flower development local modulation of cell division inside the flower meristem leads to the formation of the individual flower organs: sepals, petals, stamens and carpels. As organs grow, an intimate control of pattern and number of cell divisions ensures the establishment of different organ shapes and sizes [10].

Although there is a wealth of information on the genetic control of flower meristem initiation and flower organ identity, little is known of genes that control flower organ shape and size.

We have tried to define cell division patterns during floral organ formation by investigating the expression patterns of cell cycle genes. We report the cloning of a mitotic-like *Petunia hybrida* cyclin and its spatial and temporal expression pattern in developing floral organs. Sequence comparison to other cloned plant cyclins indicates that this cyclin can be classified as a B1 cyclin. Expression analysis demonstrates that its transcription is restricted to flower organs and developmentally regulated.

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2. Materials and methods

2.1. Plant materials

The *Petunia hybrida* line W115 plants used for this study were grown under normal greenhouse conditions.

2.2. Isolation of Pethy;CycB1;1 cDNA

Two oligonucleotides were designed on the basis of two conserved regions present on the published sequences of plant cyclins [3]. The upstream primer was a 16-fold degenerate 26-mer encoding the amino acids ILDWLV(E/Q)V, the sequence of which was 5′-ATI(C/T)TIG-TIGATTGG(C/T)TIGTI(G/C)A(A/G)GT-3′. Deoxyinosine was used to reduce degeneracy. The downstream primer was a 32-fold degenerate 26-mer, antisense for KYEE(I/M)YPP-(D/E) the sequence of which was 5′-TCTGG(T/G)GG(A/G)TA(G/C)AT(C/T)TC(C/T)TCA-TATTT-3′. 1 μl of a cDNA library (1×10^6 pfu/μl) constructed with Poly $(A)^+$ RNA extracted from ovaries of *Petunia hybrida* line W115 were used for the PCR with 20 pmol of each primer, 200 μM each deoxynucleotide-triphosphate in 50 μl total volume. The reaction also contained 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$ and 0,01% (w/v) gelatin. Reactions were automatically cycled through time/temperature cycles as follows: 94°C for 1′, 45°C for 1′ and 72°C for 50″ for 35 times and a final extension time increased to 10′. 10 μl of the amplified product were loaded on a 1% agarose gel and a fragment of about 180 bp was purified from the gel and subcloned into Topo cloning vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Two clones were sequenced by the method of Sanger [11]. Approximately 400 000 plaque forming units from a *Petunia* ovary library were screened under high stringency conditions using the ^{32}P radiolabelled 180 bp fragment as a probe according to standard procedures [12]. In vivo excision of the plaque-purified cDNA clones in the pBluescript KSII+ plasmid was performed as described by the manufacturer (Stratagene, La Jolla, CA, USA). The cDNA sequence was determined by the dideoxy-method of Sanger [11].

2.3. DNA and RNA blot analysis

Plant DNA was isolated from *Petunia* leaves according to Koes [13] and total RNA was extracted from leaves, roots and flower organs according to Verwoed [14]. 10 μg of total DNA were digested with *Eco*RI, *Eco*RV, *Bam*HI, *Hin*dIII, electrophoresed and blotted into Hybond N+ membrane (Amersham, Little Chalfont, UK). For RNA gel blot analysis 10 μg of total RNA were fractionated by gel electrophoresis under denaturing conditions on agarose gel with formaldehyde and transferred to Hybond N+ membrane. The complete cDNA clone was radioactively labelled by random oligonucleotide priming [15]. Blots were hybridised as described by Angenent [16] and washed with 0.1×SSC at 60°C for 20′ (three times) prior to autoradiography.

2.4. In situ hybridisation

Tissue preparation and hybridisation conditions were the same as described by Angenent [17]. The 3′ end 900 bp fragment of *Pethy;CycB1;1* cDNA was amplified from pBluescript KSII+ plasmid using T7 and an internal primer. Dig-UTP antisense riboprobe was synthesised from this fragment by the T7 RNA polymerase. The 5′ end 1100 bp fragment of *Pethy;CycB1;1* cDNA was amplified from pBluescript KSII+ plasmid using SP6 and an internal primer. Dig-UTP sense probe was synthesised from the latter fragment by the Sp6 polymerase. Transcripts were partially hydrolysed by incubation at 60°C in 0.1 M $NaCO_3/NaHCO_3$ buffer, pH 10.2 for about 35′. Immunological detection was performed as described by Canas [18]. Slides were treated with aniline blue as described by Eschrich [19].

3. Results

3.1. Isolation and characterisation of a Petunia hybrida cyclin cDNA

Mitotic cyclins have two regions in their amino acid sequences that display a particularly high level of conservation. Based on these sequences, two degenerate oligonucleotides were synthesised and used in a PCR reaction carried out on an ovary specific cDNA library. A fragment of about 180 bp was amplified, cloned and sequenced. The deduced amino acid sequence strongly resembled that of cyclin boxes of other cloned mitotic cyclins.

To isolate the full length cDNA, the 180 bp fragment was used as a probe to screen the ovary specific cDNA library of *Petunia hybrida*. Out of 400 000 phages screened, four were selected on the basis of their hybridisation signals and after one round of plaque purification, only the most strongly hybridising one was selected for further analysis. The cDNA insert was subcloned by in vivo excision in the Bluescript KSII+ plasmid and sequenced.

The analysed cDNA was 1838 bp in length and contained a 1335 bp open reading frame expected to encode a protein of 445 amino acids with a molecular mass of 48.5 kDa. Comparison of the deduced amino acid sequence with sequences in data bases showed significant similarity with B1 type mitotic cyclins. Consequently, the *Petunia hybrida* cyclin gene was designated *Pethy;CycB1;1*.

To determine the copy number of the *Pethy;CycB1;1* gene, a Southern blot analysis of genomic *Petunia* DNA digested with *Eco*RI, *Eco*RV, *Hin*dIII and *Bam*HI restriction enzymes was performed. A single hybridising signal was detected with *Eco*RV, *Hin*dIII and *Bam*HI whereas two bands were revealed by *Eco*RI (data not shown). On the basis of these findings and considering that among the enzymes used in the analysis only

Table 1
Flower development in *Petunia hybrida* line W115

Stage	Gynoecium and androecium	Petals and sepals	Petal size (mm)
1	Male archesporium differentiation.	Cell divisions in sepals and petals. Beginning of trichome and papillae development in petals.	<1.5
2	Microspore mother cells.	Cell divisions decrease in sepals.	1.5–2.0
3	Microsporogenesis.	Trichome development in sepals.	2.0–4.0
4	Ovule primordia. Microspores vacuolated.	Cell divisions are mostly localised in the petal tip and along the epidermis.	4.0–7.5
5	Integuments growth. Megaspore mother cell.	Beginning of parenchyma cell expansion. Increased rate of cell division along petal epidermis.	8.0–9.5
6	Megasporogenesis. Microspore bicelled.	Cells of proximal part of epidermis fully differentiated. Parenchyma cell expansion continues in the middle and distal part of petals.	10.0–14.5
7	Megasporogenesis. Pollen grains.	Petal mesophyll cells highly expansed.	15.0–19.5
8	Embryo-sac formation.	Intercellular space in petal parenchyma. Cell divisions at the petal tip and along the distal pigmented part of petal epidermis.	20.0–29.5

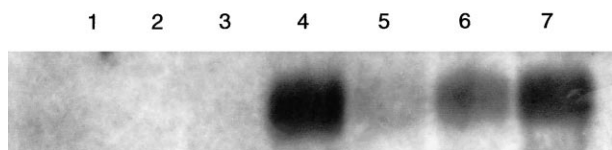


Fig. 1. Northern blot analysis of *Pethy;CycB1;1* to RNA from various organs of *Petunia hybrida* plants. Lane 1 and 2 contain 10 μg of total RNA extracted from young apical leaves and roots, respectively. Lanes 3–7 contain 10 μg of total RNA pooled from RNA samples extracted from *Petunia* floral organs (3: sepals; 4: petals; 5: anther; 6: styles and stigma; 7: ovaries) pooled from stage 4–8.

*Eco*RI cuts in the cDNA sequence (once) we concluded that there is only one copy of the *Pethy;CycB1;1* gene per haploid genome of *Petunia hybrida*.

3.2. *Pethy;CycB1;1 expression is restricted to flower organs and developmentally regulated*

As a first step towards studying *Pethy;CycB1;1* expression in different plant organs, total RNAs, extracted from young apical leaves, roots and flower organs (sepals, petals, anthers, stigma and style and ovaries) collected from *Petunia* flowers at various developmental stages (stages 4–8 see Table 1) were subjected to Northern analysis (Fig. 1).

Hybridising signals were strong in petals (Fig. 1 lane 4) and ovaries (Fig. 1 lane 7), weaker in anthers (Fig. 1 lane 5), stigma and style (Fig. 1 lane 6) and undetectable in sepals (Fig. 1 lane 3) young roots (Fig. 1 lane 1) and young leaves (Fig. 1 lane 2). We thus conclude that *Pethy;CycB1;1* expression is restricted to flowers.

The cellular localisation of *Pethy;CycB1;1* transcript was studied by in situ hybridisation with a Dig-UTP labelled *Pethy;CycB1;1* antisense probe on longitudinal and transverse sections of *Petunia* flowers at developmental stages spanning from male archespore development to embryo-sac formation. For a brief morphological description of the various developmental stages see Table 1. At low magnification, *Pethy;CycB1;1* transcripts appeared to be localised in numerous small patches of all floral organs (Fig. 2). Higher magnification revealed that these patches consisted of single or two adjacent cells containing label (data not shown).

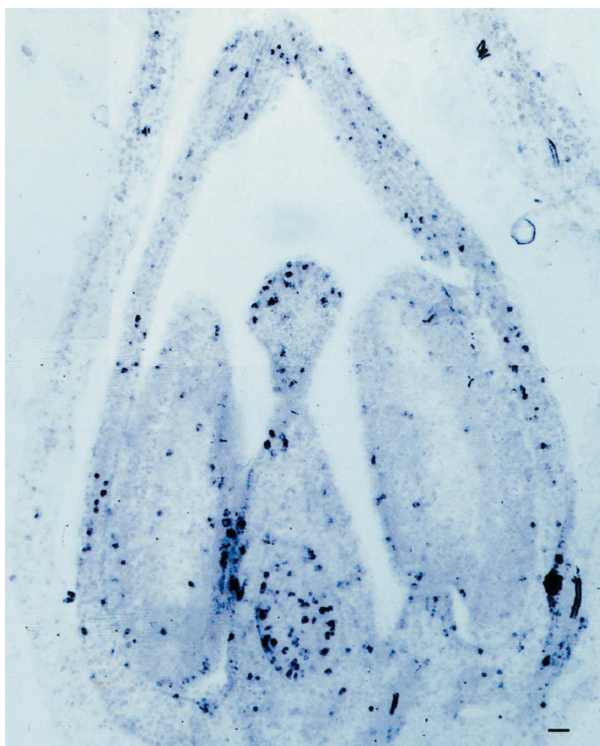


Fig. 2. In situ hybridisation of *Pethy;CycB1;1* to a longitudinal section of *Petunia* flower at stage 1. (Bar = 25μm).

The expression pattern of *Pethy;CycB1;1* was analysed in detail from the first to the fourth whorl of the *Petunia* flower. The results can be summarised as follows.

3.2.1. Sepals. *Pethy;CycB1;1* transcript was detected at stages 1–3 in sepals and never at later developmental stages. Labelling was localised in isolated cells of sepal parenchyma and epidermis (data not shown).

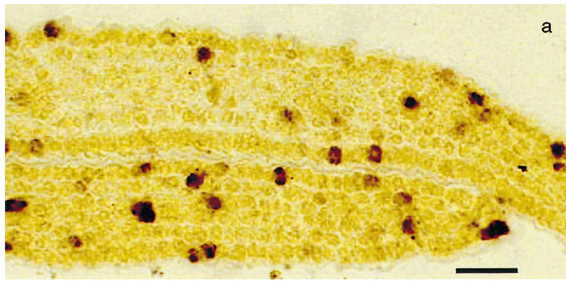
3.2.2. Petals. There was a high degree of labelling in epidermis and in parenchyma cells at the first developmental stages (1–4) in the second whorl (Fig. 3a). However, as petal development proceeded (stages 5–8) the proportion of labelled cells progressively decreased at the base of the petal and increased toward the petal rim (Fig. 3b). Spaced signals were detected along the epidermis (Fig. 3b,c). Cell divisions are required in petal epidermis during these developmental stages not only to ensure petal growth but also for trichome (abaxial epidermis) and papilla (adaxial epidermis) development. Consequently, some of the spaced signals detected along the epidermis could have marked cells which were dividing periclinally to give rise to such structures.

3.2.3. Stamens. *Pethy;CycB1;1* expression was detected in anther tissues only at stages 1μ4. Thereafter (stage 5–8), it became mostly confined to the anther filament (data not shown). Two types of labelling patterns were seen: patchy in anther epidermis, in the endothecium and in the anther filament and fairly uniform in the tapetum (Fig. 3d).

3.2.4. Carpels. In whorl four, *Pethy;CycB1;1* mRNA was detected in cells of the developing style and stigma at stage 1–3 (Fig. 2), at later developmental stages (5–8) it was mainly located in the secretory zone of the stigma (Fig. 3e) and in some of the cells of the transmitting tract of the style (Fig. 3f). The expression pattern in the ovary was striking. Signals were detected at all developmental stages. They were especially strong in the ovules but somewhat weaker in the septum and ovary wall (Fig. 3g). *Pethy;CycB1;1* expression was studied during female meiosis by combining ISH with aniline-blue staining (Fig. 3h). No signals were recorded in either megaspore mother cells or inside dyad, triad or tetrad. By contrast, strong labelling was visualised in some of the cells of the integuments which are growing to encapsulate the developing embryo-sac (Fig. 3i).

4. Discussion

In the present paper we described the cloning of a *Petunia hybrida* mitotic-like cyclin and its spatial and temporal expression pattern. Northern analysis showed that *Pethy;CycB1;1* expression was mainly restricted to flower organs. Although previous studies have demonstrated organ/tissue differential expression for cyclin genes [20–22] *Pethy;CycB1;1* is, to our knowledge, the first mitotic cyclin gene found to be highly expressed in flowers only. The analysis of the molecular determinants of this differential expression should shed lights on the links that occur between cell division and flower development. We also carried out in situ hybridisation studies on the expression pattern of *Pethy;CycB1;1* on longitudinal and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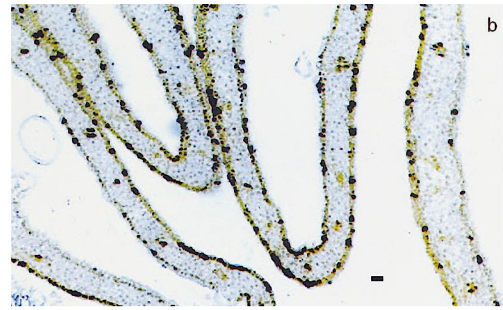
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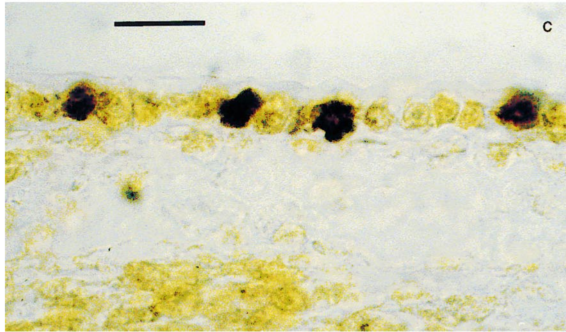
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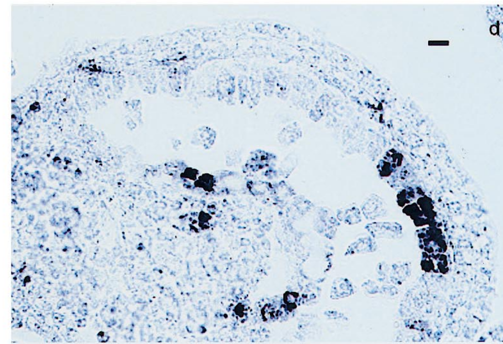
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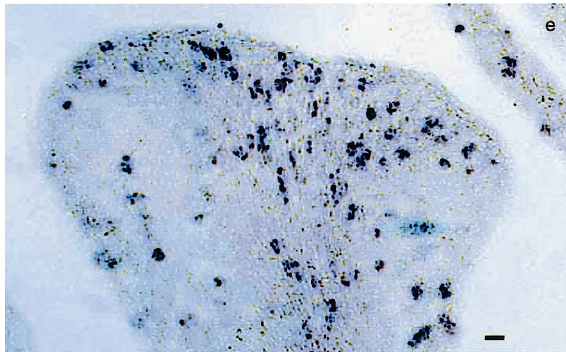
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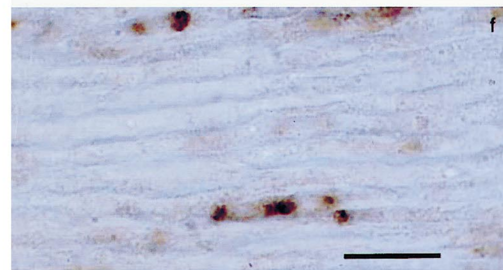
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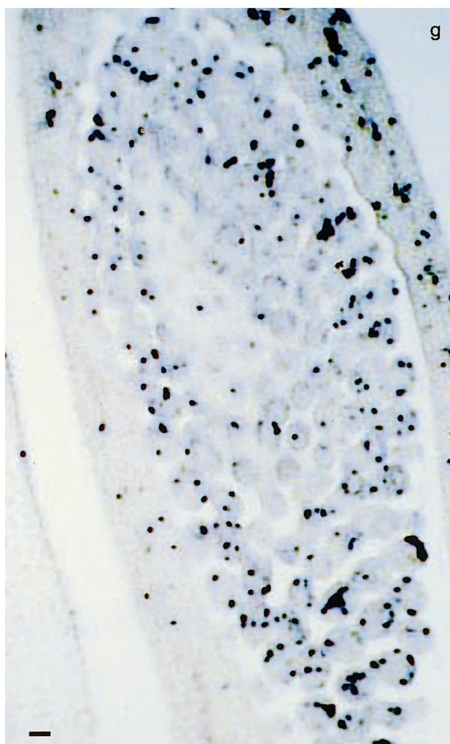
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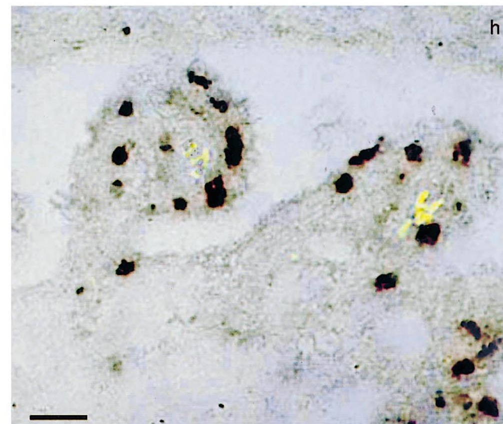
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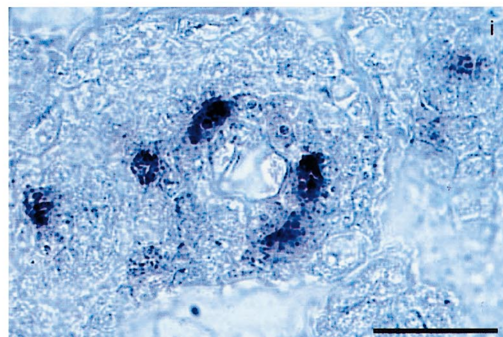
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Fig. 3. Localisation of *Pethy;CycB1;1* transcripts in various floral organs of *Petunia hybrida.* (Bars are 25μm). Hybridisation is visible as purple or dark blue colour development. a: Petal tip at stage 4; b: Petal at stage 7; c: Close up of petal epidermis at stage 6; d: Anther at stage 4; e: Stigma at stage 2; f: Close up of cells of the transmitting tract of style at stage 6; g: Ovary at stage 6; h: Close up of ovules at stage 7. After in situ hybridisation sections were treated with aniline-blue, a dye which binds to callose surrounding meiotic cells, and analysed under UV light. Green fluorescence is due to aniline-blue; i: Close up of an ovule at stage 8. Integuments cells are labelled.
←

transverse sections of *Petunia* flowers at developmental stages spanning from male archespore development up to embryo-sac formation. Since *Pethy;CycB1;1* expression was readily detectable, throughout development, in all floral organs, we used it as an in situ marker for defining at least some of the cell division patterns that shape the flower. The results, subdivided for each floral organ, are discussed below.

Sepals are the first floral organs to reach their final size. Already in developmental stage 3 cell divisions cease and further growth is achieved only through cell expansion. In contrast, petal development is completed at a later stage. On the basis of *Pethy;CycB1;1* expression pattern we propose that two distinct cell division patterns exist in petal development. Petal parenchyma cells are generated by cell divisions initially spread throughout petal primordia (stage 1–4) and later localised almost exclusively at the petal tip in a formation resembling an apical meristem. Petal parenchyma growth, therefore, requires a reiterative production of new cells at the apical meristem which will subsequently increase in volume during the enlargement phase of their growth. The cell division pattern of the petal epidermis differs in that cell divisions take place not only at the 'apical meristem' but also throughout the epidermis. Since cell expansion is mainly restricted to epidermal cells of the proximal part [23] we suggest that the cell divisions which take place along the epidermis are needed to sustain the growth of parenchyma cells by expansion. Such a model of cell divisions confirms what has already been hypothesised to explain petal pigmentation patterns of *Petunia* lines bearing unstable colour genes [24]. Two categories of coloured sectors are distinguished in these genotypes depending on whether they reach the petal margin or not. Sectors which fail to reach the margin (internal) would be generated by cell divisions occurring patchwise in the developing epidermis whereas cell divisions occurring at the 'apical meristem' would give rise to sectors that extend to the corolla margin (marginal).

Pethy;CycB1;1 expression is detectable in anther tissues only from stage 1–3 and then disappears completely. Labelling was spotted inside anther epidermis and endothecium, whereas it was uniform within anther locules, very likely reflecting the high degree of cell cycle synchronisation of tapetal cells [8]. Labelling was strong in the gynoecium at all developmental stages, the highest labelling concentration being seen in the developing ovary. *Pethy;CycB1;1* expression signals in ovules at meiotic stages were localised in the developing integuments but never in meiotic cells. Although a larger number of meiotic ovules should be analysed before reaching any conclusion on the non-involvement of *Pethy;CycB1;1* in female meiosis, it is tempting to hypothesise that a different cyclin gene is implicated in female meiosis. In budding yeast, *clb2* cyclin is extremely important for mitosis but not for meiosis [25], while in male mouse cyclin *A1*, which is essential for spermatocyte passage into the first meiotic division [26] is expressed exclusively in the germ cell lineage [27]. Little is known about the expression of the cloned cell-cycle genes during meiotic cell-cycle in plants. Detailed in situ hybridisation studies with gene specific probes on meiotic cells will provide insight in the similarity and difference, and eventually evolutionary relationships, of mitosis and meiosis.

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