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ORIGINAL RESEARCH

Confocal Microscopy Study of *Arabidopsis* Embryogenesis Using GFP:mTn

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Abstract Embryogenesis in transgenic *Arabidopsis* plants with GFP:mTn, a chimeric fusion of soluble shifted green fluorescent protein and a mouse actin binding domain, was studied. Confocal laser scanning microscopy was used to determine patterns of formation and cellular responses during asymmetric cell division. Before such cells divide, the nucleus moves to the position where new cell walls are to be formed. The apical–basal axis of the embryo develops mainly at the zygote to octant stage, and these events are associated with asymmetric divisions of the zygote and hypophyseal cells. Formation of the radial axis is established from the dermatogen to the globular-stage embryo via tangential cell division within the upper tiers. Bilateral symmetry of the embryo primarily happens at the triangular stage through zig-zag cell divisions of initials of the cotyledonary primordia. All stages of embryogenesis are described in detail here.

Keywords *Arabidopsis* · Asymmetric cell divisions · Cellular responses · Embryogenesis · GFP:mTn · Pattern formation

During plant embryogenesis, the zygote transforms and begins to function as an embryo. Undifferentiated cells initially lack the features found in mature plants. However, positional and genetic information during the embryogenic process determine the apical–basal axis and the radial pattern of the pre-embryo, which comprises hundreds of cells (Laux and Jürgens 1997; Jürgens 2001; Nawy et al. 2008). Critical steps in early embryogenesis entail asymmetric or symmetric cell divisions with mutual coordinated growth. Although such divisions occur in accordance with positional and genetic information at certain stages of embryogenesis (Mayer et al. 1993; Berleth and Jürgens 1993; Torres-Ruiz and Jürgens 1994; Scheres et al. 1995; Di Laurenzio et al. 1996; Lotan et al. 1998; Willemsen et al. 1998; Berleth and Chatfield 2002), little is known about the specific cellular responses within these cells.

Plant embryogenesis is defined by three phases—early, medial, and late. In the early phase, patterns form while an apical–basal axis and tissue systems begin to develop. During the medial phase, maturation of both axes and tissues is accompanied by the storage and accumulation of various compounds. Embryos in the late phase enter into arrested development in preparation for long-term dormancy. Embryogenesis of *Arabidopsis* proceeds relatively faster than those of other plants, with the early and medial phases coming within 12 days after pollination and the late phase beginning at day 14, leading to a dried seed (Lyndon 1990). These three main phases can be subdivided into 20 stages, from zygote to mature dried embryo; such categories are utilized in genetics and molecular biology research (Jürgens and Mayer 1994; Laux et al. 2004).

Depending on the available genetic or positional information, two types of asymmetric cell division occur during embryogenesis (Liu et al. 1993; Yadegari et al. 1994; Hardtke and Berleth 1998; Willemsen et al. 1998; Ogas et al. 1999; Steinmann et al. 1999; Bowman and Eshed 2000; Helariutta et al. 2000; Wysocka-Diller et al. 2000; Nakajima et al. 2001; Takada et al. 2001; Berleth and Chatfield 2002; Casson and Lindsey 2006; Kim 2007; Spencer et al. 2007). These events eventually give rise to the formation of external morphologies, e.g., cotyledons, radicle, and hypocotyl, as well as a variety of internal

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tissues. The first division of the zygote must be asymmetric and under genetic control, producing a smaller apical cell and a large basal cell (Jürgens and Mayer 1994; Colette and Heidstra 2007). Later, the apical cell will be responsible for aboveground activities, while the basal cells will control those of the belowground plant portions. Lens-shaped cells, which appear via asymmetric division and under genetic control, are found in the 33rd cell stage of the globular embryo, as part of the underground organs (Laux et al. 2004). In contrast, positional information controls both asymmetric cell division, such as that of the inner cells during the 17th cell phase at the dermatogen stage, and also the elongated central cells that form the vascular procambium during the 65th cell phase at the mid-globular stage.

Research on the patterns of embryogenesis has focused on several plant mutants. Although the first division usually sets up the apical–basal axis of the embryo, mutated genes confer a loss of polarity and result in an undeveloped embryo. The radial tissue pattern is established during the globular stage by genes that are responsible for cell identity (Mayer et al. 1993; Przemeck et al. 1996; Vroemen et al. 1996; Hardtke and Berleth 1998; Long et al. 2006). Genes that affect the early steps in embryonic cell division include AtML1, SCR, SHR, ZLL, STM, ANT, AS1, FIL, REV, and YAB3 (Jürgens 2001, 1993). Mutations such as GNOM, AtLTP1, PIN1, and MP adversely influence the formation of the epi- and hypocotyl. Their expression can also prevent normal embryo development under extreme conditions (Hardtke and Berleth 1998).

The observational method of embryogenesis has mainly been utilized with permanent preparation slides that display special characteristics at each developmental stage, as well as various aspects of cell division and pattern formation in mutant tissues (Steeves and Sussex 1989; Goldberg et al. 1994; Brownlee and Berger 1995; Laux and Jürgens 1997; Berleth and Chatfield 2002; Casson et al. 2005). Now, researchers have introduced green fluorescent protein (GFP) as a reporter gene for conducting developmental studies (Davis and Vierstra 1998). Here, the objective was to investigate the features of cell division during *Arabidopsis* embryogenesis. Transgenic plants containing a chimeric gene between GFP and talin, an actin binding domain, were generated, and confocal laser scanning microscopy was performed with living embryos.

Materials and Methods

Plant Materials and Growing Conditions

"Columbia-O", a wild-type *Arabidopsis*, was obtained from The Arabidopsis Information Resource. After imbibition at 4°C for 5 days, seeds were germinated in pots containing a 1:1:1 mixture of top soil/perlite/vermiculite, plus approx. 1 g of 14-14-14 Osmocote (Song and Clark 2005). Plants were grown at 24°C under approx. 800 foot-candles of constant cool-white fluorescent light.

DNA Construct, PCR, and Transformation

GFP, modified according to the method of Haseloff et al. (1997), was used as a reporter protein. A chimeric fusion, GFP:mTn, was made between this green fluorescence protein and a mouse talin f-actin binding domain. The stop codon at the 3′ end of the GFP sequence was replaced with an oligonucleotide encoding a 5× Gly Ala linker followed by a multiple cloning site (MCS). The mTn binding sequence was PCR-amplified from pGST-Tn2345~2541 (McCann and Craig 1997; Kost et al. 1998) using primers FmTn (5′-GGATCCATCCTAGAAGCTGCCAAGTC-3′) and RmTn (5′-TCTAGATTAGTGCTCGTCTCGAAGCT-3′). These were inserted in-frame with GFP into the MCS of pSTblue-1 (Novagen). PCR was performed with an initial 1 min of denaturation at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 56°C, and 1 min at 72°C. The vector construct was introduced into *Escherichia coli* DH∂5 and *Agrobacterium tumefaciens* AGL1 strain by the freeze–thaw method (An et al. 1988). For *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*, a GFP:mTn expression cassette was cloned into binary vector pCB302-3 (Xiang et al. 1999) with *Ham*H1 and *Xba*1 (Fig. 1). *Arabidopsis* plants were transformed via floral dipping (Clough and Bent 1998). T_1 and T_2 transgenics were first screened for BASTA resistance before strong signals for GFP in the seedlings were detected under a fluorescence stereo-microscope (SMZ1000, Type 108; Nikon, Japan).

Confocal Microscopy

Siliques of T_3 transgenic plants were prepared with forceps and dissecting knives under a stereo-microscope. Embryos at serial stages of development were selected and mounted on hol-slide glasses with distilled water. They were then covered and sealed with transparent glue. Slide samples were examined with a 100× oil immersion lens under a Zeiss LSM 510 laser scanning microscope (Carl Zeiss, Germany). GFP fluorescence was imaged using excitation with the 488-nm line of an argon laser and a 505~530 nm band-pass emission filter.

Results and Discussion

Vector DNA containing the GFP:mTn expression cassette was 4.4 kb long (Fig. 1). This cassette comprised 716 bp of

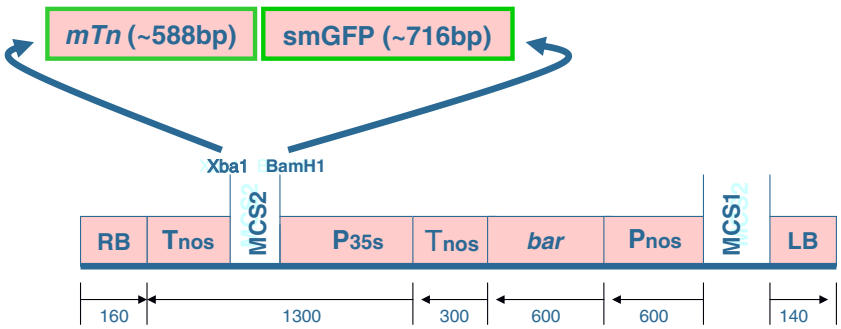

Fig. 1 DNA construct and outline of pCB302-3 vector

GFP and 588 bp of mTn. Embryogenesis was examined in T_3 plants transformed with GFP:mTn and lacking specific phenotypes. The fifth to seventh silique from the top of the inflorescence served as the control point for dissection to obtain globular-stage embryos, with the upper ones being younger than those from the lower siliques. Cell divisions in the zygote after fertilization showed nearly similar patterns in time and space during morphogenesis, activation of the primary meristem, cell differentiation, and preparation of the embryo for dormancy (Jürgens 2001). Defining features included formation of the apical–basal and radial axes and bilateral symmetry of the seedling through symmetric and asymmetric divisions.

Zygote to Octant-Stage Embryo: Formation of the Apical–Basal Axis

Within a few hours post-fertilization, the zygote elongates about three-fold along the chalaza-micropyle axis of the ovule (Mansfield and Briaty 1991). It then undergoes perpendicular cell division to the axis, giving rise to apical–basal polarity. This is manifested by a smaller apical cell that is elliptically swollen and a larger, rod-shaped basal cell (Fig. 2a). That is the first asymmetric cell division controlled by genetic information after fertilization (Mayer et al. 1993; Friml et al. 2003; Vernon and Meinke 1994). The upper cell will generate the entire embryo, with two exceptions: its very basal end and the lower hypophysis, which will produce the embryo radicle and the suspensor that provides nutrients (Yeung and Sussex 1979; Jürgens and Mayer 1994). Two cellular responses occur during zygote expansion, i.e., the cytoplasm and the nucleus are re-located toward the apical end (Mòl et al. 1994; see the basal cell in Fig. 2a). Directional expansion of the zygote may be related to the orientation of the apical–basal axis of the ovule and associated with a reorganization of the microtubular cytoskeleton (Webb and Gunning 1991). This initial round of apical cell division is characterized by the first longitudinal division, resulting in two half-moon-shaped daughter cells (Fig. 2b). The second round is the same, producing a quadrant-stage embryo and giving rise to a quarter-shaped cell that resembles a Rugby ball (Fig. 2c). The third round leads to partitioning into eight pro-embryo cells that will undergo the first periclinal cell divisions (Fig. 2d). This octant-stage embryo develops into an upper and a lower tier. The former will establish an apical domain that comprises the shoot apical meristem and most of the cotyledon, whereas the latter will contain the central domain hypocotyl, vascular primordia, and root and proximal initials of the root meristem.

Dermatogen to Globular-Stage Embryo: Formation of the Radial Axis

The first radial patterning event occurs at the dermatogen stage (Fig. 3a) and within a relatively short time. This stage consists of 16 cells that have resulted from a single round of tangential division from the octant-stage embryo (Jürgens and Mayer 1994; Berleth and Chatfield 2002). Such a division is characterized by the separation of eight inner cells plus an outer layer of eight epidermal precursor cells called the protoderm (Fig. 3a). This is the first asymmetric cell division ruled by positional information (Mansfield and Briaty 1991). Meanwhile, the uppermost derivative of the basal daughter cell undergoes asymmetric cell division controlled by intrinsic factors (Haecker et al. 2004). The daughter cell adjacent to the embryonic cell mass originates a lens-shaped cell (Fig. 3a) that will eventually cover the quiescent center and the initials of the central root cap. This early globular-stage features another round of division from the dermatogen stage, resulting in 33 cells (Fig. 3b). The vascular primordia split into a central vasculature and a surrounding layer of pericycle cells. This stage is characterized by the first appearance of primordia for vascular tissue in the central part of the lower tier. These vascular primordia are rod-shaped due to expansion along the apical–basal axis, and their nuclei are polarized to the top of the end wall (Fig. 3b). The mid-

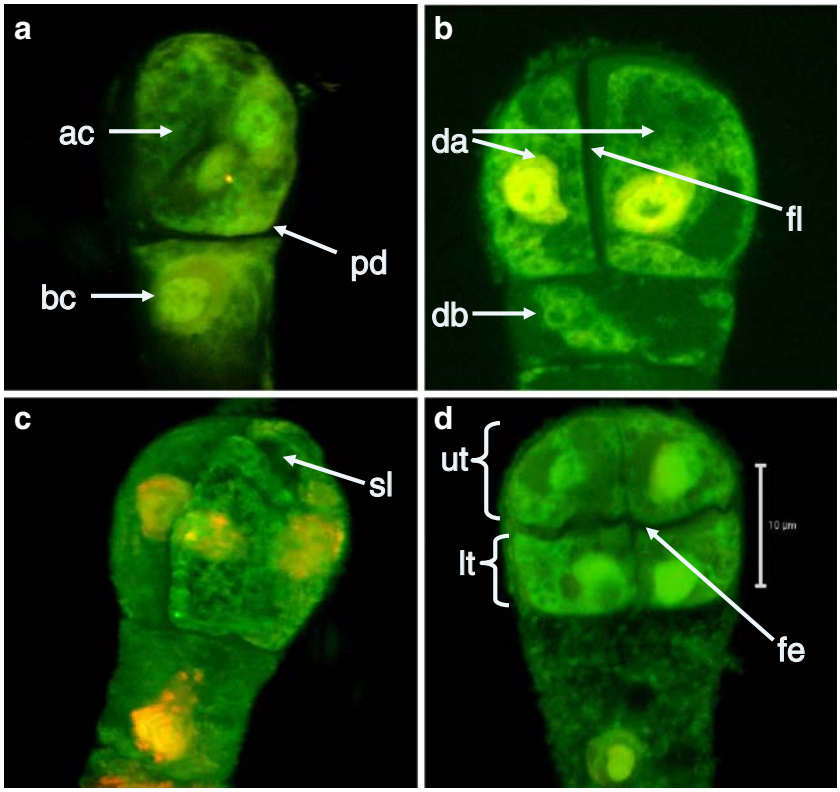

Fig. 2 Confocal micrographs from single to octant stage of embryo. **a** One-cell stage (*ac* apical cell, *bc* basal cell, *pd* first perpendicular cell division). **b** Two-cell stage (*da* derivatives of apical cell, *db* derivative of basal cell, *fl* first longitudinal cell division). **c** Quadrant stage (*sl* second longitudinal cell division). **d** Octant stage (*ut* upper tier, *lt* lower tier, *fe* first anticlinal cell division of embryonic cell). Bar=10 μm

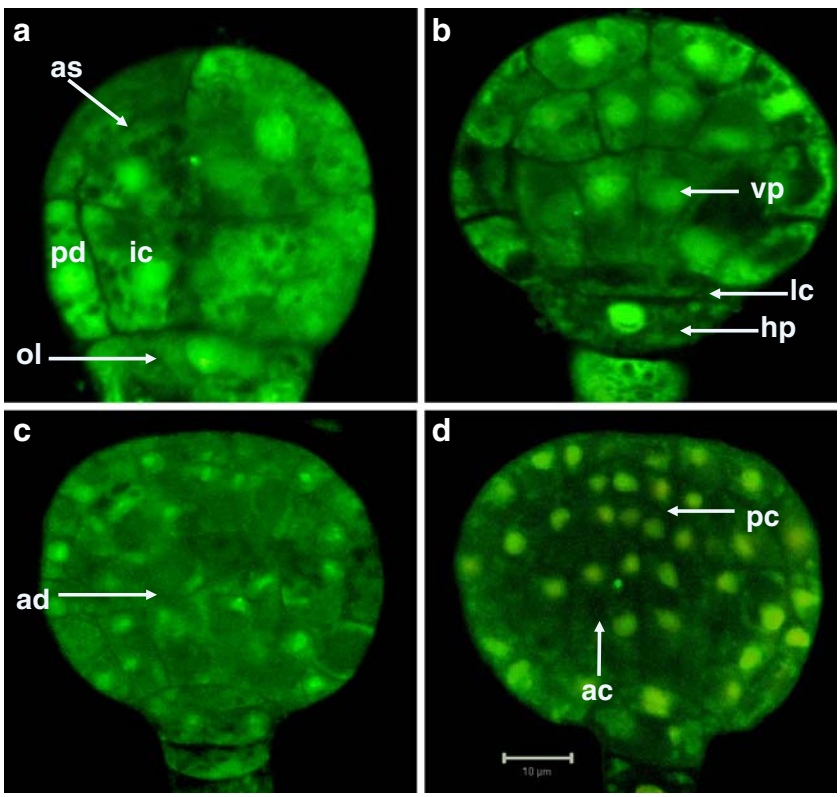

Fig. 3 Confocal micrographs from dermatogen to globular stage of embryo. **a** Dermatogen stage (*as* asymmetric cell division, *ic* inner cell, *ol* origin of lens-shaped cell, *pd* protoderm). **b** Early globular stage (*lc* lens-shaped cell, *hp* hypophysis). **c** Mid-globular stage (*ad* asymmetric cell division of vascular primordium). **d** Late globular stage (*ac* anticlinal cell division of vascular primodia, *pc* periclinal cell division of inner cell in upper tier). Bar=10 μm

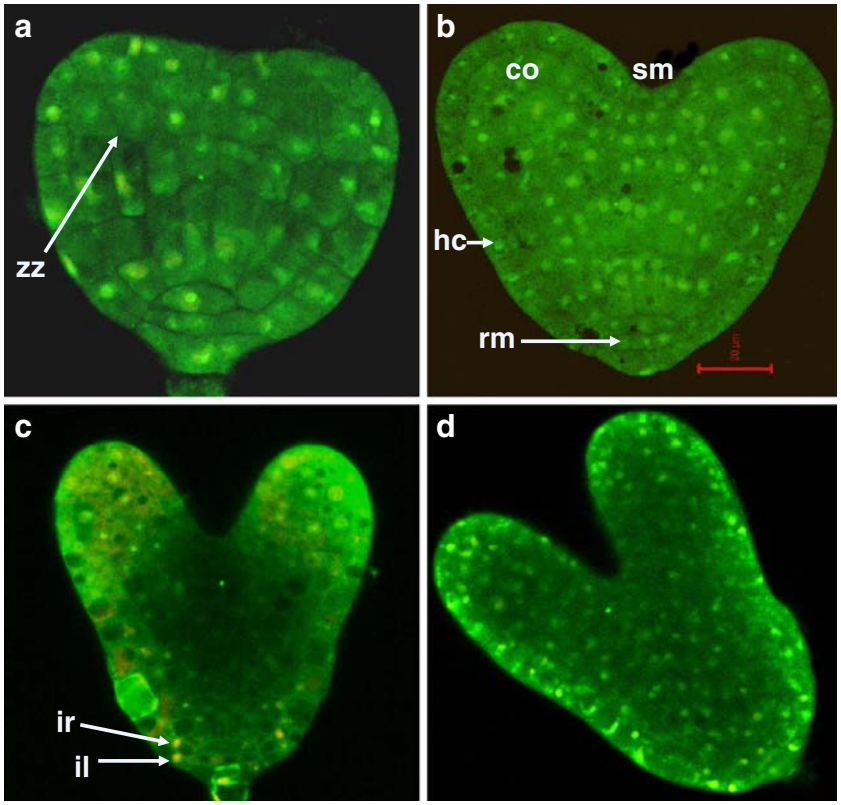

Fig. 4 Confocal micrographs from triangular to early torpedo stage of embryo. **a** Triangular stage (*zz* zig-zag cell division). **b** Mid-heart stage (*co* cotyledonary primodia, *hc* hypocotyl, *rm* incipient root meristem, *sm* incipient shoot meristem); bar= 20 μm. **c** Late heart stage (*il* initials of lateral root cap, *ir* initials of root epidermis). **d** Early torpedo stage

globular-stage embryo, with 65 cells, shows transverse and asymmetric cell division of the inner cells derived from the lower tier, making that division appear much smaller (Fig. 3c). This step is controlled by extrinsic factors (Scheres et al. 1994; Haecker et al. 2004). Interestingly, the inner cells derived from the upper tier do not undergo any transverse cell division at this stage. The radial axis is completed at the late globular stage by both perpendicular and transverse divisions throughout the tiered inner cells (Fig. 3d).

Triangular to Mature Embryo Stage: Formation of Bilateral Symmetry

The triangular stage is characterized by the development of cotyledon initials from zig-zag cell divisions in the upper tier (Fig. 4a). Upon reaching 150~250 cells, the apical surfaces of the embryo are now flattened, with their overall shape gradually becoming triangular in laterally flattened views, thereby forming a bilateral symmetry. The mid-heart stage produces four major types of seedling

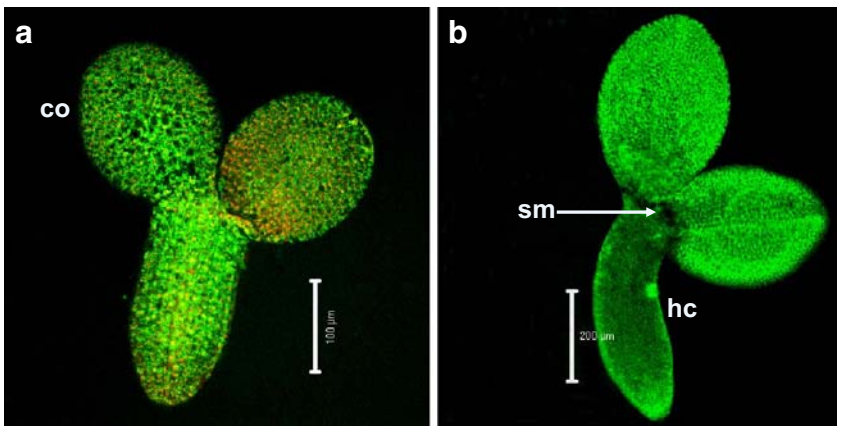

Fig. 5 Confocal micrographs of embryo from late torpedo to mature stage. **a** Late torpedo stage (*co* cotyledon); bar= 100 μm; **b** bent-cotyledon stage (*hc* hypocotyl, *sm* shoot apical meristem); bar=200 μm

structures—cotyledonary primordia, initials for shoot and root meristems, and the hypocotyl (Fig. 4b). This stage eventually numbers 500 cells (Jürgens and Mayer 1994). The late heart stage first shows initials for both the lateral root cap and the root epidermis due to periclinal divisions of the epidermal cells in the nearby hypophysis (Fig. 4c). Cotyledonary primordia in the early torpedo stage grow rapidly and are in parallel at the distal parts (Fig. 4d). The late torpedo stage is characterized by round to oval cotyledonary primordia (Fig. 5a), and the embryo is now up to 300 μm long and 100 μm wide. The mature embryo stage marks the end of embryogenesis and presents structures for an eventual seedling. Its overall length is 800 μm, with 15,000~20,000 cells (Fig. 5b; Jürgens and Mayer 1994). The cotyledonary primordia are folded back to the root cap and have developed vasculature.

Cellular Responses During Asymmetric Cell Division

Asymmetric cell divisions play an important role in morphogenesis during embryo formation and are controlled by genetic or positional information (Laux et al. 2004; Colette and Heidstra 2007). The goal of this study was to determine the cellular responses of asymmetric divisions by using GFP:mTn visualization. Before such division occurs, the nucleus is shifted to a position where new cell walls will be formed, a process that is directed by both intrinsic factors (Fig. 3a) and positional information (Fig 3b). Although the nucleus of the basal cell is still sited in the center at the beginning of that stage (Fig. 2d), it then relocates to the top of the cell (Fig. 3a). That event of asymmetric division gives rise to lens-shaped cells (Fig. 3b). In addition, the nuclei of vascular primordial cells clearly expand in the upper part of the cells (Fig. 3b), which then undergo asymmetric cell division to produce smaller apical and larger basal cells (Fig. 3c).

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