Developmental patterning of the sub-epidermal integument cell layer in Arabidopsis seeds

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ABSTRACT

Angiosperm seed development is a paradigm of tissue cross-talk. Proper seed formation requires spatial and temporal coordination of the fertilization products – embryo and endosperm – and the surrounding seed coat maternal tissue. In early Arabidopsis seed development, all seed integuments were thought to respond homogenously to endosperm growth. Here, we show that the sub-epidermal integument cell layer has a unique developmental program. We characterized the cell patterning of the sub-epidermal integument cell layer, which initiates a previously uncharacterized extra cell layer, and identified TRANSPARENT TESTA 16 and MULTICOPY SUPPRESSOR OF IRA1 Polycomb group proteins. This work demonstrates the different responses of epidermal and sub-epidermal integument cell layers to fertilization.

KEY WORDS: Fertilization, Seed coat, Integuments, Tissue cross-talk, Seed development

INTRODUCTION

In angiosperms, seed development starts with the double fertilization of the egg and central cell in the ovule that leads to the formation of embryo and endosperm, respectively. Proper seed formation is then achieved through tight spatial and temporal coordination of embryo, endosperm, and seed maternal tissues (Ingram, 2010).

In Arabidopsis, ovule primordia are composed of three functional domains: the funicular, which transports nutrients from the mother plant; the chalaza, which initiates two integuments; and the nucellus, which gives rise to the female gametophyte. Both inner (ii) and outer (oi) integuments are composed of two epidermal cell layers (ii1, ii2, oi1 and oi2) which grow by anticlinal cell divisions to progressively surround the female gametophyte. At the end of ovule development, the ii1 undergoes periclinal cell divisions to give rise to a sub-epidermal integument cell layer, the so-called ii1’ (Fig. S1) (Schneitz et al., 1995; Debeaujon et al., 2003). The fertilization-independent development of the ovule is repressed by a class of Polycomb group (PcG) proteins named FERTILIZATION INDEPENDENT ENDOSPERM (FIE) and MULTICOPY SUPPRESSOR OF IRA1 (MSI1) FIS PcG proteins act sporophytically to repress the differentiation of the integuments (Roszak and Köhler, 2011). After fertilization of the central cell, the endosperm initiates a signal, through the action of the MADS box transcription factor AGAMOUS-LIKE 62, that relieves the FIS-mediated repression and leads to the differentiation of the ovule integuments into seed coat (Roszak and Köhler, 2011). Results from Figureiredo et al. (2016) suggest auxin as the putative fertilization signal that coordinates endosperm and seed coat development. Nevertheless, exclusive fertilization of the egg cell by kokopelli or cyclin dependent kinase a;1 mutant pollen triggers partial differentiation of the ovule integuments surrounding the female gametophyte (Ungru et al., 2008; Kasahara et al., 2016). Partial differentiation of the ovule integuments is also initiated by the pollen tube content of the generative cell-specific l (gcs1) mutant, whose sperm cells fail to fertilize the female gametophyte (Kasahara et al., 2016). Furthermore, gcs1 pollen tube content induces full differentiation of the integuments of medea FIS PcG mutant ovules, which undergo fertilization-independent proliferation of the central cell. These data suggest that pollen tube rupture and not fertilization enables the central cell to initiate the signaling pathway that leads to differentiation of the seed coat. In response to the endosperm signal, the five seed coat cell layers undergo a rapid phase of cell division and expansion, and follow different cell fates (Haughn and Chaudhury, 2005). The MADS box protein TRANSPARENT TESTA 16 (TT16) works downstream of FIE and MSI1 to promote the production of proanthocyanidins (PAs) in the ii1, the innermost seed coat cell layer (also known as the endothelium) (Xu et al., 2016). Finally, endosperm and seed coat coordinate their growth through a cross-talk signaling pathway (Ingram, 2010) that was first identified in the study of the maternally acting TRANSPARENT TESTA GLABRA 2 (TTG2) and zygotically acting HAiku (IKU) genes. Both ttg2 and iku mutants show premature arrest of endosperm development and reduced seed size, indicating that the developmental interaction between seed coat and endosperm orchestrates early seed growth with limited embryo contribution (Garcia et al., 2005).

To date, all seed integuments were thought to respond to the same signaling pathway initiated by endosperm growth. Here, we show that the sub-epidermal ii1’ has a unique developmental program. We followed ii1’ cell patterning from its inception in the ovule until its differentiation in the seed. We demonstrated that the ovule ii1’
RESULTS

The ii¹ gives rise to a sixth integument cell layer

The last stage of Arabidopsis ovule development, stage 3-VI, is marked by the formation of the inner integument 1’ (ii¹) by periclinal cell divisions of the ii¹, the innermost integument cell layer (Fig. S1) (Schneitz et al., 1995; Debeaujon et al., 2003). To thoroughly characterize the process of ii¹ formation we analyzed central longitudinal sections of Arabidopsis ovules at stage 3-VI, three-dimensionally reconstructed using the modified pseudo-Schiff propidium iodide imaging technique (mPS-PI, see Materials and Methods). Cells of the ii¹ underwent periclinal cell divisions starting from the chalazal pole and progressed toward the micropyle region without interruptions (Fig. 1A-C). The first cell of the ii¹, identified as the cell following the merging of ii¹ and ii² in the chalazal pigment strand (Fig. S1), did not undergo periclinal cell divisions (Fig. 1A-C). The ii¹ arose from the second, third or fourth cell of the ii¹ and developed toward the micropyle without ever reaching it (Fig. 1A-C). At the end of stage 3-VI/beginning of stage 4-I, we observed additional periclinal cell division in the ii¹’ that gave rise to a sixth integument cell layer, which we named ii¹” (Fig. 2B, ii¹” is highlighted in green throughout). The ii¹” was limited to the chalazal area, as fertilization followed rapidly and led to the differentiation of the integuments, but persisted after fertilization showing a developmental patterning similar to ii¹’ (Fig. 2C). The ii¹” phenotype was more penetrant in the Wassilewskija accession (70% of the ovules) compared with Columbia (35% of the ovules). Both ii¹’ and ii¹” are sub-epidermal cell layers, unlike the other integument cell layers which are all L1 epidermal layers (Fig. 2B,C) (Debeaujon et al., 2003).

TT16 and STK regulate ii¹’ cellular patterning

The ovules of the Arabidopsis transparent testa 16;seedstick (tt16; stk) double mutant have been shown to carry only four integument cell layers and have been interpreted as missing the ii¹ (Mizzotti et al., 2012). The progression of wild-type integument development argues against such an interpretation, as ii¹’ is the last integument cell layer to appear (Schneitz et al., 1995). We therefore analyzed the development of the tt16;stk innermost integument cell layer by mPS-PI in ovules and seeds. We detected few periclinal cell divisions occurring stochastically along the integument proximal-distal axis (Fig. 1F,G, Fig. S2) when compared with wild type (Fig. 1D, Fig. S2). In 89% of the tt16;stk samples we observed a patchy pattern of periclinal cell divisions (Fig. 1G, Fig. S2), a phenotype never observed in wild type (Fig. 1D, Fig. S2). These data strongly suggest that tt16;stk ovules are impaired in the periclinal cell divisions of the innermost integument cell layer that, in wild-type ovules, give rise to the ii¹’. Only 9.5% of tt16;stk seeds displayed a few ii¹’ cells. Although the stk single mutant exhibited a pronounced ovule shape defect (Mizzotti et al., 2014), it did not show any ii¹’ or ii¹” aberrant phenotype (Fig. 1E, Fig. S2). However, 74% of tt16 ovules displayed a more distal ii¹’ and ii¹” (after the fourth ii¹ chalazal cell) compared with wild type.
(Fig. 2D), and 16% of tt16 ovules developed a long ii1’ that resulted in a true six-cell layered seed coat (Fig. 2G). In wild-type seeds, the ii1′ extended through the curving zone until approximately halfway towards the micropylar pole (Fig. 2C,I). As a consequence, transverse imaging of wild-type seeds beyond their midline did not show the ii1′ around the developing embryo (Fig. 3D). Conversely, in the majority of tt16 seeds, the ii1′ was still present beyond the seed midline and entered the micropylar region (Fig. 2F-I). Transverse views of tt16 seeds beyond their midline strikingly showed a shift of the ii1′ toward the side occupied by the developing embryo (Fig. 3E). Thus, tt16 developing embryos were often compressed by the concomitant mechanical action of the seed ii1′ invading the micropylar region (Fig. 2H) and the persistence of the nucellus at the chalazal pole (Xu et al., 2016). Embryo cell morphology and proliferation in the tt16 mutant appeared comparable to wild type (Fig. 2C-H). The maternal origin of the compressed embryo phenotype was confirmed by analyzing tt16 ovules fertilized with wild-type pollen (Fig. S3). This phenomenon could be partially responsible for the arrested seed phenotype that we observed in tt16 mutant siliques. Six-week-old tt16 plants carried 7-23% (Ws background) or 6-33% (Col background) arrested seeds per silique (n=20 siliques observed), whereas control wild-type plants exhibited a low rate (1.4-3.2% in Ws or 0-12% in Col) of seed arrest in the same growing conditions (n=13 siliques observed in Ws and n=20 in Col). Embryo arrest in tt16 seeds might also be caused by defects in nutrient transport through the seed coat (Chen et al., 2015). Altogether, these data indicate that STK and TT16 redundantly promote ovule ii1’ formation, whereas TT16 alone regulates the positioning of ii1′ along the ii1 proximal-distal axis.
To test if TT16 expression correlates with the development of the ovule ii1’, we created a marker line carrying the 3.4 kb TT16 promoter region and genomic sequences translationally fused to green fluorescent protein (GFP). We have previously shown that this TT16 genomic region fully complements tt16 mutant phenotypes in the nucellus and endothelium (Xu et al., 2016). We detected fluorescence in the nuclei of the proximal region of the ovule ii1’ at stage 3-V (Fig. 3A) and in the developing ii1’ (Fig. 3B) at stage 3-VI. GFP expression extended to more distal cells of the endothelium and ii1’ in seeds at 2 days after flowering (DAF; see Materials and Methods) but never reached the micropyle (Fig. 3C).

The TT16 expression pattern in the ovule ii1 marks in advance the development of ii1’ and might therefore be responsible for the correct positioning and progression of ii1 periclinal cell divisions. In one possible scenario, TT16 might define only the position of the first ii1’ cell, whereas the following periclinal cell divisions continue by default until ovule maturity. Alternatively, TT16 might define the precise ii1’ spatial window from beginning to end. To distinguish between these two hypotheses, we expressed TT16 under the control of the 1.6 kb TT16 promoter, which marks the first two or three proximal cells of the ovule ii1 and the nucellus, in a tt16 mutant background (1.6ProTT16:gTT16;tt16) (Xu et al., 2016). Three independent transgenic lines fully complemented the tt16 phenotype at the ii1’ chalazal end (Fig. 2I, Fig. S4). Nevertheless, they failed to complement growth of ii1’ into the micropylar area and produced seeds that were completely covered by five seed coat cell layers (Fig. 2I, Fig. S4). These data might highlight the limited extent of TT16 non-cell-autonomous effects (Xu et al., 2016) along the ii1 proximal-distal axis. Nevertheless, these same complementation lines fully restored the arrested production of...
PAs along the entire tt16 endothelium (Xu et al., 2016). Therefore, our analyses strongly suggest that TT16 marks the end of the ii1 periclinal cell divisions in a cell-autonomous fashion, in contrast to the non-cell-autonomous regulation of PA biosynthesis. In line with this hypothesis, early expression of TT16 in the ii1, with the exception of the micropylar zone, under control of the TTI promoter region (Fig. S4) fully complemented the tt16 ii1′ phenotype at the chalazal and micropylar regions (ProTT1:gTT16;tt16, Fig. 2I, Fig. S4). Embryos of 1.6ProTT1:gTT16;tt16 seeds, which displayed a nucellus of wild-type appearance (Xu et al., 2016) and a five cell layered seed coat at the micropylar zone, were never compressed by the surrounding seed coat (Fig. S4) as observed in and a five cell layered seed coat at the micropylar zone, were never displayed a nucellus of wild-type appearance (Xu et al., 2016). The negative effect of ii1′ cell-autonomous effects (Xu et al., 2016). The negative effect of ii1′ perpendicularly to the longitudinal axis of the seed (Fig. 3F). Thus, cell divisions non-cell-autonomously.

ovule development (Fig. 1H,I) (Mizzotti et al., 2014). The absence of ii1′ expression in the ovule outer integument (oi) and ii2 throughout ovule development (Fig. 1H,I) (Mizzotti et al., 2014). The absence of ii1′ suggests that STK affects ii1′ periclinal cell divisions non-cell-autonomously.

TT16 regulates ii1′ cell architecture
In line with previous analyses (Nesi et al., 2002), longitudinal sections of tt16 seeds showed thinner and more elongated cells in the endothelium and ii1′ when compared with wild type (Fig. 2F-H). To better characterize such morphological anomalies, we analyzed images of three-dimensionally reconstructed seeds using the mPSPI technique. In the proximal half of the wild-type seed coat, endothelium and ii1′ cells tended to be tubular in shape and oriented perpendicularly to the longitudinal axis of the seed (Fig. 3F). Thus, they appeared round in longitudinal section (Fig. 2C) and elongated in transverse section (Fig. 3D). In the tt16 mutant, endothelium and ii1′ cells appeared equally tubular but aligned along the proximal-distal axis of the seed, and thus perpendicular to wild-type cells (Fig. 3G). We noticed that the orientation of these cells changed along the proximal-distal axis in both wild-type and tt16 seeds. Longitudinal sections of wild-type ii1′ displayed cells that were more elongated in the chalazal and micropylar zones compared with the curving zone (Fig. 3H, Fig. S5). The tt16 ii1′ followed the same trend of cell elongation along the proximal-distal axis but showed cells that were strikingly more elongated than in the wild type (Fig. 3H). These analyses clearly suggest that TT16 regulates endothelium and ii1′ cell architecture, and this probably underlies the misshapen tt16 seed phenotype (Nesi et al., 2002). TT16 might establish ii1 and ii1′ cell orientation in the ovule but its mutant phenotype became evident in seeds after cell elongation. Such a phenotype accentuates the proximal-distal positional defect of the tt16 ovule ii1′ described above.

The ii1′ undergoes a drastic cell expansion in response to fertilization (Fig. 2C, Fig. 3D) (Beeckman et al., 2000). Transmission electron microscopy imaging revealed highly vacuolated ii1′ cells (Fig. 3I) at the curving zone of wild-type seeds. In tt16 seeds, these cells appeared more cytoplasmic, suggesting that their cell expansion might be impaired (Fig. 3J). Similarly, tt16 seeds fail to correctly differentiate the endothelium as they do not produce PAs (Nesi et al., 2002). Altogether, these data indicate that TT16 regulates cell orientation and differentiation of the endothelium and seed ii1′.

Unique response of ii1′ to fertilization
Fertilization of the central cell has been shown to trigger the differentiation of seed maternal tissues (Roszak and Köhler, 2011; Xu et al., 2016). To test the effect on ii1′ differentiation of each fertilization event independently, we examined the seed coat of the kokopelli (kpl) mutant, which displays random single-fertilization events (Ron et al., 2010). kpl seeds carrying only the embryo (kpl embryo-only seeds) have a small and partially differentiated seed coat (Roszak and Köhler, 2011; Kasahara et al., 2016). By contrast, kpl seeds that develop only the endosperm (kpl endosperm-only seeds) produce a large seed coat with a fully differentiated endothelium, as suggested by PA accumulation (Roszak and Köhler, 2011). In line with previous results, the ii1′ of kpl embryo-only seeds resembled that of undifferentiated ovules (Fig. 4C compared with Fig. S6). In kpl endosperm-only seeds, the ii1′ cells did not expand in coordination with the development of the other integument cell layers, creating empty spaces (Fig. 4B compared with Fig. 4A). Development of ii1′ was unaffected in kpl mutant ovules (Fig. S6). These data suggest that ii1′ differentiation requires fertilization of both the egg and central cell.

The FIE and MSI1 PcG genes are expressed in all ovule integument cell layers (Köhler et al., 2003; Xu et al., 2016) and are thought to repress their fertilization-independent development (Roszak and Köhler, 2011). frie and msi1 mutations are haploinsufficient, and unfertilized frie/+ and msi1/+ pistils carry a number of enlarged autonomous seeds that exhibit an apparently developed seed coat that accumulates PAs and a degenerated nucellus, both hallmarks of fertilization (Roszak and Köhler, 2011; Xu et al., 2016). However, we observed that frie/+ and msi1/+ enlarged autonomous seeds displayed an underdeveloped and discontinuous ii1′ made of unexpanded cells and large empty spaces (Fig. 4E,F compared with Fig. 4D; Fig. 4M). Furthermore, we found a higher number of ii1′ cells in frie/+ and msi1/+ enlarged autonomous seeds compared with wild-type seeds and ovules (Fig. 4S). By contrast, ii1′ development was unaffected in frie/+ and msi1/+ developing ovules and seeds (Fig. S6). We speculate that the differentiation of the ovule ii1′ is not solely repressed by FIE and MSI1 and might require the action of other FIS PcG proteins or a different molecular mechanism. Alternatively, it might lack any repressive mechanism and respond to the positive stimulus of double fertilization. Altogether, these results demonstrate that epidermal and non-epidermal integument cell layers are regulated by different fertilization signaling pathways.

To test if TT16 plays a role in the process, we looked at tt16;frie/+ and tt16;msi1/+ enlarged autonomous seeds. We focused our attention on the fraction of seeds that did not display a strong tt16 proximal-distal polarity defect in the ii1′ in order to better compare the results with single PcG mutants. The ii1′ of both double mutants resembled that of tt16 fertilized seeds but with less expanded cells (Fig. 4H,I compared with Fig. 4G). Since tt16 ii1′ cells are oriented perpendicularly to wild-type cells, we analyzed transverse sections of tt16;frie/+ and tt16;msi1/+ enlarged autonomous seeds. We observed that the ii1′ did not expand correctly and showed large empty spaces when compared with tt16 fertilized seeds (Fig. 4K,L compared with Fig. 4J), a phenotype similar to that observed in frie/+ and msi1/+ longitudinal seed sections (Fig. 4E,F). These results suggest that TT16 does not affect ii1′ responsiveness to the fertilization signals and to the growth of the neighboring integument cell layers.

frie/+ and msi1/+ enlarged autonomous seeds showed cells of the ii1′ physically disconnected from one another and from the ii2 (Fig. 4N). We tested whether this is also the case in wild-type seeds
by gently squeezing them in between slide and coverslip. In all our attempts \((n=20)\), ii1’ was easily detached from ii2, whereas it always remained anchored to the endothelium (Fig. 4O). The only other tissues that were occasionally separated during these experiments were ii2 and oi1, which are separated by cutin-like material (Creff et al., 2015) that allows tissue sliding (Tsuwamoto et al., 2008). These results indicate that the seed ii1’ is loosely attached to the ii2 and suggest that it might develop unique cell wall properties.
DISCUSSION

All Arabidopsis seed coat cell layers were thought to respond homogeneously to the fertilization of the central cell and to grow in a coordinated fashion with the endosperm. Our genetic analyses reveal the unique developmental program of the sub-epidermal integument cell layer. The underdeveloped ii1’ of kpi endosperm-only seeds and fie/+ and msi1/+ enlarged autonomous seeds contrasts with the growth of the other seed coat tissues and suggests a role for the embryo in early seed coat development (Fig. 4P). Furthermore, these data indicate a lack of developmental cross-talk between epidermal and sub-epidermal integument cell layers. In one scenario, the embryo might be necessary to establish developmental coordination between ii1’ and the other integument cell layers. Alternatively, the ii1’ might have evolved to grow independently of the other seed coat cell layers and arrest its development when compressed between the neighboring cell layers, solely under the constraints of mechanical forces. In line with the latter hypothesis, the seed coat of the tt16 and tt16;stk mutants grows regardless of the displaced or absent ii1’. Furthermore, the higher number of ii1’ cells observed in fie/+ and msi1/+ enlarged autonomous seeds might be interpreted as a compensation for the lack of ii1’ cell expansion driven by the absence of mechanical constraints. Compared with the epidermal integument cell layers, the ii1’ originates by periclinal cell divisions. This process might underlie the unique properties of this tissue by leading to unequal partitioning of signaling components or a change in the epigenetic state.

The morphology of the ii1’, which is highly vacuolated and free to expand on the abaxial side, resembles that of the leaf parenchyma and suggests a role in cushioning seed coat development (Fig. 4P). We speculate that the ii1’ fine-tunes seed growth by offsetting perturbations in its developmental program. For example, the ii1’ might fill the empty space left by uneven growth of the seed inner and outer integuments or adjust seed coat development to the turgor pressure exerted by the endosperm (Beauzamy et al., 2016). This function might be better achieved through mechanical constraints than tight developmental control as it would provide a level of flexibility that is highly favorable to sessile organisms, which have to adapt to environmental changes.

MATERIALS AND METHODS

Plant materials

Arabidopsis thaliana plants of ecotype Columbia (Col) or Wassilewskija (Ws) were used as wild-type controls as appropriate. kpi-1, tt16-2 and tt16-3 lines are in the Ws accession (Nesi et al., 2002; Ron et al., 2010), stk-2, stk-2; tt16-6, fie-12/+ and msi1-1/+ lines are in the Col accession (Roszak and Köhler, 2011; Mizzotti et al., 2012). The tt16-1 mutant was isolated in the Ws accession and then backcrossed to the Col accession more than three times (Nesi et al., 2002; Xu et al., 2016). Col and Ws tt16-1 mutants were used as appropriate. Unless noted, tt16 refers to tt16-1. The tt16-1; fie-12/+ and tt16-1;msi1-1/+ lines were generated in the Col accession (Xu et al., 2016).

Days after flowering (DAF) were counted starting from the emergence of the pistil from closed flowers (Xu et al., 2016). Both DAF and embryo development were used to determine seed developmental stages.

Cloning

The 3.4 kb TT16 promoter and genomic sequence was PCR amplified without stop codon using forward (5’-TCAATGGAATTCCAATGAGGAGC-GTTG-3’) and reverse (5’-ATCATCTGGGCCTGTGACTCTGTT-3’) primers that had the attB1 (5’-GGGGAAAAGTTCTACAAAAAAGGA-GGCT-3’) and attB2 (5’-GGGGACCATCTTGTACAAAAAGCTGTCG-3’) GATEWAY recombination sites at the 5’-end, respectively. The PCR product was amplified by high-fidelity Phusion DNA polymerase (Thermo Fisher Scientific), recombined into the pDONR207 vector (BP Gateway reaction) according to the manufacturer’s instructions (Thermo Fisher Scientific), and sequenced. The PCR product cloned into the DONR vector was then recombined into the pmDC107 binary vector (Curtis and Grossniklaus, 2003) (LR Gateway reaction) according to the manufacturer’s instructions (Thermo Fisher Scientific). 1.6ProTT16:gTT16;tt16-1, ProTT1: gTT16;tt16-1, ProTT1:gTT16-GUS and ProSTK:gSTK-GFP lines were described previously (Mizzotti et al., 2014; Xu et al., 2016).

Transgenic plants

The Agrobacterium tumefaciens strain C58C1 was used to stably transform Arabidopsis plants through the floral dip method (Clough and Bent, 1998). Transformants were selected by the appropriate hygromycin resistance and then checked by PCR assays. More than 20 independent transgenic lines were tested.

Modified pseudo-Schiff propidium iodide (mPS-PI) staining

This protocol allows staining of cell walls of fixed plant material (Xu et al., 2016). More than 30 independent seeds or ovules were analyzed for each genotype and time point.

Microscopy

mPS-PI and GFP fluorescent imaging was conducted with a Leica TCS-SP5 spectral confocal laser scanning microscope. Electron microscopy analyses were conducted as previously described (Xu et al., 2016).

Quantitative morphological analyses

The central longitudinal section of seeds at 4 DAF, imaged using mPS-PI, was segmented into individual cells using CellSect software (Pound et al., 2012). The length of the entire endothelium tissue and of individual ii1’ cells along the proximal-distal axis were calculated using ImageJ (Schneider et al., 2012). Area and perimeter of ii1’ cells were calculated with CellSect. Cell roundness was determined for each ii1’ cell as:

\[ R = \frac{(P^2/4\pi A) - 1}{(4/\pi) - 1} \]

where R, P and A represent roundness, perimeter and area of the cell, respectively. Length and distance from the first proximal endothelium cell were used to determine the position of each ii1’ cell along the proximal-distal axis. Since seeds display a degree of variability in length and cell number across individuals and genotypes, we arbitrarily sampled 201 points uniformly distributed along the proximal-distal axis of each seed coat analyzed. Point 0 was fixed at the proximal side of the first proximal endothelium cell, whereas point 201 was set at the distal side of the first endothelium cell reaching the embryo suspensor. For each point, we determined the area and roundness of the ii1’ cell encompassing it. We then calculated the average ii1’ cell roundness and area at each specific point. Owing to variability in ii1’ position along the proximal-distal axis in each genotype, the extreme proximal and distal points are represented by fewer cells than the points at the curving zone.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

O.C., E.F. and W.X. performed the experiments and helped to analyze the data and write the paper. D.D.V. and J.L. performed some of the morphological analyses. C.P., O.C., E.F. and W.X. performed the experiments and helped to analyze the data and write the paper. The authors declare no competing or financial interests.

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Supplementary information

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