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Visualization of microtubule organization and dynamics in living *Arabidopsis* embryonic cells

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Dear editor,

Embryogenesis is a critical developmental stage during the life cycle of flowering plants. During embryogenesis, the first round of asymmetric cell division in the zygote is followed by a series of cellular events, including cell division (symmetric or asymmetric) and directional cell expansion to generate the apical-basal axis, radial and lateral symmetry, and patterns of different cell fates for initiation of different organ primordia, which lay the foundation for post-embryonic development (Jurgens, 2001; Wendrich and Weijers, 2013). *Arabidopsis* embryogenesis is considered a morphogenesis model from which to dissect the underlying cellular basis by combining powerful genetic and genomic approaches (Wendrich and Weijers, 2013). However, direct visualization of cellular and subcellular structures in real time is challenging because the embryo is embedded deep within maternal tissues.

Microtubules (MTs) have been shown to play important roles during embryogenesis (Mayer et al., 1999; Steinborn et al., 2002). The organization of the MT cytoskeleton in fixed *Arabidopsis* embryonic cells at early stages and fixed somatic embryonic cells have been published (Webb and Gunning, 1991; Smertenko et al., 2003), and reorganization of MTs has been reported in fixed somatic embryonic cells (Samaj et al., 2003; Smertenko et al., 2003). However, direct visualization and

quantification of the dynamic behavior associated with MTs in living embryonic cells within their native developmental context are urgently needed, which will provide unique insights into the functions and mechanisms of action of MTs during embryogenesis.

To visualize the organization and dynamics of MTs in *Arabidopsis* embryonic cells, we generated a fluorescent protein fusion construct with the MT binding domain (MBD) of mammalian MAP4 as previously described (Marc et al., 1998). We selected the *KNOLLE* promoter to drive expression of the fusion construct because it is widely expressed in almost all tissues at moderate levels (Supplemental Figure 1A). *KNpro::EGFP-MBD*;Col-0 plants were indistinguishable from wild-type (WT) plants (Supplemental Figure 1B), and had normal silique development (Supplemental Figure 1C). In particular, embryos derived from *KNpro::EGFP-MBD*;Col-0 were normal compared to WT (Figure 1A; Supplemental Figure 2). The earliest intact embryo we were able to isolate with good MT decoration was at the triangular stage. Different MT arrays, including the interphase cortical MT array, the preprophase band (PPB), spindle and phragmoplast can be easily captured (Supplemental Figure 3). More embryonic cells with PPBs, spindles and phragmoplasts were observed at this stage compared to late stages, suggesting that embryonic cells actively divide at early embryonic developmental stages. Most of the embryonic cells from embryos at the heart, torpedo and cotyledon stages were in the interphase stage, and EGFP-MBD clearly decorated cortical MTs in those embryonic cells (Figure 1B). We also occasionally captured embryonic cells with PPBs, spindles or phragmoplasts at these embryonic developmental stages (Figure 1C).

We next set assay to quantify the organization of MT array in embryonic cells by determining the density of MTs as described in Kirik *et al*. (2012), and initially divided the embryo into five parts, including cotyledon primordia, shoot apical meristem (SAM), ground tissue, provasculature and root primordia, to group developmentally related cells together as previously described (Lau et al., 2012). The parts are grouped by color in the longitudinal section of the heart stage embryo (Figure 1D). The density of MTs within embryonic cells at different developmental

stages was initially determined and compared (Figure 1E). The density of MTs within embryonic cells at heart stage was higher than that within the corresponding embryonic cells at the torpedo and cotyledon stages (Figure 1E). Additionally, the density of MTs was relatively higher in SAM, ground tissue and provasculature at each developmental stage (Figure 1E), implying that tubulin dimer-polymer equilibrium shifts toward the formation of polymers in these cells. We also quantified the orientation of visually resolvable interphase cortical MTs within embryonic cells, which will provide clues regarding the regulation of directional cell expansion important for plant embryogenesis. To quantify the orientation of visually resolvable cortical MTs, we determined the angles formed between visually resolvable MTs and the long axis of cells. The long axis of cells was marked with white lines, as shown in Figure 1F. The orientation of cortical MTs within embryonic cells from ground tissues and provasculature were quantified during embryonic development. At the heart stage, more visually resolvable MTs formed angles near 90 degrees with the long axis of cells, suggesting that cortical MTs align transversely (Figure 1G), which is consistent with the need for cells to grow rapidly at this developmental stage. During progression of embryo development, the ratio of transversely aligned cortical MTs is decreased at the torpedo stage compared to the heart stage (Figure 1G), suggesting that the growth rate of embryonic primordial cells might slow down at this stage. At the cotyledon stage, the ratio of transversely aligned cortical MTs further decreased and became randomly aligned (Figure 1G), suggesting that the growth of these embryonic primordial cells might stop when the embryos reach maturation. The data suggest that EGFP-MBD clearly decorates MTs in almost all embryonic cells at different developmental stages, which allows visualization and quantification of MT organization.

We next performed drug treatment to assess the stability and turnover rate of MTs in embryonic primordial cells. Embryos at the cotyledon stage were selected for treatment because it is easy to isolate a certain number of intact embryos at this stage in a short period of time. MTs in embryonic cells are quite stable in a 9% glucose buffer for relatively long periods of time (Figure 1H and Supplemental Figure 4),

which enables subsequent drug treatment. MTs became fragmented after addition of 20 μM oryzalin, and the extent of MT fragmentation increased with longer treatment (Figure 1H and Supplemental Figure 4). By comparison, the MTs in root primordial cells are relatively more sensitive to oryzalin treatment (Figure 1H and Supplemental Figure 4). The data suggest the feasibility of assessing MT stability and turnover rates with MT drug treatment in isolated live *Arabidopsis* embryos.

We also determined whether we could trace the dynamics of visually resolvable MTs and quantify the parameters associated with them in living *Arabidopsis* embryonic cells. Embryos at the heart stage were selected for visualization and quantification because it is relatively easy to isolate intact embryos at this stage and MT images of the whole embryo can be fitted in the same microscopic field to allow comparison of parameters associated with visually resolvable MTs dynamics under the same image acquisition conditions. Dynamic events in visually resolvable cortical MTs, such as growth and shrinkage, can be tracked (Supplemental Figure 5A; see also Supplemental Movies 1 and 2), which allows us to determine the growth rates and shrinking rates associated with visually resolvable MTs in embryonic cells from the hypocotyl, cotyledon and root primordia, respectively (Figure 1I). MTs switching from growth to shrinkage or vice versa can also be captured (Supplemental Figure 5B; see also Supplemental Movies 3 and 4), allowing determination of both catastrophe and rescue frequencies associated with visually resolvable MT dynamics in embryonic cells from the hypocotyl, cotyledon and root primordia, respectively (Figure 1I). This suggests that cortical MTs from different type of embryonic cells have differentially dynamic properties. Cortical MTs in root primordia embryonic cells are more dynamic in that they grow and shrink faster and switch frequently from growth to shrinkage, which is consistent with the result of oryzalin treatment experiment. Given that the expression of *KNpro::EGFP-MBD* can also reveal MT structures within other *Arabidopsis* cells (Supplemental Figure 6A-C), it allows us to determine whether, and to what extent, *EGFP-MBD* may impact MT dynamics in *KNpro::EGFP-MBD*;Col-0 plants via performing careful comparison with *35S::MBD-GFP*;Col-0 and *35S::GFP-Tubulin*;Col-0 used routinely in the plant

cytoskeleton community. Our data showed that MTs in *KNpro::EGFP-MBD*;Col-0 pavement cells exhibit similar dynamic properties to that in *35S::GFP-TUA6*;Col-0 pavement cells (Supplemental Figure 6D), whereas MTs in *35S::MBD-GFP*;Col-0 pavement cells exhibit less dynamic (Supplemental Figure 6D), presumably due to the reason that overproduction of MBD-GFP stabilizes MTs in cells. This result also indicates that the expression of *KNpro::EGFP-MBD* can faithfully reveal MT dynamics in embryonic cells. Thus, the data suggest that visually resolvable cortical MT dynamics can be tracked and quantified in *Arabidopsis* embryonic primordial cells.

In summary, we established an assay to isolate live embryos for cytological analysis, which will complement the routinely used fixation and clearing method. This assay eliminates the potentially negative effect of fixative as well as subsequent treatments on embryonic cells and allows various cytological analyses to be performed on living embryonic cells. We also established an assay to visualize the organization and dynamics of MTs in living *Arabidopsis* embryonic cells by expressing *KNpro::EGFP-MBD*. The stability of MTs can be assessed by the application of exogenous MT drugs, and the dynamics of visually resolvable MTs can be traced and quantified within *Arabidopsis* embryonic cells. Thus, the established assay should be useful in determining the effects of some mutations that impact MTs on embryogenesis, and therefore will enrich our knowledge regarding the mechanisms of action of MTs during embryogenesis.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online*.

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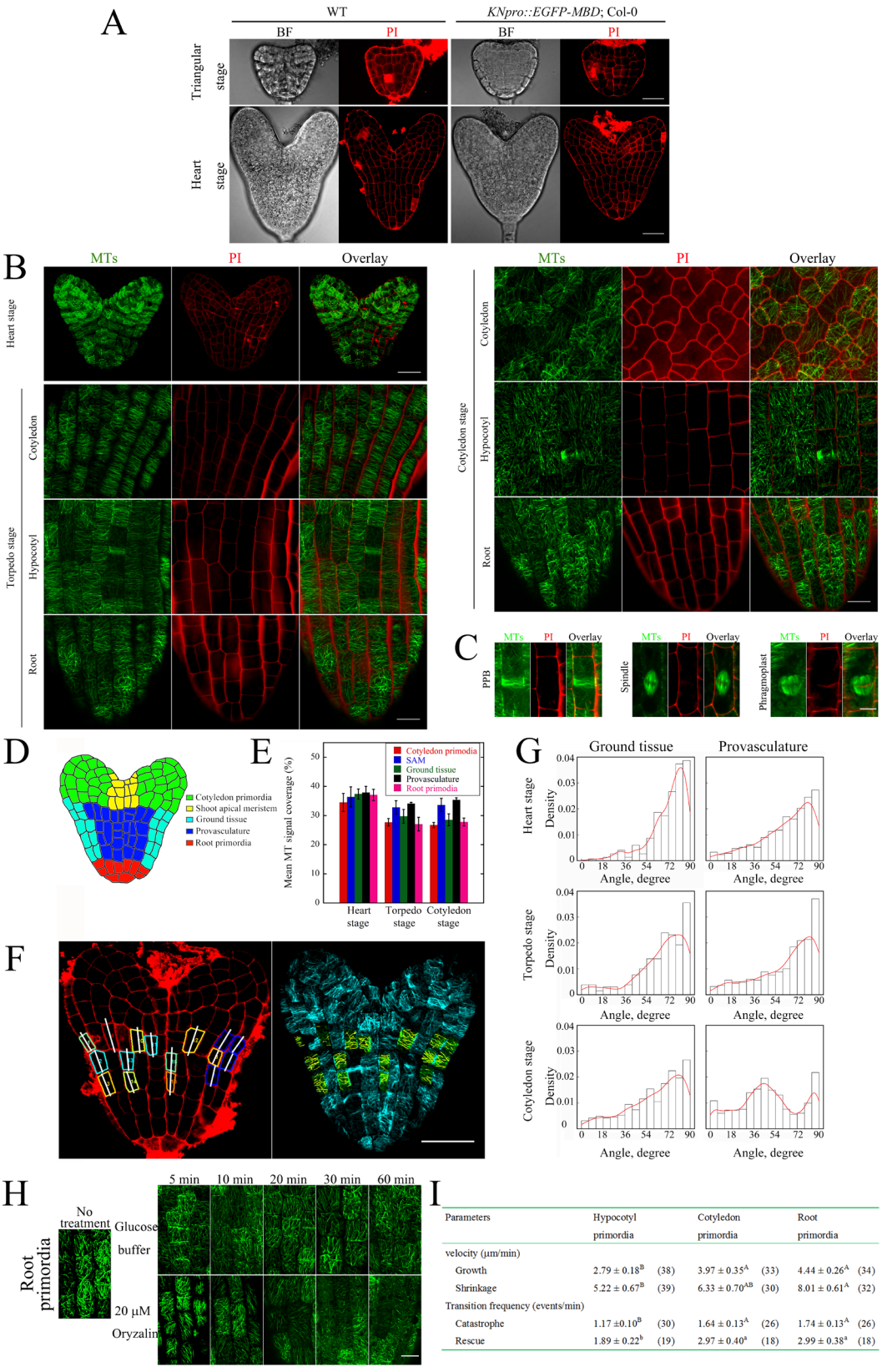

Figure 1. Visualization and quantification of microtubule organization and dynamics in living *Arabidopsis* embryonic cells

(**A**) The expression of *KNpro::EGFP-MBD* does not affect embryo development.

Isolated heart embryos were stained with propidium iodide (PI). Images from the transmission and fluorescence channels of PI staining are presented. Bar = 20 μm. (**B**) MTs in live embryos at the heart, torpedo and cotyledon stages. Cell margins were determined by PI staining. MT images were acquired under the same settings. Bars represent 20 μm, 10 μm and 10 μm for embryos at heart, torpedo and cotyledon stages. (**C**) Representative embryonic cells with PPB, spindle and phragmoplast are shown. Bar = 5 μm. (**D**) The schematic diagram shows the longitudinal section of the embryo at the heart stage. Developmentally related cells were grouped together by color. (**E**) Measurement of the density of cortical MTs in embryonic cells from five distinct portions of the embryo as described in (**D**). (**F**) Schematic diagram describing selection of the long axis of embryonic cells in the ground tissues and provasculature. The long axis of cells is marked with white lines. The right panel presents MT images, and MTs in selected embryonic cells were pseudocolored yellow. Bar = 20 μm.

(**G**) Orientation of cortical MTs was determined by measuring the angles formed between visually resolvable MTs and the growth axis of cells. Histograms of angles from ground tissue and provasculature embryonic cells are presented. Red lines display kernel density estimation of the distribution of angles. The y axis stands for the probability of microtubules formed certain angle with the growth axis of cells for a particular organ primordia. At least 500 MTs from 5 individual embryos were selected for measurement. (**H**) Oryzalin treatment caused the fragmentation and depolymerization of MTs in embryonic root primordial cells. Images before and after treatment with 20 μM oryzalin are presented. Treatment duration is indicated above each image. Bar = 10 μm. (**I**) Quantification of parameters associated with the dynamics of visually resolvable MTs in *Arabidopsis* embryonic cells. More than 40 MTs (20 cells in 3 embryos) for each type of embryonic primordial cell were selected to measure the dynamic parameters of visually resolvable MTs. The data represent the average ± SE (*n* = number of microtubules). Statistical analysis and comparison were taken between every two groups for each parameter. Different small letters indicate differences at $P < 0.05$, while different capital letters represent differences at $P < 0.01$ by one-way ANOVA through SPSS.