Plant Signaling & Behavior 5:5, 614-617; May 2010; © 2010 Landes Bioscience

A ticket for the live show

Microtubules in male gametophyte development

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Microtubule-reporter plants expressing green fluorescent protein-α-TUBULIN fusion protein (GFP-TUA6) in male gametophytic cells of tobacco and Arabidopsis provide new tools for studying the native organization of microtubule (MT) arrays during reproductive development. These plants reveal unique features of gametophytic MT arrays including a basket-like cortical MT array in polarized microspores at interphase, an asymmetric spindle and curved phragmoplast MTs at microspore division and an assembly of bundled cortical MTs during germ cell morphogenesis. The application of these MT-reporter plants has been demonstrated by RNAi-mediated knockdown of the microtubule-associated protein TMBP200, the tobacco orthologue of the conserved MAP215/Dis1 family protein. The double transgenic lines display defects in nuclear positioning, division asymmetry and cytokinesis that are associated with striking defects in spindle and phragmoplast position and organization. This study reveals native and altered MT arrays in unprecedented detail and clarifies the essential functions of MAP215/Dis1 protein function in successive steps in male germline establishment. Such gametophytic MT-reporter lines should accelerate studies of the dynamic regulation of MT arrays by microtubule associated proteins and other effectors during male gametophyte development.

Key words: microtubules, microtubule-associated protein, RNA interference, polarity, mitosis, male gametophyte

Submitted: 11/02/10

Accepted: 12/02/10

Previously published online: www.landesbioscience.com/journals/psb/article/11505

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Addendum to: Oh SA, Pal MD, Park SK, Johnson JA, Twell D. The tobacco MAP215/Dis1-family protein TMBP200 is required for the functional organization of microtubule arrays during male germline establishment. J Exp Bot 2010; 61: 969-81. PMID: 20022922; DOI:10.1093/jxb/erp367.

The male gametophytes (or pollen grains) of flowering plants are highly streamlined structures that have evolved to ensure the union of male and female gametes during sexual reproduction. For successful double fertilization each male gametophyte provides two sperm cells and a means for their long distance delivery via the pollen tube. Male gametophyte development also provides an important and tractable system that allows studies of a variety of cellular processes, such as cell polarity, asymmetric division, cell cycle control and cell fate determination.[1,2] Cellular patterning in pollen development involves only two mitotic divisions (**Fig. 1A and B**). The first mitosis is an intrinsically asymmetric division of the microspore and leads to the formation of a small male germ cell and larger vegetative cell. The second mitosis is symmetric involving division of the single germ cell to generate two sperm cells within the vegetative cell cytoplasm.

Novel MT-Reporter Plants Open a Window for Visualization of Native MT Arrays in Living Male Gametophytic Cells

The elaborate male gametophyte developmental program, involving nuclear migration, asymmetric microspore division, germ cell morphogenesis, symmetric germ cell division and formation of the male germ unit, depends upon the organization and functions of dynamic MT-based arrays. In plant somatic cells four alternating MT arrays are established. These include the conserved spindle MT array and three other plant-specific MT arrays, cortical MTs that govern directional expansion, the preprophase band (PPB) that marks the division site and the phragmoplast array to build the cell plate. Current knowledge of the MT arrays in

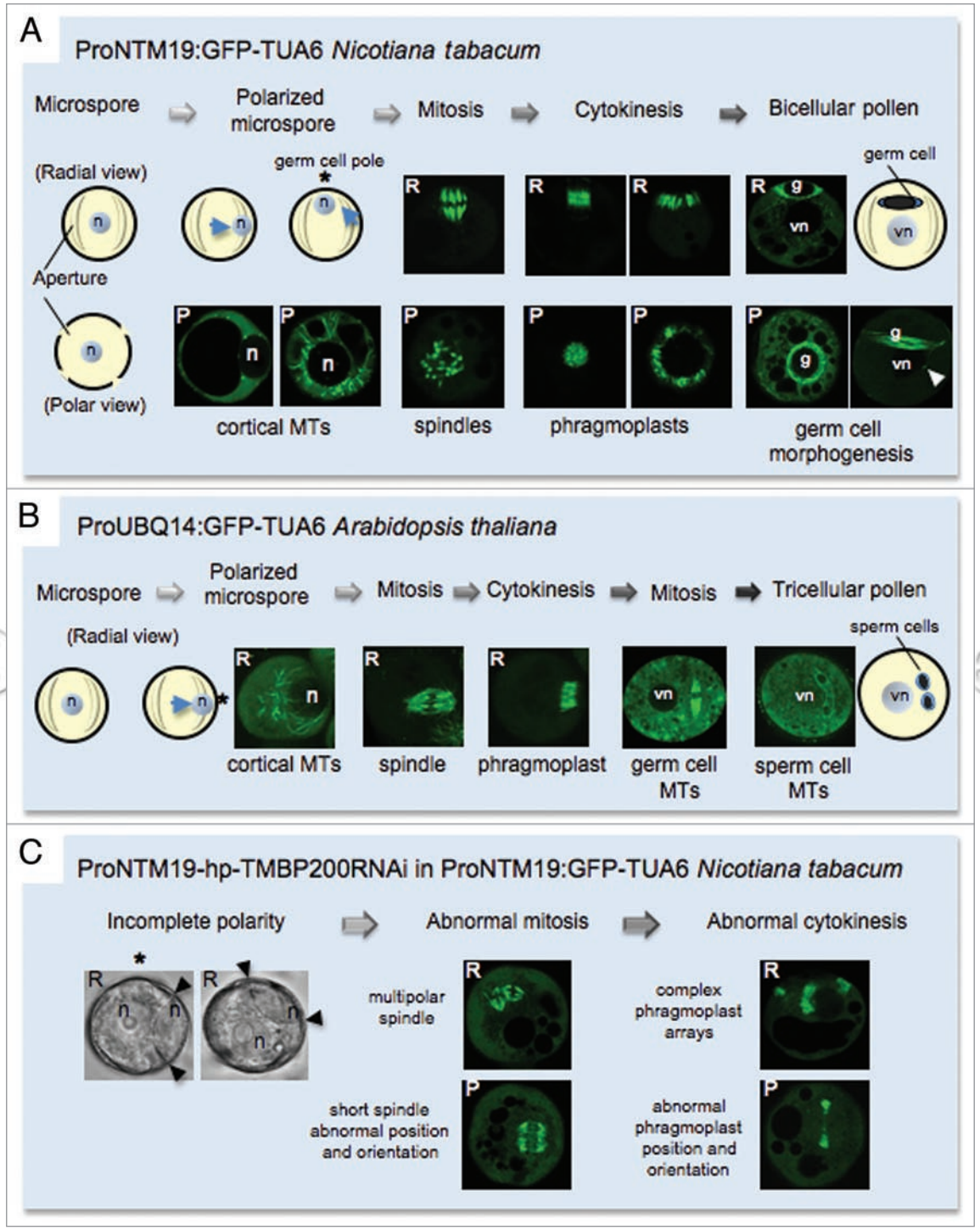

Figure 1. For figure legend, see page 3.

the developing male gametophyte is largely based on observations from immunostaining using tubulin-antibodies with or without microtubule drug pre-treatments.[3,4] These studies have shown that MT arrays in microspores display some unique features, such as the absence of the PPB, the assembly of an asymmetric spindle and a uniquely curved phragmoplast at microspore mitosis. However, compared to extensive investigations of dynamically alternating MT arrays in symmetrically dividing somatic cells and in asymmetrically dividing cells like meristemoid mother cells in the stomatal pathway,[5,6] the lack of tools to visualize MT arrays in living gametophytic cells has been a major drawback for in vivo studies of gametophytic MTs and their regulators.

To address this deficiency we constructed tobacco MT-reporter lines

Figure 1 (See opposite page). Typical stages and MT arrays observed during male gametophyte development in MT-reporter plants. Diagrams are inserted to indicate the orientation of spores where relevant. Radial view (R) shows pollen with the pollen apertures aligned from top to bottom of the image. Polar view (P) represents a transverse plane at the germ cell pole (marked with an asterisk). (A) The tobacco male gametophyte is bicellular when shed and involves a single asymmetric microspore division. Prior to this division the microspore nucleus migrates first to the radial wall and then to the future germ cell pole (asterisk). The polarized microspore undergoes asymmetric division at the germ cell pole involving tight control of the spindle and phragmoplast MT arrays. The nascent germ cell is lens-shaped and later undergoes dramatic elongation. Isolated microspores of ProNTM19:GFP-TUA6 tobacco plants revealed all of the major male gametophytic MT arrays. These include a striking basket-like array or nuclear-cap of MTs associated with the displaced microspore nucleus, an asymmetric spindle and a curved phragmoplast MT array. Bicellular pollen grains show highly bundled cortical MTs in the germ cell and a MT-enriched cytoplasmic tail connecting the germ cell and vegetative nucleus (white arrow head) to form the male germ unit. (B) The Arabidopsis male gametophyte is tricellular before pollen shed as a result of an additional mitotic division of the germ cell to form the two sperm cells. The germ cell pole in Arabidopsis is on the radial wall (asterisk). Similarly, isolated microspores of ProUBQ14:GFP-TUA6 Arabidopsis plants reveal all major gametophytic MTs including mitotic arrays during germ cell division and bundled cortical MTs in the sperm cells. (C) Double transgenic tobacco plants containing ProNTM19-hp-TMBP200RNAi and ProNTM19:GFP-TUA6 constructs produce aberrant microspores. These result from incomplete polarity, abnormal mitosis and cytokinesis that are associated with defects in spindle and phragmoplast MT position and organization. n, nucleus. g, germ cell. vn, vegetative nucleus.

expressing a green fluorescent protein-α-TUBULIN fusion protein (GFP-TUA6) in male gametophytic cells.[7] We confined GFP-TUA6 expression to male gametophytic cells using the tobacco *NTM19* microspore-specific promoter[8] to minimize non-target effects of modified TUA6 expression on MT organization during vegetative growth that have been reported previously.[9] We have also constructed transgenic Arabidopsis plants expressing the GFP-TUA6 reporter, in this case using the Arabidopsis *UBIQUITIN14* promoter to drive expression in both somatic and gametophytic cells (**Fig. 1B**).

In isolated microspores of ProNTM19:GFP-TUA6 tobacco plants we were able to visualize all of the major gametophytic MT arrays (cortical, spindle and phragmoplast). A striking basket-like array or nuclear-cap of MTs was associated with the displaced nucleus at the germ cell pole before division. Microtubules radiating from this cap structure that link the nucleus with the cell periphery may secure the eccentric microspore nucleus in place to ensure asymmetric spindle assembly and/or provide surrogate PPB functions for asymmetric cell plate positioning (**Fig. 1A**).

Similarly, the ProUBQ14:TUA6-GFP reporter enabled observation of male gametophytic MT arrays in Arabidopsis (**Fig. 1B**). Unlike tobacco microspores that undergo a two-step nuclear movement, first to a radial wall and then to the germ cell pole, the Arabidopsis microspore nucleus migrates in one-step to the radial wall prior to asymmetric division (**Fig. 1A and B**).[1] Moreover the symmetric germ cell division occurs before pollen dehiscence in Arabidopsis but during pollen tube growth in tobacco (**Fig. 1B**). These differences may involve taxa-specific MT arrays and/or MT-effectors for fine-tuning. In this regard the availability of MT reporter plants in different species using the same fusion protein will make functional analysis of MAPs or other effectors in planta more precise. Such efforts will enrich our knowledge of the conservation of MT organization in male gametophyte development, allowing the resolution of MTs in taxa-specific developmental contexts.

Further Exploitation and Future Applications

To illustrate how tobacco MT-reporter lines can be utilized to investigate the in vivo role of MT-regulators during microspore development we used a double transgenic approach, depleting the microtubule associated protein TMBP200 by RNA interference. TMBP200 is a tobacco orthologue of the conserved MAP215/Dis1 family protein (MOR1/GEM1 in Arabidopsis).[10-12] Since TMBP200 is expected to be essential for both sexes, based on the reduced transmission of *gem1* alleles in Arabidopsis, we targeted RNAi to microspores with the microspore-specific *NTM19* promoter.[8] In these lines, reduced levels of *TMBP200* leads to altered nuclear and spindle position and defects in the organization of the spindle and phragmoplast. Typical examples include short multipolar spindles, similar to those observed in cells lacking the Drosophila XMAP215/Dis1 orthologue Mini spindles,[13] and complex mispositioned phragmoplasts associated with defective chromosome segregation (**Fig. 1C**). A novel finding was that spindles were sometimes correctly positioned but misoriented leading to symmetric microspore division, indicating that nuclear migration and determination of the spindle axis are successive events, both dependent on MT growth and stability mediated by TMBP200.

Our results demonstrate that depletion of essential proteins like TMBP200 can be restricted to microspore development by RNAi without global changes in vegetative growth, providing a genetic strategy for the in vivo analysis of other proteins with essential functions. The similar application of Arabidopsis microspore-specific promoters[14] to drive cell-specific RNAi together with Arabidopsis GFP-TUA6 MT reporter plants will enable manipulation of essential Arabidopsis proteins like MOR1/GEM1,[12] and TWO-IN-ONE[15] that have microtubule associated functions in sporophytic and gametophytic cell types.

When combined with future practical improvements in in vitro microspore culture and single cell imaging, the applications of MT reporter plants will be multiplied, allowing in depth investigation of the in vivo dynamics of MT arrays unique to the male gametophyte. This effort will help to reveal real time transitions and the properties of dynamic MT arrays, so that gametophyte MT-reporter plants provide a ticket for the 'live show' to come. Challenging aspects include the dynamic monitoring of MT arrays responsible for asymmetric nuclear positioning and division plane determination (without the PPB) during microspore division, and for the organization of bundled cortical microtubule arrays directing germ cell morphogenesis and male germ unit

formation.[16] Finally we can also look forward to comparative studies of the roles of various MAPs in somatic and male gametophytic cells that possess cell type-specific differences in MT arrays.

Acknowledgements

This work was supported by BBSRC grants (91/18532, BB/E001017/1) and KRF grants (KRF-F00008, KRF-C00849).

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