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A framework for evaluating developmental defects at the cellular level: An example from ten maize anther mutants using morphological and molecular data

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ABSTRACT

In seed plants, anthers are critical for sexual reproduction, because they foster both meiosis and subsequent pollen development of male germinal cells. Male-sterile mutants are analyzed to define steps in anther development. Historically the major topics in these studies are meiotic arrest and post-meiotic gametophyte failure, while relatively few studies focus on pre-meiotic defects of anther somatic cells. Utilizing morphometric analysis we demonstrate that pre-meiotic mutants can be impaired in anticlinal or periclinal cell division patterns and that final cell number in the pre-meiotic anther lobe is independent of cell number changes of individual differentiated somatic cell types. Data derived from microarrays and from cell wall NMR analyses allow us to further refine our understanding of the onset of phenotypes. Collectively the data highlight that even minor deviations from the correct spatiotemporal pattern of somatic cell proliferation can result in male sterility in *Zea mays*.

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1. Introduction

Because of its importance to seed production and hence agricultural productivity, pollen dispersal and its control by genetic male sterility are long-standing research interests (Gómez et al., 2015). This is especially true for maize, because outcrossing is required for hybrid seed production utilized in all intensive corn farming worldwide. Because plants lack a germline, a switch from vegetative, somatic growth to meiotically competent reproductive cells is made relatively late in flower development, itself a terminal step in annual plants (Goldberg et al., 1993). To produce egg and sperm cells, specialized reproductive organs must be built to establish and maintain the correct environment for the specification, differentiation, and maturation of meiotically competent cells and the subsequent ontogeny of haploid gametophytes in which the germ cells develop. This system is parallel to similar developmental events elsewhere in plants, including root cell specification (Cruz-Ramírez et al., 2012), as well as male gamete development in animals including *Drosophila melanogaster* and *Caenorhabditis elegans*, making analysis of anther development a good example in which to test new methods of phenotypic analysis.

Beyond its agricultural importance, *Zea mays* is particularly favorable for studying anther cell fate specification, because of the relatively large organ size, accessibility, precise staging through confocal microscopy, and the ease of tissue collection for biochemical studies compared to other model plants such as rice and *Arabidopsis thaliana* (Bedinger and Fowler, 2009). In maize, anther length (Fig. 1) is reliably used as a proxy for pre-meiotic developmental stages (Kelliher and Walbot, 2011). These stages are typically described as a series of discrete events, however each stage can also be portrayed a series of processes as cell types are specified, differentiate, and then mature over developmental time (Fig. 1).

The stamen is the male reproductive organ of seed plants: a thin radial filament attaches the terminal anther to the main floral axis. Anthers are patterned from a group of undifferentiated, pluripotent cells in lobe primordia at the stamen tip. Once the anther has been initiated from the pluripotent meristematic cells, there are initially two types of cells present in each of the four anther lobes surrounding the central vascular column: Layer1-derived cells, the presumptive epidermis, and an interior group of undifferentiated, Layer2-derived cells (Fig. 1B). As an emergent property of lobe cell proliferation an hypoxic environment develops, triggering specification of the most centrally-located L2-derived cells in each lobe through a switch that requires the glutaredoxin MSCA1 (Chaubal et al., 2003; Kelliher and Walbot, 2012). Those central L2-derived cells differentiate as meiotically-competent archesporial cells and distinct specification events from individual L2-derived cells establish a column of archesporial cells in each lobe. Once archesporial cells differentiate, they secrete the

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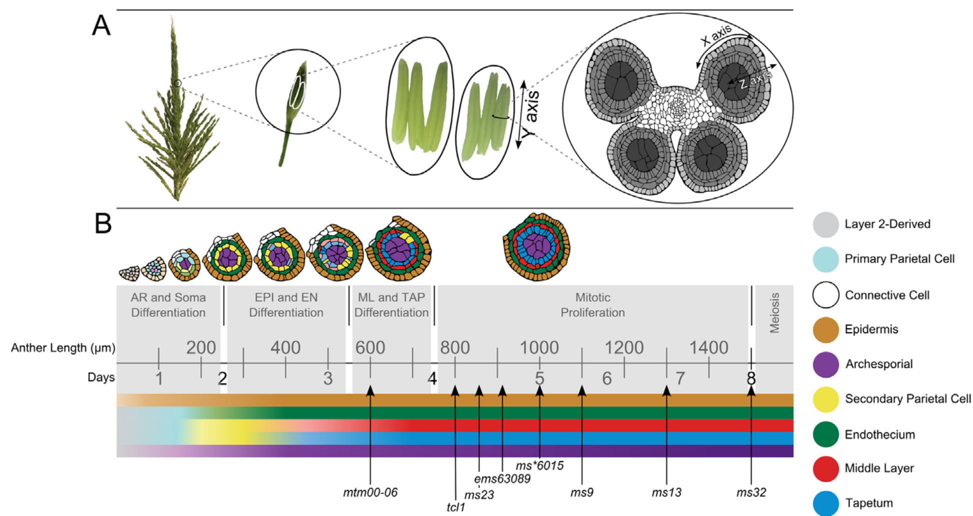

Fig. 1. Anther cell types and developmental timeline. (A) From left to right: a mature maize tassel; single spikelet; a pair of florets each with three anthers after dissection (removal of protective glume leaves) from a single spikelet; transverse section of a single anther near the start of meiosis. During the early developmental stages, the tassel is contained within the whorl of developing leaves, but eventually emerges at the top of the plant. The tassel is covered in spikelets inside each of which are two florets, each containing a group of three anthers. The upper floret is developmentally about 1 day ahead of the lower floret within a single spikelet, and spikelets range in age across the length of the tassel spike (Egger and Walbot, 2015). When viewed in transverse section each anther has 4 lobes connected to a central vascular column and connective tissues (white) with the germinal cells located at the center of each lobe (dark gray) and surrounded by a four-layered somatic niche (medium gray and light gray), with the L2-derived epidermal layer the outermost layer of this niche (light gray). (B) Pre-meiotic anther development takes 8 days in maize starting from the immature lobe stage with about 100 total cells per lobe, and anthers grow to be 1500 µm in length and lobes contain about 12,000 cells per lobe. From top to bottom: single lobes of anthers are illustrated with the cell types present at each stage; differentiation events are described in the gray boxes; colored bars representing the presence of each cell type at the corresponding stages are drawn and colors darken as cell types are first specified and then differentiate and mature with mature cell colors indicated in the color key; estimated timing of defects in 8 of 10 mutants are marked as could best be determined from the existing literature.

small protein, MAC1, signaling to the undifferentiated L2-derived neighboring cells to adopt the somatic cell fate (Fig. 1B), thus establishing a dichotomy between the somatic and germinal niches within the lobe (Wang et al., 2012). These somatically-specified cells, designated as bipotent primary parietal cells, then each undergo a periclinal division, resulting in two new subepidermal cell layers. The outer cell layer will differentiate as endothecium, a starch-storing, chloroplast-containing cell type (Esau, 1965; Murphy et al., 2015), while the inner cell layer, the secondary parietal cells, remains bipotent (Goldberg et al., 1993; Kelliher and Walbot, 2011; Sanders et al., 1999). These definitive periclinal divisions occur contemporaneously with anticlinal divisions within the existing layers to permit anther growth in length and girth as the organ expands from 150 to 250 µm in length during these specification events.

After this initial patterning within the lobe, all cell types divide anticlinally over the next 24 h, proliferating as the anther continues to grow in length along the Y-axis (Fig. 1A). The secondary parietal cells then divide periclinally over the course of 1.5 days as the anther elongates from 400–700 µm, resulting in the final two somatic cell types: the middle layer and the tapetum. The tapetum has been intensively studied as a transcriptionally active, nutritive cell layer that remodels the callose coat around the meiocytes (Wang et al., 2010) and that after meiosis will deposit the pollen coat onto the maturing pollen (Pacini, 1985; Schrauwen, 1996). From 700–1500 µm somatic cells continue to divide anticlinally, while the archesporial cells cease mitosis at the 1000 µm stage, redifferentiate as pollen mother cells and conduct pre-meiotic S-phase and other meiotic preparations, and then initiate meiosis at 1500 µm on approximately day 8 of development starting from the initial archesporial cell specification events (Fig. 1B).

Although meiosis and post-meiotic pollen maturation have been the focus of many anther studies, the molecular mechanisms underpinning pre-meiotic, somatic cellular differentiation events remain largely unexplored. Many pre-meiotic and post-meiotic male-sterile mutants have been characterized (Kelliher et al., 2014; Sanders et al., 1999; Timofejeva et al., 2013), however, only a handful of genes related to somatic cell fate specifications and differentiations have been cloned in maize (reviewed in Walbot and Egger, 2016). Four loci cloned in maize have morphological phenotypes involving somatic patterning defects (*ocl4, ms8, ms23, ms32*), and there are two with earlier defects in establishing the germinal - somatic dichotomy (*mac1, msca1*) (Chaubal et al., 2003; Moon et al., 2013; Timofejeva et al., 2013; Vernoud et al., 2009; Wang et al., 2012, 2013). More genes have been identified and described in rice (Hong et al., 2012; Jung et al., 2005; Nonomura et al., 2003; Zhao et al., 2008), and in Arabidopsis (Hord et al., 2008, 2006; Ito et al., 2004; Jia et al., 2008; Li et al., 2009; Millar and Gubler, 2005; Murmu et al., 2010; Xing and Zachgo, 2008; Zhang et al., 2006; Zhu et al., 2008), but in most of these studies the terminal phenotype of meiotic arrest has been the primary focus of analysis, rather than determining the timing and location of initial phenotypes, many of which likely involve the somatic cells.

This study seeks to accurately describe and compare mutant phenotypes involving pre-meiotic somatic cell fate defects as part of an effort to draw attention to the impact even small changes in somatic development have on successful meiotic progression and consequently on male fertility in maize. Based on initial observations and descriptions of a panel of 24 pre-meiotic male sterile mutants (Timofejeva et al., 2013), we initially anticipated that the majority would have early somatic defects in periclinal division control resulting in greater numbers of total cells within the anther lobes. The use of confocal microscopy as a tool to analyze the additional somatic layers present in many of these mutants was invaluable and by quantifying the phenotypes observed, we devised a new metric for comparing cell numbers. Combining this with molecular data through microarrays and NMR cell wall analyses we can now propose three distinct new phenotypic categories.

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