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SHEDDING LIGHT ON THE ROLE OF OPAQUE1 IN ASYMMETRIC CELL DIVISION IN MAIZE

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Title: SHEDDING LIGHT ON THE ROLE OF OPAQUE1 IN ASYMMETRIC CELL DIVISION IN MAIZE

BY

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ABSTRACT

Asymmetric cell division (ACD) is important for cell fate and tissue patterning. Many aspects of ACD in plants, however, are still unclear. The process of ACD can be broadly broken down into three phases: (1) cell polarization, (2) division plane establishment and maintenance, and (3) cell division and cytokinesis. Stomatal development in maize has proven to be a useful model for understanding the ACD mechanism. Previous studies have identified several proteins important for all three phases of ACD. Actin is important in each of the phases of ACD, suggesting an actin motor such as myosin may also be important for ACD. Opaque1 (O1) was previously identified as a gene encoding a maize myosin XI important for protein body localization in seeds (G. Wang et al., 2012). The role of O1 in ACD in maize stomata was investigated. Asymmetric stomata divisions were abnormal in o1 mutants; however asymmetrically dividing cells in o1 mutants polarize normally. This suggests O1 is required for ACD but may not be important for cell polarization. Multiple defects in microtubule-based cell division structures, which are important for division plane establishment and cytokinesis, were observed in the o1 mutant. O1 localizes to a plant-

specific cell division structure, the phragmoplast, in asymmetrically and symmetrically dividing cells. Together this data suggest that O1 plays multiple roles in asymmetrically dividing cells.

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Chapter1 Introduction

Maize stomata:

Plant stomata are ideal for studying developmental processes such as ACD. Much of the existing knowledge comes from research conducted on *Arabidopsis thaliana (Pillitteri & Dong, 2013; Ramegowda et al., 2017).* This research has provided important information that has helped understand drought tolerance and nutrient uptake to improve crop yields (Aharoni et al., 2004; Cartwright et al., 2009). Maize is an important food crop and a component of several industrial products. Therefore, it is important to gain a better understanding of maize stomata development to improve food security and sustainability.

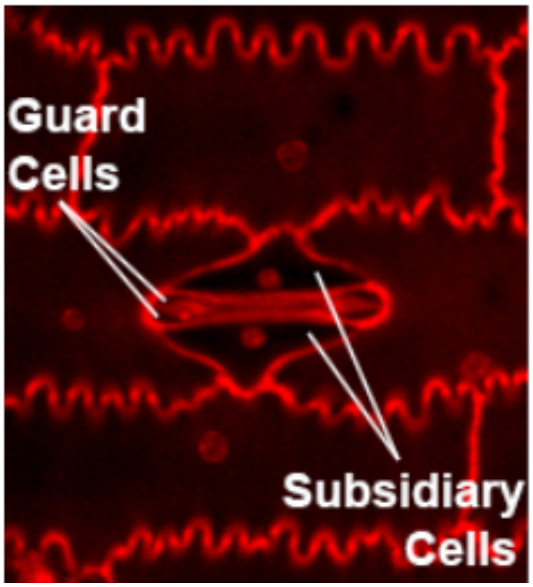

Figure 1. Maize Stomatal composition morphology: Two guard cells flanked by two subsidiary cells. Image adapted from Michelle Facette.

The epidermal layer of maize is covered in stomata that are used to absorb carbon dioxide. The opening and closing of stomata must be regulated to ensure carbon dioxide intake and prevent excess water loss or pathogen infection (Gudesblat, Torres, & Vojnov, 2009). These functions depend on proper structure of stomata, which depends on accurate execution of ACD. Maize stomata are made up of two elongated, dumbbell-shaped cells called guard cells (GCs). GCs are flanked by two lens-shaped cells called subsidiary cells (Figure 1). Together, these cell types regulate stomata opening and closing.

Plant specific cell division structures:

Plant cell division is distinct from animal cell division, largely due to the presence of the cell wall. Several plant-specific, microtubule-rich cellular structures are present during cell division (Figure 2). These structures include the preprophase band (PPB), a microtubule structure that marks the division plane. Another important structure is the phragmoplast, an F-actin and microtubules-based structure. The cell wall of daughter plant cells is formed at a structure called the cell plate. The phragmoplast anchors the cell plate to a region of the plasma membrane called the cortical division zone.

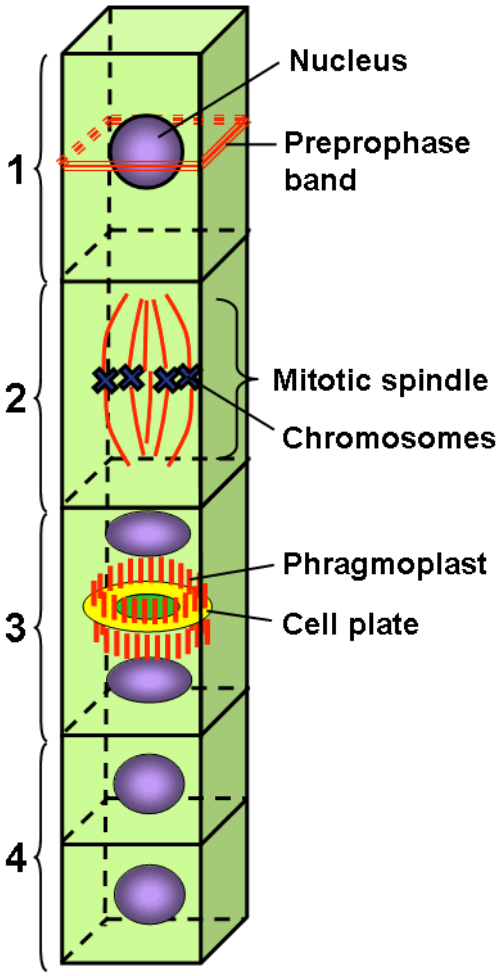

Figure 2. Plant cytoskeletal division structures. Image adapted from (Raven, 2005).

In metazoans, the mitotic spindle dictates contractile ring placement through regulation of the central spindle in complex to ensure proper cleave furrow positioning. This process can also be regulated through other mechanisms, mainly cortical polarity pathways (Cabernard, Prehoda, & Doe, 2010). For instance, in the *Drosophila* central nervous system (CNS), precursor cells called neuroblasts, utilize cortical polarity pathways to position the contractile ring (Connell, Cabernard, Ricketson, Doe, & Prehoda, 2011). Formation of the midbody in mitotic neuroblasts closely resembles the function of the phragmoplast in plant ACD.

That is, the midbody forms between the ganglion mother cell (the progeny of neuroblast ACD), and the midbody just like the phragmoplast separates the parent cell and the new daughter cell (Savoian & Rieder, 2002).

An overview of ACD and cell polarity:

Cell division is important in early development and remains an important process throughout adult stages. In both plants and animals, ACD begins with establishment of a polarity axis in the parent cell (Figure 3) (Lipka, Herrmann, & Mueller, 2015a). In the early stages, establishment of specialized tissue types depends on ACD. In this division, a parent cell divides to produce a daughter cell with the ability to differentiate. This process depends on coordination of cell polarity with mitotic spindle orientation. The cell becomes polarized by segregating cell fate determinants and other components to the different domains before entering mitosis. This is followed by a series of signals that result in orientation of the mitotic spindle in a way that ensures cell fate determinants are segregated properly during cytokinesis. Doing so ensures that the proper determinants are segregated into the self-renewing cell and the cell destined for differentiation. The resulting cells are under regulation of the respective cell fate determinants. Errors in these process result in aberrant tissue growth and may result in developmental disorders (Schweisguth, 2015).

ACD is important because it determines cell fate and tissue patterning. Many aspects of ACD in plants, however, remain to be elucidated. Previous studies have identified several actors in subsidiary mother cell (SMC) polarization including BRK proteins (regulators of actin nucleation), PAN proteins (receptor-like molecules), and ROP (a GTPase) (Cartwright et al., 2009; Facette et al., 2015; Zhang et al., 2012).

Mutations have also been identified in division plane establishment and maintenance (*dcd* and *tan*) (Kennard & Cleary, 1997; Martinez et al., 2017; Wright et al., 2009). Nuclear migration of SMC's undergoing ACD are highly dependent on the actin patch, however, its precise function remains unknown (Kennard & Cleary, 1997).

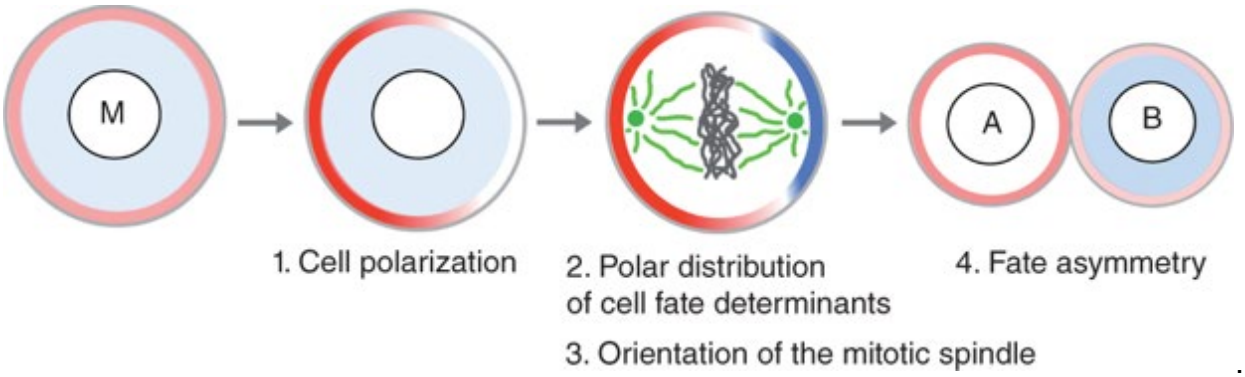

Figure 3. Conserved steps in ACD: Image adapted from (Schweisguth, 2015).

Stomata form by ACD:

Spatiotemporal gradients regulate stomatal development. That is, earlier developmental stages occur at the base of the plant and cells in the later stages are found near the leaf tip. Stomata precursor cells, divide asymmetrically to give rise to another precursor cell called a guard mother cell (GMCs). These cells go on to produce a pair of guard cells (GCs). Surrounding SMCs divide asymmetrically to produce a subsidiary cell (SC) and a pavement cell (PC). Two dumbbell-shaped GCs, two SCs, and the central pore comprise the stomatal complex (Hepworth, Caine, Harrison, Sloan, & Gray, 2018).

These ACD divisions consist of conserved steps. Initially, GMCs send a hypothetical cue to SMCs to initiate polarization. *Brk1* genes are required for correct polarized divisions of stomatal SMCs (Frank et al 2002). During ACD, the inactive PAN2 polarizes in the SMC. PAN1 is recruited and polarized followed by recruitment of ROP. ROP activates SCAR/WAVE, a five-subunit protein complex that includes the BRK1 and BRK3 proteins. Next, the actin-nucleating complex (Arp 2/3) is activated at the same time actin dense patches are formed. The formation mechanism and function of actin patches throughout ACD, however, remain unknown. During this time the nucleus begins to migrate and the PPB begins to form.

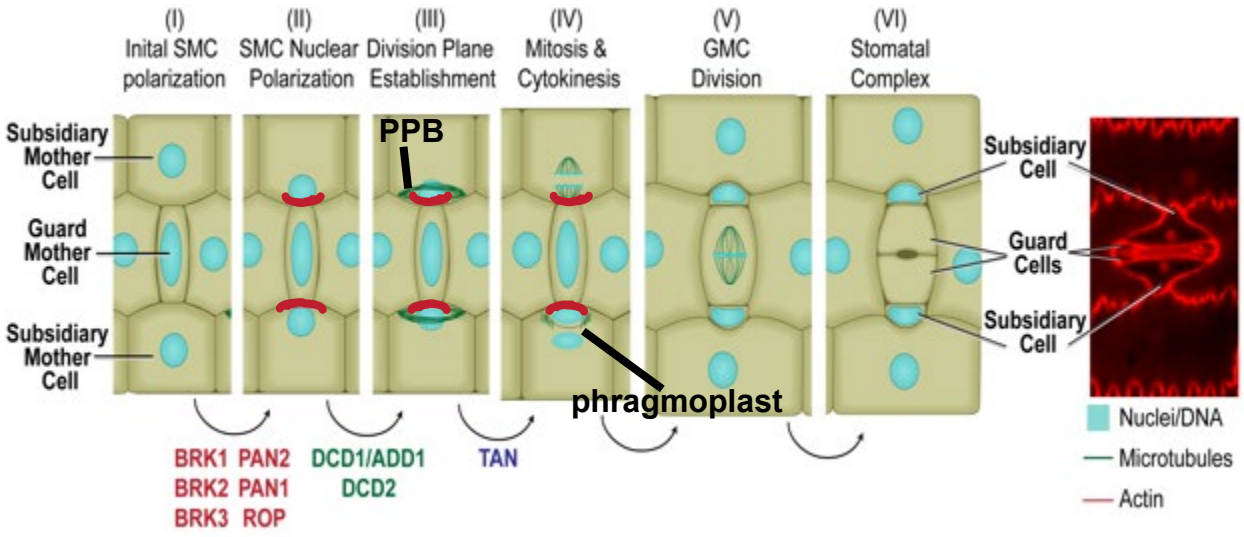

Figure 4. Formation of maize subsidiary cells via ACD. (I) The SMC receives a hypothetical polarizing cue from the GMC, initiating it to polarize. BRK, PAN and ROP proteins polarize and are required for the following steps. (II) The actin patch forms and nuclear migration occurs. (III) DCD proteins for proper preprophase band (PPB) formation, a microtubule structure, which marks the division plane. (IV) Mitosis occurs after the PPB has disassembled. TANGLED (TAN) proteins are required to mark the location of the division plane. (V) Following SMC division producing a subsidiary cell and a pavement cell the GMC undergoes a symmetric division. (VI) The stomatal complex is formed and further cell expansion results in the final stomatal maize morphology.

At the end of G2, the PPB forms and disassembles at the end of prophase (Lipka, Herrmann, & Mueller, 2015b) followed by chromosome segregation. Upon disassembly of the PPB, mitosis begins. At the end of mitosis, the phragmoplast forms (telophase) where the PPB was (Figure 4). Vesicles containing new cell wall material coalesce at the phragmoplast, which starts in the middle of the cell and expands out until it reaches the cell cortex, at the same site where the PPB was previously located. TANGLED (TAN), a unique plant-specific protein marks the previous location of the PPB (Martinez et al., 2017). TAN localizes to the cell cortex after the PPB disassembles and remains there throughout metaphase and anaphase. TAN is required for the phragmoplast to fuse at the former PPB location. TAN interacts with PHRAGMOPLAST INTERACTING KINESIN1 (POK1), which also localizes to the cell cortex at the division site throughout cell division (Lipka et al., 2014). The PPB, spindle, and phragmoplast as well as TAN and POK proteins, are present in both symmetric and asymmetric divisions (Facette, 2019). The new cell wall forms at the phragmoplast and a new stomatal complex is formed.

Myosin-actin network:

Actin, a highly conserved protein among eukaryotic cells has fundamental roles in cells (Shah, Hightower, & Meagher, 1983). Many of those roles include: assistance with cell motility, components in the cell cytoskeleton, cytoplasmic streaming, cell shape, cell division and organelle movement. In maize, there are at least six actin genes (Slajcherova, Fiserova, Fischer, & Schwarzerova, 2012) whose gene products have different roles. There are several possible roles of actin during ACD. Inhibitor studies

using inhibitors of the actin and microtubule cytoskeletons indicate that the actin cytoskeleton is required for nuclear migration of the SMC's nucleus towards the adjacent GMC (Kennard and Cleary, 1997). There is a formation of an actin patch at the same time as nuclei migration (Facette et al., 2015), however the function of the actin patch in unknown. Additionally, it was previously shown in a species with the same stomatal morphology that nuclear polarization was driven by the actin and not the nuclear cytoskeleton (Kennard & Cleary, 1997).

Myosins are motor proteins that walk along actin to perform various cellular motility functions (Reichelt et al., 1999). Plants contain two classes of myosins, myosin XI and myosin VIII (Wu & Bezanilla, 2014). Myosin XIs have been shown to be involved in organelle movement and cytoplasmic streaming (Tominaga et al., 2012). Interestingly, in *Arabidopsis*, myosin XI's have shown to play a role in nuclear migration during regular cell processes, such as movement of organelles and other cargo within the cell (Kodama, Y. et al., 2010). Thus, myosin may be required for ACD in maize and has several potential roles in ACD. Myosins might play a role during the polarization phase, such as in perception of the polarizing cue actin patch formation, or during polarization of proteins or organelles in the cell. Myosins may also play a role during division plan establishment and maintenance during PPB formation, or during formation of the spindle alignment. Finally, myosin may be involved during the final phase, division and cytokinesis, for example during phragmoplast formation.

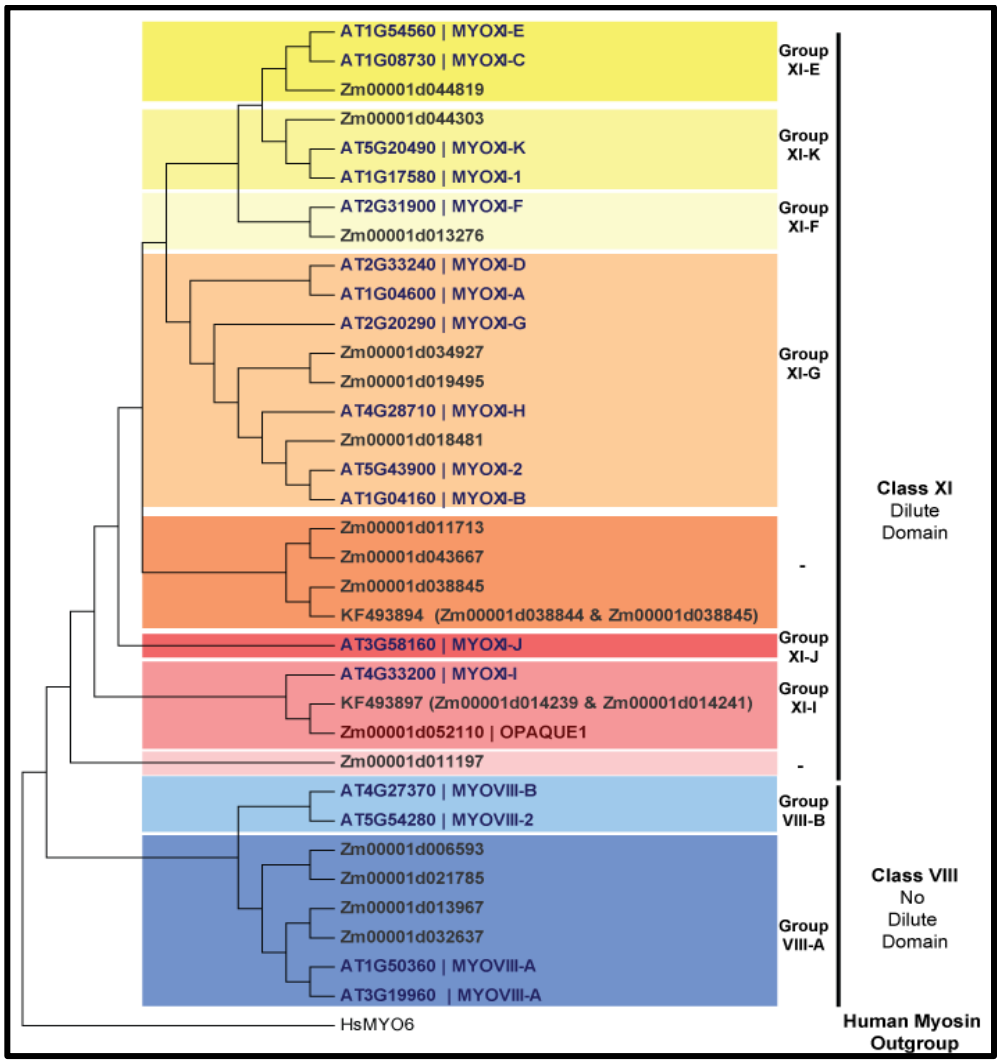

Figure 5. Classifications of myosins. Figure adapted from M.Facette
Phylogenetic tree of maize and *Arabidopsis thaliana* myosins using human myosin as out-group. Truncated myosins were not included. Protein alignments were created in Clustal Omega and a boostrap consensus tree was inferred from 1000 replicates using the UPGMA method in MEGA 7.0. Plant myosins fall into two classes: Myosin VIII's, which contain no dilute domain, and Myosin XI's, which contain a cargo-binding dilute domain.

Opaque1:

Opaque1, a myosin XI mutant has previously been identified as a kernel mutant affecting protein body localization due to decreased ER motility (G. F. Wang et al., 2012). As shown below (Figure 6), *O1* is ubiquitously expressed in maize, including in cells that are dividing. Thus, this myosin mutant is a good candidate to elucidate the role of *opaque1* in ACD.

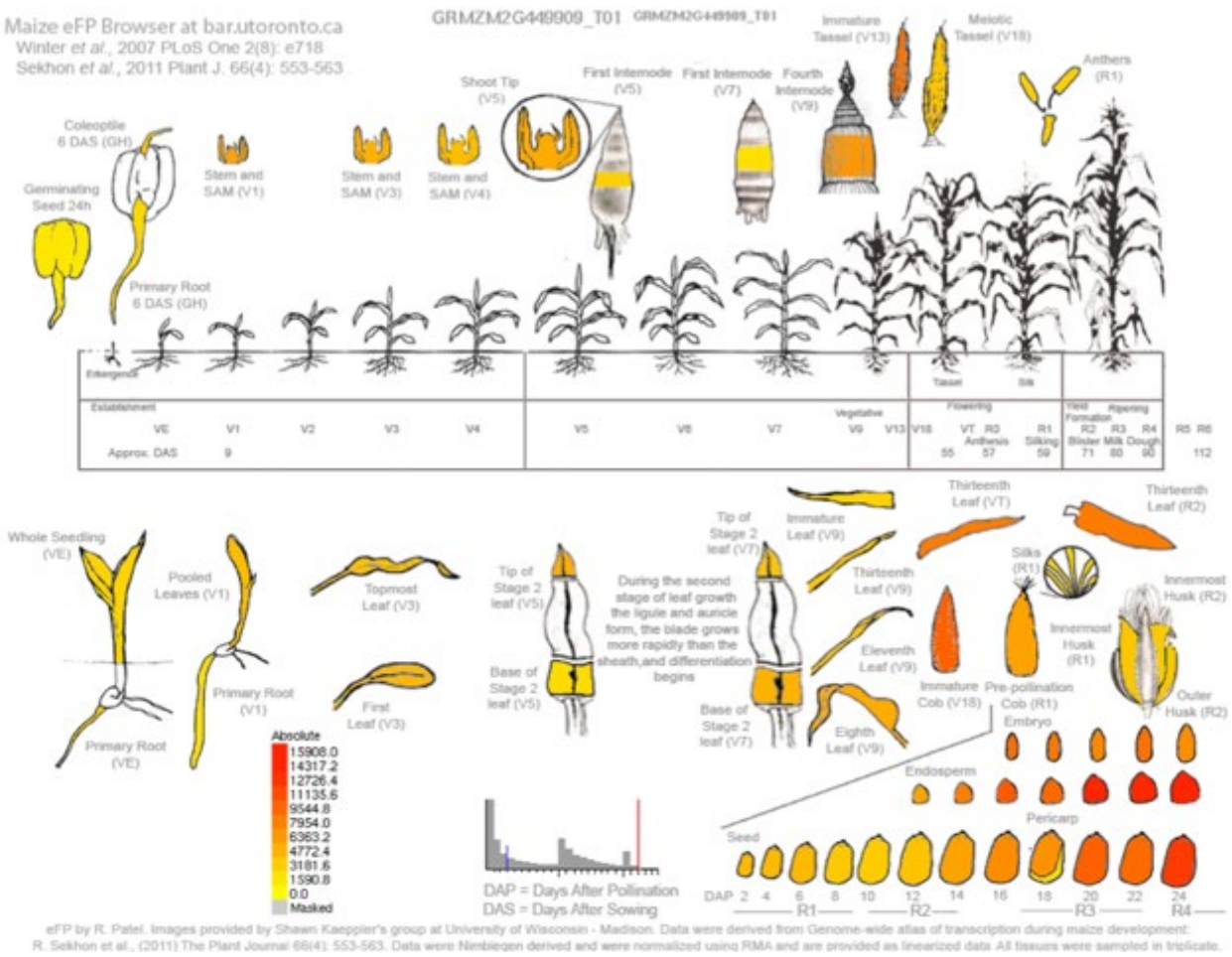

Figure 6. *Opaque1* is widely expressed in maize. Heat map of the expression of *O1* in maize adapted from (Winter et al., 2007) and (Sekhon et al., 2011).

Chapter 2 Results

Subsidiary cells in *opaque1* are abnormal

An important goal of this project was to characterize the previously unknown phenotype of *opaque1*. To determine if *O1* is involved in the asymmetric division of SMC's, expanded leaves of families segregating *o1* and wildtype plants were examined (Figure 7). Cells from fully expanded juvenile leaves (leaf 4) were examined by glue prints. These were used to count the number of abnormal cells found in a section of ~100 cells. Bean-shaped cells were considered normal and abnormal cells were defined as lacking a "bean-shaped" subsidiary cell. This analysis was performed using samples from all three alleles previously mentioned.

To determine if *Opaque1* myosin is involved in stomatal divisions, the percentage of aberrant subsidiary cells in mature, expanded leaves was quantified. Glue impressions of *opaque1* showed an abnormal phenotype compared to the wildtype (Figure 7). The mutant showed "tail" like subsidiary cells. When quantified, the three *opaque1* alleles showed about twenty-five percent of subsidiary cells were aberrant (Figure 8). Previously isolated mutants such as *pan1, pan2, brk1 and dcd2* have a similar percentage of abnormal subsidiary cells (Gallagher & Smith, 1999), (Gallagher & Smith, 2000). Notably, the long tail-like morphology of the abnormal subsidiary cell in *o1* more closely resembled *dcd1* mutants than *pan1, brk1 or tan1 mutants.*

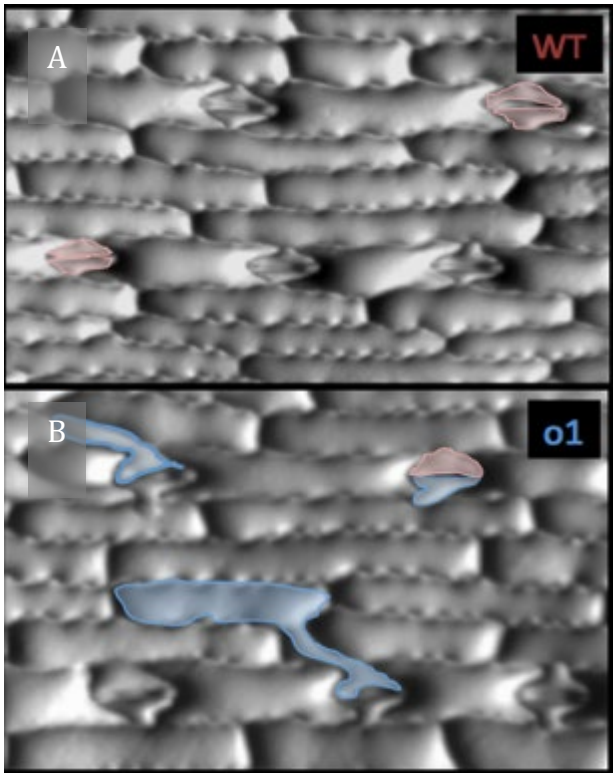

Figure 7. *opaque1* has aberrant subsidiary cells (A) Glue impression of WT plant leaf 4 showing normally shaped subsidiary cells. The outline of a normal subsidiary cell is indicated by red shading. (B) Glue impression of *o1* leaf 4 showing abnormally shaped subsidiary cells. The abnormally shaped cells have long "tails" and more closely resemble *dcd* mutants that *pan* or *brk* mutants. The outline of an aberrant subsidiary cell is indicated by a blue shading.

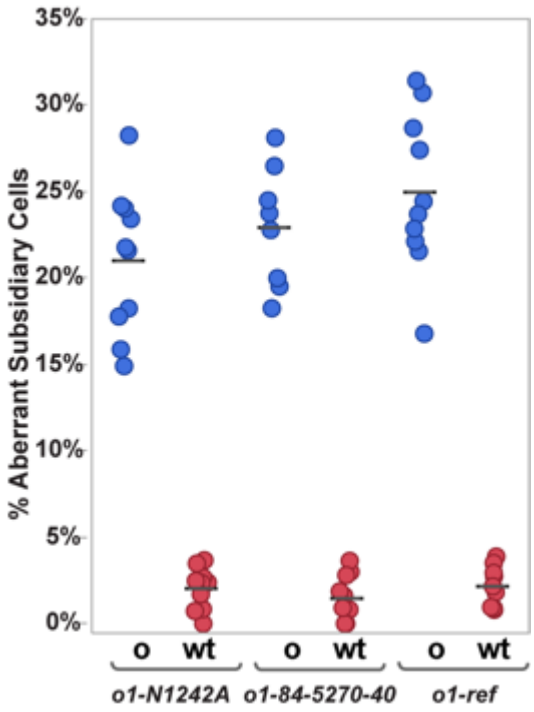

Figure 8. *Opaque1* has aberrant subsidiary cells.
Three different families segregating three different *o1* alleles were analyzed for seed and subsidiary cell defects. *o1* seeds co-segregate with a higher proportion of abnormal subsidiary cells. Grey bars indicate mean % abnormal subsidiaries. $p<0.0001$ in a ANOVA for both alleles.

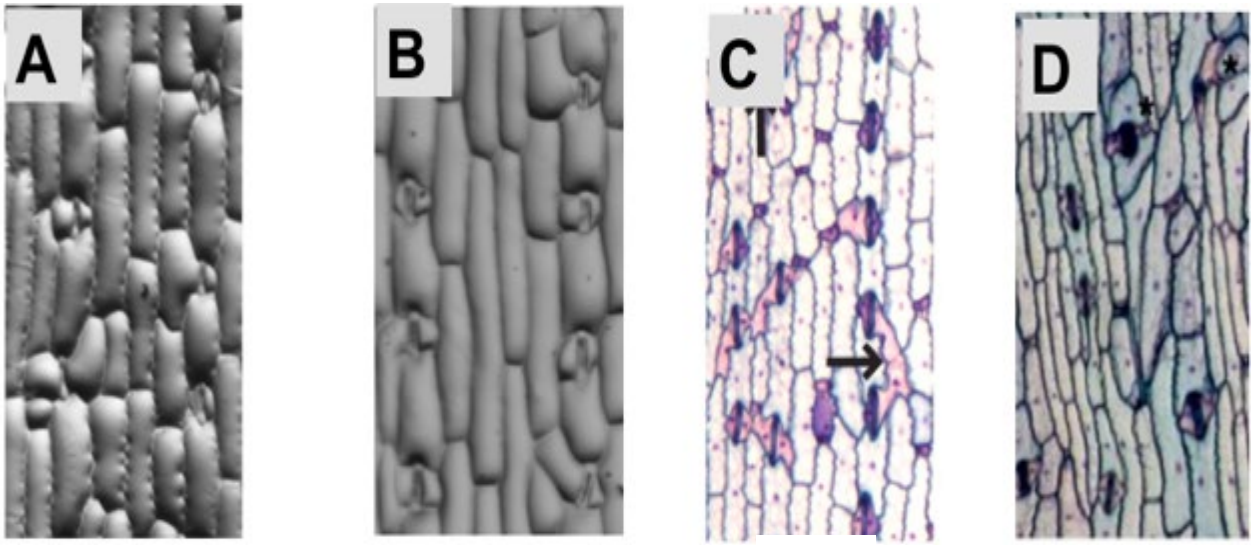

Figure 9. Abnormal cells in *o1* more closely resemble dcd mutants than pan, brk or tan mutants. (A) *pan* mutant displaying an abnormal subsidiary cells image adapted from (Cartwright, Humphries, & Smith, 2009). (B) *brk* mutant displaying an abnormal subsidiary cells image adapted from [12]. (C) *dcd1* mutant displaying subsidiary cells image adapted from (Wright, Gallagher, & Smith, 2009). (D) *tan* mutant displaying abnormal subsidiary cells cell image adapted from (Martinez, Luo, Sylvester, & Rasmussen, 2017).

opaque1 affect asymmetric cell division in multiple cell types:

During maize stomatal development, there are two asymmetric divisions. The second asymmetric division by the SMC forms the SC and a PC. However, the GMC is formed by an earlier asymmetric division that forms a GMC and an interstomatal pavement cell. In *pan1, pan2, brk1, dcd1* and *dcd3* mutants the first asymmetric division is normal, while in *dcd2* mutants this first GMC division is sometimes aberrant, resulting in an aborted GMC. One of the additional phenotypes seen in the mutant were aborted GMC's (Figure 7), suggesting *opaque1* influences more than one type of ACD. The mutant showed about ten percent-aborted GMC's compared to wildtype (Figure 8).

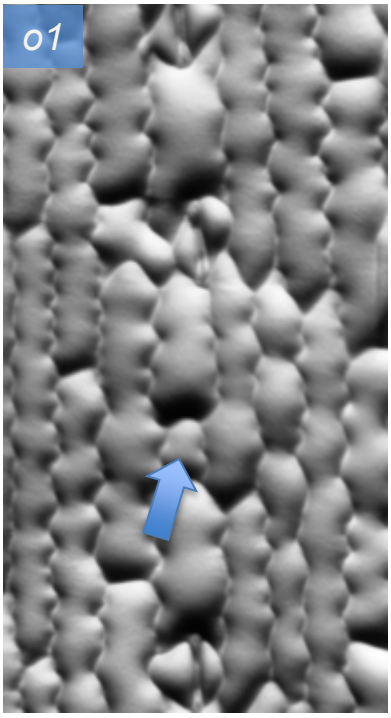

Image 10. *opaque1* shows aborted GMC's. Glue impressionf of leaf four showing aborted GMC, indicated by blue arrow.

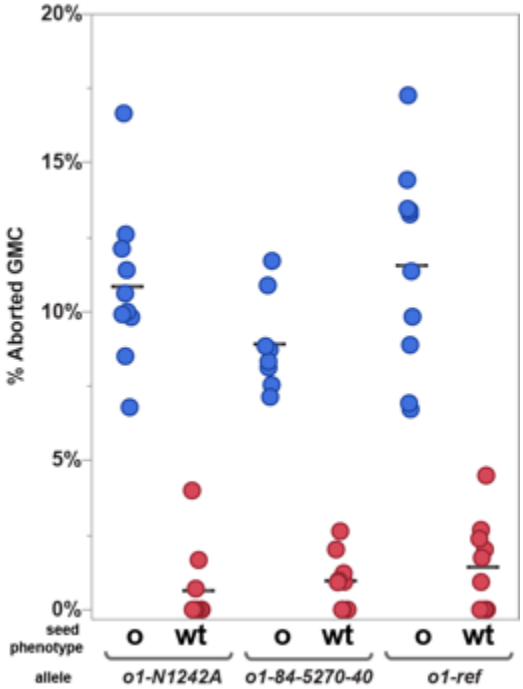

Figure 11. Opaque1 has aborted GMC's Three different families segregating three different *o1* alleles were analyzed for seed and subsidiary cell defects. *o1* seeds co-segregate with a higher proportion of aborted GMC's. Grey bars indicate mean % aborted GMC's. $p<0.0001$ in a ANOVA for the three alleles.

Defects in mature leaves are likely due to cell division defects:

Aberrantly shaped subsidiary cells suggest there is a division plane defect in *o1*. Therefore, cells were examined earlier in development, when the cell divisions that form the stomata occur (Figure 12). Stomata further away from the base, later developmental stages, of the plant were obtained for nuclear polarization analysis using propidium iodide and aniline blue. The proximity of the nuclei to the GMC was then assessed. As development progressed the nuclei migrated towards the GMC in both mutant and wildtype plants. It was concluded that o1 does not play a role prior to nuclear polarization.

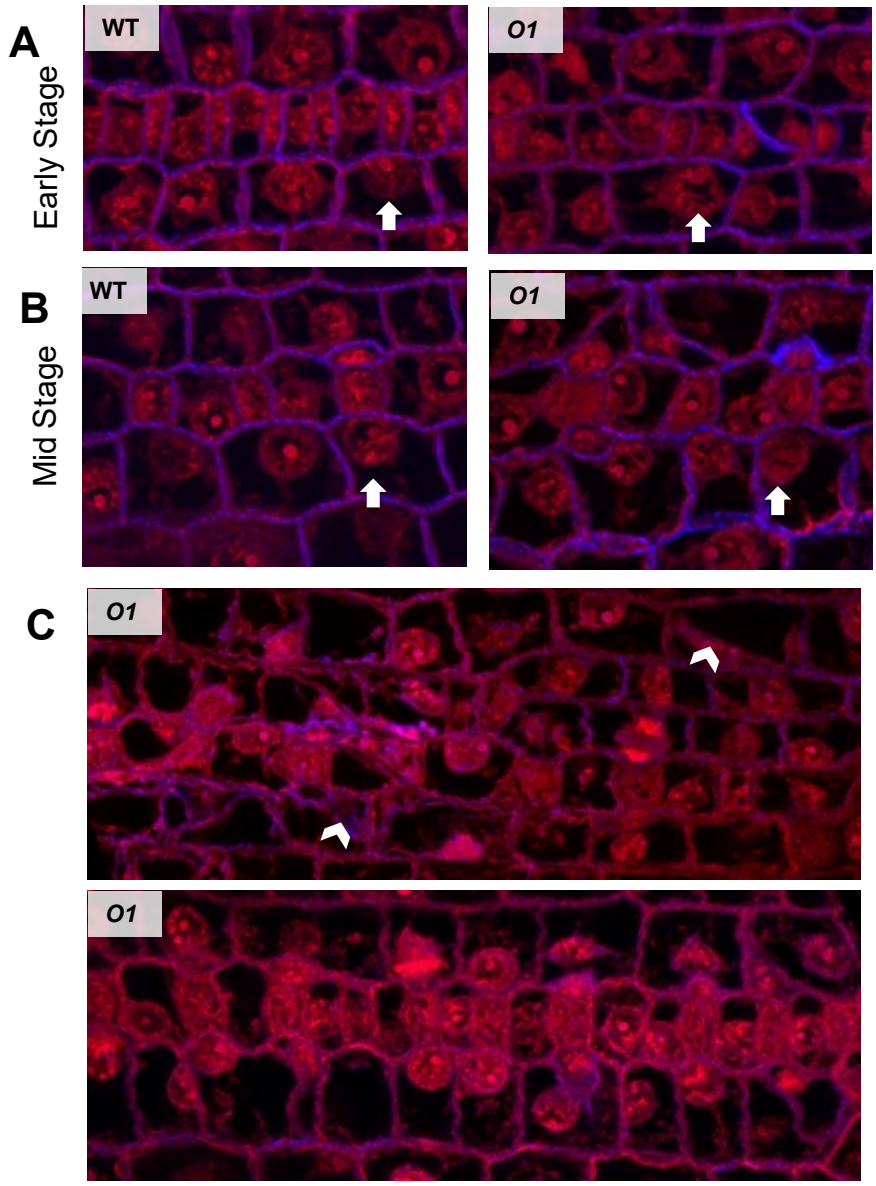

Figure 12. *opaque1* shows aberrant cell divisions during stomatal development. (A) *o1* and WT stomata row development showing the migration of the nucleus towards the GMC at the early stage (shown by the white arrows). (B) *o1* and WT stomata row development at the mid stage. (C) *o1* stomata row show aberrant cell division indicated by the white arrowheads.

To further characterize the role of O1 in ACD, cells were examined to assess cell polarization. To determine if the ACD defect was during polarization or post-polarization, division plane in wild type and *o1* was assessed. Division planes in *o1* were abnormal compared to wildtype. This is likely due to the incorrect orientation of the PPB. As development progresses the width of the GMC increases. The nucleus of the SMC migrates toward the GMC. GMC developmental stage (width) was used a marker to track nuclei polarization. Three different mutant alleles of *o1* were each compared to their wildtype siblings to determine if there was a nuclear polarization defect. Nuclei were quantified as polarized or unpolarized during GMC developmental stages. The data below show that all three mutant alleles' nuclei polarized nearly at the same stage as the wildtype (Figures 13-15). In conclusion, *o1* does not have a cell polarization defect. This suggests that the ACD defect in *o1* is post-polarization

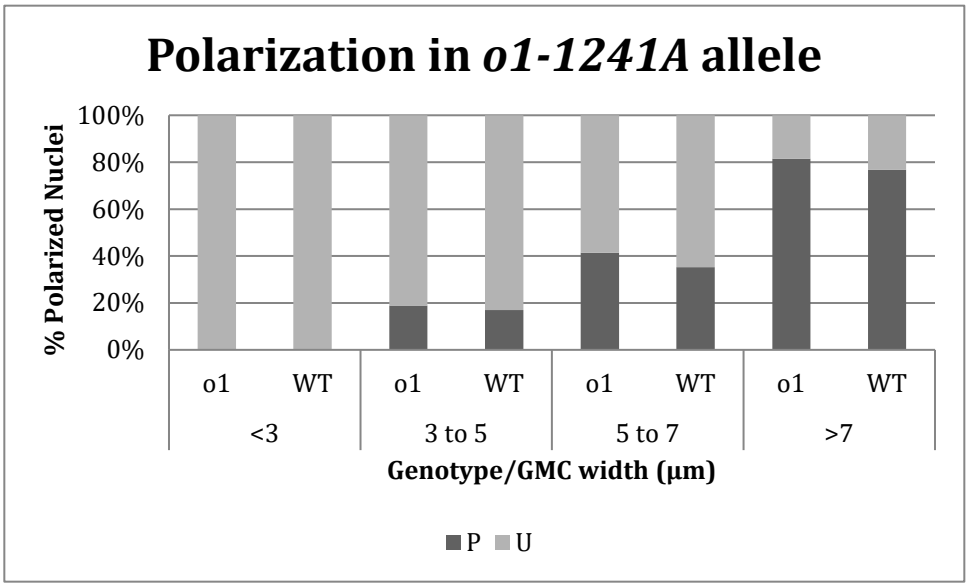

Figure 13. *Opaque1* (allele *o1-1242A)* Polarizes at the same time as wildtype. Measurements of GMC were taken and quantified for allele *o1-1242A*. It was concluded that *o1* and WT polarize at relatively the same stage. P=polarized nucleus. U=unpolarized nucleus.

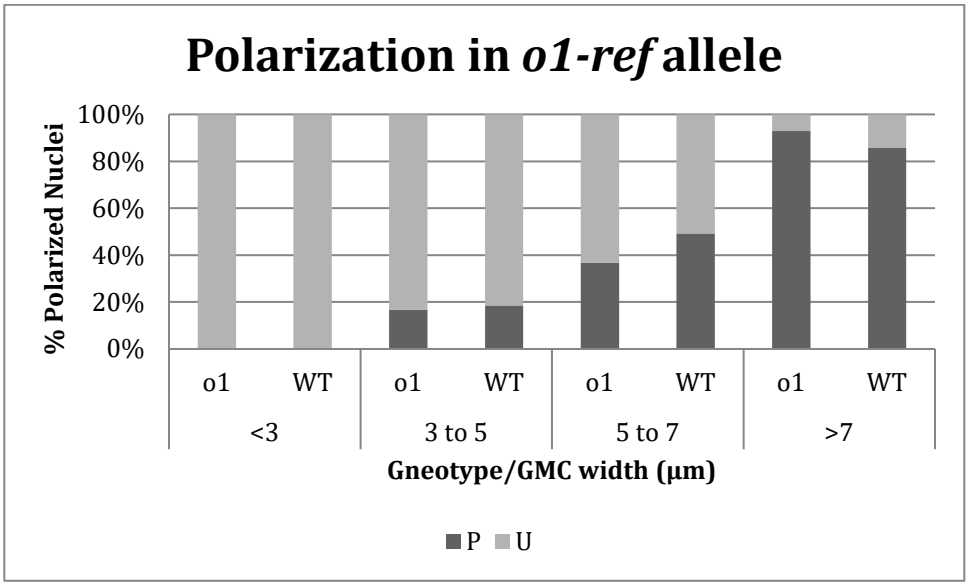

Figure 14. *Opaque1* (*o1-ref*) Polarizes at the same time as wildtype. Measurements of GMC were taken and quantified for allele *o1-ref.* It was concluded that *o1* and WT polarize at relatively the same stage. P=polarized nucleus. U=unpolarized nucleus.

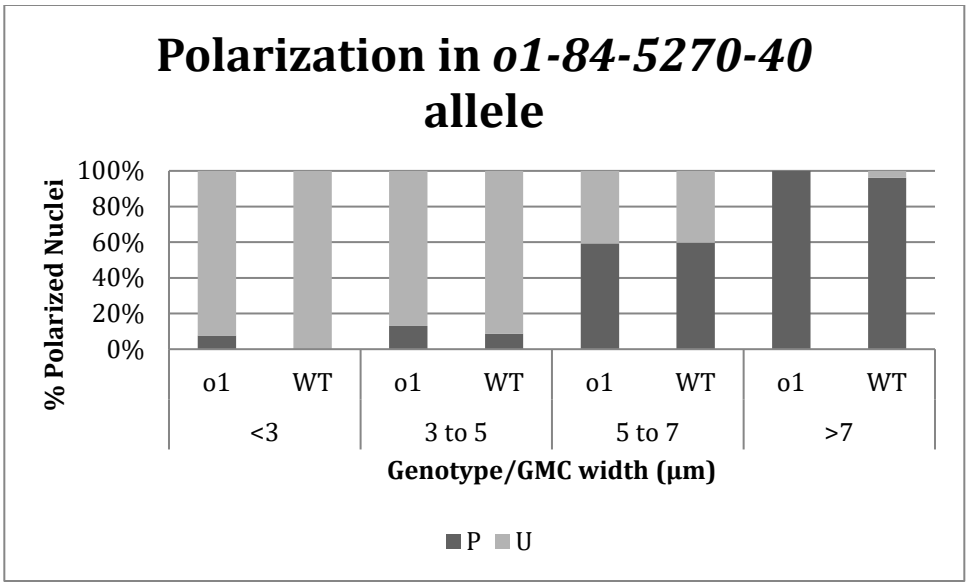

Figure 15. *Opaque1* (allele *o1-84-5270-40)* Polarizes at the same time as wildtype. Measurements of GMC were taken and quantified for allele *o1-84-5270-40*. It was concluded that *o1* and WT polarize at relatively the same stage. P=polarized nucleus. U=unpolarized nucleus.

PPBs are abnormal in *opaque1*:

Because *o1* did not have a nuclear polarization defect, this suggested that the aberrant subsidiary cells were likely to be caused by a defect in division plane establishment, division plane maintenance, or cytokinesis. To determine if *O1* is important for division plane establishment, the microtubule PPB was examined in wildtype and *o1* using immunofluorescence microscopy. The PPB was abnormal in *o1* in both symmetric and ACD. In symmetrically dividing WT cells, PPBs divide the cell in 2 equal parts and are anchored around the circumference of the cell in a single band (Figure 17A). More than 78 PPBs were observed in symmetrically dividing cells from 5 plants, and no abnormal PPBs were observed. In symmetrically dividing *o1* cells, two types of abnormal PPBs were observed. Diverged PPBs occurred when the PPB was anchored in multiple spots, and/or was not represented by a single band but rather

formed multiple bands (unpublished data). Misaligned PPBs occurred when only a single band was present, but it was thick, misaligned or did not surround the nucleus of the cell (Figure 17B). In symmetrically dividing cells that had abnormal PPBs, the PPBs were diverged 92.3% of the time and were misaligned 7.7% of the time. Similarly, in asymmetrically dividing wildtype cells where the nucleus was polarized, the PPB was always observed to be a single band surrounding the nucleus (n=53 from 6 plants; Figure 16A). In asymmetrically dividing *o1* cells that had abnormal PPBs, the PPB was diverged 79.1% of the time (Figure 16D) and misaligned 20.9% of the time (Figure 16C).

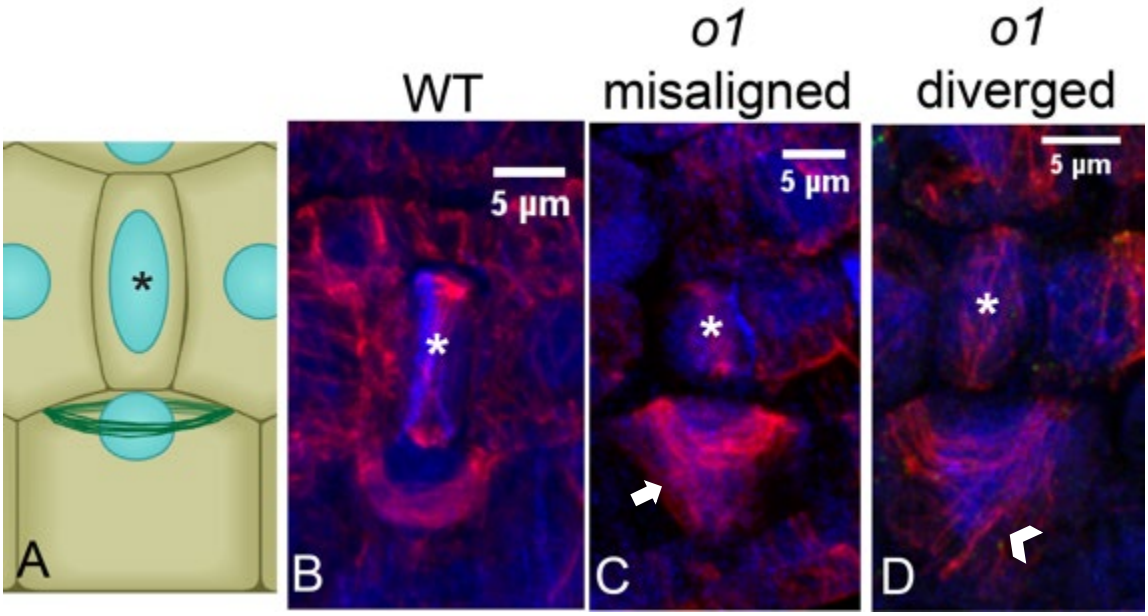

Figure 16. *Opaque1* shows abnormal PPB orientation during ACD (A) schematic cartoon showing the formation of the PPB, asterisk indicates GMC. (B) wild type PPB. (C) Misaligned PPB. The PPB is formed but there is an aberrant diagonal band as indicated by the arrow. (D) Shows a diverged PPB. The PPB is not completely formed resulting in loose microtubule strands diverging from the emerging PPB, as indicated with an arrowhead. DAPI (blue), anti-tubulin (red).

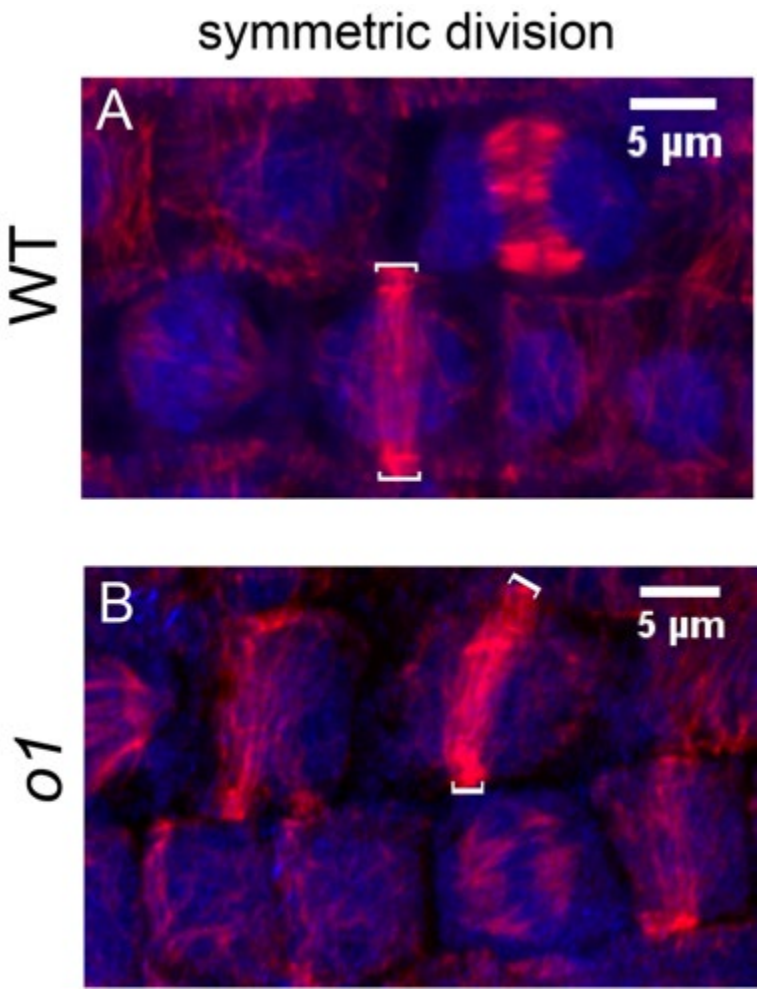

Figure 17. *Opaque1* shows defects in PPBs undergoing symmetric division (A-B) PPB formation in symmetrically dividing cells. (A) Shows the vertical formation of the PPB. (B) Shows the angled formation of the PPB, relative to wild type. White brackets highlight the orientation of the PPB. DAPI (blue), anti-tubulin (red).

Opaque1 is localized to the phragmoplast and the spindle:

opaque1 localized to the spindle in twelve independent cells from at least five independent plants; this was not seen in the mutant (Figure 18B). Plants were grown to early developmental stages (leaf 4). These were then prepared for immunofluorescence and visualized using a confocal microscope. Images were stained for DAPI, alpha tubulin, and anti-o1. Mitotic cells were used to count phragmoplasts and o1 localization

was assessed. Similar data was collected for cells with visible spindles. O1 was found to localize to the mid-section of the phragmoplast and to the spindle periphery and within the spindle (Figure 18B).

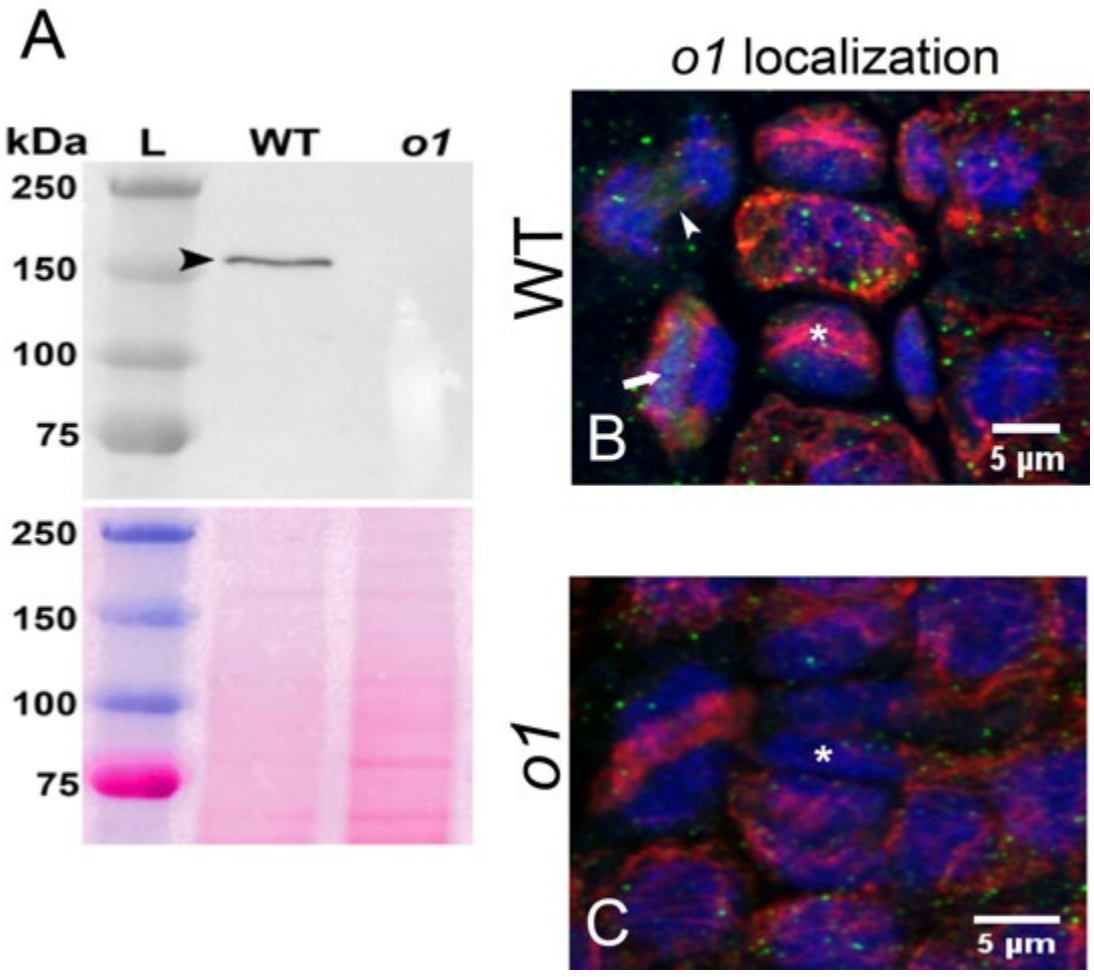

Figure 18, Localization of *Opaque1* in phragmoplast and spindle. (A) generation of anti-O1 body specific for a protein of the correct size (image adapted from Michelle Facette). (B) shows the localization of *O1* to the spindle as indicated by an arrowhead. Localization of *O1* in the phragmoplast is indicated by an arrow. (C) No *O1* present in the phragmoplast. Asterisk indicates the nascent GMC. DAPI (blue), anti-tubulin (red) and, anti-o1 (green).

Phragmoplasts are abnormal in *o1*:

Preliminary data suggest that abnormal phragmoplasts were formed in both symmetrically and asymmetrically dividing cells (Figure 19 B, D). The abnormal formation of the phragmoplasts may be affecting the location of the new cell wall, thus leading to defective subsidiary cells. This phenomenon, however, requires further studies.

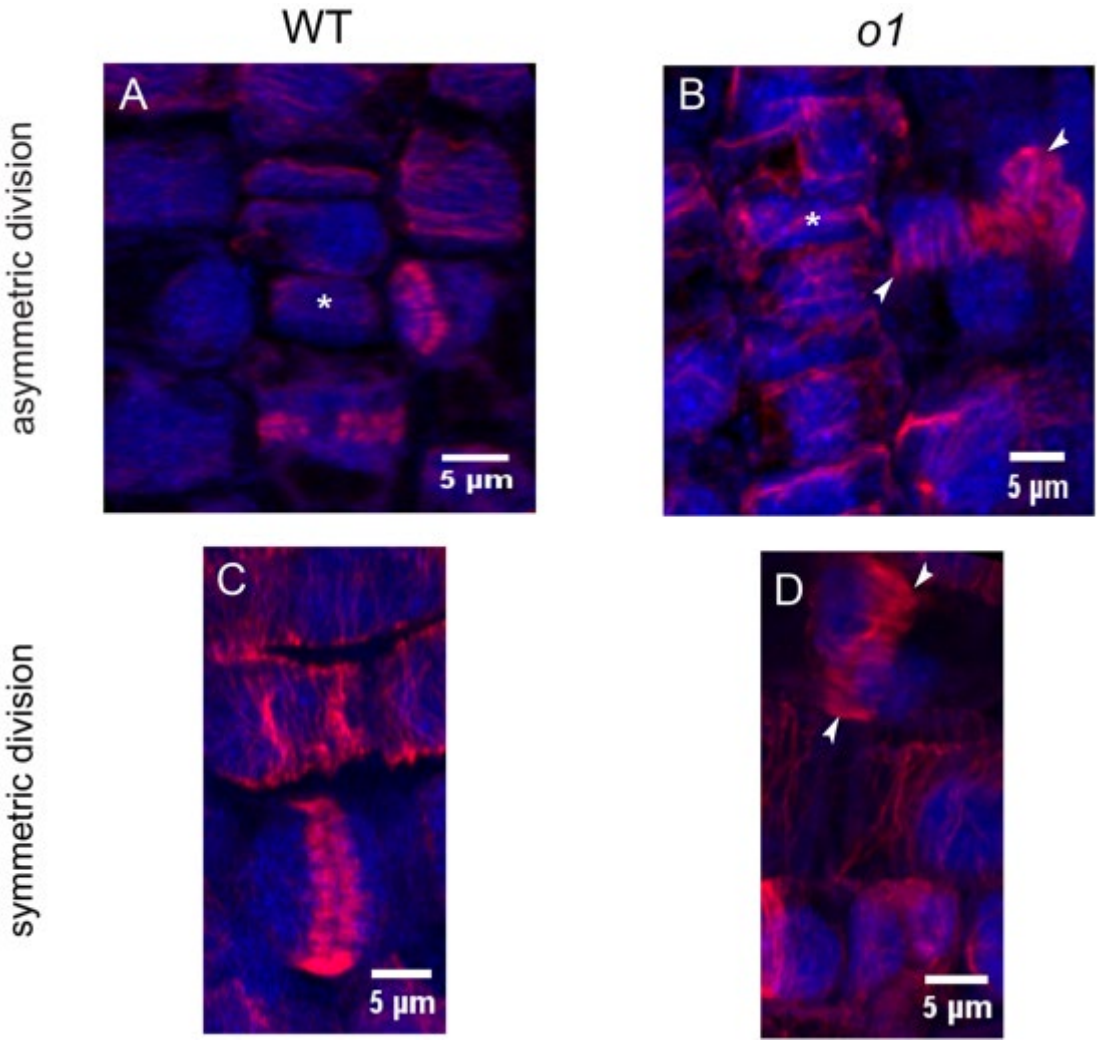

Image 19. *Opaque1* shows abnormal phragmoplast formation (A-B) show ACD of subsidiary cells. GMCs are indicated by an asterisk. (A) Wildtype formation of the phragmoplast. (B) Abnormal formation of a phragmoplast as indicated by the two arrowheads. (C – D) symmetric divisions. (C) formation of a wild-type phragmoplast,

The phagmoplast is aligned with the nucleus. (D) abnormal orientation of a phragmoplast in *opaque1*. The phragmoplast is positioned diagonally across the nucleus, as indicated by the arrowheads. DAPI (blue), anti-tubulin (red).

Chapter 3 Discussion

Development and patterning of plant stomata have been studied extensively (Martinez et al., 2017; Shao & Dong, 2016) in other organisms such as *Arabidopsis thaliana.* Maize is an important crop, providing nourishment and serving as an important component in the production of fuel, oil, alcoholic beverages, and other products. It is also important to global economies as it is one of the most traded agricultural commodities. In recent years, growing concerns of food security and climate change have prompted a search for ways to improve yield and enhance drought tolerance. An important step in doing so is to understand maize stomata development. Doing so will provide a better understanding of the implications of manipulating certain factors that may confer drought tolerance or increased nutrient uptake.

We found that *opaque1* localized to the mid zone of the spindle and phragmoplast, consistent with other studies. Sun and colleagues found that (Sun, Furt, & Vidali, 2018) myosin XI in *Physcomitrella patens* localizes to the mid zone of the spindle and phragmoplast. Furthermore, Yokota et al., showed that myosin XI also localizes to the phragmoplast (Yokota et al., 2009). Localization in the above instances could be actin-mediated. While this has not been studied in maize, Vidali and colleagues (Sun, Furt, & Vidali, 2018) recently identified actin-dependent and independent localization mechanisms. F-actin is sufficient for Myosin XI accumulation at

the cell plate; however, myosin is present in lower quantities when F-actin is depleted. In the absence of F-actin, Myosin XI localization to the chromosomes is likely kinesin7-mediated, as suggested by their co-localization.

The aforementioned studies showed formation of an abnormal cell wall in *o1* compared to wildtype. This suggests that loss of *o1* does not ablate cell wall formation, but merely interrupts the structure. Redundant mechanisms, such as those identified by Vidali and colleagues, could be mediating this phenomenon. In the absence of O1 kinesin-7 may be arbitrating transport of cell wall component-carrying vesicles to the phragmoplast. While vesicles are still being delivered to the location of the nascent cell wall, the incorrect orientation of the phragmoplast leads to improper assembly of the cell wall.

Others have shown the requirement of *dcd* and *tan* proteins in localization of the PPB and the phragmoplast. *O1* exhibited abnormal localization of the phragmoplast caused by a diverged PPB. Thus, it is highly probable that myosin XI has a role in the formation of the new cell wall since the final position of the cell wall is defined by the phragmoplast.

Chapter 4 Future Direction

Myosins are molecular motors that are involved in important cellular processes such as cell polarity, contraction, and transport. The previously discussed experiments defined some of the previously unknown roles of O1 in maize stomata development. Myosin XI is important for the formation of a normal cell wall and thus overall proper stomata

development. Interestingly, loss of Myosin XI does not prevent formation of the cell wall, rather, this results in an atypical cell wall. One possibility is that similar pathways are employed to assist during ACD. That is, other components with similar functions, such as kinesins, may compensate for the lack of Myosin XI. This can be tested by performing immunofluorescence of early stage (leaf 4) leaves and staining for kinesin-7, o1, and DAPI. Co-localization of o1 with kinesin-7 would be indicative of temporal linkage. To determine interaction full length, or truncated, Myosin XI could be purified and incubated with leaf extract. These samples could then be visualized using SDS-PAGE. Additionally, samples could also be analyzed via mass-spec to identify other protein-protein interactions. Doing so would provide further insight about redundant mechanism that may compensate for errors in phragmoplast orientation. This could be tested further by mutating Myosin XI's ATP binding site followed by localization analysis. If Myosin XI localizes to the phragmoplast then Myosin XI localization is not actin dependent. Co-localization with kinesin-7 would point to movement mediated by kinesin-7.

While it is known that Mysoin XI transports vesicles that carry components necessary for cell wall construction, the precise contents are unknown. One way to identify these would be to perform a microtubule assay. Microtubules can be collected and incubated with extract containing vesicles isolated by ultracentrifugation. These could then be analyzed further using mass spectrometry (Momen-Heravi, 2017). Further analysis will provide more information about the precise requirement of Myosin XI in stomata development.

Chapter 5 Conclusion

ACD in plants is a process consisting of nuclear polarization, PPB formation, and phragmoplast demarcation of the nascent cell wall. Actin-vesicle secretion is critical for the latter. Errors in these processes lead to abnormal subsidiary cells and in turn errors downstream processes.

The data obtained in this study showed that *opaque1* has abnormal subsidiary cells. The shapes of the abnormal subsidiary cells closely resemble previously characterized mutants such as *dcd,* suggesting *opaque1 has defects post-polarization*. Nuclear migration, actin formation, and *Opaque1* localization were also analyzed. Overall, the data suggested *opaque1* is localized to the phragmoplast and spindle and may have an important role in the formation of new cell walls after nuclear polarization. Further studies are needed to determine if *opaque1* is associated with other plan-specific organelles. This will provide a better understanding of the role of opaque1 in maize ACD.

Chapter 6 Materials and Methods

Generation of Opaque1-specific antibody:

A custom anti-O1 antibody was generated using a pair of synthetic peptides [(1078-1092): Cys-NSEPKHIYESPTPTK (16aa) (1282-1309): KSTRGQSGKASKSSGVGAHPASNSNWDN-Cys (29aa)]. Both peptides were co-injected into two different rabbits and the resulting sera were affinity purified. Western blots of maize membrane protein preps using the antibody purified from rabbit 11759 gave a single band of appropriate size, which was absent in the *o1* mutant (M. Facette, personal communication).

Immunolocalization: Leaf segments were fixed and immunolocalized according to (Cartwright et al., 2009) with the exception of the antibody dilutions and tyramide amplification. The dilutions used for anti-*o1* (custom made antibody) and anti-tubulin (Sigma Aldrich) antibodies were at 1:1000. Alexa Flour 488-conjugated anti mouse and Alexa Flour 568-conjugated anti rabbit (Invitrogen) were used at a dilution of 1:500. Washes after primary and secondary antibody incubations were left over night at room temperature.

Propidium iodide staining: Leaves four, six and eight were stained with propidium iodide according to (Wright et al., 2009).

Toluidine Blue O staining: Toluidine Blue O staining was done on leaves four, six and eight. Protocol was adapted from (Gallagher & Smith, 1999).

Leaf epidermis glue impressions: To identify phenotypes leaf glue prints that were then viewed under a microscope to assign their phenotype. Glue impression protocol was adapted from Frank, Cartwright, & Smith (2003).

Confocal Imaging: Imaging was performed on a Zeiss 780 using an 63X oil objective. Data were quantified from 3D renditions using Zeiss software and analyzed further using ImageJ.

Appendix 1

Genotyping of seeds Using PCR:

For future experiments, *o1* seeds expressing fluorescent markers are desired. Plants expressing fluorescent protein markers were crossed to homozygous *o1* plants to get families of plants segregating the marker gene and that were heterozygous for *o1.* These heterozygous plants were crossed to *o1* again to obtain a maize line that contained the markers of interest. Genotyping was done on seeds to confirm Actin Binding Domain2-Yellow Fluorescent Protein (ABD2-YFP). In addition, genotyping was done on seeds to confirm Tubulin Yellow Fluorescent Protein (TUB-YFP). Positive seeds will be used to examine actin though live cell microscopy. Seeds that are positive for the fluorescent marker are beneficial for the tagging, tracking and isolation of cells containing the protein of interest.

Method:

A small “chip” of seed was cut with a razor blade and DNA was extracted according to standardized procedures for maize DNA. PCR was done on seeds F603-2x468 to identify ABD2-YFP fluorescent marker. Each reaction consisted of 4µl of DNA, 1µl of primer 512 and 513, 10µl of New England Biolabs Taq master mix, 8.5µl of water and 0.5µl of DMSO. The initial denaturation temperature was 95 degrees for one minute. The denaturing temperature was 95 degrees for 15 seconds. The annealing temperature was 54 degrees for 15 seconds. The extension step was 72 degrees for 1 min except for a final extentension step at 72 degrees for 5 minutes. The number of reaction cycles was 30. Once the PCR was complete the DNA samples were loaded into a 1% agarose gel. Each well was loaded with 10µl of PCR product and 4µl of DNA dye. Samples were separated from other fragments using gel electrophoresis. The buffer used was a 1M TBS. The loaded samples were ran for 45 minutes at 200 volts.

Marker gene	Forward Primer	Reverse Primer	Expected Product Size
ABD2-YFP	gcgacgtaaacggccacaagttcag	cttgtacagctcgtccatgc	656bp
TUB-YFP	gcaaggtttcgatttccgta	ggtttcgggtgatccctatt	1.5kb

Table1. Primers used for genotyping. Table shows the primers and sequences used for genotyping.

Genotyping Results:

PCR identified ABD2-YFP positive seeds. Below is an example gel showing the DNA samples used for the genotyping. Twelve seeds are shown in the gel. Ten of the twelve seeds contained the ABD-YFP gene, as indicted by a band at about 600bp. The expected product weight with the fluorescent marker is approximately 656bp. As shown below all lanes with the exception of 3, 6 and 9 show a band at about 600 bp, confirming the ABD2-YFP fragment of interest. Lanes 3, 6 and 9 were negative.

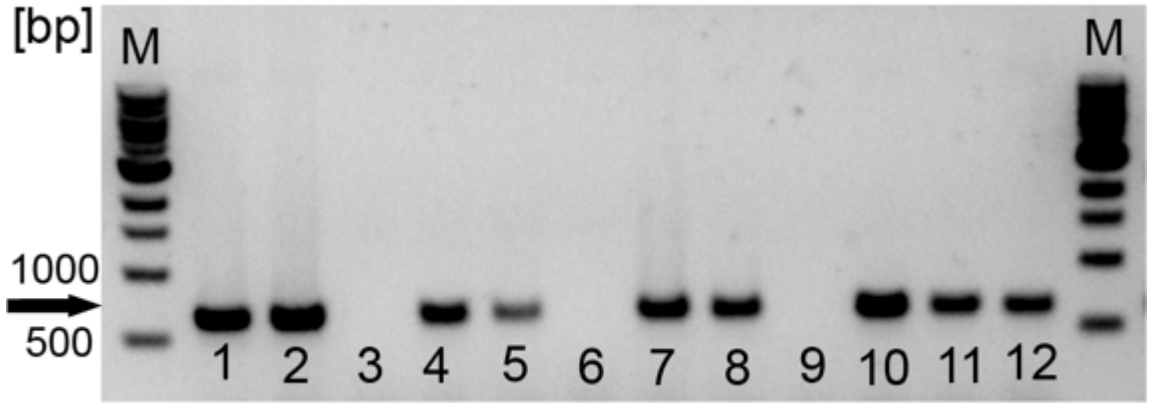

Figure 21. PCR Genotyping of maize seeds that contain ABD2-YFP marker. [Do you need this paragraph? It seems to repeat what is described above.] Gel image of 12 DNA seed samples. Lanes 1 through 12 contain PCR product from F603-2x468. The expected weight of the product with the fluorescent marker is approximately 656pb. The arrow is indicates the estimated weight of the product of interest. The DNA ladder is indicated by the letter “M”. Lanes 3, 6 and 9 are negative for the florescent-marker gene product. The other lanes are positive for the correct fragment.

Appendix 2

Characterization of stomatal function in stomatal ACD mutants

Plants absorb carbon dioxide to synthesize nutrients through the process of photosynthesis. During photosynthesis, carbon dioxide enters through the stomata and water escapes. The uptake of carbon dioxide is also known as assimilation (McAusland et al., 2016). Plants such as maize have stomata that are composed of two inner guard cells flanked by two subsidiary cells. In other commonly studied plants such as *Arabidopsis thaliana*, stomata consist only of two guard cells. The result of only having guard cells and no subsidiary cells affects stomatal performance compared to plants whose stomata are composed of both guard cells and subsidiary cells (Raissig et al., 2017). Previous work suggests that guard and subsidiary cells are crucial for proper stomatal function, although the function and relative contribution of subsidiary cells is poorly understood. Examining stomatal function in asymmetric-division mutants with aberrant subsidiary cells may help determine the relative contribution of subsidiary cells to overall stomatal function. Plants respond to various stimuli such as light that result in stomata closing and opening (McAusland et al., 2016). Carbon dioxide uptake and the release of water vapor can be measured to determine if subsidiary cells are important for proper stomatal conductance (Vialet-Chabrand, Dreyer, & Brendel, 2013). The role of subsidiary cells is in stomatal function is also something important to pursue.

To help answer the central question, four maize genotypes with abnormal subsidiary cells and wildtype were analyzed. The genotypes employed were wildtype, *brk1-1*, *o1 ref.* and *pan2-2.* The percentages of abnormal subsidiary cells were quantified for each genotype at specific stages of development.

Previous work has indicated that juvenile leaves have a stronger stomatal defect than adult leaves in *pan1* and *brk1* mutants, which both have cell polarization defects (M. Facette, personal communication). Leaf four is a juvenile leaf. Leaf six is a transition leaf which shows both juvenile and adult characteristics. Finally, leaf eight is an adult leaf. The % of abnormal cells in *pan2, brk1* and *o1* in a juvenile, transition and adult leaves were quantified. Each of the mutants however has a distinct role in subsidiary cell formation. Both *brk1-1* and *o1* are related to the actin cytoskeleton. On the other hand, *pan2-2* plays a role in cell polarity and is not an actin cytoskeleton mutant. The different genes that encode for each mutant can give us greater insight on the role subsidiary cell formation and its overall role in stomatal performance.

brk1-1 encodes for a small subunit of a five subunit SCAR/WAVE regulatory complex (WRC) that promotes actin nucleation (Facette et al., 2015). I am interested in this actin cytoskeleton-nucleating protein because it plays a role in guard cell function such as in *Arabidopsis* (Isner et al., 2017). As previous studies have shown actin nucleation is of great importance for guard cell function that overall affects photosynthesis performance. I used mutants such as *brk1-1* to ask if actin nucleation is important for subsidiary cell formation and if so does it affect stomatal conductance?

o1 encodes for an actin motor myosin XI. O1, as other myosin's are commonly known for walking among actin (Okten, Churchman, Rock, & Spudich, 2004) thus, we can use this mutant to elucidate its role if any in subsidiary cell formation. Other studies have shown that *o1* has endoplasmic reticulum defects (G. F. Wang et al., 2012) that overall affects the synthesis of ziein protein bodies. This leaves another plausible

question, whether *o1* delivers proteins that are required for subsidiary cell formation and if they are being delivered correctly.

A non-actin cytoskeleton mutant, *pan2-2* is a receptor like kinase that plays a role in cell polarity (Zhang et al., 2012). I am using *pan-2*-2 as a non-actin cytoskeleton mutant to elucidate its non-actin nucleation role in subsidiary cell formation. In other words, if *pan-2-2* has a higher number of abnormal subsidiary cells in leaf four compared to those in leaf six and eight it could be assumed that cell polarity has defects at the younger stages of leaf development. If there are a higher number of abnormal subsidiary cells in the mature leaves, cell polarity is malfunctioning at the beginning stages of development and is gradually being corrected as the leave undergoes further development. Overall, this mutant can reveal if cell polarity is essential for subsidiary cell formation and foremost elucidate its role in stomatal conductance.

The overarching goal is to determine if subsidiary cells are important in stomatal response when a stimulus such as light is introduced. We can examine this by measuring carbon dioxide intake and water vapor effluxes through these stomatal pores, with appropriate machines such as the LI-COR. LI-COR instruments allow measurement of CO_2 uptake/assimilation (A) and stomatal conductance to water vapor (g_{sw}) under controlled light conditions. We will address this question by analyzing the rate of assimilation, g_{sw} conductance in response to light exposure. We can compare the time it takes for sixty-three percent of the stomata to close and open once light has been introduced. Since all genotypes, with the exception of wild type, have defective subsidiary cells we can use comparative assimilation and stomatal conductance to

water to assess the importance of subsidiary cells in overall stomatal conductance across different genotypes.

From the data gathered it is hypothesized that there is a difference in stomatal conductance for each leaf and genotype. There should be a slower stomatal response in the younger leaves where there is an increased number of abnormal subsidiary cells. As leaf number gradually increases the percentage of abnormal subsidiary cells decreases thus, stomatal conductance increases as well. If the hypothesis is supported by these Licor measurements it can be concluded that subsidiary cells have an overall important function in stomatal conductance.

Results:

As the leaf matures there is a decreased percentage of abnormal subsidiary cells (Figure 22B) in all mutants. Interestingly, even in wildtype B73 plants where a small number of cells divide abnormally, fewer abnormal cells were observed in adult leaves. Overall, this project was used to help determine the function of subsidiary cells in stomata but new adjustments will be made to repeat the study and determine the precise role of subsidiary cells in overall stomata conduction.

To determine the role of stomatal function, stomatal conductance was measured using a Licor gas exchange analyzer under high light. Then, the light was removed to induce stomatal closure. Once the total transpiration reached steady state again (as indicated by the gas analyzer) the light was turned on again. The rates in opening and closing will give an indication of efficacy of stomatal function. Unfortunately, the data gathered were inconsistent due to high noise in the reading and no conclusions were made regarding stomatal function.

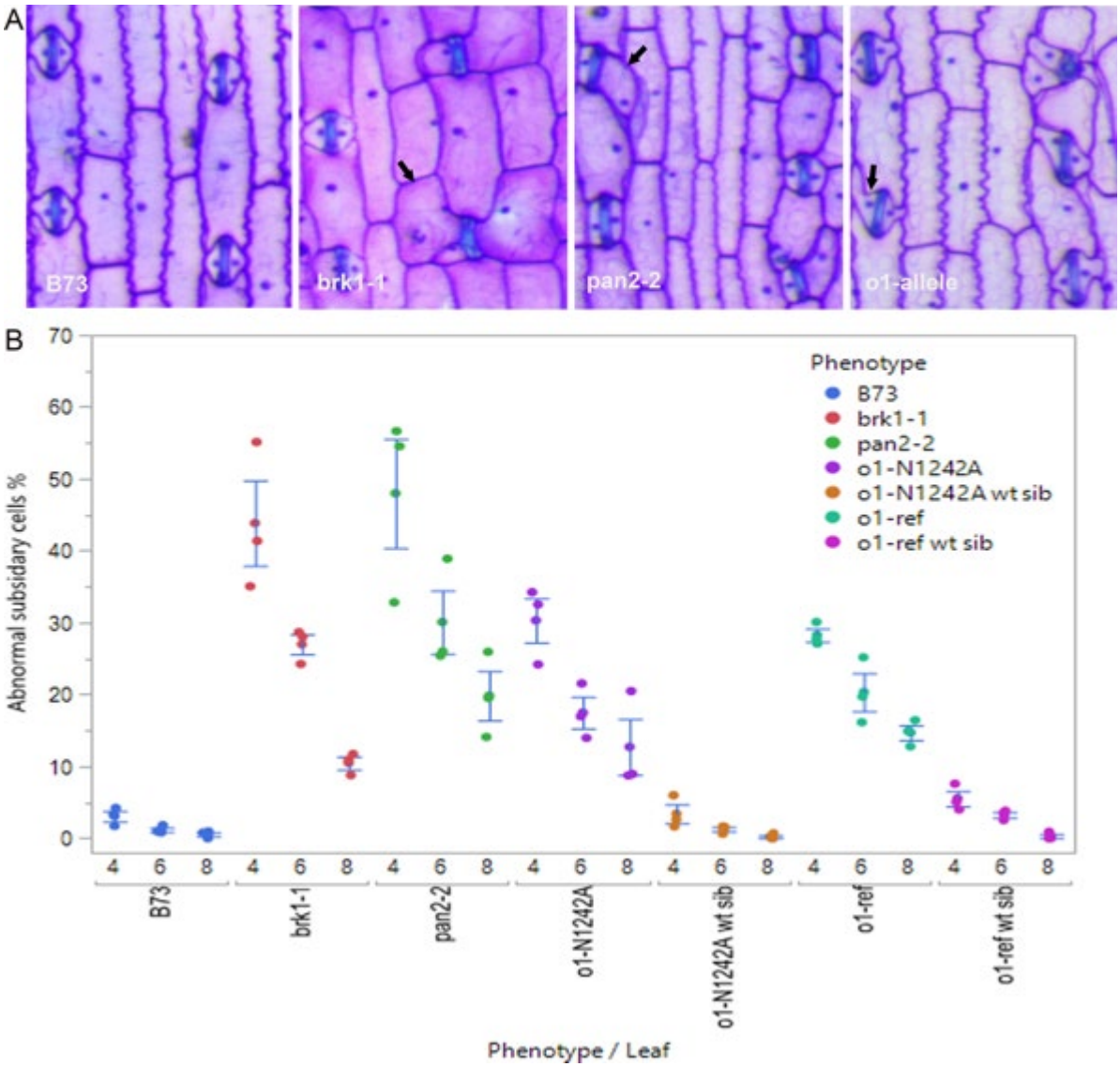

Figure 22. Across various genotypes, abnormal subsidiary cell percentage decreases as leaf number increases. (A) Displays a panel of four different genotypes stained in a toluene blue stain. From left to right reading *B73*, *brk1-1*, *pan2-2* and *o1-*allele. (B) Graph generated from counting of abnormal subsidiary cells in leaf four, six and eight.

Materials and Methods:

Seeds for each genotype were planted under the same environmental and supplemental conditions. LiCior machines were used to measure assimilation and stomata water conductance (g_{sw}). Assimilation was recorded until it reached a stable phase, took an average of 25 minutes to reach that point. Once stabilization was reached for both assimilation and (g_{sw}) light was introduced. Then within 30 minutes the machine would once again reach a stabilized phase and the measurement would be final. Plants were measured during the juvenile stage; leaf 4. The transition stage; leaf 6 and, the adult stage; leaf 8. Prior to taking any measurements every plant was phenotyped using glue impressions. After measurements were finalized for each individual leaf analysis using RStudio was used. RStudio used a script written by Elisa Gagliano to transform excel files into csv files. Once the csv files were obtained they were ran again by RStudio to create a file for (g_{sw}) and assimilation. Those files were then placed in JMP were a nonlinear program was run to best fit the parameters from equation used by (McAusland et al., 2016). The files were cleaned up to fit the best line of fit. They were then divided by the parameters and a graph for each genotype was obtained. From the data collected another variable was calculated, t63.

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