MUSTACHES REGULATES BILATERAL SYMMETRY IN STOMATA

by

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Abstract

*Arabidopsis* stomata develop from specialized stem cells in a dedicated cell lineage which harbours both asymmetric and symmetric divisions. This pathway terminates in the production of two bilaterally symmetrical guard cells (GCs) surrounding a pore, which are essential for shoot gas exchange. Morphogenesis of each GC continues in parallel after the symmetric division, indicating that development is tightly coordinated in time and space. Morphogenesis involves key events, such as the organization of distinctive microtubule arrays and the deposition of ordered cellulose microfibrils, which generate the bilateral symmetry of the mature stomatal valve. Although several genes are known to be required for the symmetric division of the guard mother cell (GMC), the genes and events required for coordinated GC development are largely unknown. MUSTACHES (MUS) is a leucine-rich repeat receptor-like kinase (LRR-RLK) required for stomatal morphogenesis that acts primarily after the two young GCs form. Analysis of the range of phenotypic defects in mature stomata in several loss-of-function mus alleles revealed disruptions in the mirror-like symmetry of microtubule arrays, GC walls, and the stomatal pore. Radial microtubule arrays in developing and mature stomata display a polarity with respect to the trajectories of the microtubule End Binding protein, EB1. In wild-type GCs, most EB1 trajectories move away from the stomatal pore. However in a mus background, many more EB1 comets move towards the stomatal pore, suggesting that the outbound to inbound ratio of microtubule trajectories might regulate or be part of a feedback loop that generates stomatal symmetry. A ProMUS:MUS-GFP translational fusion, transformed into wild-type and mus mutant lines, exhibited GFP localization in cell plates during both symmetric and asymmetric divisions throughout the plant. The only cell type observed that did
not show MUS distribution in the cell plate was the GMC where GFP distribution was instead confined to the cell periphery, a distribution that persisted in young stomata. Because the division of the GMC appears to be normal in *mus*, it is likely that MUS function from the cell periphery is required to generate an appropriately shaped, bilaterally symmetric, and functional stomatal valve.
Preface

Experiments were conceived and designed by Fred Sack, Jeannette Nadeau, Jessica Lucas, and Sandra Keerthisinghe. Identification and cloning of MUSTACHES (MUS) was performed by J. Nadeau and T. Nakagawa. T. Nakagawa also provided the mus-2 allele and a ProMUS:MUS-GFP construct. Initial work on mus mutant phenotypes, microtubule organization phenotypes, and MUS localization analysis was performed by J. Nadeau and J. Lucas. Further examination of mus phenotypes, cellular localization of MUS, and microtubule organization defects were performed by Sandra Keerthisinghe. Observation and statistical analysis of microtubule dynamics in mus and wild-type stomata was also performed by Sandra Keerthisinghe. All writing of this thesis was completed by Sandra Keerthisinghe, with input and advice from F. Sack.
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Dedication

To my parents
1. Introduction

Stomata are two-celled structures that regulate gas and water exchange across the plant epidermis. They are located on the surface of aerial organs such as leaves and stems and function as turgor operated hydrostatic valves. They regulate the entry of carbon dioxide (CO$_2$) used in photosynthesis, as well as the exit of water vapor which is important for cooling the plant. Stomata are considered to be major adaptations that fostered plant occupancy of the land during evolution, and they are integral to contemporary global CO$_2$ and water cycles (Willmer and Fricker, 1996; Berry et al., 2010).

Although the specific morphology of stomata can vary widely among different plant groups, a typical mature stoma consists of a microscopic pore, surrounded by a pair of bilaterally symmetrical cells, ‘guard cells’ (GCs), which are kidney-shaped in many taxa. In addition to periclinal walls, stomata contain cell walls perpendicular (anticlinal) to the surface of the epidermis. The dorsal anticlinal wall (away from the pore) adjoins other epidermal cells, whereas the ventral walls surround and extend from the pore. The dorsal wall is curved throughout most of its circumference, but the ventral wall is usually only curved at the pore. The differential cell wall thickenings in mature GCs allow them to alter shape in response to changes in turgor pressure, and this in turn enables GCs to act as valves. Bilateral symmetry of the two GCs ensures that changes in hydrostatic pressure are mechanically translated into pore opening and closing (Wilmer and Fricker, 1996).

The bilateral symmetry originates from a series of highly regulated genetic and morphogenetic processes. The development of an undifferentiated precursor cell into a
functional stoma spaced one cell apart from another stoma involves two major phases. First, they form via a dedicated developmental pathway characterized by two key types of cell divisions, an initial asymmetric division followed by a symmetric division. The second major phase involves guard cell morphogenesis, which is regulated, in part, by specific arrangements of the cytoskeleton and cellulose microfibrils which determine final guard cell shape (Esau, 1977; Lucas et al., 2006; Bergmann and Sack, 2007).

Entry into the stomatal pathway in most plant taxa seems to involve an asymmetric division in a relatively undifferentiated epidermal cell (Bergmann and Sack, 2007). In some plants, such as Arabidopsis, this asymmetric division produces a small triangular meristemoid (M) and a larger sister cell (Bergmann and Sack, 2007). The cell that divides unequally can be termed a meristemoid mother cell (MMC). Upon unequal division, the larger sister cell can follow one of two cell fates: it can differentiate into a pavement cell (a generic cell of the leaf), or it can itself undergo an asymmetric division (act as an MMC). By contrast, the meristemoid (M) usually becomes committed to forming a stoma by developing into an oval-shaped guard mother cell (GMC). This cell-type undergoes a symmetric division, and ultimately results in two GCs and a terminally differentiated stoma (Bergmann and Sack, 2007).

Both the asymmetric as well as symmetric divisions in the stomatal pathway are regulated by gene sets that appear to be specific to each type of division. The asymmetric division is regulated by a network of genes, including negative regulators, such as the receptor-like protein (RLP) TOO MANY MOUTHS (TMM), the ERECTA (ERL) family of receptor-like kinases (RLKs), and the small peptide ligands EPIDERMAL PATTERNING FACTOR 1 (EPF1), and EPIDERMAL PATTERNING FACTOR 2 (EPF2), and the positive regulator STOMAGEN (Nadeau, and
Negative regulators generally enforce the ‘one-cell spacing rule’ by orienting the plane of asymmetric divisions so that stomata do not differentiate in contact with each other. In addition, stomatal formation and fate are mediated by downstream targets including YODA, a MAPKK kinase, as well as the MAPK kinases MKK4 and MKK5, and the MAP kinases MPK3 and MPK6 (Bergmann et al., 2004; Wang et al., 2007). One target of the YODA-mediated phosphorylation cascade, originally initiated by the binding of ligands to negative regulators such as TMM and the ERL family, is likely to be SPEECHLESS (SPCH), a transcription factor expressed early in the stomatal lineage (Lampard et al., 2008). SPCH is required to initiate the stomatal cell pathway and to promote early asymmetric divisions in the lineage. The phosphorylation of SPCH by MPK3/MPK6 appears to regulate SPCH action such as in the initiation of new asymmetric divisions (MacAlister et al., 2007; Lampard et al., 2009).

A separate set of genes controls the symmetric division of the GMC. Two of these genes, CYCLIN DEPENDENT KINASE B1;1 (CDKB1;1) and CYCLIN DEPENDENT KINASE B1;2 (CDKB1;2) promote the symmetric division of the GMC (Boudolf et al., 2004; Xie et al., 2010). The MYB transcription factors FOUR LIPS (FLP) and MYB88, limit the number of symmetric divisions that GMCs undergo to one, as does FAMA, another basic helix loop helix (bHLH) transcription factor (Ohashi-Ito and Bergmann, 2006).

In addition to controlling the number of symmetric divisions, FAMA is required for a GC fate and differentiation after GMCs divide (Ohashi-Ito and Bergmann, 2006). FAMA, along with four other bHLHs, SPEECHLESS (SPCH), MUTE, ICE1/SCREAM1 (ICE/SCRM1) and SCREAM2 (SCRM2), appear to positively regulate cell fate transitions during the stomatal pathway. While
SPCH regulates the transition from MMC to M, and MUTE regulates the transition from M to GMC (MacAlister et al., 2007; Pilliteri et al., 2008), ICE1/SCRM1 and SCRM2 seem to regulate all three cell fate transitions in the stomatal pathway i.e. MMC to M, M to GMC, and GMC to GC (Kanoaka et al., 2008).

The process of stomatal morphogenesis begins after the symmetric division occurs and the initial bilateral symmetry is established. This process involves cell wall thickening, GC shaping, and pore formation. The specific shape of GCs and their cell walls is most likely generated by cytoskeletal-mediated and localized cell wall deposition, including cell wall thickenings. Localized cell wall thickenings first appear on both ends of the GMC. These thickenings might be positioned by the pre-prophase band (PPB), a cytoskeletal structure consisting of microtubules, which forms just prior to the division of the GMC (Galatis et al., 1982; Zhao and Sack, 1999; Lucas et al., 2006). The cell wall thickenings might regulate GMC shape such as by restricting local cell wall expansion at either end of the GMC, thus promoting the elongated oval shape characteristic of mature stomata (Zhao and Sack, 1999). These wall thickenings persist in mature stomata, and might facilitate GC opening and closing by restricting turgor-induced cell wall expansion at the end of the each guard cell, thereby allowing the rest of the guard cell to expand outward (Lucas et al., 2006).

GC shaping and morphogenesis are also correlated in time and space with the organization of microtubules in the cortical cytoplasm and with the arrangement of cellulose microfibrils in the cell wall. GCs contain a distinct radial array of microtubules (that “focus” on the stomatal pore) and cellulose microfibrils that are similarly arranged (Palevitz and Hepler, 1976, Lucas et al., 2006). These radial cellulose arrays might disperse the force created by
turgor pressure and also help form the distinctive kidney shape of GCs (Aylor et al., 1973). In addition to regulated stomatal opening and closing, these arrays also function in stomatal development. In general the disruption of microtubule and cellulose arrays in mutants, or using drug treatment has frequently been shown to induce defective morphogenesis and cell expansion (Himmelspach et al., 2003; Sugimoto et al., 2003; Wasteneys and Ambrose, 2009).

Pore formation appears to take place concurrently with GC shaping. After the GMC has finished dividing symmetrically, pore formation initiates in the center of this new (ventral) wall. The first visible sign of pore formation is the presence of a lens-shaped thickening across the two developing guard cells. Transmission electron microscopy has shown that the middle lamella of the pore cell wall thickening changes in electron density and becomes stretched as the stomatal pore forms (Zhao and Sack, 1999).

Several stomatal mutants are known to disrupt the symmetric division or GC morphogenesis, or both. For example, a disruption of the symmetric division in turn alters subsequent GC morphogenesis. Thus, a cdkb1;1/cdkb1;2 double mutant prevents many GMCs from dividing symmetrically resulting in the absence of a normal pore (Xie et al., 2010). Mutations in the FAMA locus, which encodes a bHLH protein, form GMCs that divide symmetrically, but these do not form guard cells since FAMA is required for a stomatal cell fate (Ohashi-Ito and Bergmann, 2006). However, no mutants appear to have been described that disrupt GC morphogenesis or bilateral symmetry.

Previous work in F. Sack’s lab identified a locus, MUSTACHES (MUS), which is required for the bilateral symmetry of the stoma. Mutations in this gene disrupt GC morphogenesis (Nadeau, unpublished) in that stomata display abnormal symmetry, cell wall defects, altered
microtubule organization, as well as altered pore formation and morphogenesis in GCs. MUS encodes a Leucine-Rich Repeat Receptor-Like Kinase (LRR-RLK) (Nadeau, unpublished).

Receptor-like kinases (RLKs), often act in signal reception and transmission and they constitute a large gene family in Arabidopsis; more than 600 RLKs have been identified to date in the Arabidopsis genome. The most common types of RLKs in Arabidopsis are the transmembrane RLKs. In addition to possessing a transmembrane domain, these RLKs have an extracellular (receptor) domain, as well as a cytoplasmic kinase domain (Becraft, 2002). However, many of these RLKs and their signaling pathways have not yet been characterized.

Extracellular signals such as ligands are often perceived by the RLK receptor domain, which is thought to induce the dimerization of the RLK with various partners, such as another RLK, or a receptor-like protein (RLP) (Jeong et al., 1999; Diévart et al., 2003; Wang et al., 2006). Dimerization of an RLK with its partner subsequently induces signal transduction through the cell, which often involves a phosphorylation cascade. Despite their common structures, plant RLKs are able to transmit signals from many types of ligands, suggesting that there is variety in the types of extracellular domains present in RLKs (Shiu and Bleeker, 2001).

Approximately half of all Arabidopsis RLKs identified to date contain Leucine-Rich Repeat domains (LRR). LRR domains mediate protein-protein interactions, such as ligand binding in the extracellular domain (Shiu and Bleeker, 2001). LRR-RLKs regulate diverse functions including shoot meristem development (CLAVATA1), pathogen resistance (FLAGELLIN SENSITIVE2), pollen self-incompatibility (S RECEPTOR KINASE), and hormone signaling (BRASSINOSTEROID INSENSITIVE1) (Diévart et al., 2003; Zipfel et al., 2004; Stein et al., 1991; He et al, 2000).
RLKs also act in the stomatal pathway. The ERECTA family of LRR-RLKs appears to partner with the LRR-RLP TMM, and are involved in the regulation of asymmetric division placement (Bergmann and Sack, 2007). TMM was a founding member of genes known to act during stomatal development (Nadeau and Sack, 2002). The identification of TMM facilitated the elucidation of a network of genes regulating the spacing of asymmetric divisions during stomatal development. Perhaps, the definition of MUSTACHES function may lead to defining the signal transduction pathways that regulate bilateral symmetry formation in stomata and in plants.

Stomata, located in the epidermis, are an easily identifiable and accessible cell type, which makes them an excellent model system for studying processes such as cell division, differentiation, and symmetry generation. The identification of mutants that affect stomatal symmetry should lead to further definition of the molecular and cellular events involved in stomatal and cellular morphogenesis.

This chapter analyzes aspects of the function and distribution pattern of the Arabidopsis LRR-RLK, MUSTACHES (MUS). MUS signaling seems to be required for controlling the development, placement, and symmetry of the stomatal radial microtubule array, as well as the dynamics and direction of microtubule polymerization in GCs. The analysis of MUS function should help identify how cellular processes and signaling pathways generate cell symmetry, morphogenesis and polarity.
2. Results

2.1 MUS is required for correct wall and pore symmetry

The MUSTACHES (MUS) locus and its involvement in stomatal development was identified using microscopy-based screening of a population of EMS mutagenized Arabidopsis seedlings to identify abnormal stomatal phenotypes (Jeannette Nadeau and Fred Sack, unpublished). One line showed abnormal stomata with a distorted shape and skewed stomatal pores, an allele designated mustaches-1 (mus-1). Professor Tsuyoshi Nakagawa at Shimane University, Japan independently identified a mutant (mus-2), whose phenotype resembled mus-1, and which through collaboration was found to be allelic to mus-1. Most stomata in both alleles appear normal (Fig. 2.1A), but a subset of stomata showed skewed pore walls indicating a disruption in bilateral symmetry and pore morphogenesis.

The phenotypes of mus-1 stomata range from mild (skewed walls and pores), to medium (irregularly-shaped cells), to severe (ectopic or missing pore) Fig.2.1 (B-D). In addition mus-1 GCs sometimes display ectopic cell wall thickenings (Fig.2.2).

Figure 2.1. Wild-type and a range of mus-1 stomatal phenotypes. (A) Wild-type (Col-0) stoma showing strong bilateral symmetry in guard cell shape, arrangement, and in pore size and location. (B) mus-1 stoma displaying skewed pore and offset new wall. (C) mus-1 stoma that lacks a pore, but that displays a lopsided and asymmetrical pore wall thickening. (D) mus-1 stoma that lacks both a pore and as well as a mid-cell wall thickening. Scale bar in (A) = 5 µm; (B-D) are at same magnification.
Figure 2.2. Some mus-1 stomata display ectopic wall thickenings. (A) Mature wild-type stoma (picture from Jessica Lucas). End wall thickenings (arrows) are present in GMCs as well as in mature stomata. (B-C) mus-1 stomata expressing ProKAT-GUS, a marker for a mature stomatal fate, exhibit ectopic wall thickenings (arrows) as well as misshapen guard cells (unpublished pictures from Jeanette Nadeau).

2.2 MUS is required to maintain the bilateral symmetry of stomatal microtubule arrays

Microtubules in mature wild-type stomata are arranged in fan-shaped arrays that focus visually on the stomatal pore, and most microtubules are roughly aligned along the radii of the arc-shaped GCs (Lucas et al., 2006). As a result, microtubules near the stomatal pore are closer together than along the periphery (outer anticlinal cell wall) (Fig. 2.3 A-B). Because the microtubule foci are localized opposite each other on either side of the pore, these radial arrays display a mirror like symmetry across the two GCs. This symmetry is in addition to that of the shape and arrangement of the pore and the GCs.

To determine whether the mus-1 mutation also affects the bilateral symmetry of microtubule arrays, a Pro35S: TUBULINA-GFP construct (Ueda et al., 1999) was introgressed into wild-type (Col-0) and mus-1 plants. Stomata in a mus-1 background displayed skewed microtubule arrays in addition to skewed pores (Fig. 2.3 C-G). The severity of array disruption in mus-1 varied between individual stomata. Some showed mildly skewed radial arrays and
roughly straight ventral (future wall containing pore) cell walls, while others showed two twisted guard cells, with a resulting offset in the foci of the microtubule arrays (Fig. 2.3 D-E). In addition, some mus-1 stomata displayed foci which were no longer located exactly opposite each other, and appeared to be skewed (Fig.2.3F). A range of phenotypic severity is often visible in the same leaf, or among different leaves of the same plant, and this range is roughly consistent in plants harboring the same mus allele. These cell wall and microtubule phenotypes suggest that MUS is involved in regulating the alignment of the symmetric division, and in controlling the shape, as well as the presence of the pore. Thus, MUS controls stomatal morphogenesis.

Figure 2.3. Mutations in MUS disrupt the arrangement and symmetry of radial microtubule arrays in stomata. Maximum Z-projections of Col-0 (A-B) and mus-1 (C-G) stomata. White arrows indicate the location of the cell wall normally produced by symmetric division. (A) Young wild-type stoma displaying radial and opposite microtubule foci. (B) Mature wild-type stoma containing characteristic broader radial microtubule arrays. (C) Young mus-1 stoma, shortly after symmetric division, displaying non-radial microtubule distributions. (D) Young mus-1 stoma with newly developing pore and with slight skewing of microtubule foci. (E) Later stage mus-1 stoma containing ectopic and skewed microtubule foci. (F) Mature mus-1 stoma displaying skewed walls, and slightly skewed or offset microtubule arrays. (G) Mature mus-1 stoma exhibiting severely disrupted microtubule array in enlarged, pore-less GC at right compared to a mostly normal array, half pore, and GC at left. Scale bar in (A) = 5 µm, and also represents magnification in (B-G).

2.3 MUS encodes an LRR-RLK

Nadeau and Nakagawa (unpublished) established the identity of the MUS gene through collaborative positional cloning of two alleles and by transgenic complementation. MUS was
found to be \textit{At1g75640}, which encodes an LRR-RLK in the RLK family VII (b) (Shiu and Bleeker, 2001) (Fig. 2.4-2.5).

The \textit{MUS} gene is 3,586 bp long, and does not contain any introns. \textit{MUS} encodes a typical LRR-RLK in that it contains of an extra-cellular domain consisting of Leucine-Rich Repeats, a transmembrane domain, and a cytoplasmic kinase domain (Fig. 2.6) (Nadeau, unpublished).

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<tr>
<td>4082-4321</td>
<td>SKESEVYFSC ILLEILTGK KAVMTEDDE IVKWWKRQLQ KCGIVELLEP GLLELDPESS</td>
</tr>
</tbody>
</table>

Figure 2.4. Wild-type MUS amino acid sequence and predicted protein domains (modified from Plants P database). Blue highlighting represents the potential transmembrane spanning region, pink represents leucine rich repeat domains, yellow represents a putative serine/threonine protein kinase active-site signature, and green highlighting represents the predicted protein kinase domain. The black arrows represent the location of \textit{mus-1} and \textit{mus-2} substitutions. Red highlighting represents the predicted location of the DFG motif which is defined as a sequence of three amino acids, D (aspartic acid), F (phenylalanine) and G (glycine). This sequence is
present in all functioning kinases analyzed to date (Castells and Casacuberta, 2007). However, in the wild-type MUS protein, glutamic acid (E) is substituted for aspartic acid (D) at amino acid 983 (i.e. EFG instead of DFG).

Figure 2.5. Phylogenetic relationship of MUS to other LRR RLKs in *Arabidopsis*. Figure
reproduced from Tax et al., 2004 with slight modifications.

Figure 2.6. Structure of the MUS LRR-RLK and location of three characterized mutations. MUS encodes a Leucine-Rich Repeat Receptor-Like Kinase which contains: (i) 25 LRRs, (ii) a transmembrane domain, and (iii) a kinase domain (predicted to be non-functional, see Fig.2.4).

2.4 Three mus alleles exhibit a broad phenotypic range

The phenotypes of mus-1 and mus-2 were previously characterized. The mus-1 allele induces a glycine-to-arginine point mutation in the kinase domain (Nadeau, unpublished). The mus-2 allele has a tryptophan-to-stop mutation in the first LRR domain (Nakagawa, unpublished). The SALK_003283 T-DNA insertion is located in the sixteenth LRR (Fig.2.6), whereas a SAIL T-DNA insertion (SAIL_82_D11 CS80388) is located in the MUS promoter, suggesting a possible null mutant (Fig.2.7). Due to the nature of the insertions in mus-2 and SALK_003283, these lines potentially represent functionally null alleles.

Figure 2.7. Position of SAIL_82_D11 CS80388 T-DNA insertion in MUS. Red line represents the MUS upstream region (promoter), green box represents MUS coding region, and the blue arrowhead represents the location of the T-DNA insertion (TAIR online database; Swarbreck et
Quantitative PCR (qPCR) was performed to determine the relative levels of expression in the above four mus alleles (Fig.2.8). The primers utilized were located in the kinase domain, and were specific to an area located before both the EFG/DFG domain, and the mus-1 point mutation. Relative to Col-0 plants (100% expression), transcript levels were approximately 27% ± 8% (SE) in the SALK line, 60% ± 7% (SE) in mus-1, and 82% ± 5% (SE) in mus-2. Thus, these three alleles appear to be ‘knock-down’ alleles, consistent with the relatively low phenotypic expressivity and penetrance of these alleles.

The SAIL_82_D11 CS80388 allele, which harbors a T-DNA insertion near the 5’ end of the gene, showed 154% ± 41% overexpression compared to Col-0 plants. Ectopic overexpression can be induced when T-DNA insertions, which contain 35S promoter and enhancer elements, become located adjacent to the native promoter (Ren et al., 2004).

Although MUS expression is only down-regulated by 40% in mus-1, this allele displays the highest number, and most diverse types, of stomatal defects. In contrast, the SALK insertion (SALK_003283) line, which causes a 73% decline in MUS expression, has a mild phenotype similar to that caused by mus-2, which induces an 18% reduction in MUS expression. These data suggest that the severity of the MUS phenotype is not simply due to different transcript levels, and that the location of the disruption in the MUS coding sequence might contribute as well.
RNA was extracted from whole seedlings 10 days after germination, and qPCR was performed on two biological replicates for each allele. Levels of *mus* expression were calculated relative to the *Col-0* (representing 100% MUS expression), by utilizing the deltaCT method (Livak *et al.*, 2001). Expression values from the two replicates were then averaged to obtain the mean expression level (%) for each allele. Error bars represent standard error (SE).

A higher percentage of stomata with shape, symmetry and pore defects were observed in *mus-1*, in comparison to *mus-2* (Lucas, unpublished data). Therefore, although the *mus-2* mutation occurs near the beginning of the MUS gene, its stomatal phenotype is weaker than that of the *mus-1* allele, which harbors a point mutation in the kinase domain (in addition to the pre-existing ‘natural mutation’ in the DFG motif). The *mus-2* allele was generated in a Landsberg erecta (*Ler*) background. The LRR-RLKs ERECTA, ERL1, and ERL2 genes control stomatal number and patterning along with the LRR RLP TMM through a complex set of interactions (Shpak *et al.*, 2005). Therefore, it cannot be ruled out that these quantitative differences in the phenotypes of *mus-1* and *mus-2* are due to secondary effects such as the genetic backgrounds of *Col-0* (*mus-1*) and Landsberg erecta (*mus-2*). In contrast to both *mus-1* and *mus-2*, plants homozygous for the *SAIL_82_D11* promoter insertion, which exhibits MUS overexpression, displays a completely wild-type stomatal phenotype.

Figure 2.8. Mean relative MUS expression in *mus-1*, *mus-2*, SALK_003283 and SAIL_82_D11. RNA was extracted from whole seedlings 10 days after germination, and qPCR was performed on two biological replicates for each allele. Levels of *mus* expression were calculated relative to the *Col-0* (representing 100% MUS expression), by utilizing the deltaCT method (Livak *et al.*, 2001). Expression values from the two replicates were then averaged to obtain the mean expression level (%) for each allele. Error bars represent standard error (SE).
Notably, *mus*-1 contains a glycine (G) to arginine (R) point mutation in a residue located at position 1030 of the kinase domain. The mutation in the *clavata1*-2 (*clv1*-2) allele is also caused by a substitution in the same residue. CLV is an LRR-RLK that controls the size of the *Arabidopsis* shoot apical meristem (Clark et al., 1993). Heterozygotes containing one mutant *clv1*-2 allele (*clv1*-2/*CLV1 (-/+)) have been shown to function as a dominant negative allele (Diévart et al., 2003). Although the defective CLV1 receptor binds to the other two LRR-RLK members of the complex, BAM1 and BAM2, the presence of the dominant negative kinase form of *clv1*-2 blocks phosphorylation overall. Moreover, heterozygous *clv1*-2/*CLV1 (-/+)) plants also show larger apical meristems compared to homozygous wild-type *CLV1/CLV1* plants, again likely indicating the influence of a single dominant negative allele on the CLV1 complex (Diévart et al., 2003; DeYoung et al., 2005). Despite *mus*-1 containing a mutation resembling that of *clv1*-2 allele which confers a dominant negative phenotype on shoot apical meristems, plants containing one *mus*-1 allele and one wild-type MUS allele appear phenotypically normal in stomatal development, suggesting that the *mus*-1 allele does not function as a dominant negative.

In summary, while MUS is annotated as an “LRR-RLK”, the wild-type MUS protein sequence harbors a substitution in the DFG motif, a motif invariantly required for kinase activity, suggesting that MUS is not a functional kinase. The nature of mutations in the *mus* alleles suggests that *mus*-2 and *SALK_003283* may be functional nulls. In contrast, *mus*-1 may not be a functional null. However, regardless of the nature of the mutation, all three *mus* mutant alleles have comparable phenotypes, which suggest that MUS is involved in a signaling pathway which regulates stomatal bilateral symmetry.
2.5 ProMUS:MUS-GFP is distributed in dividing cells throughout the plant

Based upon online databases, MUS is likely to be expressed in a variety of organs throughout the Arabidopsis plant (Winter et al., 2007). This bioinformatics source indicates that MUS is strongly expressed in the shoot apical meristem, as well as in developing carpels, seeds, and embryos. In contrast, MUS is reported to be weakly expressed in mature guard cells, leaves, and roots (Winter et al., 2007) (Fig.2.9). The wide range of organs that express MUS suggests that MUS might act in a common, developmental process found in many cell types.

To help understand the role that MUS plays in development, a ProMUS:MUS-GFP translational fusion was constructed and transformed into wild-type and mus-1 plants (see Materials and Methods). Wild-type plants containing the ProMUS:MUS-GFP construct did not display over-expression phenotypes. The ProMUS:MUS-GFP construct complemented the mus-1 stomatal mutant phenotype, and these complemented mus-1 plants were utilized for all analyses of MUS distribution patterns. ProMUS:MUS-GFP distribution was observed in embryonic cotyledons, as well as throughout the mature leaf and root epidermis (Figs. 2.10, 2.11 and 2.12). However, MUS was primarily detected around cell plates and newly formed cell walls. MUS-GFP fluorescence was also found in parts of the phragmoplast close to the cell plate (Figs.2.10 and 2.11). A similar distribution pattern was found in asymmetric divisions in the stomatal pathway (Fig.2.12).
Figure 2.9. Sites of MUS expression in Arabidopsis (figure modified from Winter et al., 2007). MUS is expressed at high levels in structures shaded in red, whereas it is expressed at medium levels in structures shaded in orange, and at very low levels in yellow-shaded regions.

Figure 2.10. MUS distribution in the root of mus-1 complemented lines. Longitudinal optical sections of roots, where green fluorescence represents ProMUS:MUS-GFP distribution and red fluorescence represents propidium iodide-stained cell walls. (A) MUS appears to be distributed near many cell walls - especially transverse walls - in the root. (B) Deeper optical section showing MUS distribution in the cell plates of the root cap, epidermis, ground, and vascular tissues. No ProMUS:MUS-GFP fluorescence was detected in divisions of columella cells (n = 10 roots). Scale bar in (A) = 30 μm, and also represents (B).
Fig. 2.11. MUS distribution in embryonic cotyledons dissected from immature seeds contained in siliques. Green linear fluorescence represents ProMUS:MUS-GFP, while red fluorescence represents cell walls stained with propidium iodide. Note that chloroplasts show autofluorescence in green channel. (A) Epidermis of entire cotyledon showing green lines representing MUS localization during cytokinesis in, or around cell plates. (B) Higher magnification showing GFP fluorescence in over eight different divisions. White arrow indicates MUS cell plate localization in an asymmetric division. Scale bar in (A) = 5 µm, and also represents (B).

2.6 **MUS shows distinct subcellular distribution patterns in GMCs and developing stomata**

Since MUS affects the symmetry of the stomatal pore and the stoma, one possibility was that MUS would localize around the cell plate that leads to the symmetric division of the GMC. This localization would be expected based upon where MUS is distributed in the rest of the plant. A cell-plate localization would also be consistent with a role of the MUS receptor in mediating signaling between the two guard cells, thus correctly placing the wall thickenings opposite each other to form the pore.

As indicated, MUS is localized around cell plates and new cell walls in asymmetric divisions in the stomatal cell lineage (Figs. 2.13A and 2.14A). In addition, MUS localization continues into the GMC stage. Notably, however, unlike in newly forming meristemoids in the
stomatal cell lineage, and in other cell types in the plant, MUS was not localized in the cell plate during the symmetric division of the GMC. Similarly, there was no MUS around the newly formed (ventral) wall which later forms the stomatal pore. Instead, strong MUS distribution was found in the periphery of the GMC. Similarly, MUS was observed in the periphery of newly formed guard cells just after symmetric division. MUS localization weakens as the stoma matures and as pore morphogenesis begins, but in agreement with e-FP browser data, MUS appears to be weakly localized in mature stomata (Figs. 2.13C and 2.14G). Because MUS mediates bilateral symmetry formation and pore morphogenesis at the center of the cell, these data suggest that MUS localized in the guard cell periphery controls events that occur at the developing pore wall, rather than mediating it directly from the center.

Figure 2.12. MUS is distributed in divisions throughout the developing leaf epidermis. Red fluorescence (PI stain) signifies epidermal cells, while green fluorescence (ProMUS:MUS-GFP) corresponds to MUS localization. White arrows mark ProMUS:MUS-GFP localization in the cell plates of asymmetric divisions, while white stars mark localization in GMCs. Scale bar = 5 µm
Figure 2.13. MUS distribution in the stomatal lineage. Red fluorescence (propidium iodide stain) signifies epidermal cells, while green fluorescence (ProMUS:MUS-GFP) corresponds to MUS localization. White stars indicate GMCs exhibiting peripheral MUS localization, and white arrows indicate cell plate localization of MUS in asymmetric divisions including an ectopic one at arrow in (C). Scale bar = 5 µm, and also represents (B-C).

Figure 2.14. MUS localization changes during stomatal development. Maximum Z-projections are shown. Red fluorescence (propidium iodide stain) corresponds to epidermal cell outlines, while green fluorescence (ProMUS:MUS-GFP) corresponds to MUS localization. (A) ProMUS:MUS-GFP is distributed around the cell plate during asymmetric division. The developing cell plate shows red propidium iodide staining. (B) MUS becomes peripherally distributed in early GMCs. (C-E) MUS remains peripherally distributed in late GMCs. Note two curved cell plates in (E lower left) showing MUS distribution at an angle, with the plate in the middle bordering an ectopic asymmetric division. (F) MUS is not present in the wall of a recently divided young stoma which is developing pore cell wall thickenings. (G) MUS is weakly localized in mature stomata. Scale bar = 5 µm, and also represents (B-G).

2.7 ProMUS:MUS-GFP distribution appears to be polarized in GMCs, and young stomata

Some late stage GMCs and young stomata show a polarized localization of MUS. In 67% of GMCs MUS distribution appeared to be concentrated on opposite ends of the cell in both maximum Z-projections and single optical sections (n= 15 GMCs) (Fig.2.15). Some young and mature stomata also displayed MUS localization sites near the pore walls (Fig.2.16).
Figure 2.15. MUS distribution appears to localize in a polar manner in late-stage GMCs and young stomata in wild-type plants (*mus-1* transformed with *ProMUS:MUS-GFP*). Red fluorescence (PI stain) marks all epidermal cells, while green fluorescence (*ProMUS:MUS-GFP*) corresponds to MUS localization. (A-C) show same field with both channels overlaid in (A), and separate channels shown in (B) and (C). The peripheral localization of MUS is visible in the GMC at the right in A and C, although MUS distribution is highest at the poles where the new symmetric division will occur. MUS localization in cell plates is evident during cytokinesis in the same asymmetric division visible at the left of each micrograph. Scale bar in (A) = 5 µm, and also represents (B-C).

Figure 2.16. MUS localization appears to be polarized in developing and mature stomata. (A-F) *ProMUS:MUS-GFP* localization during wild-type stomatal development. Each pairing contains a maximum Z-projection on the left, and a single optical section on the right. (A) Early GMC. (B) Late GMC. (C) Young stoma undergoing a symmetric division (white arrows). (D) Young stoma (recently divided GMC). Note the lack of MUS in the newly formed pore wall. (E) Young, stage 2, stoma containing a pore thickening. (F) Mature stoma. Several chloroplasts also show faint
green fluorescence (e.g. in C). Scale bar in (A) = 5 µm, and also represents (B-F).

2.8 MUS appears to localize to and near the cell membrane

The ability of many transmembrane Receptor-Like Kinases (RLKs) to respond to extracellular signals presumably depends upon their localization in the plasma membrane or cortical endoplasmic reticulum. MUS is distributed in the periphery of GMCs and young stomata, as well as in the cell plate of new asymmetric divisions. The peripheral distribution of MUS is consistent with MUS localizing to the plasma membrane. However, MUS also appears to be faintly distributed in other regions of the cell (Fig.2.14), which may suggest one of two possibilities: (i) MUS is distributed diffusely throughout the cortical cytosol, or (ii) MUS is distributed in the cortical endoplasmic reticulum (cortical ER). Subsequent comparison of stomata expressing a plasma membrane marker (Pro35S:Q8-GFP), an ER marker (Pro35S:ER-GFP), and ProMUS:MUS-GFP suggests that peripherally localized MUS, in addition to being located in the plasma membrane, is also located partly in the cortical ER (Fig.2.17).

In wild-type precursor cells and stomata, Pro35S:Q8-GFP distribution appears as a faint but continuous peripheral pattern. ProMUS:MUS-GFP distribution is less continuous, grainier, and thicker in appearance than Q8-GFP. Pro35S:ER-GFP is distributed in cortical ER, as well as internal ER (Fig. 2.17B). ProMUS:MUS-GFP is thus likely to be localized in the cell membrane and perhaps in some cortical ER as well. This cell membrane localization of MUS in developing stomata differs from its absence from cell plates and pore cell membranes in newly divided GMCs.
2.9 EB1 trajectories are altered in a mus-1 background.

A defining feature of mature wild-type Arabidopsis stomata in many species is that their microtubule arrays show a radial distribution pattern (Apostolakos and Galatis, 1999; Lucas et al., 2006; Apostolakos et al., 2009). The minus (nucleating) ends of microtubules in these arrays appear to be located at the developing and the mature stomatal pore (McDonald et al., 1993; Marc, 1997). As a result, the trajectory (direction and rate of growth) is outwards from the pore (in the ventral cell wall) towards the dorsal guard cell wall. The likely nucleation of minus ends of microtubules close to the stomatal pore was established by immunofluorescence...
studies of γ-tubulin (McDonald et al., 1993; Marc, 1997), which showed fluorescent ‘foci’ near mature stomatal pores.

The End Binding Protein 1-GFP (Pro35S:EB1-GFP) has been shown to mark microtubule initiation at the pore of wild-type stomata (Dixit et al., 2006). As well, Pro35S:EB1-GFP has been used to track the growth of microtubule plus ends from the pore outwards, towards the stomatal periphery (Dixit et al., 2006).

As shown above, mus-1 disrupts the mirror-like symmetry of the radial microtubule arrays. To determine whether MUS also controls microtubule trajectory and velocity, mature stomata in wild-type and mus-1 seedlings were compared utilizing Pro35S:EB1-GFP. As expected, observation of Pro35S:EB1-GFP in mature wild-type stomata showed that microtubule growth initiates near the pore and continues towards the cell periphery (outward growth) (Fig.2.18A). However, closer inspection of time lapse images also revealed that a fraction of EB1 comets grow in the opposite direction (from the periphery towards the pore) (Fig.2.18A). In wild-type stomata, about 81% ± 2 (SE) of the EB1 comets moved outward, and the remainder (19% ± 2 (SE)) moved towards the pore (Figs.2.18A and 2.19). In mus-1, EB1 also displayed ‘inward’ and ‘outward’ comet movement. However, the proportion of comets moving inward in mus-1 was much higher than in the wild-type with 53% ± 4 (SE) of comets moving ‘inward,’ and the remainder, 46%± 4 (SE) moving outward (Fig 2.18B, 2.18C and 2.19).

Some EB1 movement in mus-1 stomata was neither inward nor outward. Here the microtubules, regardless of where they initiated, appeared to converge at the center of the guard cell during the course of their growth (Fig.2.18C). This atypical movement was most often observed in guard cells which had undergone improper morphogenesis that produced a circular
or oval shaped guard cell rather than a kidney-shaped one.

Figure 2.18. Time-lapse imaging indicates that the polarity of microtubule trajectories is disrupted in mus-1. (A-C) Frames one to four from a time-lapse series, taken at two second intervals, are shown. Trajectory of six representative EB1 comets are marked, where red arrows represent EB1 movement inwards towards the pore, and yellow arrows represent EB1 movement outwards from the pore. White arrows mark the dividing cell walls. These data indicate a relationship between trajectory disruption and degree of skewedness in stomata. (A) Col-0 stoma (wild-type stoma, no disruption). (B) mus-1 stoma displaying slight skewing of cell walls (slightly skewed mild-mus-1 phenotype). (C) mus-1 stoma exhibiting GCs with abnormal morphogenesis (severe-mus-1 phenotype). Scale bar in (A) = 5 µm, and also represents (B-C).
Figure 2.19. MUS regulates net movement of EB1 to and from the stomatal pore (n = 300 comets per genotype). Trajectory of EB1 movement was observed using time-lapse imaging of whole Arabidopsis cotyledons. Imaging of trajectories persisted for a maximum of ten minutes after mounting. Scoring was performed by observing the movement of six EB1 comets in each of 50 mature wild-type stomata and 50 mature mus-1 stomata (n = 300 comets scored per genotype). Maturity was determined by considering factors such as stomatal size, presence of wall thickenings, presence of a pore or a partial pore, and the extent of guard cell expansion. In wild-type stomata (light grey bars), approximately 81% ± 2 (SE) of the EB1 comets moved outward, and the remainder (19% ± 2 (SE)) moved towards the pore. In mus-1, (dark grey bars) 53% ± 4 (SE) of comets move ‘inward,’ and the remainder, 46%± 4 (SE) move outward. Additional procedural details can be found in the Materials and Methods section.

2.10 The outbound to inbound ratio of microtubule growth trajectories is strongly related to guard cell symmetry

The above results indicate a distinct relationship between the direction of microtubule growth and the disruption of stomatal symmetry (Fig 2.19). Therefore, the direction of EB1 movement in mus-1 stomata was analyzed by visual assignment to four categories related to the severity of disruption in stomatal morphology: (1) WILD-TYPE-LIKE (normal pore and wall), (2) MILD (similar to the wild-type, but with slight skewing/misalignment of wall and pore) (3) STRONG (severely skewed, where the pore and wall are completely misaligned), and (4) SEVERE (missing or ectopic pores, improper morphogenesis, or abnormally expanded guard
This analysis demonstrates that the more asymmetric the placement of the two guard cells was with respect to each other in *mus-1* stomata, the lower the number of outward-, and the higher the number of inward-directed microtubule comets (Fig 2.20).

The association between the severity of the *mus-1* disruption and comet direction was tested utilizing ANOVA (Table 2.1a; Table 2.1b). A significant difference ($p < 0.01$) in the percentage of outbound and inbound microtubule growth trajectories, between wild-type and *mus-1* was found. Tukey Kraemer mean analysis demonstrates that stomata showing the greatest level of symmetry disruption (category 3 and 4 *mus-1*) also displayed the highest levels of disrupted EB1 trajectories, meaning that a significantly smaller percentage of outbound and a higher percentage of inbound EB1 growth trajectories were present compared to wild-type, as well as to wild-type-looking stomata in *mus-1* (category 1) and MILD-*mus-1*, (category 2) (Table 2.1A; Table 2.1B). By contrast, there was no statistical difference between comet direction in category 3 and 4 *mus-1* stomata.
Figure 2.20. The relationship between the direction of EB1 comets and the extent of symmetry disruption (degree of skewing) in mus-1 stomata. The Y-axis represents direction (expressed as the number of inbound and outbound comets), and the X-axis represents morphological symmetry disruption (the degree of skewing), with 1 being no disruption or skewing, and 5 being severe disruption or skewing. The trend indicates that higher levels of symmetry disruption are associated with an increase in the number of inbound comets (blue line), and a decrease in the number of outbound comets (red line).
Table 2.1. ANOVA of percent inbound and outbound comets for MUS and four mus-1 categories. Categories sharing the same letter are not significantly different. CV= coefficient of variation, SE = standard error.

A. Mean percent outbound EB1 comets

<table>
<thead>
<tr>
<th>DISRUPTION CATEGORY</th>
<th>MEAN % OUTBOUND</th>
<th>SIGNIFICANCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>WILD-TYPE MUS (control)</td>
<td>78.2</td>
<td>c</td>
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<tr>
<td>WILDTYPE-LIKE mus-1 (category 1)</td>
<td>70.0</td>
<td>bc</td>
</tr>
<tr>
<td>MILD-mus-1 (category 2)</td>
<td>61.6</td>
<td>bc</td>
</tr>
<tr>
<td>STRONG- mus-1 (category 3)</td>
<td>43.2</td>
<td>ab</td>
</tr>
<tr>
<td>SEVERE-mus-1 (category 4)</td>
<td>20.1</td>
<td>a</td>
</tr>
</tbody>
</table>

F = 9.97  (p < 0.01)  SE = 1.92  CV = 28.5%

B. Mean percent inbound EB1 comets

<table>
<thead>
<tr>
<th>DISRUPTION CATEGORY</th>
<th>MEAN % INBOUND</th>
<th>SIGNIFICANCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>WILD-TYPE MUS (control)</td>
<td>21.8</td>
<td>c</td>
</tr>
<tr>
<td>WILDTYPE-LIKE mus-1 (category 1)</td>
<td>30.0</td>
<td>bc</td>
</tr>
<tr>
<td>MILD-mus-1 (category 2)</td>
<td>38.4</td>
<td>bc</td>
</tr>
<tr>
<td>STRONG-mus-1 (category 3)</td>
<td>56.8</td>
<td>ab</td>
</tr>
<tr>
<td>SEVERE-mus-1 (category 4)</td>
<td>79.9</td>
<td>a</td>
</tr>
</tbody>
</table>

F = 9.97  (p < 0.01)  S.E. = 1.92  CV = 32%

Although this ANOVA suggests that the percentage of inbound and outbound EB1 comets is associated with symmetry disruption in GC morphogenesis, inbound as well as outbound EB1 movement was present in both wild-type and mus-1. Therefore, the observed symmetry disruption may not be associated solely with either inbound or outbound movement. Instead, it may be useful to look at a combined effect based on both trajectory directions, such as the outbound to inbound ratio.

To test whether this ratio correlates significantly with symmetry disruption, a Chi Square test for independence was performed. Comet direction and symmetry
disruption were found to be closely associated ($\chi^2 = 51.61; p < 0.01$). There was a highly significant difference overall between the outbound:inbound ratios of microtubule growth trajectories (Table 2.2). Individual 2x2 contingency tables indicate that all mus-1 mutant stomata, except WILD-TYPE-LIKE mus-1 (category 1), displayed significantly lower outbound:inbound ratios (or conversely significantly higher inbound:outbound ratios) compared to the wild-type. Of the four stomatal categories present in mus-1, SEVERE-mus-1, which displays the highest degree of disruption, showed an outbound:inbound ratio that was lower than each of the other four categories, at a very high significance level ($p<0.01$) (Table 2.3a). There were no significant differences in the outbound:inbound ratios between other combinations (SEVERE-mus-1 and MILD-mus-1; MILD-mus-1 and WILD-TYPE-LIKE-mus-1; MILD-mus-1 and WILD-TYPE; and WILD-TYPE-LIKE-mus-1 and WILD-TYPE) (Table 2.3 A and B).

Since the outbound: inbound ratio and symmetry disruption parameters are not independent of each other, the Chi-Square analysis indicates that it is the outbound to inbound EB1 ratio which is significantly associated with symmetry disruption in mus-1 stomata, rather than either the inbound or the outbound EB1 movement by itself.
TABLE 2.2. Chi Square analysis of outbound: inbound ratio of EB1 trajectories

<table>
<thead>
<tr>
<th>CATEGORY</th>
<th>Chi-square</th>
<th>probability</th>
</tr>
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<tr>
<td>WILD-TYPE MUS</td>
<td>51.61</td>
<td>P &lt; 0.01**</td>
</tr>
<tr>
<td>WILD-TYPE-LIKE mus-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MILD mus-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STRONG mus-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEVERE mus-1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2 x 2 CONTINGENCY TABLES

| (1) SEVERE x WILD-TYPE MUS        | 40.845 | p <0.01** |
| SEVERE x WILD-TYPE-LIKE           | 30.303 | p <0.01** |
| SEVERE x MILD                      | 18.502 | p <0.01** |
| SEVERE x STRONG                    | 7.5481 | p <0.01** |

| (2) STRONG x WILD-TYPE MUS         | 15.424 | p < 0.01** |
| STRONG x WILD-TYPE-LIKE            | 8.6878 | p < 0.01 **|
| STRONG x MILD                      | 2.7008 | p >0.01 n.s.|

| (3) MILD x WILD-TYPE MUS           | 5.5456 | P > 0.01 n.s.|
| MILD x WILD-TYPE-LIKE              | 1.7759 | p > 0.01 n.s.|

| (4) WILD-TYPE-LIKE x WILD-TYPE     | 1.0824 | P > 0.01 n.s.|

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Table 2.3. The relationship between comet direction and symmetry disruption (n=10 stomata per category). Categories sharing the same letter are not significantly different

A. Outbound:Inbound ratio of microtubule trajectories (n=10)

<table>
<thead>
<tr>
<th>DISRUPTION CATEGORY</th>
<th>OUTBOUND:INBOUND RATIO</th>
<th>SIGNIFICANCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>WILD-TYPE MUS</td>
<td>3.60</td>
<td>c</td>
</tr>
<tr>
<td>WILD-TYPE-LIKE mus-1 (category 1)</td>
<td>2.33</td>
<td>c</td>
</tr>
<tr>
<td>MILD-mus-1 (category 2)</td>
<td>1.40</td>
<td>bc</td>
</tr>
<tr>
<td>STRONG-mus-1 (category 3)</td>
<td>0.76</td>
<td>b</td>
</tr>
<tr>
<td>SEVERE-mus-1 (category 4)</td>
<td>0.25</td>
<td>a</td>
</tr>
</tbody>
</table>

\[ X^2 = 51.61^{**} \]
\[ (p < 0.01) \]

B. Inbound:outbound ratio of microtubule trajectories (n=10)

<table>
<thead>
<tr>
<th>DISRUPTION CATEGORY</th>
<th>INBOUND:OUTBOUND RATIO</th>
<th>SIGNIFICANCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>WILD-TYPE MUS</td>
<td>0.28</td>
<td>c</td>
</tr>
<tr>
<td>WILD-TYPE-LIKE mus-1 (category 1)</td>
<td>0.43</td>
<td>c</td>
</tr>
<tr>
<td>MILD-mus-1 (category 2)</td>
<td>0.71</td>
<td>bc</td>
</tr>
<tr>
<td>STRONG-mus-1 (category 3)</td>
<td>1.31</td>
<td>b</td>
</tr>
<tr>
<td>SEVERE-mus-1 (category 4)</td>
<td>4.00</td>
<td>a</td>
</tr>
</tbody>
</table>

\[ X^2 = 51.61^{**} \]
\[ (p < 0.01) \]

To analyze ratios in the context of MUS function these two EB1 ratios were plotted against the degree of disruption of stomatal symmetry (Fig.2.21 A and B). The first signs of disruption appeared as the outbound:inbound microtubule direction ratio approached 1.5 and then gradually increased until the ratio decreased below 1, when the highest levels of disruption, as seen in STRONG-mus-1 and SEVERE-mus-1, occur (Fig.2.21 A). As would be expected, a very similar, but opposite, trend was observed in the inbound:outbound ratio vs. degree of disruption plot where, the first signs of disruption start to appear in the 0.5 ratio region and gradually increased until the ratio increased above 1,
when the highest levels of disruption, as seen in STRONG-\textit{mus-1} and SEVERE-\textit{mus-1} occur (Fig. 2.21 B). The highest level of disruption, as seen in SEVERE-\textit{mus-1} seems to occur when the outbound:inbound ratio reaches 0.25 (1 outbound:4 inbound) or, conversely, the inbound:outbound ratio rises to around 4 (4 inbound:1 outbound). The 1 outbound:4 inbound ratio appears to be associated with very high levels of disruption. Interestingly, the outbound:inbound ratio of 4:1 in the WILD-TYPE was reversed to 1:4 in the most severely abnormal \textit{mus-1} guard cells (SEVERE).

Overall, these data (along with the Chi-Square analysis), suggest that the specific 4:1 ratio of outbound to inbound EB1 movement is tightly associated with correct morphogenesis.

Fig. 2.21. Relationship between outbound: inbound ratios of EB1 movement and the extent of symmetry disruption (skewing) in \textit{mus-1} stomata. The Y-axis represents the ratio of outbound: inbound EB1 comets (trajectory direction ratio), and the X-axis represents symmetry disruption (degree of skewing), with 1 being no disruption or skewing and 5 being severe disruption or skewing. (A) High ratios of outbound to inbound comets
correspond to normally formed stomata in mus-1. (B) High ratios of inbound to outbound correspond to more abnormal (skewed) mus-1 stomata.

2.11 The growth rate of outbound or inbound microtubules is not related to guard cell symmetry

Because the direction of microtubule trajectories strongly correlates with stomatal symmetry, the rate of growth of microtubules was investigated by capturing the velocity of microtubule (+) end movement in wild-type and mus-1 stomata, with respect to the degree of disruption of symmetry. The effect of mus-1 on the velocity of microtubule growth was analyzed using the Kymograph tool (ImageJ software) (additional procedural details can be found in the Materials and Methods section). This tool generates a plot of the position of an object over time (on the y-axis) against the distance the object moved (x-axis). The angle of a line drawn in a kymograph represents the velocity of the object of interest.

A t-Test analysis of the initial data (n = 20 stomata) indicated that the mean inbound and outbound velocities for both wild-type and mus-1 were not significantly different (Table 2.4).

<table>
<thead>
<tr>
<th>EB1 direction</th>
<th>WILD-TYPE</th>
<th>mus-1</th>
<th>t</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inbound</td>
<td>1.07 (±0.10)</td>
<td>1.08 (±0.10)</td>
<td>-0.058</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Outbound</td>
<td>1.30 (±0.11)</td>
<td>1.14 (±0.12)</td>
<td>0.937</td>
<td>&gt; 0.05</td>
</tr>
</tbody>
</table>

These observations were confirmed by a more detailed ANOVA of inbound and outbound velocities of WILD-TYPE and 4 categories of mus-1, which also indicated no significant difference overall between either the inbound or outbound microtubule growth.
growth rates in WILD-TYPE and mus-1, or between the 4 categories of phenotypic severity in mus-1 (Table 2.5A and 2.5B).

### Table 2.5 A. Mean inbound EB1 comet (+end) velocities

<table>
<thead>
<tr>
<th>DISRUPTION CATEGORY</th>
<th>MEAN VELOCITY INBOUND</th>
<th>SIGNIFICANCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>WILD-TYPE MUS (control)</td>
<td>0.960</td>
<td>n.s.</td>
</tr>
<tr>
<td>WILD-TYPE-LIKE mus-1 (category 1)</td>
<td>0.918</td>
<td>n.s.</td>
</tr>
<tr>
<td>MILD-mus-1 (category 2)</td>
<td>1.310</td>
<td>n.s.</td>
</tr>
<tr>
<td>STRONG- mus-1 (category 3)</td>
<td>1.122</td>
<td>n.s.</td>
</tr>
<tr>
<td>SEVERE-mus-1 (category 4)</td>
<td>1.052</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

F = 0.6103 (p > 0.05)  S.E. = 0.10  CV = 41%

### Table 2.5 B. Mean outbound EB1 comet (+end) velocities

<table>
<thead>
<tr>
<th>DISRUPTION CATEGORY</th>
<th>MEAN VELOCITY OUTBOUND</th>
<th>SIGNIFICANCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>WILD-TYPE MUS (control)</td>
<td>0.886</td>
<td>n.s.</td>
</tr>
<tr>
<td>WILD-TYPE-LIKE mus-1 (Category 1)</td>
<td>0.984</td>
<td>n.s.</td>
</tr>
<tr>
<td>MILD-mus-1 (Category 2)</td>
<td>1.148</td>
<td>n.s.</td>
</tr>
<tr>
<td>STRONG- mus-1 (Category 3)</td>
<td>1.366</td>
<td>n.s.</td>
</tr>
<tr>
<td>SEVERE-mus-1 (Category 4)</td>
<td>0.990</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

F = 0.8727 (p > 0.05)  S.E. = 0.10  CV = 42%

Since the plus end growth rates of inbound or outbound MTs do not seem to be affected by mus-1, the rate of polar MT growth appears to be independent of direction as well as the degree of stomatal skewing and disruption. Together these analyses show that MUS is required primarily for the location and polarity of microtubule growth rather than for the growth rate of microtubules.
2.12 Microtubules in *mus* are disrupted prior to pore thickening.

As indicated, many *mus-1* stomata show abnormal microtubule arrays. The degree of their disruption varies from mild skewing of microtubule foci and microtubules, to complete disruption of the radial array (Fig. 2.2). As well, *mus* disrupts pore morphogenesis. While these two effects might be independent, it is not clear which process is primary (*e.g.* whether the disruption of microtubule arrays alters pore morphogenesis or vice versa). To analyze this relationship, young cotyledons were used to define stages of development in young stomata.

Stages of pore morphogenesis were first analyzed in wild-type stomata (*n* = 20 stomata for each stage). Six developmental stages were defined using confocal microscopy, ranging from GMCs to pore formation and maturation (Fig 2.22). The first stage represents newly divided GMCs producing two new GCs separated by a narrow dividing wall. Stage 2 is marked by a developing pore thickening on both sides of the new (ventral) cell wall. In stage 3, the pore wall thickenings become wider, but no opening is yet formed. In stage 4, a partial pore opening appears either on the periclinal wall facing the atmosphere, or the periclinal wall facing the mesophyll. By stage 5 stomata have pores developing on both (paradermal) sides of the stomata. Finally, stage 6 stomata represent mature stomata which have fully developed pores that overly a sub-stomatal cavity in the mesophyll.
Figure 2.22. Stages of pore morphogenesis in stomata from wild-type plants (Note that changes in stomatal and pore size as well as cell wall thickness through time are not shown).

In *mus-1*, all stage 1 stomata observed, displayed symmetric divisions that resembled the wild-type, as they did not show any skewing (*n* = 100 stage-1 stomata per genotype). The absence of skewing in very young *mus-1* stomata was confirmed by observing new symmetric divisions in stage-1 stomata, utilizing the cell plate marker *ProSYP111:GFP-SYP111* (*SYP111*, also known as *KNOLLE*; Enami *et al.*, 2009). In both wild-type and *mus-1*, newly formed cell plates were straight and properly intersected the end wall thickenings (*n* = 20 stage-1 stomata per genotype) (Fig. 2.23).

However microtubules visualized at this stage (using *Pro35S:TUA-GFP* and *Pro35S: EB1-GFP*) were altered in distribution and dynamics (*n* = 10 stage-1 stomata) (Figs. 2.24 and 2.25). Skewed pore thickenings appeared in stage 2 in about 30% of *mus-1* stomata sampled (*n* = 100 stage-2 stomata per genotype). This observation suggests that microtubule disruptions in stage-1 *mus-1* stomata might cause the pore skewing observed in stage-2 stomata.

Alternatively, microtubule disruption might not be the primary cause of pore disruption, and
instead these two phenotypes might result from independent defects caused by mutations in 

*MUS.*

Fig. 2.23. New symmetric divisions in wild-type and mus-1 GMCs are normal. Green fluorescence represents *ProSYP111:GFP-SYP111* localization, while red fluorescence represents cell walls stained with propidium iodide. (A) Wild-type stomata. The left micrograph shows a GMC undergoing a symmetric division, as denoted by a partial cell plate (white arrows). The GMC in the right micrograph displays an older cell plate, suggesting that GMC division is almost complete. As well, *ProSYP111:GFP-SYP111* marks the stomatal periphery. (B) Two developing mus-1 stomata. The GMC in the left micrograph is undergoing a symmetric division, as denoted by the partially detached cell plate (white arrows). The GMC in the right micrograph displays an older cell plate, suggesting that GMC division is almost complete. As well, *ProSYP111:GFP-SYP111* marks the stomatal periphery. Scale bar in (A) = 5 µm, and also represents (B).

Figure 2.24. The mus-1 microtubule defect starts after symmetric division. White arrows indicate position of ventral pore wall. (A) Young wild-type stoma displaying roughly bilaterally symmetrical microtubule foci and radial microtubule arrays opposite each other in the two guard cells. (B-C) Two different young mus-1 stomata (stage two), shortly after symmetric division, displaying disrupted, non-radial microtubule arrays. Note that the plane of focus is close to the outer paradermal walls in order to capture microtubule arrays in the cell cortex. In this plane of optical section, the dorsal walls are oblique and thus not as sharply in focus as in the mid-depth of the stoma. Scale bar in (A) = 5 µm, and also represents (B-C).
Figure 2.25. The mus-1 defect in EB1 movement starts after the symmetric division. Red circles represent EB1 comets exhibiting abnormal movement (towards the pore, or circumferential movement). Yellow circles represent EB1 movement from the center to the periphery. (A) Young wild-type stoma. Four consecutive frames of a time lapse series taken at 2 second intervals are shown. Green fluorescence represents Pro35S:EB1-GFP, while red fluorescence (in A) derives from propidium iodide staining of the cell wall. As in mature stomata, most microtubules move outward from the center of the young stoma. (B-C) Young, mus-1 stomata with disrupted microtubule dynamics. Although only a few microtubule ends are shown in each optical section, the directions of EB1 movement are consistent with quantitative data from larger samples. The first four frames in each of B and C are from a time lapse series taken at two second intervals. The fifth frame is a micrograph of the stoma shown in the first four frames except that the plane of focus is at the mid-depth and shows the absence of cell wall skewing in these particular mus-1 stomata. (B) mus-1 stoma. In the young GC on the left, the majority of microtubules appear to move inward from the periphery to the center, whereas in the young GC on the right microtubules exhibit both inward and outward movement. (C) mus-1 stoma. In the young GC on the top, the majority of microtubules appear to move inward from the periphery and into the center, whereas in the young GC on the bottom the majority of microtubules exhibit circumferential movement. Scale bar in (A) = 5 µm, and also represents (B-C).
2.13 No evidence that other cytoskeletal and endomembrane components are polarized in wild-type or mus

The possibility of mus-1 affecting the distribution of other cellular components was also investigated, since such data might reveal additional information about MUS function. Thus, a variety of other markers were used including those related additional cytoskeletal components as well as to membrane compartments.

Transgenic plants containing an ACTIN BINDING DOMAIN 2 GFP construct (Pro35S:GFP-ABD2) (Sheahan et al., 2004), were analyzed in wild-type and mus-1 seedlings. This construct only labels large actin bundles, and has been reported to induce abnormal actin bundle formation (Sheahan et al., 2004; Era et al., 2009). mus-1 stomata that resembled wild-type stomata in shape, showed many actin bundles arranged across the short diameter of the guard cells, a pattern that is partially radial (fig. 2.26A). Abnormally shaped mus-1 guard cells, showed actin arrays to be mostly non-radial in distribution, a disrupted pattern that is roughly similar to that of mus-1 microtubule arrays (Fig. 2.26B-C). Thus, MUS also appears to be required for the arrangement of actin filaments in stomata. However, since the bundling caused by the Pro35S:ABD2-GFP construct distorts normal actin organization, analysis of this probe alone is not sufficient to determine whether the disruption is an effect solely of the mus-1 mutation.
Figure 2.26. Actin organization appears disrupted in mus-1 stomata. (A-C) Maximum Z-projections of Pro35S:GFP-ABD2 in mature mus-1 stomata (n=15). (A) Wild-type-like stoma in a mus-1 mutant background showing a partially radial pattern. (B) Severely skewed mus-1 stoma. (C) Disrupted mus-1 stoma displaying abnormal morphogenesis. Scale bar in (A) = 5 µm, and also represents (B-C).

As shown, the mus-1 mutation can induce ectopic wall thickenings to form on the dorsal walls (wall opposite the pore) of some stomata (Fig.2.2). This observation suggests that MUS might be involved in targeting, or correctly positioning, cellular components required for cell wall synthesis including Golgi-mediated secretion. This possibility was tested by examining the placement of Golgi stacks using the marker pro35S:SIALYLTRANSFERASE-mGFP (Pro35S:ST-mGFP) (Boevink et al., 1998). No difference could be detected between the distributions of Golgi in wild-type and mus-1 stomata in the small sample of wild-type and mus-1 seedlings (n = 5 wild-type, n = 16 mus-1) observed (Fig.2.27). It was only feasible to view a small sample of young or mature stomata since the construct underwent rapid silencing (within one day of germination). Thus, because of the limitations of the actin and Golgi probes, it was not possible to detect additional defects in the distribution of components other than microtubules and the cell wall.
Figure 2.27. *Pro35S:ST-mGFP* localization does not appear to be altered in *mus-1*. (A) Young wild-type stoma (lower left). (B) Mature wild-type stoma. (C) Developing and mature *mus-1* stomata (center and left respectively). Scale bar in (A) = 5 µm, and also represents (B-C).
3. Discussion

MUSTACHES is a Leucine Rich Repeat Receptor-Like Kinase required for generating stomatal symmetry. Based upon loss-of-function phenotypes, MUS acts specifically during guard cell morphogenesis by regulating the symmetry of the pore and stoma. While MUS expression is broadly distributed throughout the leaf and root, phenotypic defects were only found in stomatal morphogenesis. These defects likely result from altered microtubule arrays and dynamics, suggesting that MUS regulates guard cell microtubule organization directly or indirectly.

The generation of symmetry in shape of both the pore and the two guard cells suggests the involvement of a complex signaling network. MUS likely acts in signaling via LRR domains, a putative kinase domain, and a transmembrane domain. The eventual elucidation of this pathway should help in understanding how interactions between molecular and cellular components, such as a receptor complex, the cytoskeleton, and the cell wall, are translated into specific events that control the construction of a functional valve with strong bilateral symmetry.

3.1 MUS regulation of stomatal bilateral symmetry is likely mediated through microtubule array organization and dynamics

Previous studies in the Sack and Nakagawa labs showed that mature mus mutant stomata display abnormal phenotypes ranging from mild skewing of radial microtubule arrays and of microtubule foci across the two guard cells, to a complete disruption of the array (Nadeau and Lucas, unpublished). The work here identifies additional defects in microtubule arrangement and dynamics.
Mutations in MUS alter microtubule polarities, since in many mus-1 stomata about half of the microtubule plus ends grow from the periphery of stomata towards the pore, instead of mostly away from the pore as in the wild-type. This suggests that in wild-type stomata most microtubules are nucleated at the pore and that MUS is required for net polarity. Since γ-TURCS are involved in microtubule nucleation and most minus ends of these microtubules appear to originate near the pore wall (Marc et al., 1989), MUS appears to be required for coordinating the polarity of γ-tubulin ring complex formation at the pore (pore wall nucleation) (Fig. 3.1).

Alternatively, or in addition, MUS might control microtubule dynamics and organization by limiting the extent of microtubule growth in stomata (microtubule length limiting). Cortical microtubules in stomata appear to nucleate from ‘microtubule organizing centers,’ or ‘MTOCs’ located in the center of the pore wall (McDonald et al., 1993; Willmer and Fricker, 1996; Marc, 1997). By focusing throughout the depth of living stomata, it appears that microtubules grow from the pore towards both the top and the bottom of the stoma (Willmer and Fricker, 1996). Thus it is possible that in wild-type stomata, MUS limits the length of microtubule growth such that, regardless of whether microtubules grow towards the top or bottom of the stoma, only microtubule growth equivalent to the distance from the pore to the guard cell periphery is permitted. In a mus-1 background, microtubules might grow longer until they reach the dorsal wall and grow along the other paradermal wall back towards the stomatal pore. This type of increase in microtubule length would make it appear as if the microtubule were nucleating from the periphery and growing back into the pore. Confirmation of a microtubule length-limiting hypothesis would require extended time lapse studies.

Support for the pore wall nucleation hypothesis might be found in the observation that
microtubule foci in highly skewed mus-1 stomata, as determined by the concentrations of microtubules near the stomatal pore in the wild-type (Marc et al., 1989), appear to be skewed as well. If the function of MUS were restricted to limiting microtubule length, then this might not explain the observation of the skewing of microtubule foci. Attempts were made to test the pore wall nucleation hypothesis by utilizing antibodies against γ-tubulin (obtained from B. Oakley), but were unsuccessful.

Recent studies in both Arabidopsis and Drosophila show that regulation of γ-TURC localization is mediated through phosphorylation. In Drosophila, the localization of γ-TURCs to the centrosome is mediated through a gamma complex protein containing WD40 repeats (GCP-WD), a component of the γ–TURC (Luders et al., 2005). The GCP-WD contains amino acid sequences characteristic of cyclin dependent kinase (CDK)-mediated phosphorylation sites (SPIR motifs), suggesting that CDK phosphorylation directly targets γ-TURC to the centrosome. The localization of the Arabidopsis GCP-WD orthologue, AtNEDD1, also depends upon on CDK phosphorylation (Zeng et al., 2009). However, in contrast to animals, plants do not contain centrosomes, and instead γ -TURCs in plant cells have been found to localize along microtubules, the periphery of epidermal cells, and the pore in wild-type stomata (McDonald et al., 1993; Murata et al., 2005; Seltzer et al., 2008). Therefore, γ-TURC localization in plants might require signaling proteins and pathways in addition to CDKs. The effect of mus on the direction of microtubule movement might indicate that a putative MUS complex might control the localization of γ-TURCs in stomata. However, further investigations are needed to determine the extent to which MUS signaling affects γ-TURC localization, and/or microtubule length.
Figure 3.1 Model showing how MUS mutations might affect polarity of EB1 movement through disruption of γ-TURCs localization. Black ovals represent localization of γ-TURCs, green arrows represent EB1 moving out from the pore, and purple arrows represent EB1 moving towards the pore. In wild-type stomata (left) the majority of EB1 comets move outwards from the pore, suggesting that the majority of γ-TURCs are located near the pore. In mus-1 stomata (right), a large number of EB1 comets move inward, suggesting that more γ-TURCs are localized at the dorsal wall (outer peripheral wall).

This work shows that it is the ratio of inbound to outbound EB1 trajectories that correlates most strongly with the extent of disruption of guard cell symmetry, rather than the number of individual inbound or outbound trajectories themselves. Whereas wild-type Col-0 plants display 4:1 ratio of outbound to inbound trajectories, this ratio was reduced to about 1:4 in the most morphologically skewed/disrupted category of mus-1 stomata. Although highly correlated, these data do not establish cause and effect such as whether microtubule polarity induces wall skewing or vice versa.
3.2 The first defect detected in mus stomata development occurs after symmetric division and before pore formation

Microtubules regulate localized secretion and deposition of materials into the cell wall. Examples include wall thickenings in tracheids and the mucilage pockets found in Arabidopsis seed coats (Ingold et al., 1988; McFarlane et al., 2008). Microtubules also appear to function in regulating stomatal pore morphogenesis, as they are abundant at the site of the developing pore thickening where they might contribute to ordered wall deposition in developing stomata (Palevitz and Hepler, 1976; Galatis and Mitrakos, 1980; Sack and Paolillo, 1983; Zhao and Sack, 1999). Similarly the disruption of the symmetrical placement of pore thickenings and pores by mus-1, suggests that MUS is also required for microtubule organization and dynamics in pore building. In some developing mus-1 stomata which had divided symmetrically, microtubule arrays were disrupted prior to the formation of the pore thickening itself. However, the skewing of the new cell wall only seemed to occur after pore thickening took place. Nor was skewing observed in the cell plates of newly established symmetric divisions in mus-1, further indicating that the first defect in bilateral symmetry in mus-1 occurs after a normal symmetric division, but prior to pore formation.

The actual opening of the pore likely occurs developmentally when the wall thickenings are torn apart during guard cell expansion when turgor increases. Localized and enzymatic wall breakdown might also contribute to the opening process. Turgor forces in both developing and in mature GCs might be distributed throughout the stoma by the radial cellulosic array (Palevitz and Hepler, 1976; Galatis and Mitrakos, 1980; Sack and Paolillo, 1983; Zhao and Sack, 1999). Due to the likely central role played by microtubules during pore morphogenesis, any disruptions in the organization or dynamics of microtubules occurring in this early stage might
be expected to lead to the altered placement of pore thickenings and of morphogenesis. Because both guard cells still enlarge after symmetric division, the asymmetrical thickening of the young pore wall in mus might alter the dispersal of turgor pressure. This in turn could amplify the mechanical imbalance between the two guard cells. Although some stomata in mus-1 show straight walls between the ends of the guard cells and the pore thickening, the pore axis itself can be oblique, suggesting an accommodation to mechanical stresses by pore skewing. While much remains to be determined about MUS function, this protein seems to be required for ensuring that the newly formed cell wall stays straight during pore formation, a control that is essential for producing a bilaterally symmetric valve for gas exchange.

3.3 MUS encodes a leucine rich repeat receptor-like kinase, which is potentially kinase defective

Receptor-like kinases typically mediate signal transduction by phosphorylating downstream targets. However, there are also several examples of ‘atypical’ receptor-like kinases, such as STRUBBELIG (SUB) and MAIZE ATYPICAL RECEPTOR KINASE (MARK), which do not function as kinases, but are still able to mediate signalling (Chevalier et al., 2005; Llopmart et al., 2003). Both plant and animal kinases exhibit a generic core organization which consists of a two-lobed structure, where one lobe anchors and orients ATP molecules, while the other lobe binds protein substrates and initiates phosphate transfer (Castells and Casacuberta, 2007). The two lobes making up a kinase can be further divided into 12 sub-domains (I – XII). Each sub-domain contains invariant, conserved amino acid residues which are essential for phosphorylation. Atypical kinases lack, or contain substitutions in these invariant amino acids, and therefore may lack, or have reduced kinase activity.
Castells and Casacuberta (2007) conducted a systematic study of kinase domains in plant RLKs and classified (in their supplemental data) the wild-type MUS kinase as an ‘atypical kinase,’ which contains a substitution in an essential Aspartic acid (D) in lobe 2 of the kinase. In the wild-type MUS protein Glutamic acid (E) is substituted for the Aspartic acid (D) residue in the DFG motif in kinase sub-domain VII, (fig.2.4) (Castells and Casacuberta, 2007). The presence of this substitution suggests that MUS contains a defective or non-functioning kinase. Similarly, another atypical receptor, Trans-Membrane Kinase-Like (TMKL1) (Valon et al., 1993), shows a DFG to EFG substitution and contains an inactive kinase.

Comparison of amino acid sequences encoded by the wild-type MUS gene and the most phenotypically severe allele, mus-1, revealed that mus-1, harbors a point mutation (substitution) in residue G1030 (which is downstream of the DFG/EFG motif). G1030 is located in kinase sub-domain IX, which is involved in the stabilization of the catalytic loop in kinases. IX has two invariant amino acids, one of which is the G (glycine) that is substituted in mus-1. A substitution in the same residue (G1030) in the CLAVATA1 gene, the clavata 1-2 (clv1-2) allele, induces an intermediate dominant negative phenotype. The CLAVATA1 gene, encodes an LRR-RLK that normally limits the size of the shoot apical meristem. Null clv1 alleles (clv1-6, clv1-7, clv1-11, clv1-12, and clv1-13) display relatively weak phenotypes with somewhat enlarged meristems and club-shaped siliques (Diévart et al., 2003). Notably, clv1-2/CLV1 heterozygotes show more severe apical meristem and silique phenotypes than homozygous clv null mutants (Diévart et al, 2003). Since mus-1, like clv1-2, contains a point mutation that is predicted to alter the G1030 residue, it is possible that a single mus-1 allele might also exhibit a dominant negative phenotype. However, heterozygous mus-1/MUS1 plants only contain wild-type stomata, consistent with the mus-1 allele behaving as a recessive.
The weaker phenotype observed in \textit{clv} null mutants compared to the \textit{clv1-2} dominant negative allele is due to the presence of two other LRR-RLKs, BARELY ANY MERISTEM 1 (BAM1) and BARELY ANY MERISTEM 2 (BAM2) which are closely related to CLV1. This redundancy is shown by the ability of BAM1 and BAM2 to partially rescue meristem size in \textit{clv} mutant plants, indicating that BAM1 and BAM2 exhibit partial functional redundancy with CLV1 (DeYoung \textit{et al.}, 2005). When CLV function is absent, as in the case of \textit{clv} null mutants, BAM1 and BAM2 likely compensate. By contrast, in plants heterozygous for \textit{clv1-2}, the mutated CLV1 receptor is still able to dimerize with its partner (CLV2) thereby inhibiting the binding of BAM1 and BAM2 to CLV2, blocking downstream signalling, and inducing a severe phenotype.

Phylogenetic analysis indicates that BAM1 and BAM2 are closely related to CLV1 (Deyoung \textit{et al.}, 2005). The \textit{Arabidopsis} genome also contains a gene, \textit{At1g43680}, which has high sequence similarity to MUS (MUS-like, or “MUSL”) based upon a phylogenetic tree generated by Tax \textit{et al.} (2004) (Fig. 2.4). However, the stomata in \textit{musl} homozygous mutants appear normal, and \textit{mus/musl} double mutants resemble \textit{mus} mutants in stomatal phenotype (Nadeau, unpublished data), suggesting that \textit{Arabidopsis} lacks other receptors that are functionally redundant with MUS.

The phenotypes of three different \textit{mus} alleles have been characterized and they display phenotypes of varying severity. The most severe phenotype was found in \textit{mus-1}, which contains a point mutation in the kinase domain. The phenotypes of the two other alleles- \textit{mus-2}, which contains a stop codon near the beginning of the gene, and SALK 03282, which contains a T-DNA insertion in the LRR domain, display phenotypes with decreasing levels of severity. Despite this range in severity, all three alleles exhibit low phenotypic expressivity and
penetrance. This lack of penetrance might be due to additional pathways that are independent of MUS, which also promote stomatal bilateral symmetry. Alternatively, the low penetrance might be due to the ‘atypical’ kinase contained in MUS. Atypical kinases, which appear to lack, or have reduced kinase activity, might act via phosphorylation-independent signalling (Castells and Casacuberta, 2007). However, some ‘atypical’ plant kinases function indirectly in phosphorylating downstream targets by forming complexes with proteins that are capable of phosphorylation. A well known example of such a signalling module is MAIZE ATYPICAL RECEPTOR KINASE (MARK), an atypical kinase. Although lacking a functional kinase, MARK interacts with the GCK-like kinase MIK in vivo, an interaction that enables MIK to phosphorylate substrates (Llopmart et al., 2003).

Thus, MUS might not function as a kinase, and might instead interact with and perhaps stabilize the conformation of a partner protein, which is then able to phosphorylate downstream targets. During interactions between partner proteins, the stability of signaling complexes is likely to depend upon protein-protein interactions and precise binding. By this reasoning, mus might disrupt specific protein-protein interactions, thereby decreasing the stability of MUS-dependent protein complexes. Despite a decrease in complex stability due to the mus mutation, binding of the partner protein with downstream targets might still be possible in some, but not all stomata. Such a partial disturbance in binding would cause only certain stomata to display phenotypes, thereby explaining the incomplete penetrance observed in the mus stomatal phenotype (fig.3.2).
Figure 3.2. Model for *mus* disruption of symmetry. In wild-type plants MUS interacts with an unknown partner (X), shown here as another LRR-RLK. This interaction might allow the X partner to attain the correct conformation to bind and phosphorylate (red circle) an interactor (Y), thereby mediating downstream signal transduction. The *mus* mutation might disrupt binding interactions so that in some stomata, X does not achieve the right conformation to bind Y, and signal transduction fails. However, in most *mus* stomata, X might be able to bind Y, thereby mediating signal transduction, and allowing bilateral symmetry to be achieved.

### 3.4 Model for MUS action in stomatal development

Components of the MUS signaling pathway, as well as the molecular functions of MUS remain unknown. However, analysis of *MUS* distribution has revealed highly stage-specific distribution patterns that can also depend upon the cell type. MUS localizes to the cell plates of all dividing leaf epidermal cells, except for the division which produces the new dividing cell wall of the stoma (fig.3.3). Because the only abnormal phenotype detected in *mus* plants was detected after this division, the absence of *ProMUS:MUS-GFP* distribution in the cell plates that produce the stomatal pore is both unexpected and intriguing. MUS exhibits a specific subcellular distribution pattern which has not previously been described for stomata. The
absence of MUS from the newly formed wall of young stomata, in addition to its peripheral
distribution in GMCs and young stomata, suggest that MUS signaling mediates bilateral
symmetry generation and the positioning of the pore from the cell periphery.

Plants provide several examples of signaling pathways that direct internal processes
from the cell periphery. One such example is the ROP-GTPase-mediated control of pavement
cell morphogenesis. Pavement cells consist of lobes, which undergo expansion, and of
indentations, which do not expand. Lobes harbor predominately diffuse F-actin, and contain relatively few microtubules (Fu et al., 2005). Since microtubules might restrict cell expansion, the lack of microtubules in lobes might facilitate lobe expansion. In contrast, the indentations predominately contain organized microtubule arrays, and very little F-actin, consistent with the inability of indentations to expand. The exclusion of microtubules from lobes and vice-versa is mediated through crosstalk between peripherally localized ROP2/4 GTPases and their corresponding Rop-interactive CRIB motif-containing proteins (RICs) (Fu et al., 2005). Therefore, the ROP signaling pathway demonstrates how peripherally localized proteins can affect the organization of internal components of the cell.

Yeast also provides an example of the regulation of internal cellular processes by peripherally localized proteins. Schizosaccharomyces pombe (fission yeast) also undergoes a tightly regulated symmetric division. The placement of this division results from a signaling cascade initiated by Pom1, a peripherally localized DRYK kinase. Pom1 signaling from the periphery mediates division site placement by negatively regulating an internally-located kinase, Cdr2, which controls the localization of Mid1p, an anillin-like protein that in turn marks the future division site. Thus Pom1 signaling from the periphery determines the internal site of cell
A model of how MUS might regulate bilateral symmetry by transmitting signals from the periphery of the developing stoma would involve regulation of microtubule nucleation and growth in stomata. As mus might mis-localize γ-TURCs near the pore, the ratio of gamma tubulin complexes at the pore to those at the cell periphery might be important for maintaining bilateral symmetry. MUS might control microtubule organization by positioning gamma tubulin ring complexes at the site where a pore thickening will later form. During cell division, gamma tubulin is distributed to different positions in the cell, such as the metaphase spindle. After the symmetric division has occurred, gamma tubulin complexes must be correctly redirected to the site where the pore will form. The MUS signaling pathway, therefore, might be responsible for ensuring that the correct ratio of gamma tubulin complexes is maintained at the site of the pore thickening, thus ensuring the maintenance of stomatal bilateral symmetry.

Figure 3.3. MUS distribution throughout stomatal development. Red represents cell walls, dark green represents ProMUS:MUS-GFP localization, and light green represents areas in the GMC and young stoma where polar localization of MUS can be observed. MUS is localized to the cell
plate in early and late meristemoids. As the meristemoid transitions into a guard mother cell, MUS cell plate localization also begins to transition into peripheral, cell membrane, localization during the late meristemoid-early GMC stage. Strong peripheral MUS localization persists in the GMC and young stomatal stage. During these stages, some stomata also display polar localization of MUS near the sites of end wall thickenings, as represented by the light green shading. Weak MUS localization can be observed in mature stomata.
4. Materials and methods

4.1 Growth of plant material

*Arabidopsis thaliana* seeds (Col-0 ecotype) were planted onto sterile ½ strength Murashige and Skoog (MS) media containing 0.08% w/v agar. Seeds were germinated in a 21° C incubator, with a 16 hour day and 8 hour dark photoperiod.

4.2 Source of *mus* alleles

The *mus*-1 (*Col-0 gl-1* background) and *mus*-2 (*Ler* background) alleles were identified by Jeanette Nadeau and Tsuyoshi Nakagawa from seed populations mutagenized with fast-neutron bombardment and EMS respectively. The SALK_03283 and SAIL_82_D11 alleles were obtained from the SIGnal-SALK collection of T-DNA insertional mutants.

4.3 Introgression of marker lines into *mus*-1

Plants containing *Pro35S:EB1-GFP* (Komaki et al., 2010) were introgressed into *mus*-1 by Dr. Jie Le. Plants harboring constructs of *Pro35S:TUA-GFP* (Ueda et al., 1999), *Pro35S:ABD2-GFP* (Sheahan et al., 2004), and *Pro35S:ST-mGFP* (obtained from Chris Hawes) were introgressed into *mus*-1 by Jessica Lucas. Seeds harboring *Pro35S:ER-GFP* and *Pro35S:ΔTIP-GFP* were provided by David Ehrhardt and Chris Somerville respectively and introgressed into *mus*-1 (Cutler et al., 2000).
4.4 Construction of ProMUS:MUS:GFP lines

Seeds containing ProMUS:MUS:GFP-GUS that complemented mus-1 were provided by T. Nakagawa (Shimane University, Japan). To validate MUS distribution patterns observed in these lines, two independent ProMUS:MUS-GFP constructs were produced. One construct was generated using the primers: 5’-CACCAAACATTAGAAGGCATGTCTA - 3’ (Forward), 5’-CCGCCGCAACCGCGTTTCT-3’ (Reverse) and was identical to T.Nakagawa’s construct. Since T. Nakagawa’s construct contains a 3’ end portion from the gene preceding MUS, a second construct containing sequences belonging solely to MUS was also generated. Primers used to generate the second construct were: 5’- CACCTCTTCACCGATGTCGAAGATATA - 3’ (Forward), 5’-CCGCCGCAACCGCGTTTCT-3’ (Reverse). PCR products obtained from both sets of primers were cloned into pCR2.1-TOPO entry vectors. The resulting constructs were chemically transformed into TOP10 competent cells. TOP10 colonies containing the construct were identified through selection on LB media containing 100 µg/ml Kanamycin. Purified pCR2.1-TOPO from successfully transformed colonies was recombined with destination vector pGWB4. The resulting constructs were then transformed into chemically competent DH5α E coli. Efficiently transformed DH5α colonies were isolated using selective screening on LB media containing 100 µg/ml Kanamycin and 100 µg/ml Hygromycin. Purified pGWB4 from the selected DH5α colonies were transformed into electro-competent Agrobacterium strain GV301. Agrobacteria containing pGWB4 were identified utilizing LB media containing 50 µg/ml hygromycin and 50 µg/ml rifampicin. Successfully transformed colonies were used in Agrobacterium-mediated transformation of 5-6 week old wild-type (Col-0 ecotype containing gl-1), and mus-1 plants.

T1 generations were selected under growing conditions outlined above, except that ½ strength MS media containing 25µg/ml hygromycin was used to select seedlings containing the
ProMUS:MUS-GFP constructs. Analysis of T1 transformants provided one independent line from a Col-0 gl-1 background, containing T. Nakagawa’s construct. For the short construct, 8 independent T1 lines were identified in a wild-type background and 3 independent, complemented lines were isolated in a mus-1 background.

4.5 Quantitative PCR

RNA was extracted from whole seedlings of Col-0, mus-1, mus-2, SALK, and SAIL alleles 10 days after germination. Reverse transcriptase (RT-PCR) was performed on two biological replicates for each allele utilizing Oligo-dT primers. Quantitative PCR was performed with the following MUS gene-specific primers: 5’ CGT TTA GAG ATG GGA TGG TTC 3’ (Forward), and 5’ CCG TGA TGT TCT TGT GCT TC 3’ (Reverse). Levels of mus expression were calculated relative to wild-type (Col-0), with wild-type representing 100%, by utilizing the deltaCT method (Livak et al., 2001). Expression values from the two replicates were then averaged to obtain the mean expression level (%) for each allele.

4.6 Microscopy

4.6.1 Image acquisition

Images were obtained using a Zeiss-Pascal Excite laser scanning confocal microscope, or a Nikon C1 laser scanning confocal microscope. Both microscopes were equipped with 488 nm and 543 nm lasers. Images obtained on the Zeiss-Pascal confocal were captured using LSM Image Browser software, whereas images obtained from the Nikon were captured using Nikon-EZ C1 software. All images were further analyzed using either LSM Image Browser software, or ImageJ software.
4.6.2 Imaging and data analysis of Pro35S:EB1:GFP in Col-0 and mus-1

Samples were obtained from young cotyledons, or first leaves. All time-lapse images were obtained on the Zeiss-Pascal Excite confocal, using a 63X oil DIC Plan Apochromat objective (NA 1.4). Time lapse images were taken for 15 frames at 2 second intervals. To minimize tissue stress and damage, each leaf observed was exposed to laser illumination for a maximum of 10 minutes. Net direction of EB1 movement in wild-type and mus-1 was analyzed utilizing ImageJ software. Estimates of net EB1 movement were obtained by observing 6 EB1 comets per stoma observed (n = 300 comets per genotype). The Kymograph plug-in, provided by ImageJ software, was used to calculate velocity of EB1 movement in wild-type and mus-1 stomata. Again, 6 comets per stomata were selected to determine mean velocity differences. In order to determine whether velocity differences existed between inbound and outbound comets in wild-type and mus-1 stomata, an additional sample of 1 inbound EB1 comet, and 1 outbound comet was obtained from each of five stomata per genotype.

4.6.3 Imaging of other fluorescent markers in mus-1

Pro35S:TUA-GFP, Pro35S:ST-GFP, Pro35S:ER::GFP, were visualized using the 63X oil DIC Plan Apochromat objective (NA 1.4) objective on the Zeiss-Pascal Excite confocal. In order to visualize cell boundaries, an aqueous solution containing 1mg/ml propidium iodide (PI) was applied to samples for 2-3 minutes. PI solutions were washed away with distilled water prior to initiation of imaging. All optical sections obtained were captured at 0.37 µm intervals. 3D projections were generated utilizing LSM image browser software, while Z-projections were obtained utilizing ImageJ software.
Images of Pro35S:ΔTIP-GFP, and Pro:SYP111:SYP111GFP, in mus-1 were obtained using the 60X oil Plan Apo VC objective (NA 1.4) on the Nikon CLSM. Imaging was performed as detailed above for the Zeiss-Pascal. Time lapse movies were generated using ImageJ software.

4.7 Statistical analysis

4.7.1. Categorization of the degree of skewedness

For the purpose of data analysis the stomatal phenotypes were categorized as WILD-TYPE (normal pore and wall) as well as in 4 stomatal types found in mus: WILD-TYPE-LIKE (phenotypically wild-type in a mus-1 background), MILD (phenotypically close to wild-type, but slight skewing/misalignment of wall and pore), STRONG (severely skewed and misaligned pore wall) and SEVERE (missing or ectopic pores, improper morphogenesis and abnormally expanded guard cells) (Fig. 2.1).

4.7.2. Polarity of microtubule trajectory direction (movement)

Percent inbound and outbound comets: Ten stomata each were selected from WILD-TYPE and the four mus phenotypic categories. Six comets selected from each stoma were observed for the direction of their plus end movement, which was categorized as inbound for comets moving towards the pore from the periphery, and as outbound for comets moving towards the periphery from the pore. The numbers of outbound as well as inbound comets per stoma were then calculated as a percentage of all comets observed per stoma (TABLE B1).

Inbound: As the analysis involved five treatments (i.e. WILD-TYPE, WILD-TYPE-LIKE mus-1, MILD mus-1, STRONG mus-1, and SEVERE mus-1), ANOVA was used to compare means. Percent inbound comets in the WILD-TYPE category, as well as the four mus categories, were
then converted to proportions, weighted by adding \( \frac{3}{4} \) to the numerator and \( \frac{1}{4} \) to the denominator (Stephens, 2006), in order to correct sample proportions to be representative of population proportions for samples of \( n \leq 50 \). Corrected proportions were then transformed into arcsine values (degree) and subjected to ANOVA, to test for real differences in the mean percentages of inbound comets between the above five categories. Tests for assumptions of ANOVA indicated that the data met the assumptions for normality and homogeneity of variances. Comparisons between mean pairs were conducted using the Tukey-Kramer pairwise comparison test. All statistical analysis was performed using the PAST statistical package.

**Outbound**: The above procedure for inbound was repeated for outbound.

**Ratio of inbound:outbound comets**: The ANOVA indicated significant differences in the proportions of inbound and outbound comets between WILD-TYPE and the four mus categories. Thus differences were tested for significance in the ratio of inbound:outbound comets between stomatal phenotypes exhibiting different degrees of skewedness by the 5 categories (WILD-TYPE (MUS), WILD-TYPE-LIKE mus-1, MILD mus-1, STRONG mus-1 and SEVERE mus-1). For this purpose, the total outbound/total inbound comet ratios from 10 stomata of each of these 5 categories (TABLE B 9) were subjected to nonparametric Chi-square analysis both for independence and pair-wise comparison (2x2 contingency tables) using the PAST statistical package.

4.7.3. Velocity of microtuble trajectories

**Inbound velocities**: One inbound MT comet was selected from each of 20 wild-type stomata, by placing a pointer blindly on one spot and choosing the first comet to pass that spot. The same procedure was repeated for 20 stomata of mus) (Table A1). The velocity of each
As the analysis involved five treatments, ANOVA was the statistical test of choice for comparing means. Samples of one inbound comet each from five selected stomata of WILD-TYPE (MUS) and the four categories of mus were used to test for real differences between the mean inbound velocities of these five categories (Table A2). Tests for assumptions of ANOVA indicated that the data met the assumptions for normality and homogeneity of variances. As the ANOVA did not indicate any significant differences between the 5 categories no pairwise comparison test was conducted.

Because the ANOVA did not indicate any differences in the mean velocities between the 4 categories of mus phenotypes, these samples were pooled into a larger sample of (n = 20 stomata) and compared to a similar-sized wild-type sample using a paired mean t-TEST. Both wild-type and mus comet velocity samples (n=20) were checked for normality using Normal Probability Plot as well as the Shapiro Wilks Test via the PAST Statistical Package, and were found to fit normality.

All statistical analyses were performed using the PAST statistical package. The above procedure was repeated for outbound comet velocities.
5. Conclusion

5.1. Summary of research and significance of findings

Stomatal development, particularly processes involved in GC formation, provides an excellent model system to investigate how complex morphogenetic processes are coordinated between cells. Much current research in stomatal development concerns how stomatal formation is initiated and patterned. In contrast, relatively little research has been conducted on genes and underlying mechanisms involved in the stomatal morphogenesis.

Unlike other genes known to affect GC morphogenesis, MUS only appears to act after the symmetric division has been established. The observation that MUS distribution is peripherally-localized rather than in the GMC cell plate, suggests that the MUS LRR-RLK and the signaling pathway that it mediates, act in a novel way compared to other known LRR RLKs or other known stomatal genes. While some RLKs act directly at the site of their target, the MUS pathway most likely acts indirectly from outside the target site. The discovery of MUS function and expression reveals a significant new pathway controlling stomatal development. Knowledge of MUS function provides an entrée into dissection of highly coordinated processes, such as pore morphogenesis and GC shaping. In addition, the disruption of microtubule organization by mus provides a potential link between cytoskeletal dynamics and the construction of highly specialized cell wall structures, and shows that MUS likely controls morphogenesis by regulating microtubule behavior. These findings add significantly to a body of research on the organization of microtubule arrays in stomata, which have been a model cell type for studying the relationship between the cytoskeleton and morphogenesis (Apostalakos and Galatis, 1999).
Finally, this work demonstrates that microtubule dynamics, in particular, the proportion of outbound to inbound polarity of microtubule growth, is significantly correlated with stomatal morphogenesis. These findings are not only useful in understanding how microtubules regulate guard cell morphogenesis, but might also aid in understanding of morphogenesis in other plant cell types as well.

5.2 Future directions

Key areas for future research include the identification of components of the MUS signaling pathway, with particular reference to interactors and to downstream components, and the detection of additional pathways that regulate bilateral symmetry. Database analyses (ATTED) indicate that MUS is co-expressed with several microtubule associated proteins, such as the kinesins HINKEL and PAKRP1 (PHRAGMOPLAST-ASSOCIATED KINESIN-RELATED PROTEIN 1) and, as well as ANP3 (ARABIDOPSIS NPK1-RELATED PROTEIN KINASE 3), a kinase involved in cortical microtubule organization (Beck et al., 2010). Since one of the primary defects observed in mus pertains to microtubules, the above-mentioned genes represent potential microtubule interactors, and thus future analysis of mutants in these genes would be a high priority. Co-immunoprecipitation might also be used in combination with mass spectrometry to identify potential MUS interactors.

Since MUS appears to encode an atypical non-functioning kinase domain, it would be important to determine whether MUS is indeed incapable of phosphorylating downstream targets. One possible mode of action for a MUS atypical kinase is to partner with a functioning kinase. *Arabidopsis* contains one MUS-like-kinase (“MUSL”) which might be functional based
upon sequence. However a musl single mutant and mus musl double mutant do not exhibit an abnormal stomatal phenotype (J. Nadeau, unpublished). Again, analysis of co-expressed genes will be important here.

Bioinformatic analysis of the MUS promoter indicates the presence of several putative transcription factor binding sites. Therefore, future work might explore whether or not predicted binding sites can be shown to be functional. In addition, it would be valuable to understand how MUS is targeted at the sub-cellular level to the stomatal periphery. Finally, further work should analyze whether altered microtubule dynamics in mus are caused by γ–TURC mis-localization and/or by an increase in microtubule length.

Ultimately, work on MUS might reveal the extent to which both intercellular as well as intracellular coordination lead to correct symmetry generation in cellular morphogenesis.
Works cited


