Transcription Factor Control of Myrosin Cell Development in
Arabidopsis

by

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B.Sc. Biotechnology in Life Science, Peking University, 2009

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

The Faculty of Graduate and Postdoctoral Studies
(Botany)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

August 2013

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Abstract

*Brassicaceae* shoots harbor a glucosinolate-myrosinase system that acts as a chemically induced defense against insect predation and microorganisms. In *Arabidopsis*, myrosinase, also known as Thioglucoside Glucohydrolase (TGG), accumulates in the tonoplastic of guard cells (GCs) as well as of myrosin phloem cells (MPCs). The basic-Helix-Loop-Helix (bHLH) transcription factor FAMA is essential for stomatal development, where it is strongly expressed just before and after the final division that generates the two-celled stoma. FAMA enforces guard cell fate and ensures that stomata contain just two guard cells, but no other function for FAMA has been demonstrated. I report that FAMA and a second guard cell fate marker are also expressed in shoots in developing and mature MPCs. MPCs were found to arise from the ground meristem, not the procambium, and thus are not part of the phloem. The loss of FAMA function abolishes MPCs and abrogates the expression of the myrosinase genes, *TGG1* and *TGG2*. In addition, MPC development is regulated by auxin-related signaling. The loss of function of the *GNOM* gene as well as the chemical inhibition of auxin efflux, disrupt MPC patterning and morphogenesis. Thus FAMA confers MPC fate as well as the expression of myrosinase genes. In addition, polar auxin efflux is required for MPC development and patterning.
Preface

This dissertation is original, unpublished, independent work by the author, M. Li.
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List of Abbreviations

ARF-GEF  ADP ribosylation factor–GTP exchange factor
ATHB8  ARABIDOPSIS THALIANA HOMOBOX 8
BFA  brefeldin A
bHLH  basic-Helix-Loop-Helix
CBB  coomassie brilliant blue
DIC  differential interference contrast
DMSO  dimethyl sulfoxide
ESP  epithiospecifier protein
GC  guard cell
GFP  green fluorescence protein
GMC  guard mother cell
GUS  β-Glucuronidase
h  hour
HD-ZIP  homeodomain-leucine zipper
min  minute
MMC  meristemoid mother cell
MPC  myrosin phloen cell
MS  murashige and skoog
NPA  1-N-naphthylphthalamic acid
PI  propidium iodide
PIN1  PIN-FORMED PROTEIN 1
sec  second
SPCH  SPEECHLESS
TGG  Thioglucoside Glucohydrolase
UTR  untranslated region
WT  wild type
X-gluc  5-bromo-4-chloro-3-indolyl-d-glucuronide
YFP  yellow fluorescence protein
Acknowledgements

I feel fortunate that I joined the lab of Dr. Fred Sack and became a graduate student under his supervision. He guided me to develop the insight and important capability for designing experiments, to identify scientific hypothesis, and to acquire numerous plant cell biology skills, especially microscopy, under his incredible experience.

Thanks to Eunkyoung Lee who provided seeds harboring proFAMA:FAMA-GFP constructs, and other lab members Sandra Keerthisinghe, Xiaolan Chen and Lei Qin for sharing their experience with me.

I thanks to Jie Le for seeds of dynamic auxin markers, and for valuable discussions about the role of auxin in GC development, and thanks to Enrico Scarpella at the Department of Biological Sciences, University of Alberta, Canada, for seeds of proATHB:HTA6-YFP and as well as Q0990 constructs.

Special thanks go to my committee members, Dr. Jim Mattsson and Dr. Carl Douglas for their kindness, help and suggestions on my project.

Finally, I want to thank my family for supporting me at any moment.
1 General Introduction

1.1 Glucosinolate-myrosinase cell system in Crucifers

Crucifers harbor an injury-induced defense pathway termed a ‘Mustard Oil Bomb’, a glucosinolate-myrosinase system that reduces predation by forming products toxic to microbes and insects. Myrosinase is a family of glucosinolate hydrolases present at high levels in many Brassicaceae species (Rask et al., 2000).

In B. napus and Sinapis alba seeds, myrosinase is found in myrosin cells in the form of water soluble ‘myrosin grains’ located in protein storage bodies in cotyledons and in the embryonic axis (Bones et al, 1991). In plants, myrosinase and glucosinolate are synthesized and stored separately in adjacent cells termed myrosin cells and S-cells respectively (Ahuja et al., 2009; Eriksson et al., 2001; Kissen et al., 2009). Crucifers contain two types of myrosin cells that inhibit predation, guard cells (GCs) in stomata and specific cells that are reported to be located in the phloem (Myrosin Phloem Cells, MPCs) (Husebye et al., 2002; Andreasson et al., 2001).

The glucosinolate-myrosinase system can produce a range of anti-predation chemicals whose activity derives from the structure of different glucosinolates, the pH, and from cofactor catalysis (Kissen et al., 2009). During predation or unnatural cell breakage, myrosinase can hydrolyze glucosinolate from damaged plant tissues yielding a glucose molecule and an unstable aglycone. The latter is either converted to an isothiocyanate at neutral pH, or diverted to a nitrile/epithionitrile at acidic pH (<3) in the presence of an epithiospecifier protein (ESP), all of which are toxic to insects and microorganisms (Figure 1.1; Wittstock et al., 2002). Brassica napus plants lacking myrosinase activity due to the ablation of myrosin cells showed more active feeding by animals consistent with reduced toxicity (Borgen et al., 2010).
Figure 1.1 Model for glucosinolate-myrosinase defense against herbivory. Diagram modified from Winde and Wittstock (2011). ESP, epithiospecifier protein.

1.2 Arabidopsis myrosinase genes

Arabidopsis myrosinase, a synonym for thioglucosidases, is also referred to as thioglucoside glucohydrolase (TGG) (Chadchawan et al., 1993; Xue et al., 1992), and accumulates specifically in the tonoplast of myrosin cells (Ueda et al., 2006). Six myrosinase genes have been identified in Arabidopsis, of which two, TGG1 (At5g26000) and TGG2 (At5g25980), are strongly expressed in the shoot, with TGG3 likely to be a pseudogene (Andersson et al., 2004; Zhang et al., 2002).

The functions of the three other myrosinase genes, TGG4, TGG5, and TGG6, are still not well-described. TGG4 (At1g47600) and TGG5 (At1g51470) encode functional myrosinases
and appear to be expressed specifically in roots, while TGG6 (At1g51490) shows expression only in pollen and does not appear to exhibit myrosinase activity (Andersson et al., 2004; Kissen et al., 2009).

The expression patterns of TGG1 and TGG2, as well as the distribution of myrosin cells, have been described in Arabidopsis leaves, inflorescences, sepals, petals, and gynoecia (Barth and Jander, 2006). TGG1 is expressed in stomata as well as in MPCs, whereas TGG2 is expressed exclusively in MPCs (Barth and Jander, 2006). Using a loss-of-function tgg1 tgg2 double mutant, the feeding experiments show a reduction in growth rate and weight of Crucifer-specialist insects such as sexta larva, a decrease that is much stronger in either the tgg1 or tgg2 single mutants alone. Thus TGG1 and TGG2 function synergistically in reducing predation (Figure 1.2; Barth and Jander, 2006).

Figure 1.2 Growth of newly hatched Manduca sexta larvae on tgg1 and tgg2 single mutant plants compared to tgg1 tgg2 double mutants in a feeding experiment (Barth and Jander, 2006). Asterisks indicate differences in fed insect dry weight between WT and mutants. n, number of counted larva.
1.3 Development of two types of myrosin cells

1.3.1 Myrosin phloem cell

MPCs are defined as a type of idioblasts in that they differ in size and morphology from adjacent cells (Andreasson et al., 2001). MPC number is regulated by the Arabidopsis Q-SNARE protein AtVAM3 (Ueda et al., 2006). AtVAM3 is constitutively expressed in meristematic and developing cells and controls the docking and fusion of vesicles with target membranes, especially the tonoplast (Ueda et al., 2009). The loss of ATVAM3 function in an atvam3 allele mutant (atvam3-4) induces the formation of excess MPCs that fuse into an abnormal and continuous network (Figure 1.3; Ueda et al., 2006).

Figure 1.3 MPC distributions in wild-type and in the atvam3-4 mutant. Image from Ueda et al., (2006).

(A) and (B) Asterisks show MPCs in light micrographs from rosette leaves stained with coomassie brilliant blue (CBB) solution.

(C) and (D) MPCs (gray) and leaf veins (solid lines) in (A) and (B), respectively.
1.3.2 Stomata

Stomata, which are myrosin cells in Crucifers, regulate gas exchange between the shoot and the environment, and are present in nearly all plant taxa, bryophytes and above. Many aspects of stomatal development are well defined, including patterning and division regulation in the cell lineage (Figure 1.4; Pillitteri and Torii, 2012). In Arabidopsis, stomata originate from protoderm with the formation of a meristemoid mother cell (MMC) that divides asymmetrically, producing two daughter cells with distinct sizes and fates. The smaller cell, the meristemoid, can undergo several asymmetric divisions before differentiating into an oval guard mother cell (GMC), which divides once symmetrically and then produces a pair of mature guard cells (Pillitteri and Torii, 2012).

Stomata develop in part via three master basic-Helix-Loop-Helix (bHLH) transcription factors, SPEECHLESS (SPCH), MUTE and FAMA, which successively confer key cell fates and regulate proliferation in the pathway (Figure 1.3; Dong and Bergman, 2010). Each of these bHLH genes is expressed at a specific stage of stomatal developmental (Vatén and Bergmann, 2012). SPCH is expressed in MMCs and young meristemoids, and is required for entry into the stomatal lineage (Vatén and Bergmann, 2012). MUTE is expressed in late meristemoids, and promotes their transition to GMCs (Vatén and Bergmann, 2012). FAMA, which is expressed in late GMCs and in young GCs, limits the symmetric division of the GMC to one, and also promotes the acquisition of GC fate (Hachez et al., 2011).

Figure 1.4 Developmental stages in the Arabidopsis stomatal lineage. Diagram modified from Vatén and Bergmann (2012). Meristemoid shown in yellow.
1.4 Objectives

FAMA has been shown to confer stomatal cell fate, but little is known about whether it plays other roles in *Arabidopsis*. I predicted that FAMA might also be expressed outside the stomatal cell lineage and have other functions in plant development. Thus I characterized the expression pattern of FAMA and used it to identify other possible cells or tissues. If other regions or cell types were found to exhibit FAMA expression, then the effects of the loss of FAMA function would be determined with respect to fate and function. A second major goal was to use FAMA reporters as a cell marker tool in order to characterize how possible target cells or tissues might develop.
2 FAMA Controls Myrosin Cell Fate and Myrosinase Activity

2.1 Introduction

2.1.1 Structure of FAMA bHLH protein and \textit{fama} mutant alleles

bHLH proteins are ubiquitous in eukaryotes, constitute one of the largest transcription factor families in \textit{Arabidopsis}, and function in multiple signaling, hormone, and stress-related response pathways (Carretero-Paulet \textit{et al.}, 2010). The \textit{FAMA} (At3g24140) gene encodes a peptide of 414 amino acids (Ohashi-Ito and Bergmann, 2006). Similar to other bHLH transcription factors, FAMA harbors a conserved bHLH signature domain that contains the HLH domain and a basic region (Figure 2.1A). The HLH region contains approximately 60 amino acids arranged in two amphipathic $\alpha$-helices with hydrophobic residues and is linked by a variable loop region (Massari and Murre, 2000). The HLH domain promotes protein-protein interactions and the formation of homodimeric or heterodimeric complexes (Massari and Murre, 2000). The basic region, consisting of 15 to 20 conserved basic amino acid residues, has been shown to recognize the E-box motif element that harbors a hexanucleotide consensus sequence (Murphy \textit{et al.}, 2009).

Molecular and protein structural analyses show that bHLH dimer interactions take place between the two HLH regions in the two proteins, and also that each basic region from these two bHLHs, binds to half of the DNA element sequence (Massari and Murre, 2000).

Loss-of-function mutants in \textit{FAMA} have been described by Ohashi-Ito and Bergmann (2006). The mutant \textit{fama-1} (SALK_100073) allele eliminates the \textit{FAMA} transcript, a mutation resulting from a T-DNA insertion in the first intron (Figure 2.1B). A second allele, \textit{fama-2} (SALK_010525), is produced by a T-DNA insertion near the 3’ untranslated region (UTR) of \textit{FAMA} gene (Figure 2-1B; Ohashi-Ito and Bergmann, 2006).
Figure 2.1 The structure of bHLH FAMA protein, and mutations in FAMA. Data from Ohashi-Ito and Bergmann, (2006).

(A) The sequence of the bHLH domain in the FAMA clade. Boxed areas show basic regions, and shaded regions indicate HLH domains.

(B) The T-DNA insertion sites in two fama alleles. Black boxes show exons and gray triangles indicate T-DNA insertions.

2.1.2 Previous research on FAMA regulation of stomatal formation

The bHLH gene FAMA is expressed just before the final stage of stomatal development, and is required to confer a guard cell fate as well as to restrict precursor cell division (Hachez et al., 2011). FAMA is strongly expressed in late GMCs and young guard cells, but not in mature Arabidopsis stomata. The fama-1 mutant has been shown to lack of FAMA transcriptional activity, and instead of stomata, produces abnormal clusters of small and narrow cells that lack guard cell fate and morphology (Ohashi-Ito and Bergmann, 2006). However, the fama-2 allele shows a slight defect in stomatal patterning, but does not disrupt GC fate (Ohashi-Ito and Bergmann, 2006).

Analyses of FAMA regulatory networks show that FAMA might activate stomatal differentiation genes (Hachez et al., 2011). FAMA also directly targets and regulates cell cycle genes such as CDKB1;1 which is essential for the symmetric division of GMCs. Thus
FAMA acts as both a transcriptional activator and a repressor in the stomatal pathway (Hachez et al., 2011).

The transformation of a native FAMA gene into wild type or fama-1 mutant plants can induce a gain-of-function phenotype where a stoma forming inside a pre-existing guard cell (Lee et al., 2013). Such transformants also disrupt epigenetic marks on stem cell genes in the stomatal lineage, suggesting that guard cell fate is normally maintained by histone trimethylation on genes required early in stomatal development.

2.2 Material and methods

2.2.1 Plant material and growth conditions

Wild-type Arabidopsis thaliana Columbia (Col-0) plants were used as the background for transgenes and all mutants. The fama-1 (Salk_100073) mutant alleles were described in Ohashi-Ito and Bergmann (2006) respectively, and were confirmed by genotyping and mutant phenotypes. The proTGG1:GUS reporter line was obtained from Barth and Jander (2006). The E1728 enhancer trap line, as well as other stomatal reporter lines including proFAMA:GUS, proFAMA:GFP, and proFAMA:FAMA-GFP, were as described (Lee et al., 2013). Primers used for genotyping are in Table 2.1.

2.2.2 Generation of transgenic plants

fama-1 homozygous plants are not fertile. Plants harboring this mutation as well as reporters (proTGG1:GUS, proFAMA:GUS, proFAMA:GFP, and E1728:YFPer) were obtained by crossing reporter lines with fama-1/+ heterozygotes, and then selecting F1 and F2 plants by genotyping and reporter visualization. Individual F2 lines were selected on plates using kanamycin (50ug/ml) and hygromycin (25ug/ml) and then confirmed using PCR-based genotype analysis with primers described above.

2.2.3 Analysis of GUS activity

To check β-glucuronidase activity, all samples were dissected and vacuum-infiltrated in a 5-bromo-4-chloro-3-indolyl-d-glucuronide (X-Gluc) solution for 15 min. at room temperature, and then incubated at 37°C in the dark for 16-24h as described (Malamy, 1997). X-Gluc-
treated samples were rinsed with water, and then fixed in ethanol:acetic acid (6:1 [v/v]) for 2 h at room temperature. Samples were rinsed with 95% (v/v) ethanol and placed successively in 70%, 50%, and 30% (v/v) ethanol each for 10 min, and then mounted in chloral-hydrate:glycerol:water (8:1:2 [g/v/v]) as described (Berleth and Jurgens, 1993). Tissue samples were mounted on microscope slides and observed using differential interference contrast (DIC) optics.

2.2.4 Microscopy

Selected low magnification images of the leaf vasculature were acquired using dark-field optics and an Olympus SZX10 stereomicroscope. Samples stained for GUS were visualized using oil immersion DIC objectives on an Olympus AX-70 microscope or a Nikon ECLIPSE 80i confocal microscope. For 2-channel fluorescence imaging using GFP/YFP and propidium iodide (PI) fluorescence filter sets, fresh tissue was immersed in 1mg/ml PI solution for 5 min and then rinsed briefly with water. For the co-expression analysis of GFP and GUS, living tissues were mounted in water and visualized for GFP fluorescence, then stained using X-gluc solution for 2h, and discolored before visualization (as described in chapter 2.2.3). Confocal fluorescence images were processed using ImageJ and Adobe Photoshop 5.0 software.

2.2.5 RNA extraction, RT-PCR, and real-time PCR

Total RNA was extracted from 3 week-old whole shoot tissues using an RNeasy Plant Mini Kit (Qiagen) and then treated with DNase (Invitrogen) to remove genomic DNA. First-strand cDNAs were generated by 1mg RNA using Thermo Script Reverse Transcriptase and an oligo (dT) 20 primer (Invitrogen). Real-time PCR was performed with 1μl of the first-strand template at 98°C for 30 sec, 56°C for 30 sec, and 72°C for 30 sec with 25 cycles. The primers TGG1-F(ex7) and TGG1-R(ex8) were designed to amplify TGG1 transcripts, TGG2-F(ex7) and TGG2-R(ex8) to amplify TGG2 transcripts, and ACT2-F and ACT2-R to amplify ACTIN transcripts. See Table 2.2 for primer details. PCR products for semi-quantitative RT-PCR underwent electrophoresis using 1% agarose gels, and were visualized by staining with Safe View Nucleic Acid Stain (NBS). Real-time quantitative PCR based on SYBR-Green chemistry was performed using the same conditions and gene-specific primers as described
above. Real-time quantitative PCR was monitored over 40 cycles using a Bio-Rad iCycler (iQ™5) Multicolor Real-Time PCR Detection System. Gene expression levels are shown (in Figure 2.8) as the ratio relative to the wild type control and were calculated using the $2^{\Delta \Delta Ct}$ method. Two biological replicates were performed for each real-time PCR experiment.

Table 2.1 Primers and DNA sequences used for fama-1 genotype analysis.

<table>
<thead>
<tr>
<th>Name of Primer</th>
<th>DNA sequence (5'-to-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAMA-LP</td>
<td>TCATTCATTTGCTTCCTACGG</td>
</tr>
<tr>
<td>FAMA-RP</td>
<td>CAATACAAAAAGCTCCCCTCAC</td>
</tr>
<tr>
<td>LBa1</td>
<td>TGGTTCACGTA GTGGGCCATCG</td>
</tr>
</tbody>
</table>

Table 2.2 Primers and DNA sequences used for real-time PCR.

<table>
<thead>
<tr>
<th>Name of Primer</th>
<th>DNA sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Act2-F</td>
<td>CTGGATCGGTGGTTCCATT</td>
</tr>
<tr>
<td>Act2-R</td>
<td>CCTGGACCTGCCTCATCATA</td>
</tr>
<tr>
<td>TGG1-F(ex7)</td>
<td>TAAGGATGACAAAAAGGTATGA</td>
</tr>
<tr>
<td>TGG1-R(ex8)</td>
<td>CACTGAACTCTGGAAGCCGA</td>
</tr>
<tr>
<td>TGG2-F(ex7)</td>
<td>AAAACAGCAAACCTTGGAGGGCC</td>
</tr>
<tr>
<td>TGG2-R(ex8)</td>
<td>AGGGCGTGGGCGTATTGAGT</td>
</tr>
</tbody>
</table>
2.3 Results

2.3.1 Guard cell fate markers are expressed in myrosin phloem cells

Stomatal-related reporter gene transcriptional fusions such as proFAMA:GFP, proFAMA:GUS, and the E1728:YFPer enhancer trap, have previously been shown to be expressed in GMCs and in young GCs (Ohashi-Ito and Bergmann, 2006). In addition to their expression during stomatal development (Figure 2.2 A-C), I found that all of these markers were also expressed in long, isolated, and irregularly shaped cells (Figure 2.2 E-G). These cells were located near the vasculature throughout the shoot, including in petioles, leaves, sepals, petals and carpels (Figure 2.3).

To determine the identity of this cell type, expression patterns driven by the FAMA and E1728 reporters were compared to that of a myrosin cell-type specific marker, a TGG1-GUS transcriptional fusion (proTGG1:GUS). In leaves transformed with all three reporters, FAMA and E1728 were expressed in distinct and isolated cell types also marked by TGG1 expression (Figure 2.4). Thus FAMA, E1728, and TGG1 are all co-expressed in both types of myrosin cells i.e. MPCs as well as guard cells.

Notably, unlike the transcriptional fusion, a proFAMA:FAMA-GFP translational fusion was only expressed strongly in nuclei of young but not mature MPCs (Figure 2.2 D and H). The absence of FAMA expression from mature MPCs parallels the lack of proFAMA:FAMA-GFP fluorescence from mature GCs, and also parallels its presence just before and after GMC symmetric division (Figure 2.5). These results indicate that FAMA acts before MPCs mature, and also suggest that FAMA expression during MPC maturation is negatively and post-transcriptionally regulated like RNA interference or Proteolysis.

2.3.2 fama-1 mutant plants show defects in growth

To define the function of FAMA in MPC development, the phenotypes of a fama loss-of-function mutant (fama-1 allele, Ohashi-Ito and Bergmann, 2006) was evaluated. Compared to a bolting wild-type (WT) plant (Figure 2.6 A), fama-1 mutant plants arrested growth as pale and stunted seedlings that contain 7-8 small leaves (Figure 2.6 B). Using dark-field microscopy, in contrast to the rich vein network in the wild-type (e.g.14 DAG, mature, first
rosette leaves) (Figure 2.6 D), *fama-1* only contained a mid-vein and first and second loop of secondary veins (Figure 2.6 E). Thus this mutant displays significant defects in growth and vascular development.

### 2.3.3 *FAMA* is required for myrosin phloem cell fate and *TGG* expression

To further define *fama* phenotypes in MPC development, I transformed three MPC markers, *FAMA*, E1728, and *TGG1*, into wild-type and *fama-1* mutant plants. In contrast to reporter-based expression in MPCs in wild-type rosette leaves (Figure 2.7; Figure 2.9 A), in *fama-1*, *FAMA* promoter activity was only detected in clusters of narrow cells located in place of stomata (Figure 2.7). This mutant completely lacked any recognizable and definable MPCs near procambial strands or the phloem (Figure 2.7; Figure 2.9 B). By contrast, the *tgg1 tgg2* double mutant harbored MPCs (as well as stomata) that were normally distributed (Figure 2.10).

Additionally, the complementation of *fama-1* mutant plants with a pro*FAMA*:FAMA-GFP translational fusion (referred to as “*FAMA*trans”, Lee *et al*., 2013), completely restored plant growth and reproduction, the leaf vascular network, as well as the presence of MPCs (Figure 2.6 C and F; Figure 2.9 C). Thus, the loss of FAMA function blocks the fate conferral MPCs as well as their expansion and morphogenesis.

Compared with both semi-quantitative and qRT-PCR analyses of 3 week-old shoot tissues from the WT, *fama-1* mutants eliminated the transcription of both *TGG1* and *TGG2*. By contrast, *FAMA*trans *fama-1* plants harbored low levels of *TGG1* and *TGG2* transcription (Figure 2.8 A-C). Thus, FAMA promotes the transcription and activity of myrosinases, as well as MPC differentiation in *Arabidopsis*.
Figure 2.2 Guard cell markers are also expressed in myrosin phloem cells.
(A) GCs show epidermal proFAMA:GUS expression.
(B) GCs exhibit E1728:YFPer expression.
(C) Epidermal proFAMA:GFP expression in Guard Mother Cells and GCs.
(D) Young GCs harbor proFAMA:FAMA-GFP expression.
(E) MPCs express proFAMA:GUS near a leaf vein in the same field shown in the epidermis in (A).
(F) Three MPCs harboring E1728:YFPer expression near leaf veins.
(G) MPCs with proFAMA:GFP expression (near leaf veins, not shown).
(H) Young MPCs near leaf vein exhibit nuclear proFAMA:FAMA-GFP expression. Arrow indicates a large MPC that no longer exhibits fluorescence. Cell wall is stained by propidium iodide (PI).
All images are from rosette leaves at similar developmental ages. All scale bars = 50 um.
Figure 2.3 A FAMA-GUS transcriptional fusion is expressed throughout the shoot in GCs and MPCs.
(A) 8 DAG first rosette leaf showing expression in MPCs (e.g. at arrow) and in stomata (e.g. at black line).
(B) No GUS expression was found in roots.
(C) GUS expression in an MPC (arrow), as well as in GCs (oval GUS-stained dots).
(D) Inflorescence flower stalk. Note that the large blocks of GUS staining in (D), as opposed to GCs and MPCs, appear to be an artifact, perhaps due to over-staining.
(E) Flower bud
(F) Carpel
(G) Sepal
(H) Petal
Arrows indicate GUS expression from MPCs located near the vasculature.
Figure 2.4 Co-expression of \( FAMA \) and \( E1728 \) with \( TGG1 \) in MPCs. Arrows indicate specific MPCs that co-express fluorescence (from either \( proFAMA:GFP \) or \( E1728:YFPer \)), as well as \( proTGG1:GUS \), in each of two different leaves (“A” and “B”). All scale bars = 0.1mm.
Figure 2.5 A FAMA-GFP translational fusion (proFAMA:FAMA-GFP) is expressed in young but not mature GCs and MPCs.

(A) Young GC just after symmetric division exhibits GFP fluorescence.
(B) A newly formed stoma lacks FAMA expression.
(C) Expression is also absent from mature GCs.
(D) FAMA expression is present in the nucleus of a young (unelongated) MPC.
(E) Expression is also present in two elongating MPCs (arrows).
(F) No FAMA expression was detected in this older, larger, and more elongated MPC

Bars = 10um.
Figure 2.6 Plant and vasculature phenotypes of three genotypes compared: wild-type, fama-1, and FAMAtransfama-1

(A) - (C) Plants one week after bolting are diminutive in size in the fama-1 mutant, but appear almost normal when complemented by the proFAMA:FAMA-GFP gene fusion (FAMAtrans).

(D) - (F) Dark-field microscopy showing apparently normal vasculature when complemented. Compare with the highly reduced vasculature in the fama-1 mutant. All samples from 14DAG first rosette leaves. All scale bars = 1mm.
Figure 2.7 Loss of function phenotypes of MPCs in fama-1 leaves.

(A), (E) and (I) Wild-type showing proTGG1:GUS and E1728:YFPer expression in stomata (A) and in MPCs (A, E and I).

(B), (F) and (J) fama-1 lacks proTGG1:GUS and E1728:YFPer expression since fama abrogates MPC fate. The elongated cells are part of the phloem.

(C) and (K) WT showing proFAMA:GUS staining and proFAMA:GFP fluorescence in MPCs.

(D), (G) and (L) proFAMA:GFP and proFAMA:GUS expression is absent from fama-1 which lacks MPCs. The globular structures showing GUS expression are out of focus epidermal cell clusters shown in (H).

(H) proFAMA:GUS is expressed in clusters of cells (e.g. at arrow) that arise from excess symmetric divisions in GMCs in fama-1, but that do not form stomata. Scale bars = 50um
Figure 2.8 RT-PCR analysis of wild-type, *fama-1*, and \( \text{FAMA}^{\text{trans}} \) *fama-1* plants.

(A) Semi-quantitative RT-PCR shows *TGG1* and *TGG2* expression in wild-type, *fama-1*, and in the *fama-1* mutant complemented with \( \text{FAMA}^{\text{trans}} \).

(B) Data from qRT-PCR showing that *TGG1* transcription is abolished in *fama-1* but is slightly higher in *fama-1* plants harboring \( \text{FAMA}^{\text{trans}} \).

(C) As in (B) except for *TGG2* transcript levels.
Figure 2.9 Test for complementation of wild-type, fama-1, and FAMA\textsuperscript{trans} fama-1 plants.

(A) \textit{proFAMA:FAMA-GFP} fluorescence in MPCs in wild-type (arrows).

(B) No expression from the transcriptional \textit{proFAMA:GFP} reporter was found near the phloem since MPCs are absent from the \textit{fama-1} mutant.

(C) Transformation with the \textit{FAMA}\textsuperscript{trans} construct into the \textit{fama-1} mutant restores FAMA-GFP expression to MPCs (arrows).
Figure 2.10 MPCs and GCs are still present in a \( tgg1-3 \ tgg2-1 \) double mutant.
(A) 4 week-old bolting plant. Bar = 1mm
(B) 10 DAG first rosette leaf. Cell walls visualized using PI fluorescence. Arrows indicate MPCs in focus. MPCs can be identified, in part, by their lack of chloroplasts and by their larger size compared to surrounding mesophyll cells in this field. Bars = 50um.
(C) Epidermis from a different 10DAG first rosette leaf with cell walls visualized using PI fluorescence. Arrows indicate examples of developing or mature GCs. Bars = 50um.

2.4 Discussion

2.4.1 The transcription factor FAMA controls the fate of both types of myrosin cells

In this study, I showed that the expression of \( FAMA \), as well as that of the enhancer trap \( E1728 \), mark MPC development. Previously both markers were only known to be expressed in guard cells (Hachez et al., 2011). Thus FAMA displays novel functions in promoting both MPC identity and development. Moreover, loss-of-function mutants in other stomatal regulatory genes, such as \( SPCH \) and \( MUTE \), do not disrupt MPC formation (data not shown). Thus, FAMA is the only transcription factor known to confer and control the cell fate of both types of myrosin cells, as well as the expression of two key myrosinase genes, \( TGG1 \) and \( TGG2 \). Our study provides possible entrée into analyzing the regulation of myrosin cell formation and of events upstream of myrosinase expression.

Transformation of a native \( FAMA \) gene (\( FAMA^{\text{trans}} \)) into wild type or \( fama-1 \) plants has been shown to induce a novel gain-of-function phenotype in which guard cell fate is reset to that
of stomatal lineage stem cells resulting in GCs converting into an MMC that then divide unequally to form a new ectopic stoma within the shells of the old (Lee et al., 2013). This might explain why FAMA\textsuperscript{trans} in a fama-1 background only partially rescue myrosinase genes.

Orthologs of FAMA genes have been identified in many flowering plants including in rice and maize (Liu et al., 2009). Like Arabidopsis, rice fama loss-of-function mutants show defects in GC fate acquisition as well as abnormal clusters of small cells instead of stomata, suggesting that FAMA function is conserved in stomatal development in various land plants (Liu et al., 2009). It is thus possible that FAMA might regulate the fate and identity of MPCs and GCs in other Crucifers.

2.4.2 Potential FAMA targets in MPC development

Conserved amino acids in the basic region of most bHLH proteins show a high affinity for recognizing E-box motif elements involved in promoter regulation (Murphy et al., 2009). The promoter regions of TGG contain multiple E-box elements, such as the 5’-UTR sequences of the TGG1 promoter region that contains four types of E-box motifs (5’-CATGTG-3’, 5’-CATTTG-3’, 5’-CATCTG-3’, and 5’-CAACTG-3’). In addition, 5’-UTR sequences in the TGG2 promoter region also contain classic E-box motifs e.g. 5’-CATGTG-3’, 5’-CATTTG-3’, and 5’-CAACTG-3’ (TAIR). Consistent with a requirement for FAMA in the transcription of both the TGG1 and TGG2 genes, these data suggest that FAMA might contribute to activating TGG transcription specifically via E-box motif elements in the TGG promoter.

Moreover, previous reports show that the inducible overexpression of FAMA in Arabidopsis can up- or down-regulate the expression of multiple stomatal development regulator genes (Hachez et al., 2011). Candidate FAMA targets have been identified via microarray analysis, and include cell cycle regulators, cell wall enzymes, signaling receptor kinases, as well as cytokinesis-related transcription factors (Figure 2.11; Hachez et al., 2011). Thus these findings might also help in identifying MPC-specific genes or factors that act in common for GCs and MPCs as well as those that directly regulate MPC differentiation and number, such as those related to cell cycling and MPC cell growth.
Figure 2.11 Mechanistic model for FAMA-mediated guard cell development. Diagram reproduced from Hachez (et al. 2011).
3 Myrosin Phloem Cells are Differentiated Idioblasts that Arise from the Ground Tissue Meristem

3.1 Introduction

3.1.1 Three primary shoot meristems

Meristems are undifferentiated, embryonic cells present throughout the life of the growing plant. Meristematic cells can divide and differentiate into various types of cells and tissues. The apical meristem is present at the tips of roots and shoots as well as in leaf axils. The apical meristem is responsible for plant organ growth and development. In the primary growth of shoots, this meristem differentiates into three types of primary tissues meristems, *i.e.* the protoderm, procambium, and ground meristem (Fletcher and Elliott, 2000). The protoderm produces all epidermal cells, while the procambium induces cell proliferation that leads to the differentiation of vascular tissues (Fletcher and Elliott, 2000). All other parenchyma tissues arise from the ground meristem (Fletcher and Elliott, 2000).

Figure 3.1 *Arabidopsis* shoot development and the origin of primary tissues (Mauseth, 2009).
3.1.2 Early vascular development

MPCs have been reported to be located in phloem parenchyma throughout the shoot (Husebye et al., 2002; Andreasson et al., 2001). During vascular development in leaves, vascular stem cells differentiate into xylem and phloem precursor cells via cell proliferation followed by the acquisition of specific cell fates. Vascular network pre-patterning in leaves starts in a single strand of ground meristem cells where auxin flows and accumulates, which can be detected in part by the expression of PIN1 (PIN-FORMED PROTEIN1), that encodes an auxin efflux carrier (Scarpella et al., 2010). Then, ARABIDOPSIS THALIANA HOMEOBOX 8 (ATHB8), a homeodomain-leucine zipper (HD-ZIP) III protein which is highly expressed in selected ground cells, can promote entry into procambial cells which become elongated and narrow (Scarpella et al., 2010). Procambial cells can be marked by the expression of ATHB8 as well as an enhancer trap Q0990 (Scarpella et al., 2010), and further undergo elongation, symmetric and asymmetric division, and then differentiation into xylem and phloem that form vascular bundles (Donner et al., 2009). Bundle sheath cells originate from the ground tissue and surround the vascular strand (Zhang et al., 2009).

3.2 Material and methods

3.2.1 Plant material and growth conditions

Two procambial markers, proATHB8:HTA6-YFP and Q0990 (an enhancer trap) were used as in Scarpella et al., (2010).

3.2.2 Confocal microscopy

To more clearly visualize cell outlines near vascular strands, juvenile fist leaves (less than 10DAG) were immersed in 1.4mg/ml PI solution for 8-10 min and then mounted in water for 1 min before observation. Confocal fluorescence images were acquired using a Nikon ECLIPSE 80i microscope and processed using ImageJ and Adobe Photoshop 5.0 software.
3.3 Results

3.3.1 Myrosin phloem cells arise from the ground tissue meristem, not the procambium

MPCs have been reported to be located in the abaxial phloem parenchyma in vascular bundles in *Arabidopsis* leaves (Husebye *et al*., 2002). However, these reports did not employ cell fate markers for the ground tissue meristem or the procambium. To define the tissue system that gives rise to MPCs, the distribution of GUS staining driven by the *FAMA* promoter was analyzed in cross-sections of primary inflorescence stems. Mature MPCs were found at the border between the phloem and the cortex, which arise from the procambium and/or the ground tissue meristem respectively (Figure 3.2).

The location of *FAMA* expression in juvenile rosette leaves was then compared with that of markers of the elongating procambium *i.e.* fluorescence from reporters of the transcription factor gene *ARABIDOPSIS THALIANA HOMEBOX8 (ATHB8)*, as well as of the enhancer trap *Q0990* (Scarpella *et al*., 2010). The localization of the MPC expression markers *FAMA* and *E1728* (Figure 3.3 E-H) was found to be different from that of the procambial markers *ATHB8* (Figure 3.3 A and B) and the *Q0990* (Figure 3.3 C and D). Thus, although MPCs are located close to the phloem, they derive from the ground tissue meristem rather than the procambium.

3.3.2 Parallel development of myrosin phloem cells and the procambium

Procambial cells and young MPCs appeared simultaneously and formed at roughly comparable times during leaf development (Figure 3.4). Procambial cells elongate and proliferate during early shoot growth and then differentiate (Scarpella *et al*., 2010). I found that *FAMA* and *E1728* expression first appeared in small ground meristem cells during the early stages of the development of leaf primordia (Figure 3.4 A and G) located under short procambial cells (Figure 3.4 D and J). These square-shaped, ground meristem cells then increased in both length and width (Figure 3.4 B and H), and were located under elongating procambial cells (Figure 3.4 E and K). MPCs then matured into irregularly-shaped and isolated idioblasts (Figure 3.4 C and I) positioned next to abaxial vascular tissue (Figure 3.4 F and L).
3.3.3 Characteristics of mature myrosin phloem cells
MPCs in mature rosette leaves achieved a length 5-6 times that of surrounding spongy mesophyll cells, and their diameter was often greater than adjacent and narrow phloem cells such as sieve tubes and companion cells (Figure 3.5B). Mature MPCs harbored a spindle-shaped nucleus, as well as large vacuoles containing soluble myrosinases (Figure 3.5 A and C, Ueda et al., 2006). MPC shape varied and these cells were found either singly or in contact (Figure 3.6), in contrast to the more continuous arrangement of e.g. the spongy mesophyll or the developing vasculature.

Figure 3.2 FAMA promoter-driven GUS (proFAMA:GUS) expression in MPCs in stem cross-section.
(A) Location of GCs in the epidermis as well as single and connected MPCs present outside vascular bundles.
(B) High magnification of a single vascular bundle in (C). Black and white arrows show GUS expression in MPCs and GCs respectively. X, Xylem; C, Vascular cambium; Ph, Phloem; Co, Cortex. All scale bars = 0.1 mm.
Figure 3.3 The GFP expression patterns of procambial and MPC markers.

Four columns showing vertically paired confocal images at two planes of focus. Each upper image is focused on procambial/provascular tissue, whereas the lower image is focused on the MPC layer (white arrows). Each column shows GFP/YFP expression from a different marker. \textit{Q0990:GFPer} marks the procambium, and at the sub-cellular level, localizes to the endoplasmic reticulum (ER). Fluorescence from \textit{proATHB8:HTA6-YFP} fusion labels the procambium and is localized to the nucleus. Note fluorescence from the MPC markers \textit{proFAMA:GFP} and \textit{E1728} at lower left images. All scale bars = 50um.
Figure 3.4 Comparison of stages of MPC and vascular development shown using two different MPC markers.

Confocal images taken 7, 8, and 10 Days After Germination (DAG) from first leaves. Pairs of vertical images (e.g. A & D, and I & L) show the same field focused on either the MPC layer (A, B, C & G, H, I) or the subjacent procambial /provascular (PC) tissues (D, E, F & J, K, L). “FAMA” denotes the transcriptional proFAMA:GFP fusion. E1728 refers to expression from the E1728:YFPer construct. All scale bars = 50um.
Figure 3.5 Visualization of MPCs with three different fluorescent markers showing positional relationships between MPCs and surrounding tissues.

(A) Fluorescence expression (related to auxin levels) from the 35S:3xvenus-N7 fusion protein that marks nuclei, is expressed in an MPC (*), as well as in the surrounding mesophyll (M).

(B) MPC (*). Cell walls visualized using PI staining. “Ph” marks the location of the phloem, indicating the close proximity of MPCs to the phloem. MPCs are not located in the phloem but derive instead from the ground tissue meristem. “M” denotes mesophyll tissue.

(C) Fluorescence from the Q5 enhancer trap marks the vacuolar membrane. The star indicates an MPC. “M” refers to a mesophyll cell.
Figure 3.6 Variations in wild-type MPC shape and distribution visualized using the *proFAMA:GUS* marker

(A) - (C) Many MPCs are isolated and separated from each other.

(D) - (F) Other MPCs normally form in contact *e.g.* in clusters of two (E) or three (F) MPCs.

All scale bars = 50um.

3.4 Discussion

3.4.1 Comparison of the origins of MPCs, stomata, and the vasculature

Although guard cells as well as MPCs are myrosin cells, their development differs significantly. Stomata arise through successive cell types that include stem cells (MMCs and meristemoids) as well as GMCs (Pillitteri and Torii, 2012). In the stomatal lineage, the loss of FAMA function leads to two different phenotypes, an absence of GC fate, as well as clusters of cells that form in place of guard cells and that arise from extra symmetric divisions. GMCs have a specific cell fate and are strictly separated from each other (Pillitteri and Torii, 2012). By contrast, MPC precursor cells arise from single or adjacent ground
meristem cells; in the latter case this can result in two MPCs forming in contact with each other in the wild-type. Since adjacent MPCs are often offset from each other, it is unlikely that both arise from the division of the same parent cell.

MPCs were found to originate from young ground meristem cells located under elongating procambial strands. Thus, although MPCs are located next to the phloem, the term ‘Myrosin Phloem Cells’ is a misnomer since they do not originate from the procambium. Instead, their position is more similar to that of a bundle sheath cell or even the mesophyll, rather than the phloem.

Mutant *fama-1* plants exhibited poor growth, reduced vascular formation, and showed an apparent absence of detectible MPCs in leaves. Clearly, the lack of stomata in the *fama-1* mutant prevents gas exchange and thus greatly reduces plant productivity. Moreover, the loss of myrosin cells and of selected *TGG* functions presumably makes *fama-1* plants more susceptible to predation, since half of the glucosinolate-myrosinase system is absent. Although MPCs do not derive from the procambium, their proximity to veins normally deters a certain degree of phloem feeding such as by insects. In addition, seedling growth is severely inhibited in *fama-1* plants presumably, in part, because vein networks are so poorly developed.
4 Polar Auxin Efflux is required for MPC Patterning and Morphogenesis

4.1 Introduction

4.1.1 Regulation of vesicular trafficking in auxin polar transport

Auxin accumulates in specialized locations such as procambial cells via directed intercellular export involving the vesicular trafficking of PIN proteins that function as auxin efflux carriers (Ohashi-Ito and Fukuda, 2010).

The Arabidopsis gene GNOM encodes an ADP ribosylation factor–GTP exchange factor (ARF-GEF), a GTPase/small G-protein, necessary for vesicle trafficking and cargo selection (Donaldson and Jackson, 2000). GNOM is required for PIN1 localization in the cell membrane where it controls polar auxin transport out of the cell.

Auxin efflux can be blocked chemically with the synthetic phytotropin 1-N-naphthylphthalamic acid (NPA), whereas the fungal toxin brefeldin A (BFA) inhibits ARF-GEF activity thereby disrupting PIN recycling as well as blocking outward auxin transport across the cell membrane (Morris et al., 2010; Geldner et al., 2003). Thus chemical inhibitions of NPA and BFA, as well as mutant gnom alleles, induce numerous auxin-mediated developmental defects including the loss of vascular polarity and continuity that results from abnormal auxin transport (Kleine-Vehna et al., 2009).

4.1.2 Auxin functions during the final stages of stomatal development

In stomatal patterning, differentiated GCs have been shown to generate inhibitory signals that discourage surrounding cells from developing into new GCs (Pillitteri and Torii, 2012). Auxin activity during stomatal development can be monitored by auxin input and output markers (Brunoud et al., 2012). Transcriptional activity from the DR5 promoter has been shown to represent auxin-mediated output activity (Figure 4.2; Vernoux et al., 2011).

Expression of the auxin-related input marker DII-VENUS is repressed when local IAA levels are elevated (Figure 4.2; Brunoud et al., 2012). DR5 activity decreases after GMCs divide symmetrically and could not be detected in mature GCs. In contrast, DII signal levels are
relatively high during GC maturation, consistent with the presence of low auxin levels and activity in late stomatal development (Jie et al., unpublished).

GNOM and PIN regulation of auxin levels in the stomatal lineage has been shown to control GC morphology and to enforce patterning, including at the stem cell stage (Jie et al., unpublished). gnom alleles and pin quadruple mutants have been shown to disrupt stomatal patterning by inducing the abnormal formation of two stomata in direct contact (Jie et al., unpublished).

![Diagram of interactions for BFA and NPA with GNOM-mediated PIN cycling and auxin efflux](image)

**Figure 4.1** A model of interactions for BFA and NPA with GNOM-mediated PIN cycling and auxin efflux (Muday et al., 2003).
Figure 4.2 Diagram showing the auxin-response pathway (Brunoud et al. 2012). The DII domain from Aux/IAA was fused to nuclear Venus fluorescence (35S:DII-3xVenus-N7). The DII domain is present in Aux/IAA proteins and is part of the early auxin signalling pathway (input activity). DR5 transcription is activated later in the auxin signalling pathway (output) (Brunoud et al. 2012).

4.2 Material and methods

4.2.1 Plant material and growth conditions

The seeds of proDR5:3xVENUS-N7 and of 35S:DII-3xVenus-N7 were described separately by Heisler et al., (2005) and Brunoud et al., (2012), respectively. Seeds harboring proPIN3:PIN3-GFP were obtained from Jie et al., (unpublished) and used as described (Zádníková et al., 2010). gnom<sup>R5</sup> mutants harboring proFAMA:GFP reporter were obtained from Jie et al., (unpublished). Individual F2 lines were selected by phenotyping and reporter visualization and then confirmed by PCR-based genotype analysis with primers in Table 4-1.

4.2.2 Genotyping

Since homozygous gnom<sup>R5</sup> plants are infertile, plants harboring this (point) mutation were obtained from progeny of gnom<sup>R5</sup>/+ heterozygous plants. Individual lines were genotyped by specific primers (Table 4-1). To amplify the region of DNA flanking the point mutation, forward and reverse primers were designed for a 206bp length fragment that spanned the
mutation. PCR products were cut overnight by DdeI and then obtained using electrophoresis with a 4% agarose gel. \textit{gnom}^{R5}/\textit{+} heterozygotes display two bands, 206 and 175bp in size, whereas only one 206bp band is present in the wild-type.

4.2.3 Chemical inhibition

Seeds were germinated on media containing 10uM NPA or 20uM BFA (Sigma). These chemicals were dissolved in dimethyl sulfoxide (DMSO, Sigma), and then added to 1/2 murashige and skoog (MS) plant growth medium. The final DMSO concentration was less than 0.1% [v/v].

Table 4.1 Primers and DNA sequences used for \textit{gnom}^{R5} genotype analysis.

<table>
<thead>
<tr>
<th>Name of Primer</th>
<th>DNA sequence (5' to 3')</th>
</tr>
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<tbody>
<tr>
<td>gnomR5-F(DdeI)</td>
<td>GAAAGTGAAAGTAAGAGGCAAACTAA</td>
</tr>
<tr>
<td>gnomR5-F(DdeI)</td>
<td>ATTCTTGATCAGGGAATAGCTCC</td>
</tr>
</tbody>
</table>

4.3 Results

4.3.1 Young myrosin phloem cells exhibit high auxin-mediated transcriptional activity

To determine whether MPC development might involve auxin-mediated transcription, YFP fluorescence from the \textit{proDR5:3xVenus-N7} was analyzed during MPC development. Signal was high in nuclei in small and developing MPCs (Figure 4.3 A and B), but decreased during MPC expansion (Figure 4.3 C and D), indicating that auxin-mediated transcriptional output activity is strongest in early MPCs.

Fluorescence from the \textit{proPIN3:PIN3:GFP} translational fusion, that encodes an auxin efflux carrier, was detected in the cell membrane (Figure 4.3 B). This result is consistent with active auxin efflux occurring during MPC development. However, \textit{DII-VENUS} from the \textit{35S: DII-3xVenus-N7} is only expressed in mature GCs and old mesophyll cells, while no expression was detected during MPC elongation and maturation (Figure 4.4), consistent with the absence of any significant decrease in local auxin levels during MPC development.
Figure 4.3 Auxin-DR5 output activity was detected only in early stages of MPC development.

(A) *proDR5:3xVenus-N7* fluorescence (*) marks nuclei in two adjacent young MPCs. Cell walls visualized with PI staining.

(B) *proDR5:3xVenus-N7* fluorescence from nuclei in two young MPCs (*). Cell membranes visualized (in different field from (A)) with *proPIN3:PIN3-GFP*

(C) As in B, except later stage of MPC (*) expansion/development that shows little DR5 fluorescence.

(D) DR5 expression is absent in an older and larger MPC (*).

All stars mark MPCs. All scale bars = 10um.
Figure 4.4 35S:DII-3xVenus-N7 activity in developing and mature rosette leaves
(A) 30DAG lower epidermis showing Venus fluorescence in the nuclei of mature GCs.
(B) MPC layer from 9DAG seedling showing absence of DII fluorescence. Arrows indicate elongating MPCs.
(C) 30DAG Spongy mesophyll cells exhibiting DII-Venus nuclei fluorescence.
(D) No DII fluorescence was detected in MPC layer from seedling 30DAG. Arrow marks a differentiated MPC. Cell walls visualized with PI staining. All scale bars = 50um.

4.3.2 Chemical inhibition of auxin efflux in myrosin phloem cells

Both the chemical inhibitors, NPA and BFA, have been shown to disrupt the distribution of the procambium as well as of the mature vascular network in leaves (Donner et al., 2009). To further characterize possible relationships between MPC development and auxin-efflux-related signaling, seedlings were treated separately with each inhibitor (Figure 4.5). As
shown by the guard cell and MPC reporter marker *E1728* in wild-type leaves, MPCs normally formed a loose network associated with the vasculature (Figure 4.6 A-D). However, the application of NPA, that disrupts the efflux function of PIN proteins, caused MPCs and vascular tissues to redistribute into the leaf margin and petioles (Figure 4.6 E and F). However, the morphology of both cell types (GCs and MPCs) appeared to be unaffected by NPA treatment (Figure 4.6 G and H).

The application of BFA, which blocks GNOM-mediated vesicle trafficking, caused the crowding of MPCs as well as the vasculature (Figure 4.6 I and J). BFA also induced both GC and MPC swelling (Figure 4.6 K and L). However, neither NPA nor BFA affected the normal location of MPCs abaxial to vascular strands (Figure 4.6). Thus, MPC shape and pre-patterning require normal auxin efflux. But the disruption of auxin efflux does not affect the position of MPCs with respect to the vasculature.

![Figure 4.5 Plants grown with or without 10uM NPA or 20uM BFA. All plants are from first leaves of 8DAG seedlings. Both chemicals, but especially BFA, inhibit seedling growth and development. All scale bars = 1mm.](image)
Figure 4.6 Disruptions of MPC, vascular, and stomatal patterning by the auxin-pathway-related chemical inhibitors NPA and BFA.

Except for the dark-field images at the left, all figures show green fluorescence from the *E1728:YFPer* construct (as well as PI staining) in 8DAG first leaves.

(A) Normal vascular patterning, dark-field microscopy.

(B), (C) and (D) Normal distribution and patterning of stomata (dots) and MPCs (lines) visualized with *E1728:YFPer* fluorescence.

(E) NPA (10uM) collapses and radializes vascular distribution in the leaf margin and petiole. Dark-field microscopy.

(F), (G) and (H) NPA radializes MPC patterning between the leaf margin and the petiole without disruption of stomatal or MPC morphology. Arrows in H mark two MPCs in contact, a grouping also found in untreated plants.

(I) BFA (20uM) shortens and disrupts the vasculature (arrow). Dark-field microscopy.

(J) BFA induces MPC swelling and increases the number of MPCs in contact.

(K) and (L) BFA induces guard cell swelling as well as MPC swelling (compare to length-to-width ratio of MPCs in (D)). All scale bars = 50um.
4.3.3 The *gnom*<sup>R5</sup> allele disrupts the distribution and morphology of myrosin phloem cells

The ARF-GEF GNOM is required for polar cell-to-cell auxin transport that regulates vascular tissue formation and continuity (Geldner *et al.*, 2003). MPCs as well as GCs formed abnormally in contact when *GNOM* function is impaired in the *gnom*<sup>R5</sup> allele that harbors a mutation in a single-nucleotide (Figure 4.7 A, 6B, 6E and 6F; Geldner *et al.*, 2003). This allele induces MPC crowding instead of their normal, mostly separated distribution in the wild-type (Figure 4.7 C and G).

The *gnom*<sup>R5</sup> allele also induced abnormal swelling of MPCs (as well as of GCs) (Figure 4.7 F and B), a phenotype also generated by BFA treatment. These mutants showed decreased seedling growth and disrupted rosette leaf shape, as well as many short vascular bundles (Figure 4.8). Quantification of the effects of the *gnom*<sup>R5</sup> allele, using a *FAMA-GFP* transcriptional-reporter fusion, showed many more MPCs in contact than in the wild-type (Figure 4.9 B). However, the number of MPCs in *gnom*<sup>R5</sup> mutant leaves was similar to that of the wild-type (Figure 4.9 A). Together these data demonstrate that the normal distribution and shape of MPCs depend upon PIN and GNOM-mediated polar auxin efflux.

However, *pin1,3,4,7* and *pin2,3,4,7* quadruple mutants showed no abnormal MPC phenotypes (Figure 4.10). Thus MPC patterning might depend upon other sets of PIN proteins.
Figure 4.7 Wild-type and gnom<sup>R5</sup> phenotypes visualized with proFAMA:GFP fluorescence in GCs and MPCs.

(A) Wild-type rosette leaf showing MPC and GC distribution using proFAMA:GFP.

(B) Higher magnification showing normal GC patterning.

(C) Normal patterning includes multiple MPCs arranged in contact linearly (vertically arranged cluster), as well as side-by-side (lower right).

(D) Wild-type showing higher magnification of the same leaf in (A) of groups of MPCs with normal, elongated shape. White lines indicate the limits of the end of a single MPC.

(E) Low magnification of MPCs in the gnom<sup>R5</sup> mutant showing many in abnormal contact with each other.

(F) Abnormally swollen GCs in gnom<sup>R5</sup>. Arrow denotes two stomata in direct contact.

(G) Loss of GNOM function increases the number of MPCs in contact. Higher magnification of the same leaf in (E).

(H) Abnormally swollen MPC in gnom<sup>R5</sup> mutant. White lines indicate the limits of the end of a single MPC.

All samples are from 10DAG first leaves. All scale bars = 50um.
Figure 4.8 14DAG wild-type and gnom<sup>R5</sup> seedlings.
(A) Wild-type, bright-field image.
(B) gnom<sup>R5</sup> bright-field image.
(C) Dark-field image of gnom<sup>R5</sup> leaf. Arrows indicate short vascular strands. Bar = 0.5mm.

Figure 4.9 Quantification of MPC number in the wild-type and in the gnom<sup>R5</sup> mutant background.
(A) Total numbers of MPCs scored from 7DAG and 14DAG leaves.
(B) Extent of single MPCs compared to those with two or three MPCs in direct contact in leaves from 7DAG and 14DAG plants.
Value is the average calculated from ten first leaves (error bars) and visualized using the proFAMA:GFP MPC marker.
Figure 4.10 MPC patterning does not appear to be disrupted in two different *pin* quadruple mutants.
Both mutants are from first leaves from 14DAG seedlings. Arrows indicate elongated and apparently normal MPCs located close to the phloem. All scale bars = 50um

4.4 Discussion

4.4.1 Comparison of auxin-mediated expression markers in MPC and stomatal development

Similar to stomatal patterning (Pillitteri and Torii, 2012), differentiated MPCs have been hypothesized to generate inhibitory signals that discourage surrounding cells from developing into new MPCs (Ueda *et al.*, 2006). GNOM regulation of auxin levels in the stomatal lineage has been shown to control GC morphology and to enforce patterning,
including at the stem cell stage (Jie et al., unpublished). The results shown here suggest that the patterning of normal MPCs, whether arranged singly or in small clusters, requires intracellular GNOM-mediated signal transport. Thus, normal IAA levels and efflux between MPCs and nearby cells might regulate MPC formation in time and space.

Fluorescence from the auxin-related input marker DII-VENUS is repressed when local IAA levels are elevated (Brunoud et al., 2012). DII signal levels are relatively high during GC maturation, consistent with the presence of low auxin levels late in stomatal development (Jie et al., unpublished). By contrast, no DII-VENUS expression was detected during MPC elongation and maturation, whereas DR5 output activity was present at these stages, indicating that the expression patterns of these two markers differ between both types of myrosin cells.

4.4.2 MPC morphogenesis and patterning is likely and normally controlled by the polar localization of PIN1 by GNOM and AtVAM3

Vascular tissues arise from procambial cells when auxin accumulates, whereas bundle sheath cells originate and differentiate directly from ground meristem cells that surround provascular tissues (Donner et al., 2009; Zhang et al., 2009). Both GNOM and AtVAM3 are required for the polar localization of the PIN1 protein that regulates directional auxin transport as well as the accumulation of IAA in the procambial/provascular cell lineages (Ohashi-Ito and Fukuda, 2010; Ueda et al., 2009). AtVAM3 promotes the docking of vesicles that then fuse with the tonoplast membrane, as well as enhancing PIN1 degradation and recycling (Naramoto et al., 2009). The ARF-GEF GNOM regulates the localization and turnover of PIN1 proteins in the cell membrane, and thus helps regulate auxin efflux and cell-to-cell auxin transport (Ueda et al., 2009). The loss of GNOM function induces gaps in the vasculature resulting in partial discontinuity (Geldner et al., 2003). This defect arises when auxin transport is disrupted and instead flows ectopically through inner tissues (Naramoto et al., 2009; Friml, 2010). In addition, the loss of AtVAM3 function reduces the number of veins as well as their junctions due to low auxin levels in the inner provascular tissue of developing leaves (Ueda et al., 2009).
Also, *atvam3* mutants appear to promote MPC formation leading to the development of excess MPCs that become abnormally arranged into a network (Figure 1.2; Ueda *et al.*, 2006). I found that the number of MPCs in contact increases in a *gnom* mutant background as well as in *atvam3*. These data suppose that GNOM and AtVAM3 normally regulating MPC distribution and provascular formation by ensuring the correct subcellular localization and recycling of PIN1 as well as the polarity of auxin transport.

While *pin1,3,4,7* and *pin2,3,4,7* quadruple mutants disrupt stomatal development (Jie *et al.*, unpublished), I found no abnormal MPC phenotypes in these quadruple mutants. Thus other sets of PIN proteins might involve in MPC patterning. Alternatively, GNOM and AtVAM3 might regulate other auxin-efflux mediated pathways.
5 Conclusions and Significance

5.1 Summary of major findings

MPC localization as well as the crystal structure and chemical activity of myrosinases have been well described (Ahuja et al., 2009). However, relatively little is known about how these cells develop, such as the genes that confer their fate as well as where MPCs originate in planta. Here I show that FAMA and the E1728 enhancer trap that mark GC fate are also expressed in developing as well as in mature MPCs, and that FAMA is required for MPC fate and TGG expression. In addition, I report that MPCs originate from the ground meristem rather than the procambium, and MPC shape and distribution depends upon auxin-related signaling.

5.2 Evolutionary significance of myrosin cells and glucosinolate-rich cells in the ‘Mustard Oil Bomb’ system

Myrosinase genes have been characterized in Brassica napus and Sinapsis alba as well as in Arabidopsis (Morant et al., 2008). The functions of these enzymes, as well as those in the glucosinolate synthesis pathway, have been well described (Wang et al., 2011), as have the spatial distributions of the cells that synthesize and store myrosinases (Kissen et al., 2009). Two types of myrosin cells have found to present in Arabidopsis, in the epidermis and outside of the phloem (Husebye et al., 2002). The rupture of these cells located in or relatively close to the epidermis, by fungi and insects, activates glucosinolate breakdown, thus deterring deeper penetration e.g. into the stem cortex and the phloem (Chen et al., 2010). Thus myrosin cells provide two lines of defense that reduce attacks to stomata and that discourage predator access to sugars in the phloem.

Similar to myrosinases in MPCs, glucosinolates are especially abundant in sulfur-rich idioblasts termed S-cells (Bones et al., 1991). S-cells are located in groups or clusters in proximity to MPCs, and are also located between the endodermis and phloem of each vascular bundle in flower stalk (Figure 5.1; Kissen et al., 2009). Glucosinolate synthesis is costly metabolically and diverts energy from plant growth (Kliebenstein, 2013). However, S-cells are limited in number, but are located strategically. Moreover, the proximity of S-
cells and MPCs means that the contents of both cell types can be simultaneously disrupted by predation. This in turn leads to interactions between myrosinases and glucosinolates that foster the enzymatic reactions that characterize the “Mustard Oil Bomb” and thus deter predation. Thus the proximity of myrosin cells and S-cells discourages predation while minimizing metabolic costs.

Figure 5.1 A diagram showing the distributions of myrosin cells and glucosinolate-rich/S-cells in a partial cross-section of an Arabidopsis stem. Diagram reproduced from Kissen (et al. 2009).

Pi, pith; X, xylem; P, phloem; M in red, MPCs; S, S-cells; M in orange, guard cells; E, epidermis.

The MPC/S-cell system has been mainly described in Crucifers. Myrosinases and glucosinolates appear to be unique beta-glucosidases and glucosides that are present in most members of the mustard family as well as in other Brassicales species such as Nasturtium (Wielanek et al., 2009). Moreover, some insect herbivores have parallel adaptations representing coevolution with their Brassica host plants. For example, some Brassica specialist aphids store myrosinases in crystalline microbodies in their digestive system, and
defend against predators by ingesting plant glucosinolates (Bridges et al., 2002). Parallel or comparable defense mechanisms have also been identified in other Angiosperm families, such as flavenoids and corresponding glucosides in legumes (Morant et al., 2008). This raises the possibility that cellular anti-predation systems comparable to that of MPCs/GCs might also have evolved in other Angiosperm species. Thus, my identification of a gene and a hormone that together regulate MPC development might help in define new genes that regulate glucosinolate-rich cells and their pathway, as well in recognizing novel anti-predation cell types in Crucifers and other Angiosperms.
Bibliography


GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth. Cell. 112(2), 219-230


Appendices

Appendix A: GUS staining solution

Table A-1 GUS Staining solution

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<td>1.8ml</td>
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<tr>
<td>200ul</td>
<td>0.5M EDTA</td>
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<tr>
<td>1ml</td>
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<tr>
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<tr>
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<td>X-Gluc</td>
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Table A-2 Phosphate buffer stock solution

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Appendix B: Confocal microscopy

Figure B.1 The Nikon ECLIPSE 80i confocal microscope.