The Plant Cell, Vol. 26: 4039–4052, October 2014, www.plantcell.org

FAMA Is an Essential Component for the Differentiation of Two Distinct Cell Types, Myrosin Cells and Guard Cells, in *Arabidopsis*[W]

Makoto Shirakawa,[a,b] Haruko Ueda,[a] Atsushi J. Nagano,[c,d] Tomoo Shimada,[a] Takayuki Kohchi,[b] and Ikuko Hara-Nishimura[a,1]

[a] Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan
[b] Graduate School of Biostudies, Kyoto University, Kyoto 606-8502, Japan
[c] Center for Ecological Research, Kyoto University, Otsu, Shiga 520-2113, Japan
[d] PRESTO, Japan Science and Technology Agency, Kawaguchi, Saitama 332-0012, Japan

Brassicales plants, including *Arabidopsis thaliana*, have an ingenious two-compartment defense system, which sequesters myrosinase from the substrate glucosinolate and produces a toxic compound when cells are damaged by herbivores. Myrosinase is stored in vacuoles of idioblast myrosin cells. The molecular mechanism that regulates myrosin cell development remains elusive. Here, we identify the basic helix-loop-helix transcription factor FAMA as an essential component for myrosin cell development along *Arabidopsis* leaf veins. FAMA is known as a regulator of stomatal development. We detected *FAMA* expression in myrosin cell precursors in leaf primordia in addition to stomatal lineage cells. *FAMA* deficiency caused defects in myrosin cell development and in the biosynthesis of myrosinases THIOGLUCOSIDE GLUCOHYDROLASE1 (TGG1) and TGG2. Conversely, ectopic *FAMA* expression conferred myrosin cell characteristics to hypocotyl and root cells, both of which normally lack myrosin cells. The FAMA interactors ICE1/SCREAM and its closest paralog SCREAM2/ICE2 were essential for myrosin cell development. DNA microarray analysis identified 32 candidate genes involved in myrosin cell development under the control of FAMA. This study provides a common regulatory pathway that determines two distinct cell types in leaves: epidermal guard cells and inner-tissue myrosin cells.

INTRODUCTION

Plants have evolved various strategies for herbivore defense, including the release of toxic compounds. The myrosinase (thioglucoside glucohydrolase [TGG])-glucosinolate defense system is characteristic of Brassicales. When herbivores damage tissues, myrosinase is released from its subcellular compartment to interact with its substrate glucosinolate, and the reaction products are toxic to herbivores (Rask et al., 2000; Wittstock and Halkier, 2002; Grubb and Abel, 2006; Halkier and Gershenzon, 2006; Hopkins et al., 2009; Kissen et al., 2009). Large amounts of myrosinase are stored in myrosin cell vacuoles (Rask et al., 2000; Andréasson et al., 2001; Husebye et al., 2002; Ueda et al., 2006), whereas the glucosinolate substrates are stored in different cells at the leaf periphery and along veins (Koroleva et al., 2000; Shroff et al., 2008). Myrosin cells were first discovered as idioblasts by Heinricher in 1884 (Heinricher, 1884). They were designated as myrosin cells by Guignard in 1890 (Guignard, 1890). *Arabidopsis thaliana* myrosin cells specifically develop along leaf veins (Xue et al., 1995; Andréasson et al., 2001; Husebye et al., 2002; Thangstad et al., 2004; Barth and Jander, 2006; Ueda et al., 2006). Several mutants with defective myrosin cell distribution have been identified (Ueda et al., 2006; Shirakawa et al., 2010, 2014). However, the molecular mechanism regulating myrosin cell development is largely unknown.

Stomatal guard cells function as specialized valves that mediate vapor and gas exchange in plants. Guard cell differentiation proceeds through a series of steps originating from meristemoid mother cells (Nadeau and Sack, 2002; Lau and Bergmann, 2012; Pillitteri and Torii, 2012; Pillitteri and Dong, 2013) and is positively regulated by two distinct basic helix-loop-helix (bHLH) transcription factor subfamilies. One subfamily contains three paralogs, SPEECHLESS (SPCH), MUTE, and FAMA, which regulate distinct developmental steps (Bergmann et al., 2004; Ohashi-Ito and Bergmann, 2006; MacAlister et al., 2007; Pillitteri et al., 2007). These three paralogs are not functionally exchangeable (MacAlister et al., 2007; MacAlister and Bergmann 2011). The other subfamily contains two paralogs, ICE1/SCREAM (SCRM) and SCRM2/ICE2, which redundantly regulate all steps of stomatal development (Kanaoka et al., 2008). Three different bHLH heterodimers, SPCH-ICEs, MUTE-ICEs, and FAMA-ICEs, are proposed to specifically promote the three distinct differentiation steps of stomatal lineages (Kanaoka et al., 2008). ICE1 and SCRM2 also function in freezing tolerance regulation (Chinnusamy et al., 2003; Fursova et al., 2009), but no other biological functions are reported for SPCH, MUTE, and FAMA.

We performed in silico analysis to identify transcription factors that were coexpressed with myrosinase-glucosinolate system genes and identified *FAMA* as an essential component for

[1] Address correspondence to ihnishi@gr.bot.kyoto-u.ac.jp.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Ikuko Hara-Nishimura (ihnishi@gr.bot.kyoto-u.ac.jp).
[W] Online version contains Web-only data.
www.plantcell.org/cgi/doi/10.1105/tpc.114.129874

myrosin cell differentiation. Before differentiation of stomatal lineages in leaf primordia, a subset of ground meristem cells transiently expresses *FAMA*; these cells subsequently differentiate into idioblasts (myrosin cells) expressing myrosinase. Differentiation of myrosin and guard cells requires *ICE1* and *SCRM2*. By contrast, guard cell differentiation requires *SPCH* and *MUTE*, but these are not required for myrosin cell differentiation. Our study elucidates the molecular mechanism underlying myrosin cell development. The data indicate that regulatory mechanisms for cell differentiation can be shared by two different developmental pathways that generate different cell types.

RESULTS

FAMA Expression in Corniculate-Shaped Cells of the Leaf Inner Layer and Stomatal Lineage Cells

To identify a key regulator of myrosin cell development, we analyzed transcription factor coexpression with genes involved in the myrosinase-glucosinolate system. We performed in silico screening with the *Arabidopsis* ATTED-II transcriptome database (Obayashi et al., 2009). We identified *FAMA* as a gene coexpressed with *EPITHIOSPECIFIER MODIFIER1* (Supplemental Figure 1), which encodes a protein in the myrosinase-glucosinolate pathway (Zhang et al., 2006). FAMA is a bHLH transcription factor that acts as a master regulator of stomatal development (Bergmann et al., 2004; Ohashi-Ito and Bergmann, 2006).

We investigated the spatial expression pattern of *FAMA* in greater detail by generating transgenic plants expressing β-glucuronidase (GUS) under control of the 3.1-kb *FAMA* promoter (*ProFAMA:GUS*). GUS activity was detected in stomatal lineage cells of the leaf epidermis (Figures 1A and 1B), consistent with previous results (Ohashi-Ito and Bergmann, 2006), and in corniculate-shaped cells with horn-like extensions that were distributed along veins in inner layer leaf tissues (Figures 1A and 1C). The characteristic shape and distribution of GUS-positive cells in inner layer tissues was similar to that of myrosin cells. Myrosin cells are localized in aerial parts but excluded from the hypocotyl in *Arabidopsis* (Husebye et al., 2002; Barth and Jander, 2006). GUS-positive corniculate-shaped cells were not observed in roots or hypocotyls (Supplemental Figure 2). These observations suggest that *FAMA*-expressing cells of the inner leaf tissues correspond to myrosin cells.

FAMA Expression in Leaf Primordia Identifies Myrosin Cells and Stomatal Cells

To determine whether *FAMA*-expressing cells of inner leaf tissues are myrosin cells and/or their precursors, we generated transgenic plants coexpressing the mature myrosin cell reporter *ProTGG2:VENUS-2sc* (Shirakawa et al., 2014) and the FAMA reporter *ProFAMA:TagRFP-FAMAg*. The FAMA reporter contained a translational fusion of *TagRFP* and a full genomic *FAMA* sequence; this reporter was functional because expressing *ProFAMA:TagRFP-FAMAg* rescued growth defects of *fama-1* mutants (Supplemental Figure 3). The Venus signals of mature myrosin cell reporters were detected in cells with TagRFP-FAMA-positive nuclei in leaf inner tissues (Figure 2A). The maturing and/or mature myrosin cells with high Venus fluorescence had low

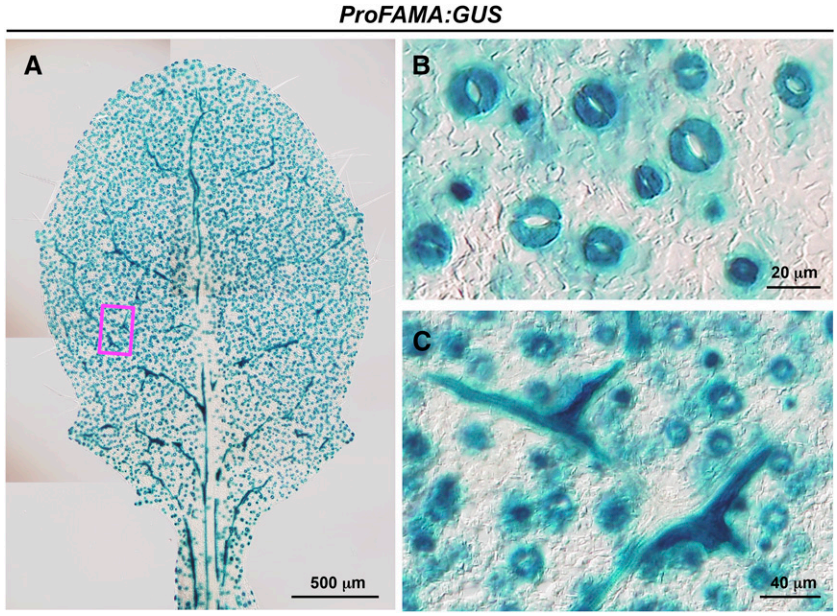

Figure 1. *FAMA* Expression in Leaf Inner Tissue Layer.

GUS staining of a rosette leaf of wild-type *Arabidopsis* (Col-0) expressing *ProFAMA:GUS*.
(A) An image of the whole leaf.
(B) Enlarged image of epidermal stomatal lineage cells.
(C) Enlarged image of the boxed area in **(A)**. Note that GUS activity is detected in the corniculate cells of the inner tissue layer.

TagRFP-FAMA expression levels, whereas immature myrosin cells with low Venus fluorescence had high TagRFP-FAMA expression levels (Figure 2B). Typically, mature myrosin cells had almost no TagRFP-FAMA signals (Figure 2B, arrowhead). These results suggest that *FAMA* is expressed in myrosin cell precursors and promotes myrosin cell development.

We examined changes in *ProFAMA:GUS* expression patterns during development of inner leaf tissues. A GUS-positive cell first emerged at the middle point of a future primary vein in leaf primordia with 85 to 140 μm length (Figure 2C; Supplemental Figure 4). At this stage, the GUS-positive cell was morphologically indistinguishable from neighboring ground meristem cells, which start to differentiate into several cell types including vascular and mesophyll cells (Kang and Dengler, 2004; Scarpella et al., 2004; Sawchuk et al., 2008). At a subsequent stage, leaf primordia with a length of 240 μm expressed the mature myrosin cell marker *ProTGG2:VENUS-2sc* at the middle point of a future primary vein axis (Supplemental Figure 5). These results suggest that *FAMA* is first expressed in a subset of ground cells and that these *FAMA*-positive cells begin to express *TGG2* in later developmental stages. At much later stages, *FAMA* was expressed in stomatal-lineage cells in leaves of ~340 μm in length (Figure 2C).

Loss-of-Function *fama* Mutants Fail to Develop Myrosin Cells

We examined the effect of the *FAMA* null mutation on myrosin cell development by generating two T-DNA insertion mutants (*fama-1* and *fama-3*) that lacked detectable *FAMA* transcripts

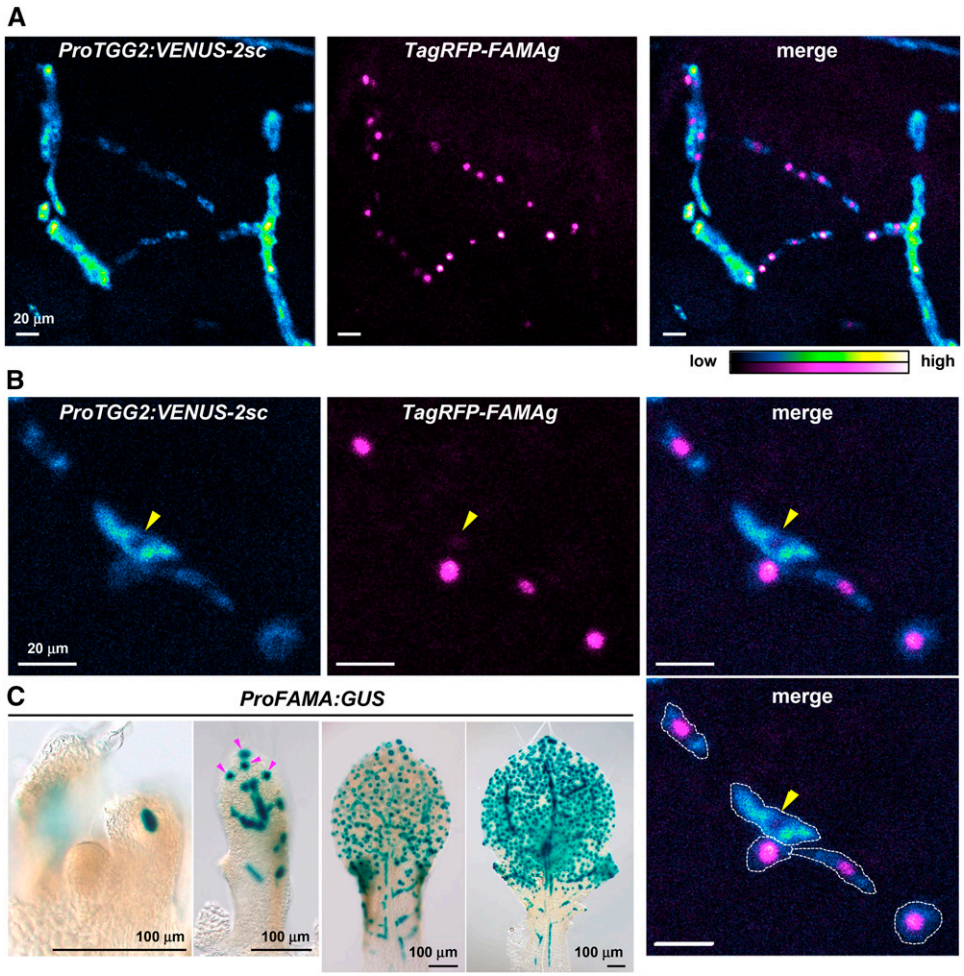

Figure 2. *FAMA* Is Expressed before a Mature Myrosin Cell Marker in Myrosin Cells.

(A) and **(B)** Confocal images of the inner tissue of leaf primordia coexpressing both *ProTGG2:VENUS-2sc* (blue-to-yellow) and *ProFAMA:TagRFP-FAMAg* (magenta-to-white). Images are maximum intensity projections of a series of images in the Z-plane. Signal intensities are shown in blue-to-yellow or magenta-to-white according to increasing intensity levels. VENUS-2sc localizes to the endoplasmic reticulum and vacuoles, whereas TagRFP-FAMAg localizes to the nucleus. Note that very low expression of *ProFAMA:TagRFP-FAMAg* was found in mature myrosin cells (arrowheads in **[B]**). The approximate border of the myrosin cell is indicated by a white outline (lower right panel in **[B]**).
(C) Developmental change in *ProFAMA:GUS* expression pattern. Arrowheads indicate stomatal lineage cells.

(Figures 3A and 3B). The myrosin cell reporters *MYR001:GUS* (Shirakawa et al., 2014) and *ProTGG2:GUS* (Barth and Jander, 2006) distributed along leaf veins and formed network patterns in wild-type plants (Figure 3C, Columbia-0 [Col-0]). When these myrosin cell markers were introduced into *fama-1*, GUS activity was not detected in leaves (Figure 3C, *fama-1*). This result was supported by the undetectable level of the *TGG1* transcript in *fama-1* and *fama-3* (Figure 3D). TGG1 and TGG2 are endogenous myrosin cell markers because they accumulate to high levels in myrosin cells. Immunoblot analysis showed that leaves, stems, and flowers of both *fama* mutants lacked detectable TGG1 and TGG2 (Figure 3E; Supplemental Figure 6). The F1 progeny of *fama-1* × *fama-3* was defective in myrosin cell reporter *MYR001:GUS* expression (Figure 3F) and accumulation of both TGG1 and TGG2 (Figure 3G), indicating that *fama-1* and *fama-3* are allelic to each other. Collectively, these results suggest that *FAMA* is essential for myrosin cell development in *Arabidopsis*.

The enhancer trap line E1728 was originally identified as a guard-cell-specific green fluorescent protein (GFP) line (Gardner

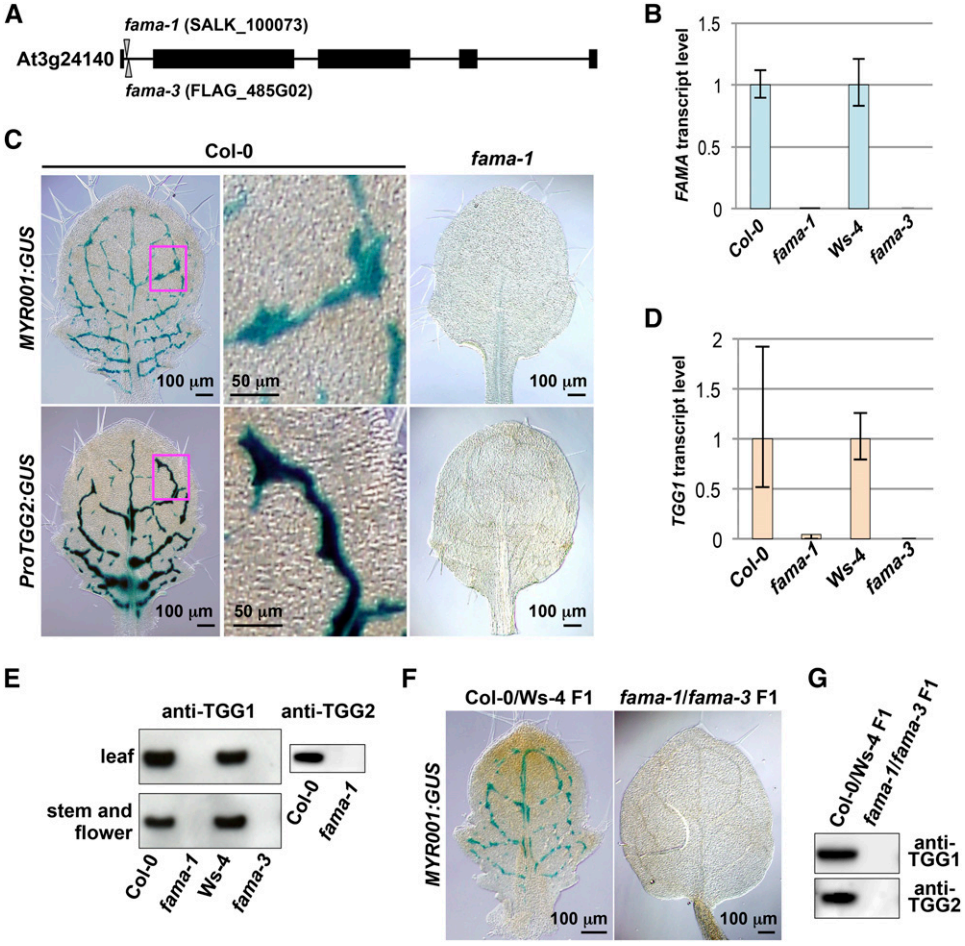

Figure 3. Myrosin Cell Development in Leaf Primordia of *fama* Loss-of-Function Mutants.

(A) Exon-intron organization of *FAMA*. T-DNA insertions are shown for *fama-1* (SALK_100073) and *fama-3* (FLAG_485G02). Closed boxes, exons; solid lines, introns.

(B) Quantitative RT-PCR of *FAMA* in 28 d after germination plants of *fama-1*, *fama-3*, and their respective wild-type lines (Col-0 and Ws-4) using *Actin2* as a control. Error bars indicate 95% confidence intervals (n = 3).

(C) GUS staining of the rosette leaves of Col-0 and *fama-1* plants expressing the myrosin cell markers *MYR001:GUS* (upper panels) and *ProTGG2:GUS* (lower panels). The boxed areas in the left panels are enlarged (middle panels).

(D) Quantitative RT-PCR of *TGG1* in 28 d after germination plants of *fama-1*, *fama-3*, and their respective wild-type lines (Col-0 and Ws-4) using *Actin2* as a control. Error bars indicate 95% confidence intervals (n = 3).

(E) Immunoblot analysis of rosette leaves (upper panel) and stems and flowers (lower panel) of *fama-1*, *fama-3*, and their respective wild-type lines (Col-0 and Ws-4) with anti-TGG1 antibody (left panels) and anti-TGG2 antibody (right panel).

(F) GUS staining of the rosette leaves of F1 progenies of Col-0 × Ws-4 (left panel) and *fama-1* × *fama-3* (right panel), both of which expressed *MYR001:GUS*.

(G) Immunoblot analysis of rosette leaves of the indicated F1 progenies with anti-TGG1 antibody (upper) and anti-TGG2 antibody (lower).

et al., 2009). E1728 also had GFP fluorescence in corniculate-shaped cells along veins in leaf inner tissue and in epidermal guard cells (Supplemental Figure 7A). GFP fluorescence along leaf veins was not detected in *fama-1* (Supplemental Figure 7B). These characteristics suggest that GFP-positive corniculate-shaped cells in the E1728 line are myrosin cells. The fluorescent activity of E1728 in the leaf inner tissues may be overlooked previously because of difficulty to detect fluorescence in inner tissues. E1728 can be used as a line for the analysis of both myrosin cells and guard cells.

FAMA Is Required for Its Own Expression in the Myrosin Cell Lineage but Not in the Stomatal Cell Lineage

To examine the mode of action of FAMA in myrosin cell development, we generated transgenic plants expressing *FAMA* fused to the ethylene response factor-associated amphiphilic repression (EAR) domain (FAMA-SRDX), under the control of the 35S promoter (Figure 4A). Transgenic plants neither accumulated endogenous TGG1 (Figure 4B) nor expressed *MYR001:GUS* (Figure 4C). These results indicate that FAMA-SRDX has dominant-negative activity. FAMA might act primarily as a transcriptional activator in myrosin cell development.

Positive feedback regulation is a common mechanism that enables many transcription factors to stabilize their own expression. A previous report indicated that positive autoregulation is not absolutely required to promote *FAMA* expression in stomatal lineage cells (Ohashi-Ito and Bergmann, 2006), although FAMA binds its own promoter (Hachez et al., 2011). In agreement, we detected GUS activity in stomatal lineage cells of transgenic plants expressing *ProFAMA:GUS* in the *fama* background (Figure 4D). However, GUS activity was not detected in the myrosin-lineage cells of transgenic plants expressing *ProFAMA:GUS* in the *fama* background (Figure 4D; Supplemental Figure 8). These results suggest that *FAMA* is required for its own expression in the myrosin lineage cells. Positive feedback regulation of FAMA expression might function in the myrosin lineage cells, but not in the stomatal lineage cells.

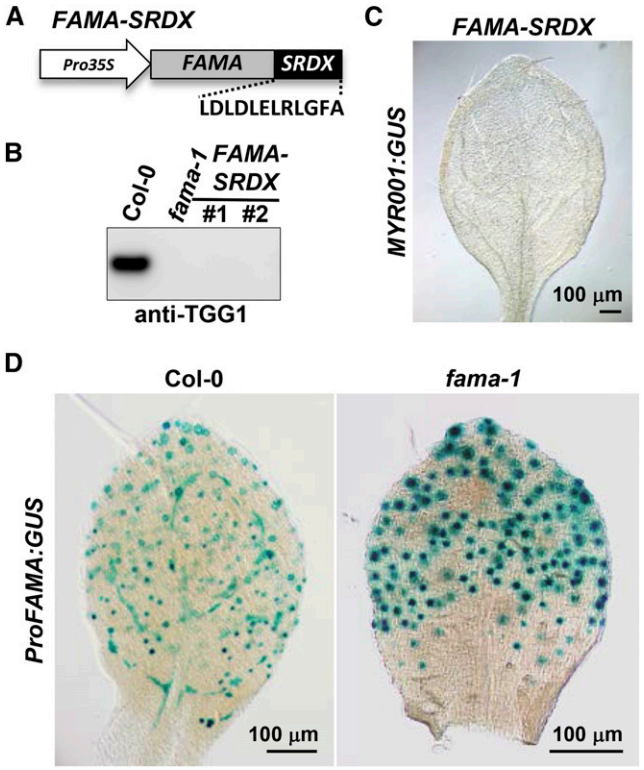

Figure 4. FAMA Is Required for Its Own Expression in Myrosin Lineage Cells.

(A) Structural organization of the *FAMA-SRDX* construct. *FAMA-SRDX* expresses FAMA fused to the EAR repression domain under the control of the cauliflower mosaic virus 35S promoter.
(B) Immunoblot analysis of rosette leaves of Col-0, *fama-1*, and transgenic plants expressing *FAMA-SRDX* (independent lines #1 and #2) with anti-TGG1 antibody.
(C) GUS staining of the rosette leaves of *FAMA-SRDX* transgenic plants expressing *MYR001:GUS*.
(D) GUS staining of rosette leaves of Col-0 and *fama-1* plants expressing *ProFAMA:GUS*.

FAMA Expression Potentially Confers Myrosin Cell Identity to Various Cell Types

To investigate whether *FAMA* is sufficient for myrosin cell development, we expressed FAMA-sGFP under the estrogen-inducible promoter in *MYR001:GUS*-expressing transgenic plants. Estrogen treatment strongly induced GUS expression in cells throughout the leaf in two independent lines (Figure 5A; Supplemental Figure 9). Consequently, the TGG1 and TGG2 protein levels in estrogen-treated leaves were more than 30-fold higher in two independent lines compared with those in untreated leaves (Figure 5B; Supplemental Figure 10). The estrogen-treated leaves became pale green during seedling growth, which is probably due to the overproliferation of myrosin cells in mesophyll tissue (Supplemental Figure 11). Estrogen treatment induced the ectopic development of myrosin cells in hypocotyls and roots, both of which lack myrosin cells in wild-type plants (Figure 5A; Supplemental Figure 9). Similarly, transgenic plants expressing both *ProEstro:FAMA* and *ProTGG2:GUS* exhibited the ectopic development of myrosin cells under the estrogen-treated condition (Figure 5C). These results suggest that *FAMA* expression potentially confers the myrosin cell identity to various cell types.

FAMA Expression in the Myrosin Cell Lineage Is Independent of *SPCH* and *MUTE*

FAMA functions downstream of the bHLH transcription factors *SPCH* and *MUTE* in the stomatal cell lineage (MacAlister et al., 2007; Pillitteri et al., 2011). To investigate the epistatic effect of *FAMA* on *SPCH* and *MUTE* during the generation of *FAMA*-expressing cells in the leaf inner tissues, *ProFAMA:GUS* was expressed in *spch-3* and *mute-2* mutants, both of which lack stomata. In these lines, GUS signal was detected in cells along leaf veins (Figure 6A; Supplemental Figure 12). This distribution pattern of the GUS-positive cells in *spch-3* expressing *ProFAMA:GUS* was very similar to that in *spch-3* expressing

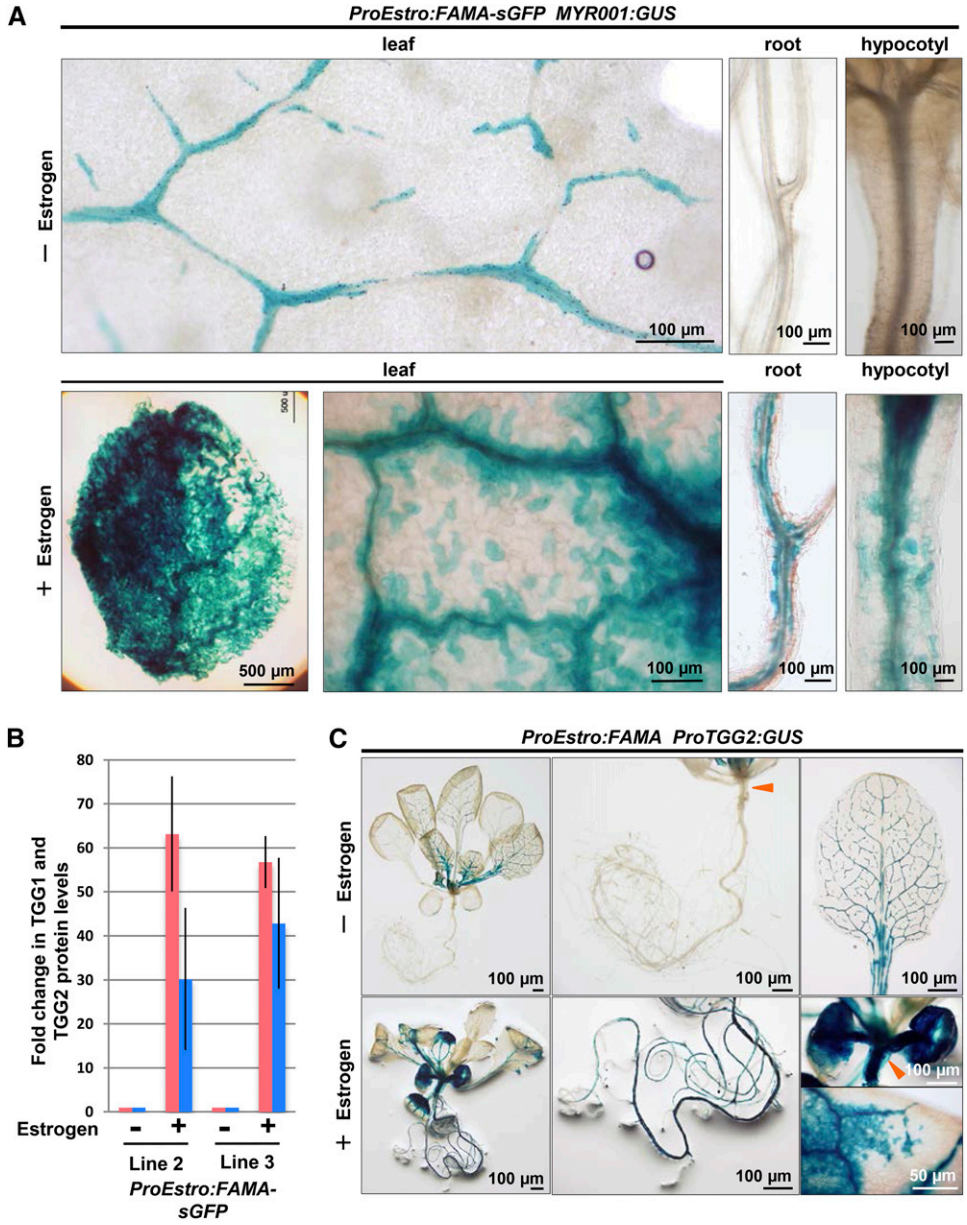

Figure 5. *FAMA* Expression Confers Myrosin Cell Identity to Various Cell Types.

(A) GUS staining of transgenic *ProEstro:FAMA-sGFP* plants expressing *MYR001:GUS*. The *ProEstro:FAMA-sGFP* plants express *FAMA-sGFP* under the control of the estrogen-inducible promoter. The 8-d-old plants were transplanted onto inductive medium containing 10 μM estrogen (+Estrogen) or no estrogen (−Estrogen) and incubated for 2 weeks.

(B) Immunoblot analysis of rosette leaves of *ProEstro:FAMA-sGFP* plants (independent lines 2 and 3) with anti-TGG1 (pink) and anti-TGG2 (blue) antibodies. The immunoblot signal intensities were quantified by densitometry. Error bars indicate standard deviations (n = 3). See also Supplemental Figure 10.

(C) GUS staining of transgenic plants expressing *ProTGG2:GUS* and *ProEstro:FAMA*. This plant expressed *FAMA* under the control of the estrogen-inducible promoter. The 8-d-old plants were transplanted onto inductive medium containing 10 μM estrogen (+Estrogen) or no estrogen (−Estrogen) and then incubated for 2 weeks. Arrowheads indicate hypocotyl.

MYR001:GUS (Figure 6B). By contrast, no GUS signals were detected in stomatal lineage cells in the epidermis of *spch-3* and *mute-2* expressing *ProFAMA:GUS* (Figure 6A; Supplemental Figure 12). RT-PCR analysis revealed that both *FAMA* and *TGG1* were expressed in *spch-3* at almost the same levels as in wild-type plants (Supplemental Figure 13). Consistent with this, TGG1 accumulation levels in *spch-3* and *mute-2* were similar to those in the wild type (Figure 6C). Notably, the TGG1 levels in both mutants were slightly less than in the wild type, which is consistent with the report that TGG1 is also expressed in guard cells

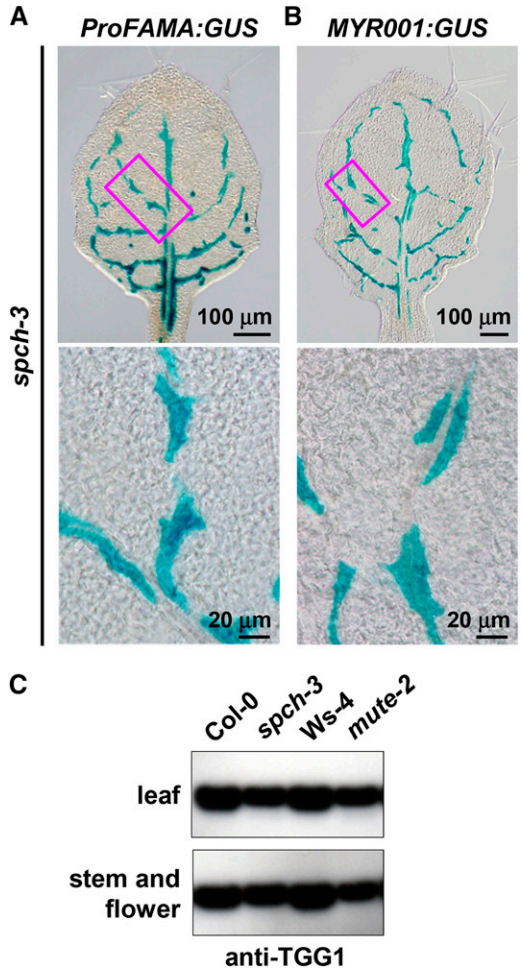

Figure 6. *FAMA* Expression in Myrosin Cells Is Independent of *SPCH* and *MUTE*.

(A) and **(B)** GUS staining of the rosette leaves of *spch-3* expressing *ProFAMA:GUS* **(A)** or *MYR001:GUS* **(B)**. Each boxed area in the upper panel is enlarged in the corresponding lower panel.

(C) Immunoblot analysis of rosette leaves (upper panel) and stems and flowers (lower panel) of wild-type lines (Col-0 and Ws-4), *spch-3*, and *mute-2* with anti-TGG1 antibody.

(Husebye et al., 2002; Barth and Jander, 2006). These results suggest that *FAMA* expression in myrosin cells is independent of *SPCH* and *MUTE*.

FAMA-Interacting Partners ICE1 and SCRM2 Are Required for Myrosin Cell Development

ICE1 and its closest paralog *SCRM2* encode bHLH transcription factors that form heterodimers with FAMA and act together in guard cell differentiation although they are expressed throughout leaves (Chinnusamy et al., 2003; Kanaoka et al., 2008; Fursova et al., 2009). We examined their involvement in myrosin cell development using the *GUS* reporter gene of *ICE1* (*ProICE1:GUS*). GUS activity was predominantly detected in corniculate-shaped cells along leaf veins (Figures 7A and 7C) and in stomatal lineage cells (Figure 7B), in addition to weak signals in mesophyll (Figures 7A and 7C). The *spch-3* mutant exhibited GUS-positive corniculate-shaped cells along leaf veins (Figures 7D and 7E) and GUS-positive mesophyll cells (Figures 7D and 7E). This expression pattern of *ProICE1:GUS* except for mesophyll cells was similar to the *ProFAMA:GUS* pattern in *spch-3* (Figure 6A). These results suggest that *ICE1* is expressed in myrosin lineage cells.

We questioned whether *ICE1* and *SCRM2* were required for myrosin cell development. The TGG1 protein level was markedly reduced in *ice1-2*, but not in *scrm2-1* (Figure 7F; Supplemental Figure 14). No TGG1 was detected in *ice1-2 scrm2-1*, similar to that observed for *fama* mutants (Figure 7F; Supplemental Figure 14). Similar defects in both TGG1 and TGG2 accumulations were detected in transgenic plants expressing an artificial microRNA targeted to both *ICE1* and *SCRM2* (*amiRNA-ICE1_SCRM2*) (Figure 7F). In addition, no GUS activity conferred by *MYR001:GUS* was detected in *amiRNA-ICE1_SCRM2* (Figure 7G). Taken together, these results suggest that *ICE1* and *SCRM2* act redundantly during myrosin cell development.

Identification of Novel Components of FAMA-Regulated Myrosin Cell Development

We identified the following novel components of myrosin cell development under the control of FAMA. First, we performed comparative microarray experiments using the wild type and the myrosin cell overproliferation mutant *syp22-4* (Ueda et al., 2006), which exhibited normal distribution and density of stomata (Supplemental Figure 15). In *syp22-4*, 931 genes were significantly upregulated relative to the wild type. Next, by comparing these microarray data with those of the 4-h estrogen-inducible *FAMA* overexpression lines (Hachez et al., 2011), we found that 32 genes were upregulated commonly in both lines (Figure 8A, Table 1). These genes were candidate components in the myrosin cell development regulated directly by *FAMA*. They did not include the myrosin cell marker genes *TGG1* and *TGG2* because both *TGG1* and *TGG2* were significantly upregulated in *syp22-4* but were not included in the 357 genes of the microarray data (Hachez et al., 2011).

Top priority genes were transcription factors, especially of the bHLH class, given the fact that this family plays key roles in many cell fate decisions in animals and plants. We found only one bHLH transcription factor, bHLH090 (At1g10610), in the candidate gene list (Table 1). Real-Time PCR analysis confirmed that the expression level of *bHLH090* in *syp22-4* was 2.5-fold higher than in the wild type (Figure 8B). To investigate the spatiotemporal expression pattern of *bHLH090* in leaf primordia, we generated transgenic plants expressing GUS under the control of the 2-kb *bHLH090* promoter (*ProbHLH090:GUS*). GUS activity was detected in corniculate-shaped cells with horn-like extensions that were distributed along veins in *ProbHLH090:GUS* lines as in *ProFAMA:GUS* lines and was not detected in stomatal lineage cells of the leaf epidermis (Figure 8C). In *syp22-4 ProbHLH090:GUS*, GUS-positive cells were more abundant than in the wild type and formed a drastically denser network than observed in the wild type (Figure 8D). These results suggest that bHLH090 is a novel component for the myrosin cell development,

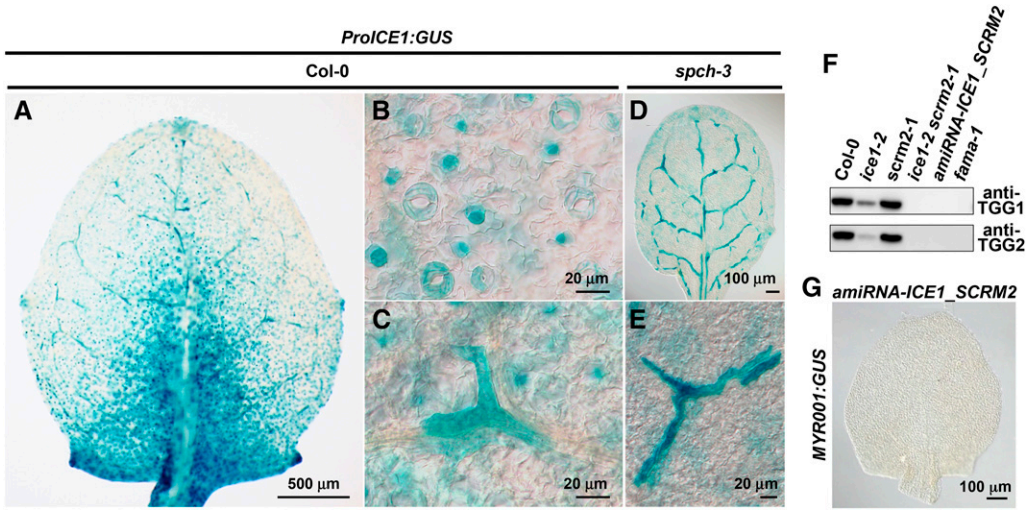

Figure 7. ICE1 and SCRM2 Are Required for Myrosin Cell Development.

(A) to **(E)** GUS staining of rosette leaves of Col-0 (**[A]** to **[C]**) and *spch-3* (**[D]** and **[E]**) expressing *ProICE1:GUS*. In the Col-0 background, GUS activity is detected in the corniculate cells of leaf inner tissue **(C)** as well as in epidermal stomatal lineage cells **(B)**. In *spch-3*, GUS activity is detected only in the corniculate cells of leaf inner tissue **(E)**.

(F) Immunoblot analysis of rosette leaves of the wild type and the indicated mutant or transgenic lines with anti-TGG1 antibody (upper) and anti-TGG2 antibody (lower).

(G) GUS staining of a rosette leaf of transgenic *amiRNA-ICE1_SCRM2* plants expressing *MYR001:GUS*.

and our comparative microarray experiments may identify new components in the myrosin cell development.

DISCUSSION

FAMA Is an Essential Transcription Factor for Myrosin Cell Differentiation

No essential component for idioblast myrosin cell development has been identified, although several factors required for the development of *Arabidopsis* myrosin cells have been identified (Ueda et al., 2006; Shirakawa et al., 2010, 2014). In this study, we show that the bHLH transcription factor FAMA is essential for myrosin cell differentiation in *Arabidopsis*. *FAMA* has a dual functional role: it regulates differentiation of myrosin cells in leaf inner tissues, and it regulates differentiation of guard cells in epidermal tissues (Ohashi-Ito and Bergmann, 2006). We propose that *FAMA* is a master regulator of myrosin cell differentiation based on three results. First, *FAMA* was expressed in the ground meristem cell at very early developmental stages of leaf primordia (Figures 1 and 2). Second, no mature myrosin cells developed in *fama* loss-of-function mutants (Figure 3). Third, ectopic *FAMA* expression was sufficient to confer idioblast myrosin cell identity to various cell types such as root cells, which do not differentiate into myrosin cells in wild-type *Arabidopsis* (Figure 5). It was reported that TGG1 was also detected in a subset of guard cells, called myrosin guard cells (Husebye et al., 2002; Barth and Jander, 2006). In *fama*, differentiations of both idioblast myrosin cells and myrosin guard cells were blocked (Figure 3) (Bergmann et al., 2004; Ohashi-Ito and Bergmann, 2006). Taken together, these results indicate that *FAMA* plays a crucial role in myrosin cell differentiation in *Arabidopsis*.

How *FAMA* Generates Two Different Cell Types in Plants

Myrosin cells are a recent innovation that is restricted to Brassicales (Rask et al., 2000). By contrast, stomata composed of a pair of guard cells represent a very old plant innovation found in all land plants except liverwort (Bowman, 2011). *Arabidopsis* has two *FAMA* paralogs, *SPCH* and *MUTE*. These three paralogs are involved in the development of stomatal lineage cells at different steps (MacAlister et al., 2007; Pillitteri et al., 2007). Phylogenetic analysis indicates that the FAMA amino acid sequence is better conserved among land plants than is SPCH or MUTE (Ran et al., 2013). The question arises how *FAMA* exerts its dual function for guard cell and myrosin cell development, without dynamic changes in its sequence.

One possible explanation is as follows: Expression of *FAMA* in epidermal guard mother cells results in guard cell differentiation, whereas expression of *FAMA* in ground meristem cells of leaf inner tissue results in myrosin cell differentiation. *FAMA* might have different downstream targets in different cell types due to differences in chromatin structure in these cells. Another possible explanation could be spatiotemporal differences in the dynamics of *FAMA* expression in the two cell types. *FAMA* expression is regulated by a positive feedback loop in myrosin lineage cells (Figure 4), but not in stomatal lineage cells (Ohashi-Ito and Bergmann, 2006). *FAMA* expression is transient in myrosin lineage cells (Figure 2), whereas *FAMA* expression is prolonged in stomatal lineage cells (Ohashi-Ito and Bergmann, 2006). Regulatory mechanisms governing *FAMA* expression dynamics

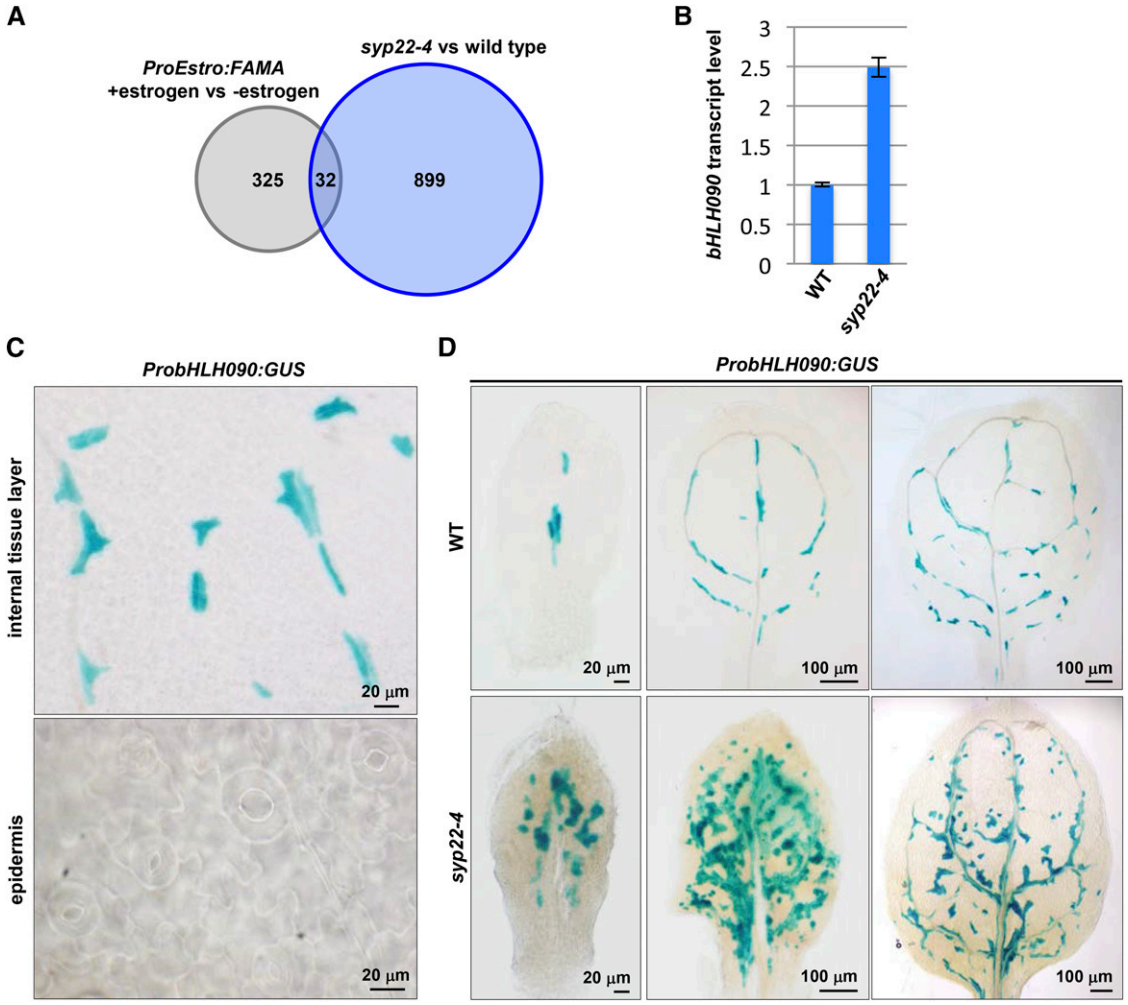

Figure 8. *bHLH090* Specifically Expressed at the Myrosin Lineage Cells in Leaves.

(A) Summary of comparative microarray results showing 32 candidate genes involved in myrosin cell development. See Table 1 for details of these genes. The genes upregulated in *syp22-4* are listed in Supplemental Data Set 1. They were compared with the genes upregulated in 4-h estrogen-treated plants expressing *ProEstro:FAMA* (Hachez et al., 2011).

(B) Quantitative RT-PCR of *bHLH090* in 21 d after germination plants of wild-type lines (Col-0) and *syp22-4* using Actin2 as a control. Error bars indicate SD (n = 3).

(C) GUS staining of the rosette leaves of *ProbHLH090:GUS*.

(D) Developmental change in *ProbHLH090:GUS* expression pattern in the first pair of rosette leaves of the wild-type lines (Col-0) and *syp22-4*.

might differ in the two cell types. Further analysis is necessary to identify the downstream targets and regulatory mechanism of FAMA.

Upstream Factors of FAMA at the Start of Myrosin Lineage Cell Specification

FAMA expression in stomatal lineage cells depends on *SPCH* and *MUTE*, indicating that *FAMA* is a downstream target (MacAlister et al., 2007; Pillitteri et al., 2011). By contrast, this study demonstrates that *FAMA* is required for myrosin cell development, but *SPCH* and *MUTE* are not. We show that myrosin lineage cells are derived from ground meristem cells. Vascular precursor cells (preprocambium/procambium) are also derived from ground meristem cells (Kang and Dengler, 2004; Scarpella et al., 2004). These two cell lineages independently arise from ground meristem cells because both *ProFAMA:GUS* and *ProAtHB8:GUS* (*AtHB8* is a master transcription factor of vascular precursor cells) are initially expressed simultaneously in a subset of the ground meristem cells with different spatial patterns (Figure 2) (Baima et al., 1995, 2001; Kang and Dengler, 2004; Scarpella et al., 2004). *AtHB8* expression in ground meristem cells is determined by the phytohormone auxin (Mattsson et al., 2003; Donner et al., 2009; Ohashi-Ito and Fukuda, 2010; Krogan et al., 2012). It is possible that *FAMA* expression in ground meristem cells also is determined by auxin. Auxin involvement in the onset of *FAMA* expression and synchronous

Table 1. Candidate Genes Involved in Myrosin Cell Development

AGI Code	Gene Name	Gene Description
Transcription factors		
AT1G10610	bHLH090	bHLH transcription factor
AT1G52890	NAC019	NAC transcription factor
AT2G40340	DREB2C	DREB subfamily A-2 of ERF/AP2 transcription factor
AT2G40350		DREB subfamily A-2 of ERF/AP2 transcription factor
AT2G47260	WRKY23	WRKY transcription factor
AT3G57600		DREB subfamily A-2 of ERF/AP2 transcription factor
AT5G65590	SCAP1	Dof-type transcription factor
Others		
AT1G03440		Leucine-rich repeat (LRR) family protein
AT1G19160		F-box family protein
AT1G27680	APL2	ADPGLC-PPase large subunit
AT1G30040	GA2OX2	Gibberellin 2-oxidase
AT2G11810	MGDC	Monogalactosyldiacylglycerol synthase type C
AT2G25780		Protein of unknown function (DUF1677)
AT2G28420	GLYI8	Lactoylglutathione lyase/glyoxalase I family protein
AT3G05640		Protein phosphatase 2C family protein
AT3G46230	HSP17.4	Heat shock protein 17.4
AT3G55840		Hs1pro-1 protein
AT3G56620		Nodulin MtN21 /EamA-like transporter family protein
AT4G09740	GH9B14	Glycosyl hydrolase 9B14
AT4G15120		VQ motif-containing protein
AT4G21730		Pseudogene of *N*-ethylmaleimide sensitive factor (NSF)
AT4G23560	GH9B15	Glycosyl hydrolase 9B15
AT4G24480		Protein kinase superfamily protein
AT4G29570		Cytidine/deoxycytidylate deaminase family protein
AT4G35070		SBP (*S*-ribonuclease binding protein) family protein
AT5G17830		Plasma membrane choline transporter family protein
AT5G43860	CLH2	Chlorophyllase 2
AT5G66400	RAB18	Dehydrin family protein
Unknown proteins		
AT2G21560		
AT3G09730		
AT3G26390		
AT4G28330		

division in stomatal lineage cells was recently reported (Le et al., 2014). The involvement of auxin in myrosin lineage cell specification is supported further by our previous observation that abnormal myrosin cell development is observed in *syp22/vam3* mutants, which exhibit an abnormal distribution of auxin (Ueda et al., 2006; Shirakawa et al., 2009). An investigation of *FAMA* in *syp22/vam3* would help reveal the fate determination mechanism of ground meristem cells. Thus, it is necessary to examine the relationship between auxin and *FAMA* expression during myrosin lineage cell specification.

bHLH090 Is a Novel Component in Myrosin Cell Development

Comparative DNA microarray experiments identified 32 candidates involved in myrosin cell development (Table 1). Indeed, one of these candidates, *bHLH090*, was expressed specifically in myrosin lineage cells in leaves (Figure 8). The expression of *bHLH090* was quickly triggered by *FAMA* (Hachez et al., 2011) and the 2-kb promoter region of *bHLH090* contains 11 G-box sequences (CANNTG) that are typical binding motifs of bHLH transcription factors. These results suggest that *bHLH090* might be a direct target of *FAMA*. Although bHLH090 is classified as an orphan bHLH protein (Pires and Dolan, 2010), bHLH090 shows high levels of sequence similarities with bHLH093, ICE1, and SCRM2, all of which interact with FAMA (Ohashi-Ito and Bergmann, 2006; Kanaoka et al., 2008), suggesting that bHLH090 might bind FAMA to modulate the activity of FAMA. Because bHLH090 homologs seem to be specific to Brassicales (Supplemental Figure 16), bHLH090 may be an evolved type of bHLH transcription factors for the production of myrosin lineage cells.

How Did Brassicales Plants Acquire Myrosin Cells during Evolution?

It is intriguing that the different cell lineages are regulated by the same FAMA transcription factor in *Arabidopsis*. *FAMA* is also expressed around leaf veins in rice (*Oryza sativa*) (Liu et al., 2009), but rice lacks myrosin cells. Although the nature of these *FAMA*-expressing cells is unknown, *FAMA* might function as an idioblast regulator in leaf inner tissue. It is possible that the *FAMA*-regulating idioblasts were specified to accumulate myrosinase and differentiate into myrosin cells when the Brassicales

acquired myrosinase during evolution. It would be interesting to study the nature of *FAMA*-expressing cells near leaf veins in rice and other plants. In an alternative scenario, transcription networks including FAMA and myrosinases might first have been established in myrosin guard cells. Subsequently, these transcription networks might have been co-opted from guard cells to idioblasts in leaf inner tissue during the evolution of Brassicales.

METHODS

Plant Material and Growth Conditions

The *Arabidopsis thaliana* Columbia (Col-0) ecotype was used for all lines except for *fama-3* and *mute-2* (Ws-4). The T-DNA insertion mutants and an enhancer trap line were obtained from the following sources: SALK_100073 (*fama-1*), SAIL_36_B06 (*spch-3*), SALK_003155 (*ice1-2*), and SAIL_808_B10 (*scrm2-1*) from the ABRC at Ohio State University; FLAG_485G02 (*fama-3*) and FLAG_225D03 (*mute-2*) from INRA; and E1728 from The European Arabidopsis Stock Center. The *ProTGG2:VENUS-2sc* and *MYR001:GUS* constructs were reported previously (Shirakawa et al., 2014). *ProTGG2:GUS* (Barth and Jander, 2006), *fama-1* E1728 (Ohashi-Ito and Bergmann, 2006), and *ice1-2 scrm2-1* (Kanaoka et al., 2008) were provided by G. Jander (Boyce Thompson Institute for Plant Research), D.C. Bergmann (Stanford University), and K.U. Torii (University of Washington), respectively. *syp22-4* was previously described (Ohtomo et al., 2005). Seeds were surface-sterilized with 70% ethanol and then sown onto 0.5% w/v gellan gum (Wako) containing 1% w/v sucrose and Murashige and Skoog medium (Wako). The seeds were incubated at 4°C for 3 to 5 d to break seed dormancy and were grown at 22°C for 20 d under continuous light. Plants were transferred onto vermiculite for subsequent growth.

Plasmid Construction and Transgenic Plants

The Gateway Cloning System (Life Technologies) was used for plasmid constructions. For transcriptional GUS fusion constructs, the 3.1-kb promoter of *FAMA*, the 2.6-kb promoter of *ICE1*, and the 2-kb promoter of *bHLH090* were cloned into pENTR D-TOPO. They were introduced into the binary vector pBGWFS7 (BASTA selection for plants) or pHGWFS7 (hygromycin B selection for plants) using LR reactions. For translational fusion constructs, the cDNA encoding TagRFP was inserted in front of the start codon of the 5.8-kb *FAMA* genomic fragment (including 3.1 kb of the 5′-flanking sequence and 0.5 kb of the 3′-flanking sequence). After cloning into pENTR D-TOPO, the construct was introduced into the binary vector pBGW (BASTA selection for plants) using LR reactions. For the *ProEstro:FAMA* and the *ProEstro:FAMA-sGFP* constructs, *FAMA* coding sequence and *FAMA* coding sequence fused to the cDNA encoding sGFP were cloned into pENTR D-TOPO and introduced into pMDC7 (Curtis and Grossniklaus, 2003) using LR reactions, respectively. An artificial microRNA (amiRNA) against both ICE1/SCRM and ICE2/SCRM2 was designed using the WMD2-Web MicroRNA designer (http://wmd3.weigelworld.org) and was amplified using the following primers from the pRS300 vector: amiRNA-F, amiRNA-R, ICE1/2_amiRNA_1, ICE1/2_amiRNA_2, ICE1/2_amiRNA_3, and ICE1/2_amiRNA_4. The primer sets are presented in Supplemental Table 1. The amplified amiRNA-ICE1_SCRM2 DNA fragment was cloned into the pENTR D-TOPO plasmid. The plasmid was introduced into binary vector pFAST-G02 (Shimada et al., 2010) using the LR reaction to generate *amiRNA-ICE1_SCRM2*. For the *FAMA-SRDX* construct, *FAMA* coding sequence fused to the EAR motif (Hiratsu et al., 2003) was cloned into pENTR D-TOPO. The plasmid was introduced into the binary vector pFAST-G02 (Shimada et al., 2010) using the LR reaction. *Agrobacterium tumefaciens* (strain GV3101) was transformed with these constructs. Plants were transformed with *Agrobacteria* using the floral dip method (Clough and Bent, 1998). T1 seeds were selected using medium containing 10 mg L^{-1} BASTA or 25 to 50 mg L^{-1} hygromycin B.

GUS Staining

Samples were first placed into ice-cold acetone for 15 min and then into GUS staining solution containing 0.5 mg/mL X-Gluc, 0.1 M sodium phosphate buffer, pH 7.0, 10 mM EDTA, 0.5 to 5 mM potassium ferricyanide, 0.5 to 5 mM potassium ferrocyanide, and 0.1% Triton X-100. Samples in the GUS staining solution were placed under a vacuum and incubated at room temperature for 4 to 24 h.

SDS-PAGE and Immunoblot Analysis

SDS-PAGE and immunoblot analysis were performed as described previously (Shimada et al., 2003). The antibodies used in this analysis were anti-TGG1 (diluted 5000-fold) (Ueda et al., 2006) and anti-TGG2 (diluted 5000-fold) (Ueda et al., 2006).

Confocal Laser Scanning Microscopy

Fluorescence micrographs were obtained with a confocal laser scanning microscope (LSM780; Carl Zeiss) using a water immersion objective (63× 1.20 numerical aperture [NA]) and dry objectives (40× 0.95 NA, 20× 0.80 NA, and 10× 0.50 NA). The laser wavelengths used include 488 nm (GFP and Venus) and 543 nm (TagRFP). The images were analyzed using LSM image software (Carl Zeiss) and were processed using ImageJ (NIH) and Photoshop (Adobe Systems) software.

RT-PCR

Total RNA was prepared from wild-type and mutant plants at 14, 21, and 28 d after germination using an RNeasy Plant Mini Kit (Qiagen). After DNase I (Invitrogen) treatment, reverse transcription was performed using a SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) with an oligo (dT)12-18 primer (Invitrogen). We performed PCR using 30 and 35 cycles with each primer set. Quantitative RT-PCR was performed using a gene-specific primer set (*FAMA*, At02279294_g1; *TGG1*, At02185835_g1; *Actin2*, At02335270_gH; Applied Biosystems) and a TaqMan Gene Expression Assay Kit (Applied Biosystems) or SYBR Premix Ex Taq II (Takara Bio) in a 7500 Real-Time PCR system (Applied Biosystems). The relative quantity of target mRNA was calculated using *Actin2* as a control. Primer sets except TaqMan Probe are presented in Supplemental Table 1.

Microarray Experiments

Total RNA was isolated from each developing leaf of both the wild type and *syp22-4* using the RNeasy Plant Mini Kit. The extracted RNA was quantified using a NanoDrop ND-1000 UV-VIS spectrophotometer (NanoDrop), and the quality of the RNA samples was confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNA (200 ng) was subjected to fluorescent labeling. Labeling was performed using an Agilent Low Input Quick Amp Labeling Kit 1-Color (Agilent Technologies) according to the manufacturer's protocol. The labeled cRNA was fragmented and hybridized on a slide of the *Arabidopsis* V4 Gene Expression Microarray 4 × 44K (Agilent Technologies; G2519F-021169) at 65°C for 17 h. Hybridization and washing of the hybridized slides were performed according to the manufacturer's instructions. Slides were scanned using a G2505B DNA microarray scanner (Agilent Technologies), and background correction of the raw signals was performed using the Agilent Feature Extraction software. All microarray data were transformed into a $\log_2$ scale and normalized using the qspline normalization method (Workman et al., 2002). The microarray data collected in this study are available in Supplemental Data Set 1.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *FAMA* (At3g24140), *ICE1/SCREAM* (At3g26744), *ICE2/SCREAM2* (At1g12860), *SPEECHLESS* (At5g53210), *MUTE* (At3g06120), *TGG1* (At5g26000), *TGG2* (At5g25980), *VSR1* (At3g52850), *EPITHIOSPECIFIER MODIFIER1* (At3g14210), *SYP22/VAM3* (At5g46860), *bHLH090* (At1g10610), and *ACT2* (At3g18780).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. *FAMA* Coexpression Network Predicted by ATTED-II.

Supplemental Figure 2. *FAMA* Expression Pattern in Wild-Type Plants.

Supplemental Figure 3. *ProFAMA:TagRFP-FAMAg* Expression Rescues *fama-1* Dwarfism.

Supplemental Figure 4. *ProFAMA:GUS* Expression Pattern in Early Developmental Stages of Leaves.

Supplemental Figure 5. *ProTGG2:VENUS-2sc* Expression Pattern in Early Developmental Stages of Leaves.

Supplemental Figure 6. The *fama*-Deficient Mutant Lacks Endogenous TGG1 Protein.

Supplemental Figure 7. Myrosin Cell Development in Leaf Primordia of *fama* Loss-of-Function Mutants.

Supplemental Figure 8. *FAMA* Expression Pattern in the *fama*-Deficient Mutant.

Supplemental Figure 9. Development of Myrosin Cells in *FAMA-sGFP* Overexpression Lines.

Supplemental Figure 10. Accumulation Levels of TGG1 and TGG2 in *FAMA-sGFP* Overexpression Lines.

Supplemental Figure 11. Plant Morphology of *FAMA-sGFP* Overexpression Lines.

Supplemental Figure 12. *FAMA* Expression in Myrosin Cells Is Independent of *MUTE*.

Supplemental Figure 13. *TGG1* and *FAMA* Transcript Levels in *spch*.

Supplemental Figure 14. Accumulation Levels of TGG1 in ICE1 and SCRM2 Mutants.

Supplemental Figure 15. Distribution of Stomata in the Wild Type and *syp22-4*.

Supplemental Figure 16. The bHLH Domain and Alignment of bHLH090 Homologs.

Supplemental Table 1. Primer Sets Used in This Study.

The following materials have been deposited in the DRYAD repository under accession number http://dx.doi.org/10.5061/dryad.m9160.

Supplemental Data Set 1. Microarray Data of Col_vs_syp22.

ACKNOWLEDGMENTS

We thank Yoichi Ogawa (Chiba University) for discussions. We thank Georg Jander (Boyce Thompson Institute for Plant Research), Dominique Bergmann (Stanford University), and Keiko Torii (University of Washington) for sharing materials. We also thank ABRC, INRA, and NASC for providing seeds of *Arabidopsis* lines. This work was supported by Specially Promoted Research of Grant-in-Aid for Scientific Research to I.H-.N. (no. 22000014) and Grants-in-Aid for Scientific Research to H.U. (nos. 21200065 and 25440132) from the Japan Society for the Promotion of Science (JSPS). M.S. was supported by a research fellowship from JSPS (nos. 20002057 and 24005453) and by a Grant-in-Aid for Plant Graduate Students from the Nara Institute of Science and Technology.

AUTHOR CONTRIBUTIONS

M.S., H.U., T.S., and I.H.-N. designed research. M.S. performed all experiments. A.J.N. analyzed DNA microarray experiments. M.S., H.U., T.S., and I.H.-N. wrote the article. T.K. and I.H.-N. supervised the study.

Received July 14, 2014; revised August 20, 2014; accepted September 23, 2014; published October 10, 2014.

REFERENCES

Andréasson, E., Bolt Jørgensen, L., Höglund, A.S., Rask, L., and Meijer, J. (2001). Different myrosinase and idioblast distribution in Arabidopsis and *Brassica napus*. Plant Physiol. **127:** 1750–1763.

Baima, S., Nobili, F., Sessa, G., Lucchetti, S., Ruberti, I., and Morelli, G. (1995). The expression of the *Athb-8* homeobox gene is restricted to provascular cells in *Arabidopsis thaliana.* Development **121:** 4171–4182.

Baima, S., Possenti, M., Matteucci, A., Wisman, E., Altamura, M. M., Ruberti, I., and Morelli, G. (2001). The arabidopsis ATHB-8 HD-zip protein acts as a differentiation-promoting transcription factor of the vascular meristems. Plant Physiol. **126:** 643–655.

Barth, C., and Jander, G. (2006). Arabidopsis myrosinases TGG1 and TGG2 have redundant function in glucosinolate breakdown and insect defense. Plant J. **46:** 549–562.

Bergmann, D.C., Lukowitz, W., and Somerville, C.R. (2004). Stomatal development and pattern controlled by a MAPKK kinase. Science **304:** 1494–1497.

Bowman, J.L. (2011). Stomata: active portals for flourishing on land. Curr. Biol. **21:** R540–R541.

Chinnusamy, V., Ohta, M., Kanrar, S., Lee, B.H., Hong, X., Agarwal, M., and Zhu, J.K. (2003). ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis.* Genes Dev. **17:** 1043–1054.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana.* Plant J. **16:** 735–743.

Curtis, M.D., and Grossniklaus, U. (2003). A Gateway cloning vector set for high-throughput functional analysis of genes in planta. Plant Physiol. **133:** 462–469.

Donner, T.J., Sherr, I., and Scarpella, E. (2009). Regulation of preprocambial cell state acquisition by auxin signaling in *Arabidopsis* leaves. Development **136:** 3235–3246.

Fursova, O.V., Pogorelko, G.V., and Tarasov, V.A. (2009). Identification of *ICE2*, a gene involved in cold acclimation which determines freezing tolerance in *Arabidopsis thaliana.* Gene **429:** 98–103.

Gardner, M.J., Baker, A.J., Assie, J.M., Poethig, R.S., Haseloff, J.P., and Webb, A.A. (2009). *GAL4 GFP* enhancer trap lines for analysis of stomatal guard cell development and gene expression. J. Exp. Bot. **60:** 213–226.

Grubb, C.D., and Abel, S. (2006). Glucosinolate metabolism and its control. Trends Plant Sci. **11:** 89–100.

Guignard, L. (1890). Recherches sur la localization des principes actifs des Cruciferes. J. Bot. **4:** 385–395.

Hachez, C., Ohashi-Ito, K., Dong, J., and Bergmann, D.C. (2011). Differentiation of Arabidopsis guard cells: analysis of the networks incorporating the basic helix-loop-helix transcription factor, FAMA. Plant Physiol. **155:** 1458–1472.

Halkier, B.A., and Gershenzon, J. (2006). Biology and biochemistry of glucosinolates. Annu. Rev. Plant Biol. **57:** 303–333.

Heinricher, E. (1884). Uber Eiweisstoffe fuhrennde Idioblasten bei einigen Cruceren. Ber. Dtsch. Bot. Ges. **2:** 463–467.

Hiratsu, K., Matsui, K., Koyama, T., and Ohme-Takagi, M. (2003). Dominant repression of target genes by chimeric repressors that include the EAR motif, a repression domain, in *Arabidopsis.* Plant J. **34:** 733–739.

Hopkins, R.J., van Dam, N.M., and van Loon, J.J. (2009). Role of glucosinolates in insect-plant relationships and multitrophic interactions. Annu. Rev. Entomol. **54:** 57–83.

Husebye, H., Chadchawan, S., Winge, P., Thangstad, O.P., and Bones, A.M. (2002). Guard cell- and phloem idioblast-specific expression of thioglucoside glucohydrolase 1 (myrosinase) in Arabidopsis. Plant Physiol. **128:** 1180–1188.

Kanaoka, M.M., Pillitteri, L.J., Fujii, H., Yoshida, Y., Bogenschutz, N.L., Takabayashi, J., Zhu, J.K., and Torii, K.U. (2008). *SCREAM/ICE1* and *SCREAM2* specify three cell-state transitional steps leading to *arabidopsis* stomatal differentiation. Plant Cell **20:** 1775–1785.

Kang, J., and Dengler, N. (2004). Vein pattern development in adult leaves of *Arabidopsis thaliana.* Int. J. Plant Sci. **165:** 231–242.

Kissen, R., Rossiter, J.T., and Bones, A.M. (2009). The 'mustard oil bomb': not so easy to assemble?! Localization, expression and distribution of the components of the myrosinase enzyme system. Phytochem. Rev. **8:** 69–86.

Koroleva, O.A., Davies, A., Deeken, R., Thorpe, M.R., Tomos, A.D., and Hedrich, R. (2000). Identification of a new glucosinolate-rich cell type in Arabidopsis flower stalk. Plant Physiol. **124:** 599–608.

Krogan, N.T., Ckurshumova, W., Marcos, D., Caragea, A.E., and Berleth, T. (2012). Deletion of *MP/ARF5* domains III and IV reveals a requirement for *Aux/IAA* regulation in *Arabidopsis* leaf vascular patterning. New Phytol. **194:** 391–401.

Lau, O.S., and Bergmann, D.C. (2012). Stomatal development: a plant's perspective on cell polarity, cell fate transitions and intercellular communication. Development **139:** 3683–3692.

Le, J., et al. (2014). Auxin transport and activity regulate stomatal patterning and development. Nat. Commun. **5:** 3090.

Liu, T., Ohashi-Ito, K., and Bergmann, D.C. (2009). Orthologs of *Arabidopsis thaliana* stomatal bHLH genes and regulation of stomatal development in grasses. Development **136:** 2265–2276.

MacAlister, C.A., and Bergmann, D.C. (2011). Sequence and function of basic helix-loop-helix proteins required for stomatal development in *Arabidopsis* are deeply conserved in land plants. Evol. Dev. **13:** 182–192.

MacAlister, C.A., Ohashi-Ito, K., and Bergmann, D.C. (2007). Transcription factor control of asymmetric cell divisions that establish the stomatal lineage. Nature **445:** 537–540.

Mattsson, J., Ckurshumova, W., and Berleth, T. (2003). Auxin signaling in Arabidopsis leaf vascular development. Plant Physiol. **131:** 1327–1339.

Nadeau, J.A., and Sack, F.D. (2002). Stomatal development in Arabidopsis. The Arabidopsis Book **1:** e0066, doi/10.1199/tab.0066.

Obayashi, T., Hayashi, S., Saeki, M., Ohta, H., and Kinoshita, K. (2009). ATTED-II provides coexpressed gene networks for Arabidopsis. Nucleic Acids Res. **37:** D987–D991.

Ohashi-Ito, K., and Bergmann, D.C. (2006). *Arabidopsis* FAMA controls the final proliferation/differentiation switch during stomatal development. Plant Cell **18:** 2493–2505.

Ohashi-Ito, K., and Fukuda, H. (2010). Transcriptional regulation of vascular cell fates. Curr. Opin. Plant Biol. **13:** 670–676.

Ohtomo, I., Ueda, H., Shimada, T., Nishiyama, C., Komoto, Y., Hara-Nishimura, I., and Takahashi, T. (2005). Identification of an allele of VAM3/SYP22 that confers a semi-dwarf phenotype in *Arabidopsis thaliana*. Plant Cell Physiol. **46:** 1358–1365.

Pillitteri, L.J., and Dong, J. (2013). Stomatal development in Arabidopsis. The Arabidopsis Book **11:** e0162, doi/10.1199/tab.0162.

Pillitteri, L.J., Peterson, K.M., Horst, R.J., and Torii, K.U. (2011). Molecular profiling of stomatal meristemoids reveals new component of asymmetric cell division and commonalities among stem cell populations in *Arabidopsis.* Plant Cell **23:** 3260–3275.

Pillitteri, L.J., Sloan, D.B., Bogenschutz, N.L., and Torii, K.U. (2007). Termination of asymmetric cell division and differentiation of stomata. Nature **445:** 501–505.

Pillitteri, L.J., and Torii, K.U. (2012). Mechanisms of stomatal development. Annu. Rev. Plant Biol. **63:** 591–614.

Pires, N., and Dolan, L. (2010). Origin and diversification of basic-helix-loop-helix proteins in plants. Mol. Biol. Evol. **27:** 862–874.

Ran, J.H., Shen, T.T., Liu, W.J., and Wang, X.Q. (2013). Evolution of the bHLH genes involved in stomatal development: implications for the expansion of developmental complexity of stomata in land plants. PLoS ONE **8:** e78997.

Rask, L., Andréasson, E., Ekbom, B., Eriksson, S., Pontoppidan, B., and Meijer, J. (2000). Myrosinase: gene family evolution and herbivore defense in Brassicaceae. Plant Mol. Biol. **42:** 93–113.

Sawchuk, M.G., Donner, T.J., Head, P., and Scarpella, E. (2008). Unique and overlapping expression patterns among members of photosynthesis-associated nuclear gene families in Arabidopsis. Plant Physiol. **148:** 1908–1924.

Scarpella, E., Francis, P., and Berleth, T. (2004). Stage-specific markers define early steps of procambium development in *Arabidopsis* leaves and correlate termination of vein formation with mesophyll differentiation. Development **131:** 3445–3455.

Shimada, T., Fuji, K., Tamura, K., Kondo, M., Nishimura, M., and Hara-Nishimura, I. (2003). Vacuolar sorting receptor for seed storage proteins in *Arabidopsis thaliana.* Proc. Natl. Acad. Sci. USA **100:** 16095–16100.

Shimada, T.L., Shimada, T., and Hara-Nishimura, I. (2010). A rapid and non-destructive screenable marker, FAST, for identifying transformed seeds of *Arabidopsis thaliana.* Plant J. **61:** 519–528.

Shirakawa, M., Ueda, H., Koumoto, Y., Fuji, K., Nishiyama, C., Kohchi, T., Hara-Nishimura, I., and Shimada, T. (2014). CONTINUOUS VASCULAR RING (COV1) is a *trans*-Golgi network-localized membrane protein required for Golgi morphology and vacuolar protein sorting. Plant Cell Physiol. **55:** 764–772.

Shirakawa, M., Ueda, H., Shimada, T., Koumoto, Y., Shimada, T.L., Kondo, M., Takahashi, T., Okuyama, Y., Nishimura, M., and Hara-Nishimura, I. (2010). Arabidopsis Qa-SNARE SYP2 proteins localized to different subcellular regions function redundantly in vacuolar protein sorting and plant development. Plant J. **64:** 924–935.

Shirakawa, M., Ueda, H., Shimada, T., Nishiyama, C., and Hara-Nishimura, I. (2009). Vacuolar SNAREs function in the formation of the leaf vascular network by regulating auxin distribution. Plant Cell Physiol. **50:** 1319–1328.

Shroff, R., Vergara, F., Muck, A., Svatos, A., and Gershenzon, J. (2008). Nonuniform distribution of glucosinolates in *Arabidopsis thaliana* leaves has important consequences for plant defense. Proc. Natl. Acad. Sci. USA **105:** 6196–6201.

Thangstad, O.P., Gilde, B., Chadchawan, S., Seem, M., Husebye, H., Bradley, D., and Bones, A.M. (2004). Cell specific, cross-species expression of myrosinases in *Brassica napus*, *Arabidopsis thaliana* and *Nicotiana tabacum.* Plant Mol. Biol. **54:** 597–611.

Ueda, H., Nishiyama, C., Shimada, T., Koumoto, Y., Hayashi, Y., Kondo, M., Takahashi, T., Ohtomo, I., Nishimura, M., and Hara-Nishimura, I. (2006). AtVAM3 is required for normal specification of idioblasts, myrosin cells. Plant Cell Physiol. **47:** 164–175.

Wittstock, U., and Halkier, B.A. (2002). Glucosinolate research in the *Arabidopsis* era. Trends Plant Sci. **7:** 263–270.

Workman, C., Jensen, L.J., Jarmer, H., Berka, R., Gautier, L., Nielser, H.B., Saxild, H.H., Nielsen, C., Brunak, S., and Knudsen, S. (2002). A new non-linear normalization method for reducing variability in DNA microarray experiments. Genome Biol. **3:** h0048.

Xue, J., Jørgensen, M., Pihlgren, U., and Rask, L. (1995). The myrosinase gene family in *Arabidopsis thaliana*: gene organization, expression and evolution. Plant Mol. Biol. **27:** 911–922.

Zhang, Z., Ober, J.A., and Kliebenstein, D.J. (2006). The gene controlling the quantitative trait locus *EPITHIOSPECIFIER MODIFIER1* alters glucosinolate hydrolysis and insect resistance in *Arabidopsis.* Plant Cell **18:** 1524–1536.