Research Article

A new loss-of-function allele *28y* reveals a role of *ARGONAUTE1* in limiting asymmetric division of stomatal lineage ground cell

Running title: AGO1 and stomatal spacing division

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Keywords: Arabidopsis; ARGONAUTE1; stomatal development; cell division.

Edited by: Stephan Wenkel, University of Tübingen, Germany

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1111/jipb.12154]

Received: November 10, 2013; Accepted: December 26, 2013

Abstract

In Arabidopsis, stomata are produced through a series of divisions including asymmetric and symmetric divisions. Asymmetric entry division of meristemoid mother cell produces two daughter cells, the smaller meristemoid and the lager sister cell, a stomatal lineage ground cell (SLGC). SLGCs can differentiate into epidermal pavement cells but have the potential to divide asymmetrically, spacing divisions, to create satellite meristemoids. Peptide ligands and TOO MANY MOUTHS (TMM) and ERECTA family receptors regulate the initiation of stomatal lineages, activity and orientation of spacing divisions. Here we reported that a natural mutant *28y* displayed an increased stomatal density and index. Using map-based cloning, we identified mutation in *ARGONAUTE1* (*AGO1*) as the cause of *28y* phenotypes. Time-lapse tracing of stomatal lineage cells reveals that stomatal overproduction in *28y* is caused by the excessive asymmetric spacing division of SLGCs. Further genetic results demonstrated that *AGO1* acts downstream of *TMM* and negatively regulates the *SPCH* transcripts, but in a BR-independent manner. Upregulation of *AGAMOUS-LIKE16* (*AGL16*) in *28y* mutants suggests that *AGO1* is required to restrict AGL16-mediated stomatal spacing divisions, a microRNA pathway in addition to ligand-receptor signaling modules.

INTRODUCTION

Stomata are specialized epidermal structures that regulate CO_2 influx used in photosynthesis as well as water vapor loss between plants and their environment (Casson and Hetherington 2010). In *Arabidopsis*, stomata develop from protodermal cells after at least one asymmetric division and a symmetric division. The meristemoid mother cell (MMC) undergoes asymmetric entry division to produce a small, triangular meristemoid and a larger sister cell, a stomatal lineage ground cell (SLGC). The meristemoid differentiates into a guard mother cell (GMC). Then the GMC undergoes a single symmetric cell division to generate a pair of guard cells (GCs). The SLGC can directly differentiate into pavement cell or undergoes an asymmetric spacing division to generate a satellite meristemoid, and eventually develops into a new stoma (Bergmann and Sack 2007).

There is at least one pavement cell between two stomata (‘one cell spacing’ rule), suggesting that signals must be transduced between cells during stomatal development. In *Arabidopsis*, plasma membrane receptors TOO MANY MOUTHS (TMM) (Yang and Sack 1995; Nadeau and Sack 2002), ERECTA (ER) family (Shpak 2013) and putative ligands (Hara et al. 2007; Hunt and Gray 2009; Sugano et al. 2010; Abrash and Bergmann 2010), i.e., EPF1, EPF2, STOMAGEN, and CHALLAH, are predicted to participate in stomatal signal transduction. For instance, EPF2 and EPF1 secreted from meristemoids or GMCs are perceived by TMM and ER family receptors in neighbor cells and provide the positional information to regulate the frequency and orientation of asymmetric spacing divisions (Lee et al. 2012).

In turn these signals are transduced via a mitogen-activated protein kinase (MAPK) cascade, which includes YODA, MEKK4/5, and MPK3/6 (Bergmann 2004; Lampard et al. 2008; Lampard et al. 2009). Three basic-helix-loop-helix (bHLH) transcription factors, SPEECHLESS (SPCH), MUTE, and FAMA, are required for successive stages of development including lineage initiation and proliferation, as well as terminal differentiation (Ohashi-Ito and Bergmann 2006; MacAlister et al. 2007; Pillitteri et al. 2007). SPCH is essential for MMC formation, stomatal entry division, and maintenance of meristemoid stem cell activity (MacAlister et al. 2007). MUTE promotes the transition of meristemoids into GMCs and is required for asymmetric amplifying division of meristemoids (Pillitteri et al. 2007). FAMA plays function in GMC differentiation and proliferation (Ohashi-Ito and Bergmann 2006). Additional bHLH proteins INDUCER OF CBF EXPRESSION1 (ICE1)/SCREAM(SCRM) and SCRM2, directly interact with SPCH, MUTE, and FAMA during stomatal development (Kanaoka et al. 2008). MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) signaling cascade likely regulates stomatal lineage entry through its control of the phosphorylation status of SPCH. Overexpression of a *SPCH*

driven by its own promoter promotes the formation of stomatal meristemoids and leads to extra stomata in clusters (Lampard et al. 2008). The phytohormone brassinosteroid (BR) regulates stomatal development through SPCH phosphorylation either directly by the serine/threonine glycogen synthase kinase 3 (GSK3)/SHAGGY-like BIN2 (BRASSINOSTEROID INSENSITIVE 2), or indirectly via YODA or MKKs in the MAPK signaling pathway (Gudesblat et al. 2012; Kim et al. 2012; Khan et al. 2013). The degradation of SPCH induced by MPK or BIN2 phosphorylation prevents epidermal cells from entering into the stomatal cell lineage (Lampard et al. 2008; Gudesblat et al. 2012).

MicroRNAs (miRNAs) play important roles in regulating gene expression in multicellular plants and animals (Carrington and Ambros 2003). The miR824 regulates asymmetric division of satellite meristemoids through repressing *AGAMOUS-LIKE16 (AGL16*) in stomatal lineage cells (Kutter et al. 2007). Recently study found that the components of the miRNA pathway *HYPONASTIC LEAVES1* (*HYL1*), *ARGONAUTE1* (*AGO1*) and *HUA ENHANCER1* (*HEN1*) genes, participate in the proximal–distal and adaxial–abaxial patterning of leaves and in stomatal production (Jover-Gil et al. 2012). Mutation of *AGO1* leads to narrow leaves, dwarf seedlings, late flowering, and low fertility, developmental defects (Baumberger and Baulcombe 2005; Vaucheret 2008). But it remains unclear how *AGO1* regulates stomatal development.

Here we identified that *28y* is a new loss-of-function allele of *AGO1*. Time-lapse analysis revealed that *AGO1,* like TMM, is another negative regulator in restricting asymmetric spacing divisions in SLGCs. We demonstrated that *AGO1* acts downstream of *TMM* in stomatal production. Furthermore, we proposed that the upregulation of *SPCH* transcript in *28y* mutants is partially mediated by AGL16-dependent miRNA pathway.

RESULTS

Overproduction of stomatal in *28y* cotyledons and hypocotyls

Dwarf seedlings with dark green and narrow cotyledons were found in the segregating population of a natural mutant. These mutant plants could form a couple true leaves but could not produce seeds (**Figure 1A, D**). This line number is 28, thus we termed this mutant as *28y*. Segregation analysis demonstrated that the mutation in *28y* mutant is recessive and monogenic (107 out of 435). To assay the function of *28Y* function in stomatal development, we analyzed the stomatal density and index of 14-day-old cotyledons and total stomatal number of hypocotyls. The stomatal density and index of wild-type were 160 ± 17 mm^{-2} and 31 ± 0.9 %, respectively. By contrast, *28y* mutant had an approximately double stomatal density of 337 ± 63 mm^{-2}, and a higher stomata index of 44 ± 4.6%.

The total stomatal number in *28y* hypocotyls was much more than that in wild type as well (**Table 1**). In addition, the stomata in *28y* mutant often formed clusters (**Figure 1B, C, E, F**; white brackets indicate stomata in direct contact). These results indicated that *28Y* negatively regulates stomatal production either in cotyledons or hypocotyls.

Excessive asymmetric spacing divisions in *28y* hypocotyls

To investigate the abnormal stomatal pattern formation in *28y* mutant, we analyzed the developmental processes of stomata in wild-type and *28y* mutant by time-lapse imaging. In wild-type hypocotyls, MMCs divided asymmetrically to produce a triangular meristemoid and a big sister cell, a SLGC (**Figure 2A, B**). Meristemoids differentiated into GMCs that then divided symmetrically to generate a pair of stomatal GCs. While the adjacent SLGCs differentiated into pavement cells (**Figure 2C, D**).

Mutation of *28Y* did not affect MMC divisions in producing meristemoids and their big sister cells, SLGCs (**Figure 2E, F**). However, unlike wild type, SLGCs in the *28y* mutant were able to acquire MMC character and executed asymmetric spacing divisions to produce satellite meristemoids and new SLGCs (**Figure 2G**). The satellite meristemoids then directly differentiated into GMCs and mature stomata eventually, but the new SLGCs continued spacing divisions to produce higher-order stomatal complexes (**Figure 2H**). Thus, the excessive spacing division happened in SLGC is the cause of overproduction of stomata in *28y* hypocotyls.

Ectopic stomatal lineage cells in *28y* cotyledons

Owing to the narrow and thick cotyledons of *28y* mutant, it is difficult to trace the epidermal cell development by time-lapse imaging. We then use stomatal lineage marker genes to analyze the process of stomatal development in wild-type and *28y* cotyledons. *TMM* encodes a leucine-rich repeat receptor-like protein controlling stomatal production and pattern (Nadeau and Sack 2002). *TMM:TMM-GFP* is highly expressed in stomatal lineage especially before and after stomatal asymmetric divisions, but not in mature GCs and pavement cells (**Figure 3A–C**). There was no obvious difference of *TMM:TMM-GFP* expression pattern between *28y* and wild-type at one day after seed germination (**Figure 3A, D**). The number of cells expression *TMM:TMM-GFP* fluorescence was gradually reduced in the wild-type cotyledons at 5 or 7 days after germination (**Figure 3B, C**). Due to the excessive asymmetric divisions occurred in SLGCs, the number of cells expressing *TMM:TMM-GFP* in *28y* cotyledons was higher than that in the wild-type plants at the same developmental stage (**Figure 3E, F**).

In contrast to the expression pattern of *TMM:TMM-GFP* expression pattern, E1728 is only

expressed in mature GCs. With the development of leaf and maturation of GCs, the number of cells expressing E1728 fluorescence reaches a constant level in wild-type cotyledons. At seven days after germination, most of stomata completed their development and acquired their maturity in wild-type (**Figure 3G–I**). Whereas, the number of mature GCs in *28y* cotyledons increased during the same observation period. The presence of immature GCs without E1728 fluorescence in 7-day-old cotyledons indicated a remained high activity of producing new stomata in *28y* cotyledons (**Figure 3J–L**).

spch is epistatic to *28y* in regulating stomatal asymmetric division

SPCH encodes a bHLH transcription factor that is required for stomatal cell lineage initiation and asymmetric entry and spacing divisions (MacAlister et al. 2007). In loss-of-function *spch-4* mutant, only pavement cells can be found in cotyledons or hypocotyls (**Figure 4A, B**). To investigate the interaction between *28Y* and *SPCH* in regulating stomatal production, *28y* was crossed with *spch-4* mutant. Although the overall growth of *spch-4 28y* double mutant seedlings is similar to that of *28y* single mutant (data not shown), no stoma was found in *spch-4 28y* epidermis from either cotyledons or hypocotyls, suggesting *spch-4* mutant is epistatic to *28y* in stomatal initiating (**Figure 4C, D**).

In wild-type epidermis, expression of a translational reporter *SPCH:SPCH-GFP* is restricted to stomatal precursor cells and big neighbor cells. In older organ, the *SPCH:SPCH-GFP* expression decreased and finally disappeared in the big neighbor cells (**Figure 4E**). In *28y* mutant, the cells expressing *SPCH:SPCH-GFP* were often formed in big clusters, indicating epidermal cells acquired stomatal lineage fate (**Figure 4F**). This is in agreement with the upregulated *SPCH* transcript levels in *28y* seedlings (**Figure 4G**).

28y is epistatic to *tmm-1* in regulating stomatal production in hypocotyls

Stomatal phenotypes in *tmm* mutants are tissue specific (Bhave et al. 2009). *tmm* mutant produce clustered stomata in leaves, whereas no stomata in hypocotyls (**Figure 5A, B**). *28y* was crossed with a null *tmm-1* mutant to generate a *tmm-1 28y* double mutant. Although the stomatal density and index have no significant difference between *tmm-1* and *tmm-1 28y* double mutant cotyledons (**Table 2**), Mutation of *28Y* suppressed the formation of big stomatal clusters (more than 4 pairs of stomata in direct contact) in *tmm-1* mutant, suggesting that *28Y* might act downstream of *TMM* (**Figure 5C, E**).

Surprisingly, unlike the ‘no stomata‘ in *tmm-1*, *tmm-1 28y* double mutant produced plenty of stomata in hypocotyls, indicating that *28y* is epistatic to *tmm-1* mutant in regulating hypocotyl stomatal production (**Figure 5B, D**). Expression of *SPCH:SPCH-GFP* in wild-type plants also displayed a

moderate stomatal overproduction phenotypes in cotyledons and hypocotyls (Figure S1A, B). Most the epidermal cells were converted into stomata when *SPCH:SPCH-GFP* was introduced into *tmm-1* cotyledons (Figure S1C). In hypocotyls, expression of *SPCH:SPCH-GFP* induced large stomatal clusters in *tmm-1* (Figure S1D). Because our real-time PCR analysis showed a significant increase of *SPCH* transcriptional level in *28y* mutants (**Figure 4I**), we speculated that *28Y* negatively regulates *SPCH* expression downstream of *TMM*.

28Y acts in a pathway parallel to BR-mediated stomatal regulation

The phytohormone BR positively regulates stomatal production through enhancing SPCH protein stability by phosphorylation. The stomatal production in hypocotyls largely increased when treated by BR hormone brassinolide (BL) (Gudesblat et al. 2012). An application of BL at a concentration of 1 μM significantly increased stomatal number in wild-type hypocotyls, whereas the BR synthesis inhibitor brassinazole (BRZ) inhibited stomatal production in hypocotyls (Figure S2A-C).

To test whether *28Y* gene regulates stomatal production through the BR-mediated SPCH signaling pathway, *28y* mutants were treated with BR and BRZ, respectively. We found that the number of stomata in *28y* hypocotyls greatly increased from untreated 182 ± 86 to 857 ± 180 (n=10) per hypocotyl after 1 μM BL-treatment (Figure S2D, E). By contrast, the stomatal number was largely reduced by BRZ-treatment to a level of 70 ± 16 (n=10) per hypocotyl (Figure S2F). Taken together, *28Y* and BR appear to act independently in regulating stomatal production in hypocotyls.

28Y functions at the early stage of stomatal development

MUTE is a bHLH transcription factor that is required for the meristemoid to GMC fate transition and for asymmetric amplifying division (Pillitteri et al. 2007). In the *mute* mutant, the stomatal development is arrested at meristemoid stage but the amplifying asymmetric division is repeated, resulting in ‘rosettes’ of larger daughter cells surrounding a triangular meristemoid at the center (Figure S3A, B). In *mute 28y* double mutant, no mature stomata were found in either cotyledons or hypocotyls. Presence of clusters of ‘rosettes’ indicates an additive interaction between *MUTE* and *28Y,* indicating that *28Y* functions at early stages during stomatal development but requires MUTE for M-to-GMC differentiation during stomatal development (Figure S3C, D).

The *MUTE:MUTE–GFP* is expressed in the nuclei of late meristemoids and early GMCs, but is not in GCs and pavement cells (Figure S3E). Ectopic expression *MUTE:MUTE–GFP* in small cells next to an existing stoma was often found in *28y* epidermis (Figure S3F). As we expected, a relatively high *MUTE* transcript level in *28y* seedlings (Figure S3G).

28Y gene encodes AGO1 protein

To find the mutated gene in the *28y* genome, we initiated map-based cloning of *28Y*. *28y* mutant was crossed with L*er* and F2 population for genetic mapping. The *28y* mutation was first delimited to a BAC clone F21D18 on chromosome 1. Analysis of genes in this fragment revealed the presence of *AGO1* (AT1G48410). As the phenotype of *ago1* mutant alleles is highly similar to *28y* mutant, we then sequenced *AGO1* in *28y*. A single C to T substitution at the seventh base and a deletion of TAGCTGA between the eighth base and the fourteenth base of the fifth exon were found within *AGO1* gene in *28y*, resulting in an early transcription termination (**Figure 6A**).

To confirm whether the stomatal phenotype is caused by *AGO1* mutation, a point mutation line CS3854 (*ago1-8*), obtained from the ABRC seed stock center, was used for further analysis. Compared with the wild type, *ago1-8* shows phenotypic characters similar to *28y* mutant, such as narrow and thick leaves, smaller seedling size, flat pavement cells without lobes, and excessive stomata in epidermis (**Figure 6B, C**). We crossed the heterozygous *28y* mutant with heterozygous *ago1-8* mutant. The phenotypic analysis of F1 seedlings demonstrated that 25% seedlings show similar phenotypes to *28y* and *ago1-8*, indicating that *28y* is allelic to *ago1-8* (**Figure 6D, E**).

Then we generated transgenic lines carrying *GUS* gene driven by the *AGO1* promoter. A preferential expression of *AGO1:GUS* was found in stomatal lineage cells, consistent with the involvement of *AGO1* in stomatal development (**Figure 6F-J**).

AGO1 is required to repress AGL16-mediated microRNA stomatal regulatory pathway

AGO1 acts in miRNA pathway in regulating gene expression during many development processes (Vaucheret et al. 2004). The miR824 regulates asymmetric division through repressing *AGL16* mRNA in stomatal lineage cells (Kutter et al. 2007). Mutation of miR824 or overexpression of *AGL16m* form causes excessive asymmetric division and results in extra stomata in epidermis, a similar stomatal phenotype found in 2*8y* mutant. Real-time PCR result showed the relative mRNA expression level of *AGL16* is significantly increased in *28y* mutants (**Figure 7**). This result suggested that *AGO1* regulates stomatal division through participating in miR824 pathway and repressing *AGL16* expression, indicating that *AGO1* is required to restrict AGL16-mediated regulation of stomatal development.

DISCUSSION

28y is a new strong allele of *AGO1*

AGO1 is the founding member of the Arabidopsis *AGO* gene family. In *Arabidopsis*, more than 100 *ago1* alleles have been used to characterize the pleiotropic functions of *AGO1* in many plant

development processes, *i.e.* embryonic meristem development, adventitious root formation, root radian patterning, leaf development and venation patterning (Bohmert et al. 1998; Vaucheret et al. 2004; Miyashima et al. 2009; Jover-Gil et al. 2012). Although abnormal stomatal overproduction was observed in two intermediate alleles *ago1-51*/*icu9-1* and *ago1-52/icu9-2*, the molecular mechanism has not been well-characterized (Jover-Gil et al. 2012).

ago1-8 (EMS-induced mutation) is a strong allele exhibiting dwarf seedlings, narrow and thickened rosette leaves (Lynn et al. 1999). However, the phenotypes in *28y* are stronger than those found in *ago1-8*. The premature termination of transcript of *AGO1* in *28y* would produce to a truncated protein that lacks the PAZ and PIWI domains that are required for RNA recognition.

AGO1 is involved in regulation stomatal ground cell differentiation and division

The stomatal development is the most complex process of epidermal development including at least one asymmetric division and a symmetric division. There are three types of asymmetric division including entry division, amplifying division, and spacing division. SLGCs are big daughter cells of new meristemoids after each kind of asymmetric divisions. The SLGCs can directly differentiate into jiasaw-puzzle-shaped pavement cells that interlock with neighboring cells. SLGCs are able to acquire a MMC fate and then divide unequally to produce new satellite meristemoids and SLGCs (Bergmann and Sack 2007). Thus, asymmetric spacing divisions taken place in SLGCs initiate more stomatal lineage cells and produce more stomata in epidermis, leading to an increase of total number or density of stomata in epidermis. By contrast, each asymmetric amplifying division happened in meristemoids regenerates a meristemoid and produces another SLGC, resulting in a decrease of stomatal index, the ratio of the number of stomata to the total number of epidermal cells. A significant increase of stomatal density, but not stomatal index, in *28y* cotyledons and a higher number of total stomata in *28y* hypocotyls indicates a function of *AGO1* in restricting stomatal spacing division instead of amplifying division.

Excessive asymmetric divisions occurred in *28y* SLGCs lead to a significant increase of stomatal density. However, the narrow and small size of pavement cells in *28y* might contribute as well to its increased stomatal density. But lineage tracing results demonstrated that excessive spacing asymmetric divisions in SLGCs are the major cause of overproduction of stomata in either cotyledon or hypocotyl epidermis. In addition, the lobe formation in *28y* pavement cells are greatly reduced, indicating AGO1 might be required as well for the cell shape control during pavement cell differentiation. Base on previous reports (Jover-Gil et al. 2012) and our observations, AGO1 is not only required in restricting asymmetric division in SGLCs but also in promoting pavement cell

differentiation. Therefore, *AGO1* is playing as a key gene in regulating SLGC division and differentiation, providing a developmental flexibility of stomatal production and distribution in epidermis in response to ever changed surrounding environments.

The *AGO1* regulates stomatal spacing division though repressing *SPCH* expression

Our genetic analysis results demonstrate that *AGO1* acts upstream of *SPCH* in stomatal production. SPCH is a key bHLH transcriptional factor required for stomatal lineage initiation (acquiring MMC fate) and subsequent asymmetric division (Lau and Bergmann 2012). We found that *SPCH* transcriptional level is negatively regulated by *AGO1.* AGO proteins are integral players in small RNA-mediated regulatory pathways. These small RNA-mediated AGO proteins regulate gene expression at various levels, such as by RNA cleavage, DNA methylation (Vaucheret et al. 2004). Although a CpG island was predicted in *SPCH* cDNA (465-707, counting from ATG), no obvious difference in methylation level was found between wild-type and *28y* plants (Figure S4), indicating that repression of the *SPCH* expression by *AGO1* may not through methylation machinery.

It was shown that miR824-mediated regulation of *AGL16* might act in stomatal development (Kutter et al. 2007). AGL16 functions as a positive regulator in promoting spacing division. Indeed, an increased transcript level of *AGL16* was found in *28y* plants, which was also observed in *ago1-52* mutant (Jover-Gil et al. 2012). Here we found that *AGO1* is expressed widely in epidermis but display a preferential high expression in MMCs, meristemoids, GMCs and young GCs but not in mature GCs. However, *AGL16* mRNA was detected only in mature GCs (Kutter et al. 2007). Thus *AGL16* transcript levels might be down-regulated by *AGO1* in stomatal precursor cells and immature GCs, but in mature GCs. Since AGO1 is proposed to restrict the division in SLGCs, the presence of miR824 in satellite meristemoids and GMCs raises the possibility that the regulating of AGO1 on *AGL16* transcript is mediated by miR824, which might move from stomatal precursor cells to the neighboring SLGCs (**Figure 8A**).

Additional AGO1-mediated signaling pathway in regulating stomatal development

A current model of stomatal signaling pathway involves TMM/ERf-MAPK and BR pathways (**Figure 8B**). Signals of secreted peptide ligands, *i.e.* EPF1, EPF2, STOMAGEN, CHALLAH, are received by TMM and ERECTA family receptors. In turn these signals are transduced via MAPK cascade which regulates stomatal lineage entry through its control of the phosphorylation status of SPCH. The phytohormone brassinosteroid (BR) regulates stomatal development also through SPCH phosphorylation. The degradation of SPCH induced by MPKs or BIN2-mediated phosphorylation

prevents epidermal cells from entering into the stomatal cell lineage (Lampard et al. 2008; Gudesblat et al. 2012; Kim et al. 2012).

Loss-of-function of *TMM* causes a region-specific stomatal phenotype. For instance, many excess stomata in direct contact form in *tmm* leaves, displaying 'too many mouths' phenotype. However, no stoma is produced in *tmm* stems and hypocotyls (Geisler et al. 1998). Extra spacing divisions and their misplaced division planes cause the formation of stomatal clusters in *tmm* mutants. Our phenotypic analysis of stomatal complexity in *tmm-1 28y* double mutants showed that *AGO1* mutation suppressed the formation of big stomatal clusters in *tmm-1* mutant, suggesting a role AGO1 in limiting spacing divisions downstream of *TMM*. *28y* complemented the no stomata phenotype in *tmm* hypocotyls, further confirmed that *AGO1* act downstream of *TMM* in stomatal production. Similarly, overexpression of *SPCH* (*SPCH:SPCH-GFP*) could compensate defects of stomatal production in *tmm* hypocotyls. Taken together, our results suggest that *AGO1* might function downstream of *TMM* but upstream of *SPCH* in hypocotyl stomatal production.

SPCH activity is regulated by MAPK or BIN2-mediated protein phosphorylation in TMM/ERf or BR signaling pathways. In *28y* hypocotyls, BR treatment has additive effects in promoting stomatal production and BR inhibitor BRZ can not completely suppress stomatal formation. Thus we propose that a transcriptional regulating of *SPCH* by AGO1-mediated miRNA pathway is an additional regulatory route to the known SPCH phosphorylation (**Figure 8**).

MATERIALS AND METHODS

Plant genotypes and growth conditions

All genotypes were in Columbia (Col-0) and L*er* ecotypic background. Seeds of *ago1-8* (CS3854) were obtained from Arabidopsis Biological Resource Center (ABRC). Seedlings were grown on half strength Murashige and Skoog (MS) medium (pH 5.8) at 22-24 °C under a 16h/8h of light/dark cycles. For the Brassinolide (BL, Sigma) and brassinazole (BRZ, TCI) treatments, seeds were sown to the surface of medium supplemented with 1 μM BL or 10 μM BRZ, respectively.

Microscopy

Two-week-old cotyledons and hypocotyls were cleared as in Malamy and Benfey (1997) (Malamy and Benfey 1997). For fluorescence, cotyledons and hypocotyls were stained with 0.1% (w/v) propidium iodide (PI), and GFP fluorescence was imaged using a confocal laser scanning microscope (FV1000-MPE, Olympus).

The density of epidermal cells and stomatal cells was measured within six regions located at the base, middle, and top of each leaf using an Olympus BX51 microscope. Stomatal index was calculated as ratio of the number of stomata to the total number of epidermal cells.

For the time-lapse imaging of stomatal development in hypocotyls, the seedlings were sown on the surface of half strength MS medium solidified with 1% agar and grown vertically. After each time-point imaging, water remained on epidermis was soaked by small pieces of filter paper and seedlings were placed back to growth chamber. Images were taken by a 60x objective and a camera mounted on an Olympus BX51 microscope.

GUS staining

To obtain *AGO1:GUS*, the promoter DNA fragment obtained by PCR using the primer shown in Table S1 and was cloned into the pMD19-T vector (TaKaRa) for sequencing. The fragment was subcloned into a pCAMBIA1300 vector (CAMBIA) and introduced into Col-0 plants. The transformants were selected on medium containing 25 mg L^{-1} hygromycin and confirmed by PCR. *GUS* staining was performed as in Lai et al (2005) (Lai et al. 2005). Seedlings were then cleared and observed using an Olympus BX51 microscope.

Quantitative RT-PCR analysis

For quantitative RT-PCR analysis, total RNA from wild-type and *28y* seedlings was extracted using TRNzol reagent (http://www.tiangen.com). Reverse transcription was performed using a Promega kit (http://www.promega.com). *TUBULIN2* was used as an internal control. Real-time quantitative PCR experiments were repeated independently three times. cDNA was amplified using SYBR Premix ExTaqTM (TaKaRa) with a Corbett Research Rotor-Gene 3000 Thermal Cycler. Primers specific to *SPCH*, *MUTE* and *AGL16* were used for quantitative RT-PCR indicated in the Table S1.

Map-based cloning and sequencing

The *28y* mutant was crossed to the wild-type L*er*. The F2 population was screened for *ago1* mutants on the basis of defective phenotypes of seedlings. *28Y* was first mapped within the BAC Clone F21D18. The mutations in *28y* were identified by sequencing.

Methylation analysis

CpG island region and primers were analyzed as in Li et al (2002) (Li and Dahiya 2002). The methylation reaction of *28y* and wild-type DNA were done according to the manufacturer instructions (ZYMO RESEARCH CORP www.zymoresearch.com), and then amplified by PCR using the primers

shown in Table S1. The PCR products were cloned into the pMD19-T vector (TaKaRa), and then were sequenced and analyzed.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Natural Science Foundation of China, 30971652 and 31271463 (J.L.), 31071198 (K.Y.), Hundred Talents Program and KSCX2-YW-N-073 from the Chinese Academy of Sciences.

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Figure legends

Figure 1. Seedling and stomatal phenotypes of *28y* mutant

(A–C) *28y* mutant. A 14-day-old *28y* seedling **(A)**. Inset, a mature *28y* plant grown in soil. DIC images showing clustered stomata from a *28y* cotyledon **(B)** and a hypocotyl **(C)**. White brackets indicate stomata in clusters. **(D–F)** Wild type. A 14-day-old wild-type seedling **(D)**. Patterned and spaced stomata in epidermis from a wild-type cotyledon **(E)** and a hypocotyl **(F)**. Bars=200 mm in **(A, D)**, 50 μm in **(B, C, E, F)**.

Figure 2. Stomatal lineage tracing in hypocotyls

(A–D) Stomatal development in a wild-type hypocotyl. Insets, illustrations of the development of stomatal lineage cells within the white frames. A MMC divides asymmetrically to produce a triangular meristemoid (red) and a big sister cell, a SLGC. Meristemoid differentiates into GMC (yellow) that then divide symmetrically to generate a pair of stomatal GCs (green). The adjacent SLGCs differentiate into epidermal pavement cells. **(E–H)** Higher-order stomatal complexes form in a *28y* hypocotyl. Excessive asymmetric spacing divisions in SLGCs produce extra satellite meristemoids and stomata. Bar=50 μm.

Figure 3. Ectopic stomatal lineage cells produced in *28y* cotyledons

(A–C) *TMM:TMM-GFP* is expressed in stomatal lineage cells and their neighbor sister cells in wild-type cotyledons, tracing at 3, 5 and 7 days after germination. **(D–F)** Number of the epidermal cells expressing *TMM:TMM-GFP* in *28y* mutant is higher than that in wild-type cotyledons at the same stages. **(G–I)** Expression of a mature stomatal marker E1728 in wild-type cotyledons. **(J–L)** E1728 expression in *28y* cotyledons. An arrow indicates a newly-formed stoma without E1728 fluorescence in a *28y* cotyledon. Bar=20 μm.

Figure 4. *spch* is epistatic to *28y* in initiating stomatal lineage

(A, B) No stomata occurred in a *spch-4* cotyledon **(A)** and a hypocotyl **(B)**. **(C, D)** Stomatal defects in a *spch-4* mutant is not rescued by *28Y* mutation. **(E)** *SPCH:SPCH-GFP* in wild-type epidermis is restricted mostly in meristemoids. An arrow points to a SLGC with weak GFP

fluorescence. **(F)** More cells expressing *SPCH:SPCH-GFP* are present in *28y* epidermis, indicated by arrows. **(G)** *SPCH* transcript level was largely increased in *28y* seedling comparing that in wild types (**P<0.01 after Student *t*-test, *28y* versus wild-type). Bar=50 μm in **(A–D)**, 20 μm in **(E, F)**.

Figure 5. *28y* is epistatic to *tmm* in regulating hypocotyl stomatal production

(A) Clustered stomata in *tmm-1* cotyledons. **(B)** No stomata produced in *tmm-1* hypocotyls. **(C, D)** Clustered stomata are present in *tmm-1 28y* double mutant cotyledons **(C)** as well as in their hypocotyls **(D)**. Note that *28y* is epistatic to *tmm-1* in rescuing the 'no stomata' phenotype of *tmm-1* hypocotyls. **(E)** *28y* mutation reduces the cluster complexity of *tmm-1* (**P<0.01 after Student *t*-test, *28y tmm-1* versus *tmm-1*). Bar=50 μm in **(A, C)**, 20 μm in **(B, D)**.

Figure 6. Mutation of *AGO1* is the cause of the defective seedling growth and stomatal production in *28y* mutant

(A) Mapping of the *28Y* gene and physical structure of the *28Y/AGO1* gene. Letters above lines indicate the position of molecular markers. BAC at F21D18 (red letters) contains the *28Y/AGO1* gene. The mutations in *28y* mutant are indicated in the bottom panel. *28y* has a T to A transition (arrow) and a deletion of bases (red letters), resulting in a premature termination of *AGO1* transcript. Gray boxes indicate the exons. **(B, C)** *ago1-8* seedlings and its cotyledon epidermis. **(D, E)** *28y* is allelic to *ago1-8. A* progeny seedling and the cotyledon epidermis of *28y* and *ago1-8* crosses. **(F–J)** *AGO1:GUS* broadly expressed in the epidermis **(F)**, especially in stomatal lineage cells, including MMC **(G)**, meristemoids **(H)**, GMCs **(I)**, young GCs **(J)**. Bars=200 mm in **(B, D)**, 20 μm in **(C, E–J)**.

Figure 7. *AGL16* transcript levels in *28y* mutants

The *AGL16* relative mRNA expression levels is up-regulated in *28y* mutant seedlings (**P<0.01 after Student *t*-test, *28y* versus wild-type seedlings).

Figure 8. Scheme of *AGO1* regulatory pathway in stomatal development

(A) Stomata lineage initiates from meristemoid mother cell (MMC, in pink color). MMC executes

asymmetric entry division and produces a meristemoid (M, in brown color) and a big sister cell, a stomatal lineage ground cell (SLGC). SPCH, MUTE, FAMA, and FLP/MYB88 are transcriptional factors in regulating key transitions during stomatal development. SLGCs can differentiate into a pavement cells. SLGCs also can reacquire a MMC fate and initiate asymmetric spacing divisions to produce satellite meristemoids. AGO1 acts in restricting spacing divisions. **(B)** *AGO1* functions downstream of *TMM* but independently from BR-mediated SPCH phosphorylating pathway. Instead, AGO1 might be involved in AGL16-meidated microRNA post-transcriptional regulatory pathway.

Tables

Table 1. Increased stomatal production in *28y* mutant

	Stomatal index (%)	Stomatal Density (Number mm^{-2})	Number of Stomata per Hypocotyl
WT	31 ± 0.9[1]	160 ± 17	24 ± 8
28y	44 ± 4.6	337 ± 63**	182 ± 86**

[1]Values are average ± standard deviation. ** P <0.01 after Student's *t*-test (n=10).

Table 2. Stomatal densities and indexes in *tmm-1* and *tmm-1 28y* mutants

mutant	Stomatal Index (%)	Stomatal Density (number mm^{-2})
tmm-1	62 ± 3.8[1]	677 ± 99
tmm-1 28y	62 ± 4.0	808 ± 192

[1]Values are average ± standard deviation (n=10).

Figure 1

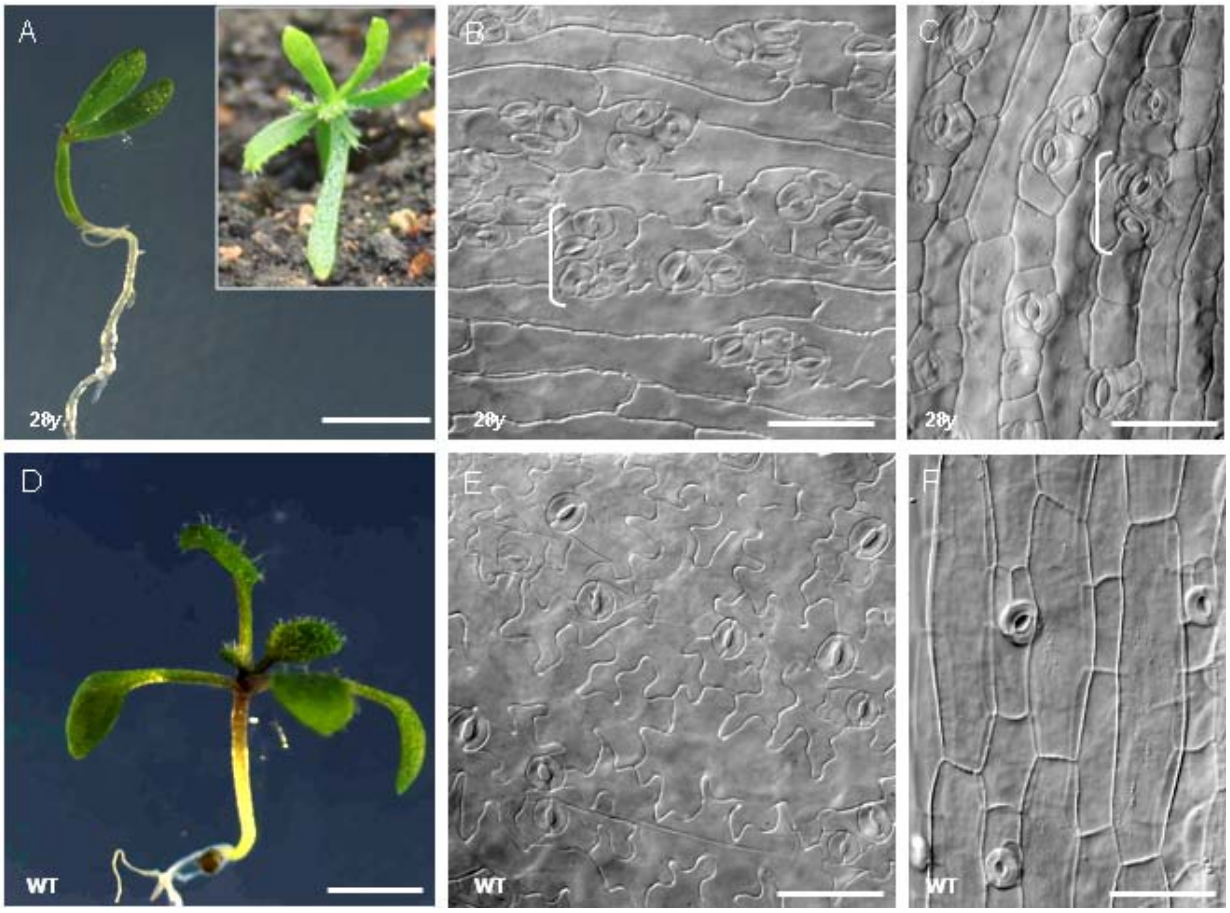
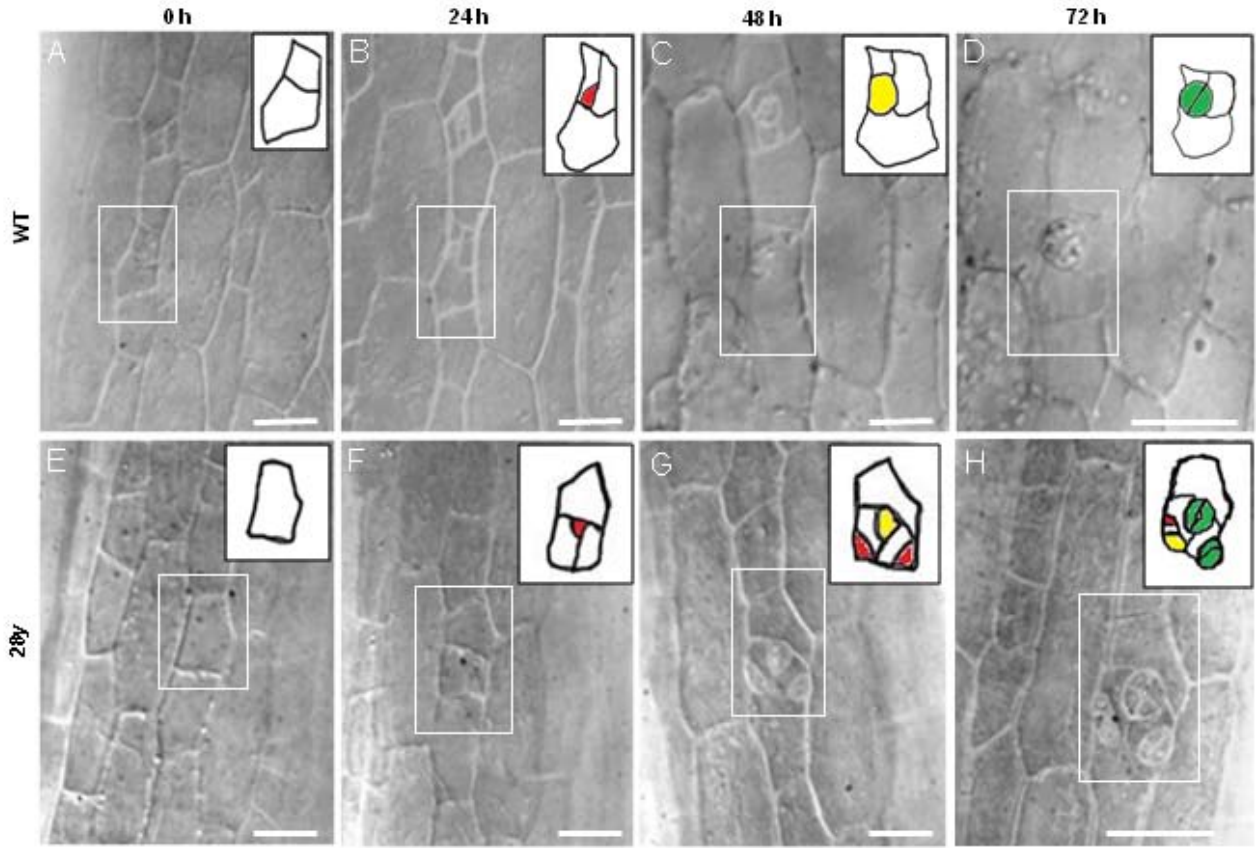


Figure 2



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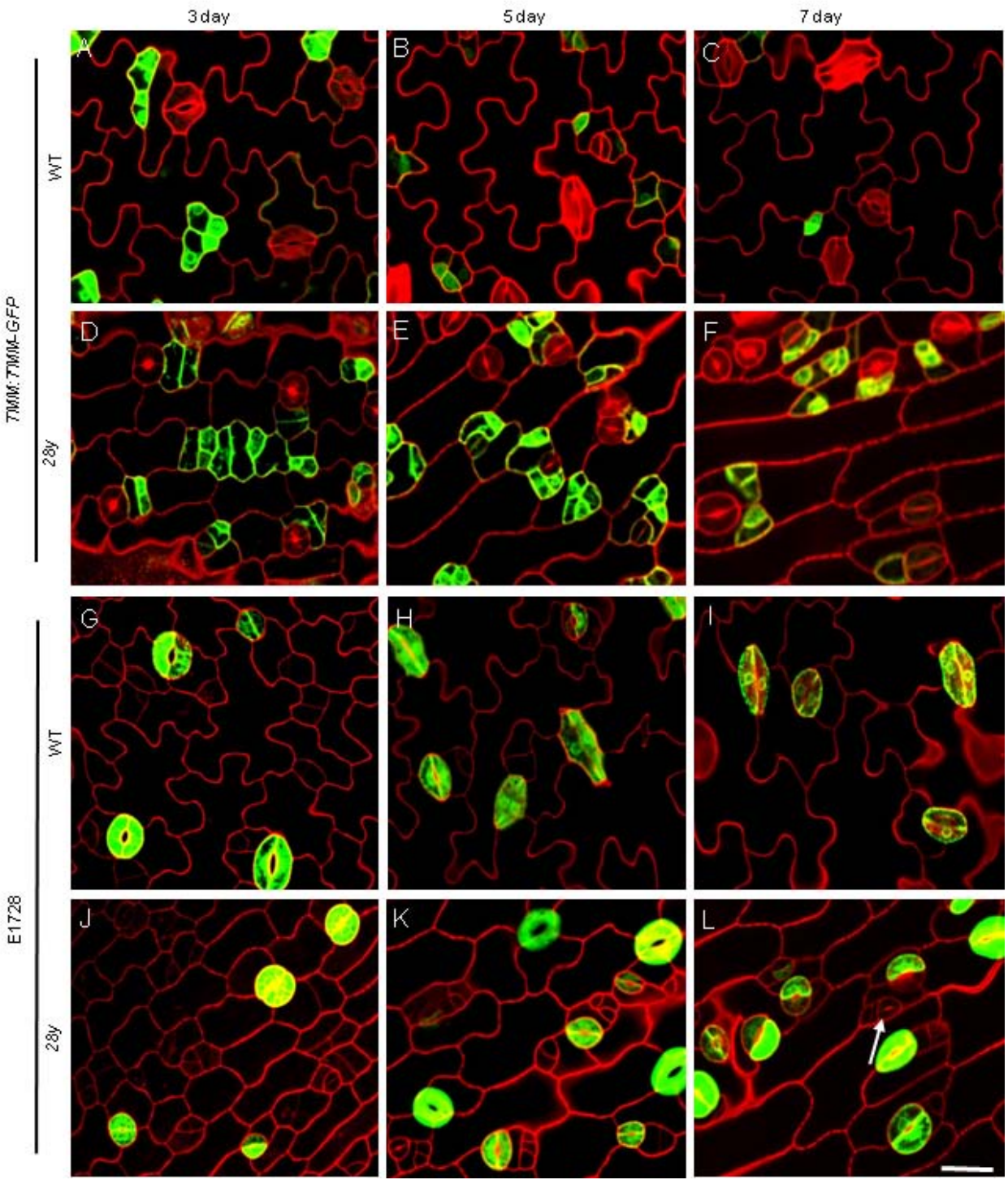

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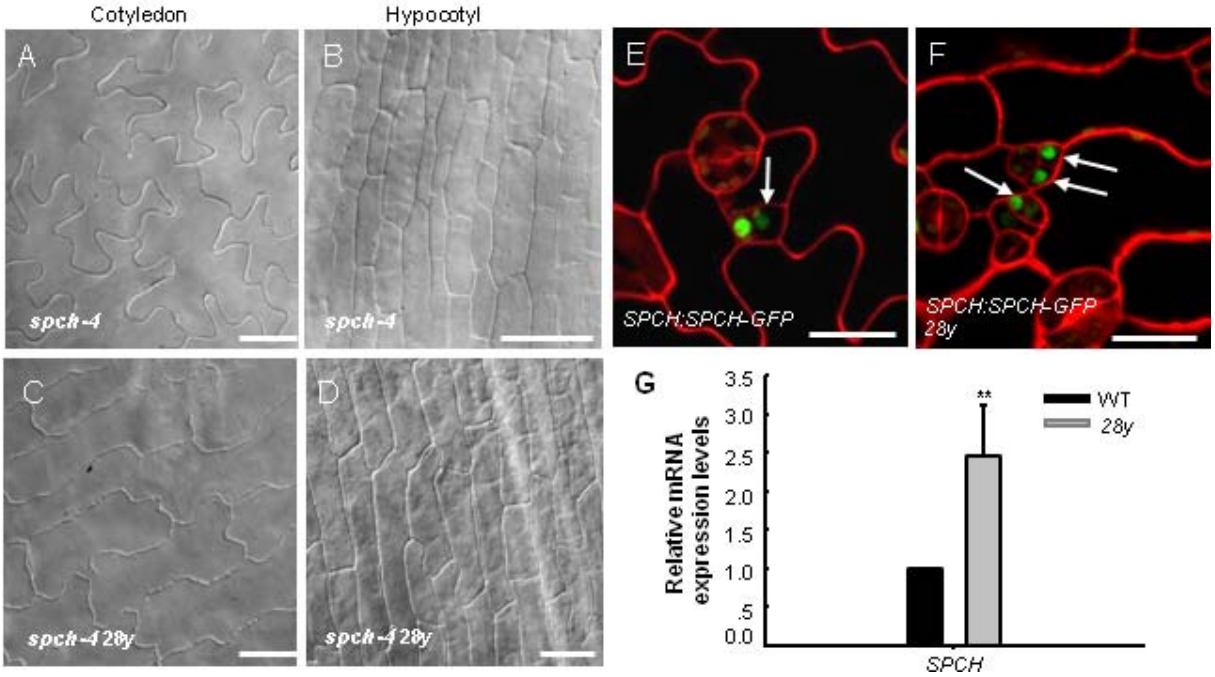

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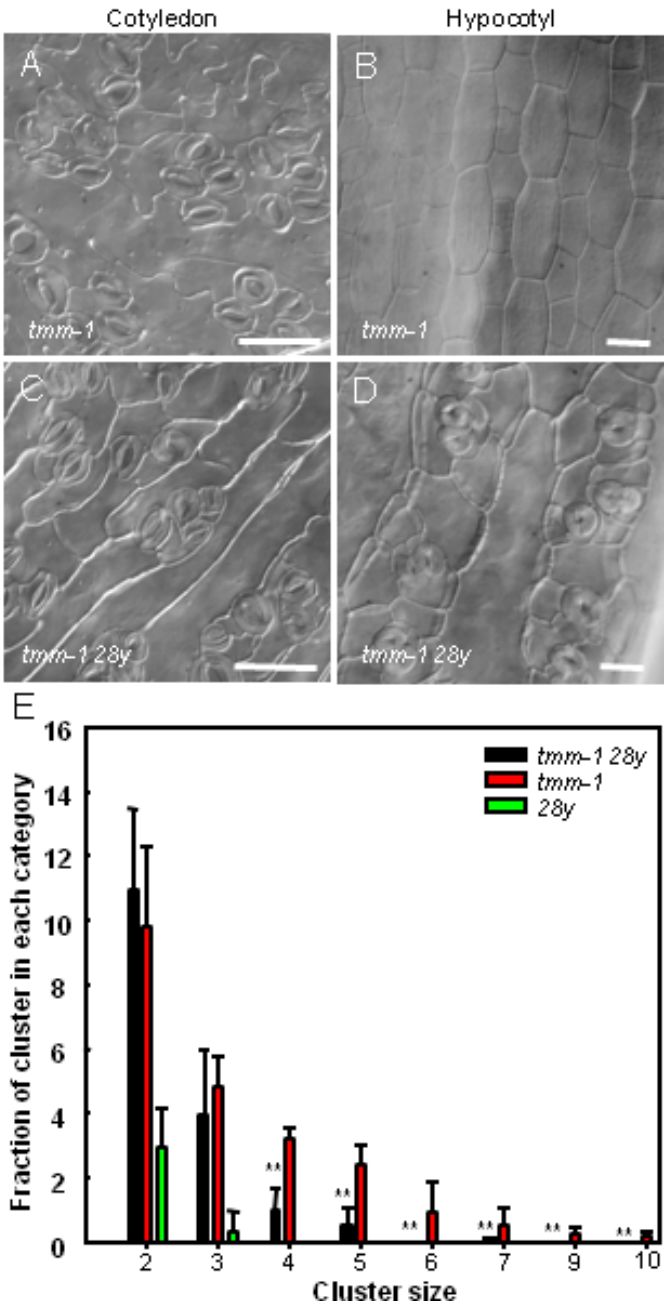

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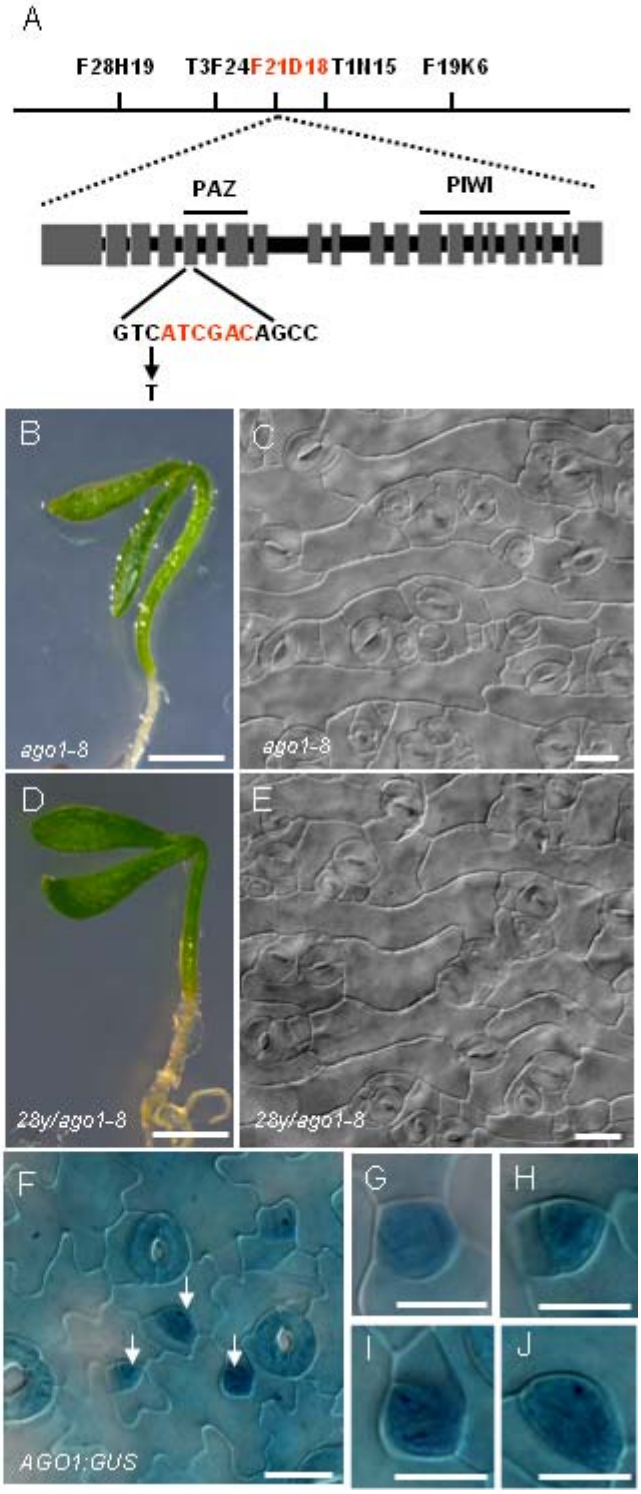

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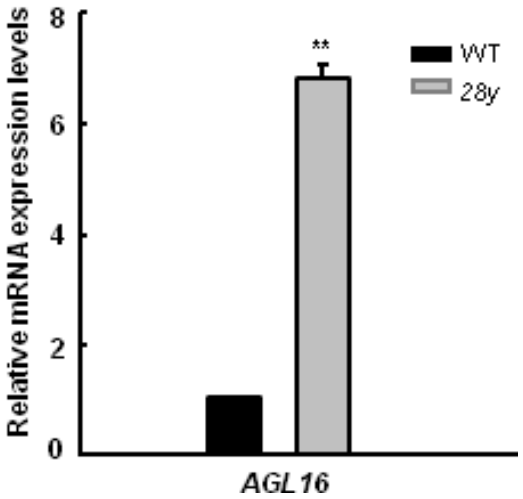

Figure 8