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RESEARCH PAPER

Light-induced STOMAGEN-mediated stomatal development in *Arabidopsis* leaves

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Abstract

The initiation of stomata, microscopic valves in the epidermis of higher plants that control of gas exchange, requires a co-ordinated sequence of asymmetric and symmetric divisions, which is under tight environmental and developmental control. *Arabidopsis* leaves grown under elevated photosynthetic photon flux density have a higher density of stomata. *STOMAGEN* encodes an epidermal patterning factor produced in the mesophyll, and our observations indicated that elevated photosynthetic irradiation stimulates *STOMAGEN* expression. Our analysis of gain and loss of function of *STOMAGEN* further detailed its function as a positive regulator of stomatal formation on both sides of the leaf, not only in terms of stomatal density across the leaf surface but also in terms of their stomatal index. *STOMAGEN* function was rate limiting for the light response of the stomatal lineage in the adaxial epidermis. Mutants in pathways that regulate stomatal spacing in the epidermis and have elevated stomatal density, such as *stomatal density and distribution* (*sdd1*) and *too many mouth* alleles, displayed elevated *STOMAGEN* expression, suggesting that *STOMAGEN* is either under the direct control of these pathways or is indirectly affected by stomatal patterning, suggestive of a feedback mechanism. These observations support a model in which changes in levels of light irradiation are perceived in the mesophyll and control the production of stomata in the epidermis by mesophyll-produced STOMAGEN, and whereby, conversely, stomatal patterning, either directly or indirectly, influences STOMAGEN levels.

Key words: *Arabidopsis thaliana*, PPFD, SDD1, STOMAGEN, stomatal density, TMM.

Introduction

The development of a bicellular structure in higher plants that controls the opening and closure of a micropore in the aerial epidermis, the stoma, proved to be an evolutionary successful adaptation for the colonization of land (Vaten and Bergmann, 2012). These stomata, hydraulically controlled micropores in the relatively watertight epidermis, regulate

Abbreviations: ANOVA, analysis of variance; HLH, basic helix–loop–helix; GC, guard cell; HL, high light; LL, low light; PPFD, photosynthetic photon flux density; RNAi, RNA interference; RT-PCR, reverse transcription PCR; SD, stomatal density; SI, stomatal index; WT, wild type; YFP, yellow fluorescent protein.

water vapour loss while enabling sufficiently high CO_2 uptake. The regulation of gas exchange occurs at both physiological and developmental levels. Whereas the relatively fast stomatal closing and opening response is controlled by the activity of ion channels in guard-cell (GC) membranes, the frequency and size of stomatal pores in the epidermis is adjusted by long-term acclimation and adaptation processes (Franks and Beerling, 2009; Haworth *et al.*, 2011).

In the last decade, cellular and molecular mechanisms for the formation of stomata have been revealed, and for this the genetic model *Arabidopsis thaliana* (L.) Heynh. has been instrumental (Geisler *et al.*, 1998; Nadeau and Sack, 2002; Nadeau, 2009; Serna, 2009; Pillitteri and Dong, 2013). At the origin of the stomatal lineage lies the acquisition of meristemoid mother cell identity by a protodermal cell. Meristemoid mother cells undergo an asymmetric cell division producing a meristemoid, a stem-cell-like triangular cell, and a larger sister cell. The meristemoid can self-renew through up to three asymmetric divisions or differentiate into a round guard mother cell, symmetric division of which generates a pair of GCs, forming a stoma. The larger sister cell, called the stomatal lineage ground cell, can generate a satellite meristemoid by another asymmetric division, usually avoiding contact between the new stoma and the existing one, or can terminally differentiate into an epidermal pavement cell (Nadeau, 2009). The 'one-cell spacing rule' (Serna *et al.*, 2002; Bergmann and Sack, 2007; Torii, 2012) stipulates that stomata are spaced by at least one epidermal pavement cell. Together with the size of the pavement cells, the frequency of meristemoids and satellite meristemoids predetermines stomatal density (SD) and co-determines the stomatal index (SI).

The development of stomata is regulated by positive and negative peptide signals from the epidermis and inner leaf tissues (Shimada *et al.*, 2011; Vaten and Bergmann, 2012). Stomata in *Arabidopsis* leaves are placed above the junctions of several mesophyll cells (Serna and Fenoll, 2000), and thus radially transmitted signals from the mesophyll could be propagated towards the epidermis and vice versa. Recently, EPIDERMAL PATTERNING FACTORs (EPF) and EPIDERMAL PATTERNING FACTOR LIKE (EPFL) proteins have been characterized (Rychel *et al.* (2010). EPF1 (Hara *et al.*, 2007) and EPF2 (Hara *et al.*, 2009; Hunt and Gray, 2009), both synthesized in the epidermis, have been identified as negative regulators of stomatal differentiation. Another negative regulator, EPFL6, known as CHALLAH (Abrash and Bergmann, 2010), and the positive regulator EPFL9, called STOMAGEN (Hunt *et al.*, 2010; Kondo *et al.*, 2010; Sugano *et al.*, 2010), are produced in internal tissues.

Putative receptors for the EPFs and EPFLs are TOO MANY MOUTHS (TMM), a leucine-rich-repeat receptor-like protein (Geisler *et al.*, 1997), and members of the ERECTA (ER) family of leucine-rich-repeat receptor-like kinases (Masle *et al.*, 2005; Shpak *et al.*, 2005; Shpak, 2013), including ER and ERECTA-LIKE (ERL) proteins. These receptors are thought to associate with each other in the plasma membrane (Shimada *et al.*, 2011). Although the structure of these ligand–receptor molecular complexes has yet to be fully resolved, Lee *et al.* (2012) suggested that TMM modulates the binding of EPF ligands to ER receptors. Mutants defective in TMM (Nadeau and Sack, 2002) and ERs form stomatal clusters on their leaves, i.e. groups of stomata in contact with each other so that the one cell spacing rule is breached. Curiously, loss of TMM resulted in loss of stomata on stems (Geisler *et al.*, 1998; Bhave *et al.*, 2009). In stomatal development, the important role of another small extracellular peptide, STOMATAL DENSITY AND DISTRIBUTION (SDD1), a subtilisin-like serine (processing) protease (Berger and Altmann, 2000; von Groll *et al.*, 2002), still remains to be fully understood. Mutation in the encoding gene causes increased SD and formation of small stomatal clusters.

The regulation of stomatal development also involves the mitogen-activated protein kinases cascade signal transduction chain (Bergmann *et al.*, 2004; Shpak *et al.*, 2005) and the action of the basic helix–loop–helix (bHLH) transcription factors SPEECHLESS, MUTE, and FAMA (Pillitteri and Torii, 2007), and SCREAM (SCRM) and SCRM2, which interact directly with and specify the consecutive action of the above-mentioned bHLH factors (Kanaoka *et al.*, 2008). In addition, protein factors that regulate asymmetric (Bergmann *et al.*, 2009; Dong *et al.*, 2009; Pillitteri *et al.*, 2011) and symmetric cell division, e.g. FLP and MYB88 factors (Yang and Sack, 1995; Lee *et al.*, 2013, 2014) are key to executing the controlled morphogenesis of stomata.

In response to the environment or developmental signalling, the apertures of the pores or the SD, frequency, and size are modulated. As such, the stomata and the stomatal lineage respond to CO_2 (Woodward, 1987; Royer, 2001; Bunce, 2007; Engineer *et al.*, 2014; Santrucek *et al.*, 2014), light quality and quantity (Royer, 2001; Casson and Gray, 2008; Casson *et al.*, 2009; Casson and Hetherington, 2010, 2014; Craven *et al.*, 2010), water availability (Aasamaa and Sober, 2011), transpiration and abscisic acid (Lake and Woodward, 2008; Tanaka *et al.*, 2013; Chater *et al.*, 2014), and drought stress (Hamanishi *et al.*, 2012). The effect of plant hormones, auxin (Zhang *et al.*, 2014), and brassinosteroids (Kim *et al.*, 2012) on stomatal development has also been reported. Photosynthetic photon flux density (PPFD) and ambient CO_2 concentration modulate SD in newly developing leaves through systemic signalling from mature leaves (Lake *et al.*, 2001; Coupe *et al.*, 2006), but the pathways involved in this systemic signalling are currently unresolved. Typically, sun leaves have a higher SD than their shaded counterparts (Schoch *et al.*, 1980; Ticha, 1982; Matos *et al.*, 2009). Moreover, the light-quantity response is at least partly wavelength specific. It relies on the density of photons in the red part of the spectrum perceptible by the PHYTOCHROME B (PHY B) photoreceptor. The response involves the downstream transcription factor PHYTOCHROME INTERACTING FACTOR 4 (PIF4) (Boccalandro *et al.*, 2009; Casson *et al.*, 2009; Kang *et al.*, 2009).

Here, we further explored the role of STOMAGEN in the regulation of stomatal formation. More specifically, we addressed on which side of the leaf STOMAGEN is active. We investigated the influence of light levels on *STOMAGEN* expression and its importance for the response to light of the

stomatal lineage. Furthermore, we explored the effect of elevated SD on *STOMAGEN* expression, presenting us with a putative feedback mechanism. Collectively, our observations indicate that STOMAGEN is part of the mechanism that links levels of the photosynthetically active light with stomatal development.

Material and methods

Plant material and growth conditions

Loss-of-function alleles of *TMM1* [Columbia (Col-0) Col-0 background] (Yang and Sack, 1995) and *SDD1* (C24 background) (Berger and Altmann, 2000) were studied. Wild-type *A. thaliana* (L.) Heynh. plants, ecotypes Col-0 and C24 were used as experimental controls. Lines with manipulated STOMAGEN/EPFL9 levels were created in the Col-0 background (Sugano *et al.*, 2010): RNA interference (RNAi)-silenced *STOMAGEN* (*ST-RNAi*), *35S::STOMAGEN (ST-Ox, overexpressing STOMAGEN)*, and *35S::STOMAGEN-VENUS [ST-Venus*, expressing *STOMAGEN* fused to the Venus yellow fluorescent protein (YFP)] were examined (Sugano *et al.*, 2010).

Except for ST-Venus, plants were grown either in controlled environment chambers in compost (Primaflora; AGRO CS, Czech Republic) (the two WTs and mutants *tmm* and *sdd1*; see Figs 1 and 6) or in hydroponic culture (all other experiments) in ¼ Hoagland solution under 10/14 h (light/dark) photoperiod with 22/18 °C day/night temperatures. For confocal microscopy, ST-Venus plants were grown *in vitro* on ½ Murashige–Skoog medium solidified with 0.8% agar.

For PPFD experiments, seeds were sown, stratified for 3 d at 4 °C in the dark and germinated at 150 ± 20 µmol photosynthetically active radiation (PAR) photons m^{-2} s^{-1}. After 14 d, the seedlings were transferred into pots and cultivated in growth chambers (Fitotron, Sanyo, UK, or Snijders Scientific, The Netherlands). Half of the plants of each genotype were grown at 250 ± 20 µmol PAR photons m^{-2} s^{-1} [high light (HL)] and the other half at 25–50 ± 5 µmol m^{-2} s^{-1} [low light (LL)] or 150 ± 10 µmol m^{-2} s^{-1} [medium light (ML)]. Relative air humidity was maintained at 50–70 %. The plants were then grown for 3 weeks. After 3 weeks, the three youngest developing leaves in the leaf rosette were harvested (leaves from five plants were sampled) at growth stage 3.5 according to Boyes *et al.* (2001), snap frozen in liquid nitrogen and stored at 80 °C. Fully expanded leaves (adult), but not senescent ones, were used for scoring of stomata (SD and SI estimation).

SD

Impressions of adaxial and abaxial leaf surfaces of the fully developed leaves were made with dental silicone resin (Stomaflex; Spofa Dental, Czech Republic). Clear nail varnish was applied to the dental impressions and varnish replicas were viewed with an Olympus BX61 microscope (magnification ×50 objective) and imaged using a EOS 1000D camera (Canon, Japan). Stomatal and epidermal cell densities were counted from 20–25 areas of 0.133 mm^2 from the middle part of the leaf for each combination of genotype and light conditions. Areas came from five to seven different plants of each genotypes (three to five mature leaves of each plant) and the experiment was repeated three times. Cell Counter in ImageJ software was used (http://rsbweb.nih.gov/ij/). The numbers of the cells were expressed per mm^2. SI was calculated with the following formula: SI=(number of stomata)/(number of stomata+number of other epidermal cells)×100. Finally, for relationships with gene expression, the results were expressed as counts averaged over both leaf sides.

Gene expression

Total RNA was extracted from up to 100 mg of frozen leaves using an RNeasy Plant Mini kit (Qiagen) according to the manufacturer's instructions. Samples were purified using RNase-free DNA (Qiagen) during isolation and additionally by Ambion DNA Free according to the manual. cDNA was synthesized from 1 µg of total RNA (DNA free) using a High Capacity RNA to cDNA kit (Applied Biosystems).

Real-time PCR was performed on a Step One Real-time PCR system (Applied Biosystems/Life Technologies) using 40 cycles with TaqMan Gene Expression Master Mix (Applied Biosystems) according to the manufacturer's instructions. TaqMan Gene Expression Assay kits for expression of *STOMAGEN* (At4g12970; assay identifier At02219575_g1), *SDD1* (At1g04110; assay identifier At 02260111_s1), *TMM* (At1g80080; assay identifier At02219649_s1), *ERECTA* (At2g26330; assay identifier At02275070_g1), *EPF1* (At2g20875; assay identifier At02178566_g1), *EPF2* (At1g34245;

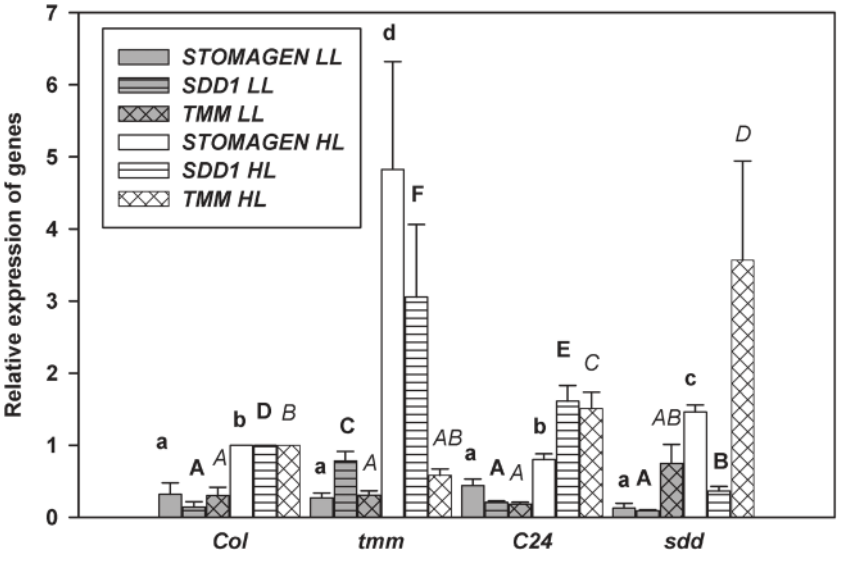

Fig. 1. Expression of *STOMAGEN*, *TMM*, and *SDD1* in WT and *tmm1* and *sdd1* mutants. Expression of *STOMAGEN*, *SDD1*, and *TMM* in young developing leaves of *A. thaliana* WT Col-0 and C24, and mutants *tmm1* and *sdd1* grown under two different light conditions (HL: 250 µmol m^{-2} s^{-1}, and LL: 25–50 µmol m^{-2} s^{-1}). Expression was estimated by real-time reverse transcription PCR (RT-PCR) relative to Col-0 HL leaves normalized to *ACTIN 8* expression (housekeeping gene). Error bars represent SEM (four biological replicates). Different letters indicate statistically significant differences: lower case, *STOMAGEN*; upper case, *SDD1*; upper case italics, *TMM* (P<0.05, one-way ANOVA, Tukey's test).

assay identifier At02193517_g1), *SPEECHLESS* (At5g53210; assay identifier At02321067_g1), *MUTE* (At3g06120; assay identifier At02235996_g1), and *FAMA* (At 3g24140); assay identifier At02279293_g1) were used.

The *ACTIN8* (At1g49240) TaqMan gene expression assay kit At02270958_gH was used for reference (housekeeping gene). To determine relative levels of gene expression, the threshold cycle method according to Livak and Schmittgen (2001) was applied. Reaction efficiency (estimated from a calibration curve) was used for precise calculation of the relative expression levels.

Confocal microscopy

The leaves of the *STOMAGEN*–Venus line were observed with a Zeiss LSM 5 Duo confocal microscope (Carl Zeiss, Jena, Germany) with appropriate filter sets for YFP–Venus detection (excitation 488 nm and emission between 525 and 560 nm) and a ×40 C Apochromat water immersion objective was used. FM 4–64 fluorescent dye was used as a plasma membrane marker.

Statistics

Statistical analyses were performed using the program SigmaPlot 11.0 (Systat Co.). One-way analysis of variance (ANOVA) with Tukey's test for post-hoc comparisons was used. In the case of non-normal distribution, a Kruskal–Wallis one-way ANOVA on ranks followed by Dunn's test for all pairwise comparisons and comparisons against a control group was applied.

Results

STOMAGEN *expression is stimulated by light*

Recently, the STOMAGEN/EPF family protein was identified as a positive regulator of stomatal formation. Intriguingly, most other members of this family act as repressors (Hunt *et al.*, 2010; Kondo *et al.*, 2010; Sugano *et al.*, 2010). Its expression and localization in mesophyll (Kondo *et al.*, 2010; Sugano *et al.*, 2010) and its positive role in stomatal development make it a good candidate to act in the systemic signalling between mesophyll and epidermis. Given the role of mesophyll as the main tissue for photosynthesis, we hypothesized that STOMAGEN might be involved in linking photosynthetic activity and epidermal patterning. In order to test this, we first examined the expression of STOMAGEN under two different levels of PPFD. We used the youngest developing leaves of 28-d-old seedlings to monitor *STOMAGEN* expression, as the overall transcription activity was reported previously to be highest in young leaves (Sugano *et al.*, 2010), and this was corroborated by our relative transcript quantification (Supplementary Fig. S1, available at *JXB* online). This suggested that *STOMAGEN* expression is associated with mitotic activity and early cellular growth rather than with mature cells. Furthermore, the influence of development on expression due to different growth conditions was minimal on young leaves compared with older leaves.

In these youngest developing leaves, transcript levels of *STOMAGEN* were markedly increased under HL compared with LL conditions (Fig. 1). No significant difference in *STOMAGEN* expression was observed between wild types (WTs) and mutants under LL conditions (Fig. 1). These observations indicated that *STOMAGEN* expression is stimulated by photosynthetic active light.

STOMAGEN modulates the expression of genes involved in stomatal development and patterning and is rate limiting for stomatal formation in the adaxial and abaxial epidermis

Expression of the *ST-RNAi* and *ST-Ox* constructs modulated the levels of *STOMAGEN* transcripts in the young leaves. For *ST-RNAi*, we observed a decrease in *STOMAGEN* transcripts to approximately 25% of WT levels, whereas the *ST-Ox* line contained approximately 35 times more *STOMAGEN* transcripts than WT (Fig. 2A). We monitored expression of *ST-Venus* by confocal microscopy and observed STOMAGEN–Venus in the mesophyll (Supplementary Fig. S2A, available at *JXB* online), as well as throughout the epidermis in the GCs and pavement cells, with a brighter signal in the pavement cells near stomata (Supplementary Fig. S2B–D), confirming that the *ST-Ox* construct was active in the mesophyll as well as in the epidermis.

The decrease and increase in *STOMAGEN* expression in the *ST-RNAi* and *ST-Ox* lines, respectively, was accompanied by coincident changes in SDD1 expression (Fig. 2A). *STOMAGEN* overexpression did not induce higher expression of its putative receptor *TMM* but did influence levels of *ER* transcripts (Fig. 2B).

A similar trend was observed for the *EPF1* gene encoding a negative regulator of stomatal formation secreted by stomatal precursors, from late meristemoids to guard mother cells, as well as GCs (Hara *et al.*, 2007) (Fig. 2C) and for two of three bHLH transcription factors genes, *MUTE* and *FAMA* (Fig. 2D). MUTE and FAMA are involved in the later stages of stomata formation, in the specification of GC precursors and their symmetric division (Pillitteri *et al.*, 2007). In contrast, no changes were observed for transcription of *SPEECHLESS* bHLH transcription factor and *EPF2* genes, both involved in the initial specification of the meristemoid in the stomatal lineage (Fig. 2C, D).

These changes in transcript levels suggested that STOMAGEN influences the expression of several genes, either directly or indirectly, which are performing a crucial role in the stomatal lineage.

Lines with suppressed *STOMAGEN* expression by RNA interference (*ST-RNAi*) and the *STOMAGEN*-overexpressing line (*ST-Ox*) were examined for stomata formation and pavement cell density (Fig. 3). Modulation of STOMAGEN transcripts levels had opposite effects on the pavement cell density on both sides of the leaf (Fig. 3B). Downregulation increased cell density in the adaxial epidermis but decreased it in the abaxial. Nevertheless, the proportion of stomata, as evidenced by the SI, in both the adaxial and abaxial epidermis was reduced in the *ST-RNAi* line and upregulated in the *ST-Ox* line (Fig. 3C). Our observations on the abaxial leaf side are in line with earlier reports (Sugano *et al.*, 2010). Given the concomitant large increase in pavement cell density in the abaxial epidermis, this had an extensive positive effect on the SD of that leaf side (Fig. 3A). The proportion of stomata in the abaxial epidermis, as reflected by the SI, was similarly affected by modulation of *STOMAGEN* expression, but the effect on SD on the adaxial side was weaker given the concomitant modulation of pavement cell density (Fig. 3A, B).

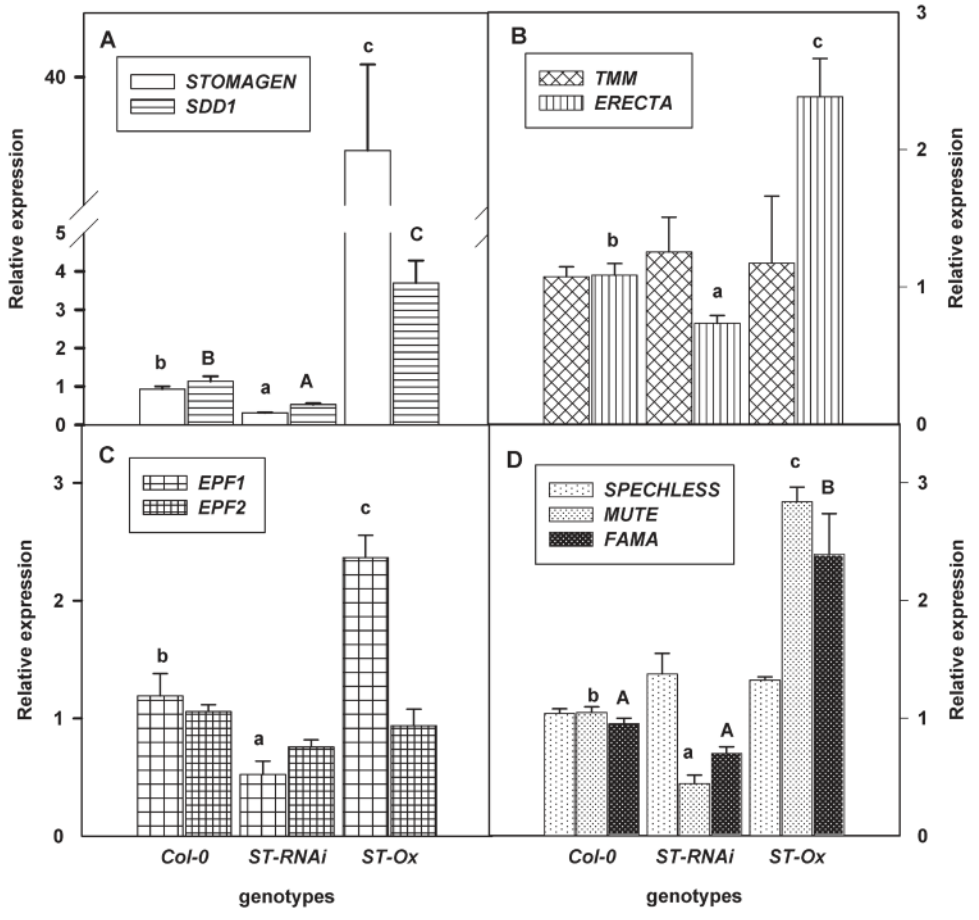

Fig. 2. *STOMAGEN* expression affects expression of a subset of genes involved in stomatal development. The relative expression levels of the selected genes *STOMAGEN* and *SDD1* (A), *TMM* and *ER* (B), *EPF1* and *EPF 2* (C), and *SPEECHLESS*, *MUTE*, and *FAMA* (D) in developing leaves of *STOMAGEN*-overexpressing (ST-Ox) and silenced (ST-RNAi) transgenic lines compared with WT Col-0. Expression was estimated by real-time RT-PCR normalized to *ACTIN 8* expression (housekeeping gene). Error bars represent SEM (data are means of three biological replicates). Letters indicate statistically significant differences ($P<0.05$, one-way ANOVA or Kruskal–Wallis one-way ANOVA on ranks; in cases where the equal variance test failed, Tukey's or Dunn's post-hoc test was used).

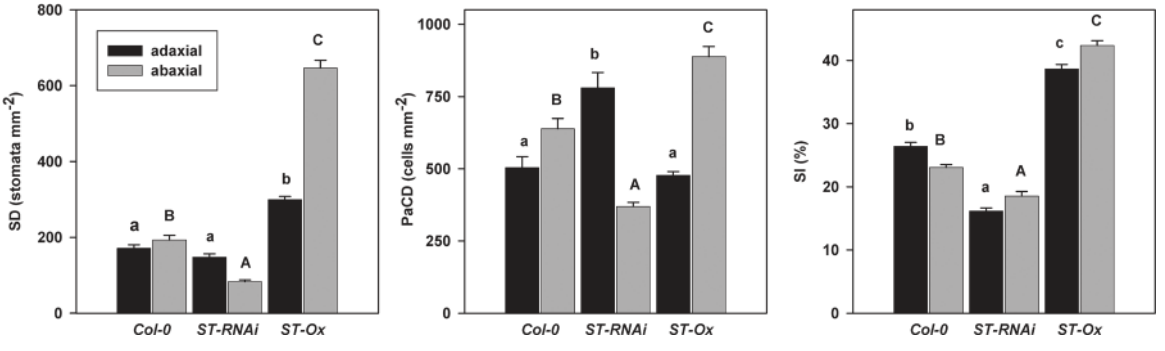

Fig. 3. Expression of *STOMAGEN* affects stomatal and pavement cells density, and the SI. SD (A), density of epidermal pavement cells (B; PaCD), and SI (C) on the adaxial (black columns) and abaxial (grey columns) leaf surfaces of fully developed leaves of *A. thaliana* WT Col-0 and *STOMAGEN*-overexpressing (ST-Ox) and silenced (ST-RNAi) transgenic lines. Data are means from 25 areas of 0.13 mm^2 (used for each genotype). Different letters indicate significant differences separately for adaxial (lower case) and abaxial (upper case) leaf sides; error bars represent SEM ($P<0.05$).

Taken together, the modifications of transcripts of genes in the stomatal pathway and the effect on SI indicated that STOMAGEN influences the formation of stomata in the epidermis of both leaf sides. In addition, modulation of *STOMAGEN* expression had an opposite effect on cell density in the adaxial and abaxial leaf epidermis, resulting in a positive effect of STOMAGEN on the density of stomata mainly in the abaxial epidermis.

STOMAGEN is rate limiting for the HL response of the stomatal lineage in the adaxial epidermis

In order to validate our experimental set-up, we performed initial experiments on the Col-0 background to establish that elevated levels of photosynthetic radiation stimulated the formation of stomata in line with previous reports (Casson and Gray, 2008; Casson and Hetherington, 2010). Indeed and as expected,

elevated light intensity increased SD (Fig. 4A and Supplementary Figs S3 and S4A, available at *JXB* online) and SI on the adaxial side of the leaf (Fig. 4C and Supplementary Fig. S4B).

In order to establish whether STOMAGEN is required to elevate the number of stomata in response to elevated photoactive radiation, we scored the stomata in *ST-RNAi* lines exposed to HL. Although the SD still responded to light (Fig. 4A), we observed that HL was unable to stimulate the SI (Fig. 4C) in the adaxial epidermis of the *ST-RNAi* line, in contrast to Col-0 where HL elevated the stomatal index in both the adaxial and abaxial epidermis. This suggests that the increase in SI by HL involves STOMAGEN in the adaxial side. The necessity of STOMAGEN for stomatal development modulated by light was also obvious from the relationship of SD (summed over both surfaces) with *STOMAGEN* relative expression shown in Fig. 4D.

SDD1 loss of function triggers a stronger light response of the stomatal lineage in the adaxial epidermis

According to the experiment discussed above, in Col-0 WT leaves grown under a relatively high PPFD of 250 µmol $m^{-2}s^{-1}$ (HL), the SD increased in the WT Col-0 when compared with shaded plants (growing at low PPFD of 25–50 µmol m^{-2} s^{-1}, LL), especially on the more exposed adaxial leaf side (Fig. 5A, B, and Supplementary Fig. S4A). Statistical analyses (one-way ANOVA followed by Tukey's post-hoc test) were performed separately for the adaxial SD (Fig. 5A) and SI (Fig. 5C) and abaxial SD (Fig. 5B) and SI (Fig. 5D) to evaluate statistically significant differences between variants under the two different light intensities.

In line with a developmental dose response of high HL in the Col-0 background, HL significantly increased the SD on both sides of Col-0 leaves even when compared with leaves grown under medium levels of PPFD (150 µmol m^{-2} s^{-1}, ML), as well as ML compared with LL. Both differences were found to be statistically significant; however, a stronger effect was observed on the adaxial side (Supplementary Fig. S4A).

In the Col-0 ecotype, again a positive effect on the proportion of stomata (SI) under HL was observed on the adaxial surface indicating that the SI responded in the more exposed epidermis (Fig. 5C and Supplementary Fig. S4B). However, this effect seems to be ecotype dependent, since although the SD was affected by HL levels, HL did not elevate the SI in either epidermis (Fig. 5C, D) of the C24 accession.

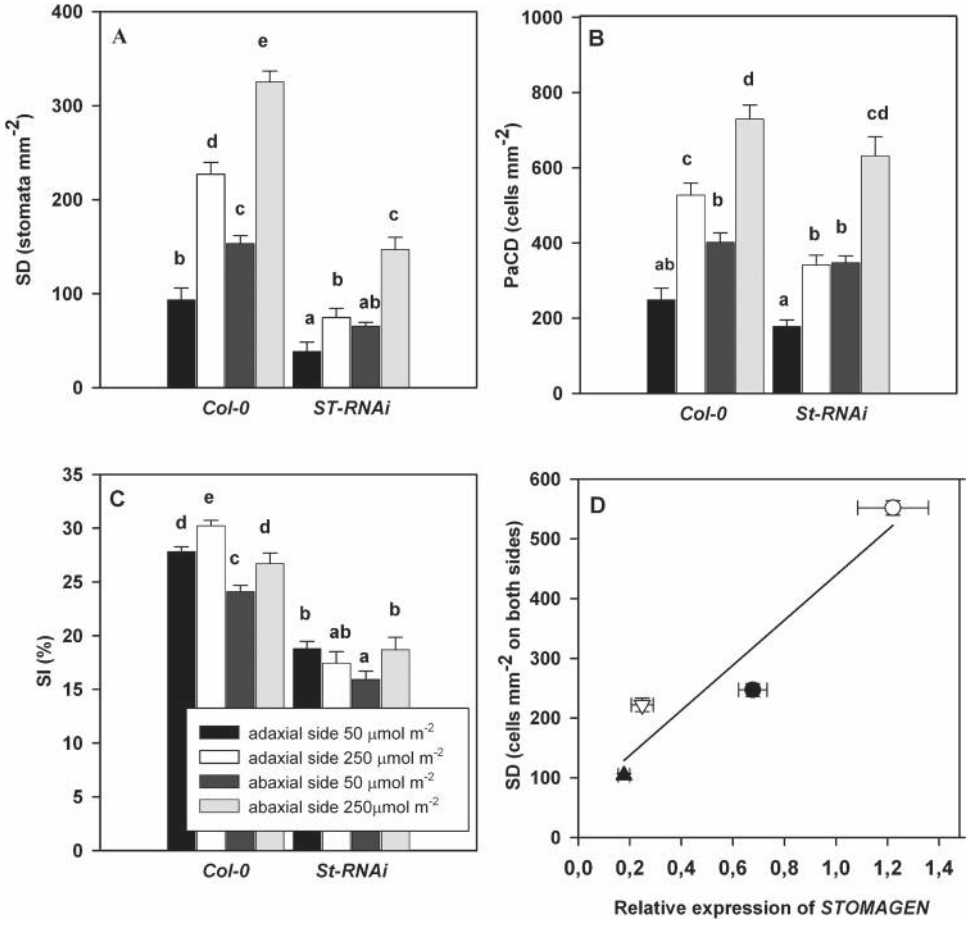

Fig. 4. SD and stomatal frequency in *STOMAGEN* loss-of-function plants under increased light levels. (A–D) SD (A), density of epidermal pavement cells (B; PaCD), and SI (C) on the adaxial (black and white) and abaxial (dark and light grey columns) leaf surfaces of fully developed leaves of *A. thaliana* WT Col-0 and the *STOMAGEN*-silenced (*ST-RNAi*) line. Data are means from 25 areas of 0.13 mm^2 (used for each genotype); error bars represent SEM. Different letters indicate significant differences ($P<0.05$). (D) Relationship between SD (summed over both leaf sides) and relative mRNA level of *STOMAGEN* of Col-0 WT (circles) and *ST-RNAi* plants (triangles) (D) under two different light conditions: HL (250 µmol m^{-2} s^{-1}; open symbols) and LL (25–50 µmol m^{-2} s^{-1}; filled symbols). Bidirectional error bars represent SEM.

To investigate the effect of light on stomatal development in genetic backgrounds with defects in stomatal patterning, two distinct stomata-clustering mutants were examined. The *tmm1* mutant epidermis contains large clusters of three to four stomata (Yang and Sack, 1995; Geisler *et al.*, 1997, 1998), especially on the abaxial leaf side, and the *sdd1* mutant has smaller, usually two-stomata-containing clusters, on both leaf sides (Berger and Altmann, 2000; von Groll *et al.*, 2002), so the single-cell-spacing rule for stomatal patterning is violated in both these mutants.

Compared with WT plants (Col-0 and C24 for mutants *tmm1* and *sdd1*, respectively), SD was greater on the abaxial side in both mutants, irrespective of light treatment. In all genotypes (WT Col-0 and C24 and both mutants), the SD increased at least 2-fold on both leaf sides in HL compared with LL conditions (Fig. 5A, B), indicating that the mutations had profound effects on the epidermis architecture.

In the adaxial epidermis, the *sdd1* mutation sensitized the C24 epidermis to HL with respect to stomatal formation. Whereas the WT C24 epidermis responded less to elevation of light, the SI of the *sdd1* adaxial epidermis increased from 40 to 50% (Fig. 5C). In the Col-0 background, both WT and *tmm1* mutant responded similar to the HL treatment (Fig. 5C).

Concerning the clusters, the stomatal clustering index (ratio of clustered stomata to all stomata) was less on the adaxial side of both mutants compared with the abaxial side, especially at under LL conditions, and increased markedly under HL conditions. The stomatal clustering index on the abaxial surface did not respond to changing light (Supplementary Fig. S5, available at *JXB* online).

In the abaxial epidermis, both stomata-clustering mutant alleles increase the stomatal formation under both light regimes, and the *sdd1* mutant again responded more strongly to elevated light. Although a small increase on average was observed in the *tmm1* mutant grown under HL levels, this was not found to be statistically significant. No positive response in the WT Col-0 or C24 abaxial epidermis was observed (Fig. 5D). The positive effect on the proportion of stomata conferred by the *tmm1* mutant allele only manifested itself in the abaxial epidermis, a tissue where stomatal proportion does not respond to high HL, suggesting that the *tmm1* mutant allele had no effect on the adaxial epidermis in terms of the overall proportion of stomata.

In summary, it therefore appeared that both *sdd1* and *tmm1* mutations had a large effect on the proportion of stomata in the abaxial epidermis. Furthermore, these observations also indicated that loss of function of *SDD1* sensitizes stomatal formation in the adaxial epidermis to elevated light levels.

tmm1 and sdd1 mutations confer increased STOMAGEN *expression*

In both WT backgrounds, Col-0 and C24, the expression levels of *STOMAGEN*, *TMM*, and *SDD1* were substantially higher in HL-treated leaves than in LL-treated leaves (Fig. 1),

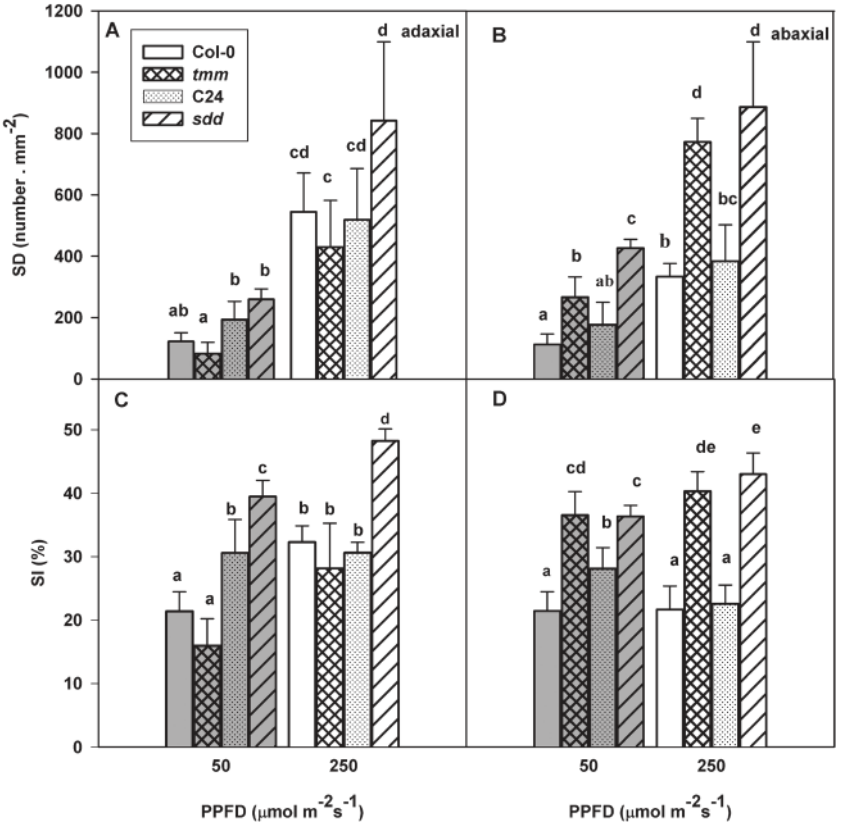

Fig. 5. The effect of photon flux density on SD and SI is modulated in *tmm1* and *sdd1* mutants. SD (A, B) and SI (C, D) on the adaxial (A, C) and abaxial (B, D) sides of the leaf surfaces of fully developed leaves of *A. thaliana* WT Col-0 and C24, and *tmm1* and *sdd1* mutants grown under two different light conditions: HL (250 μmol m^{-2} s^{-1}), and LL (25–50 μmol m^{-2} s^{-1}). Data are means of 25 replicates (five areas of 0.13 mm^2 chosen randomly on the leaf, repeated on five different plants). Error bars represent SEM. Different letters indicate significant differences over genotype×light level combinations separately for adaxial and abaxial leaf sides (P<0.05, one-way ANOVA and Tukey's test).

but more striking differences were observed in the *tmm1* and *sdd1* mutants. In the *tmm1* mutant, levels of *STOMAGEN* and *SDD1* transcripts were increased, indicating that in *tmm1* mutants *SDD1* is upregulated. This could be due to direct regulation of *SDD1* expression by the TMM receptor-mediated pathways or due to the increased proportion of stomatal lineage cells. Nevertheless, in the *sdd1* mutant with an even stronger increased proportion of stomatal lineage cells, evidenced by the elevated SI (Fig. 5C, D), we observed only a marginal increase in *STOMAGEN* transcripts, although this was statistically significant. These observations under HL conditions indicate that the TMM1 and SDD1 pathways cross-react, and that TMM1 and SDD1, either directly or indirectly by modifying the epidermal pattern and thereby influencing gas exchange, influence *STOMAGEN* expression.

Overall, we observed a positive correlation between *STOMAGEN* expression and SD (summed over both leaf sides) in WT and *tmm1*, *sdd1* and *STOMAGEN* loss-of-function mutants grown under HL and LL conditions (Fig. 6), supporting a functional relationship between *STOMAGEN* levels and SD. In the *STOMAGEN* overexpression line, this correlation was less clear, probably due to effects conferred by the ectopic expression.

Discussion

A strong candidate for communication between the developing epidermis and photosynthetically active mesophyll is STOMAGEN, a positive regulator of SD that was recognized only recently (Kondo *et al.*, 2010; Sugano *et al.*, 2010). This small peptide (45 aa when fully processed) is a member of the EPFL family of proteins (Rychel *et al.*, 2010) and probably competes with two members of the EPF family, EPF1 (Hara *et al.*, 2007) and EPF2 (Hunt and Gray, 2009), for the putative receptor proteins on the plasma membrane, TMM and ER (and/or ERECTA-LIKE) (Shimada *et al.*, 2011). STOMAGEN is synthesized in the leaf mesophyll and is probably secreted into epidermis (Meng, 2012). Our observations on lines with suppressed and elevated levels of *STOMAGEN* expression confirmed the role of STOMAGEN as a positive regulator of stomatal formation. *STOMAGEN* was highly expressed in the youngest leaves, supporting its role as a regulator of epidermal development (Sugano *et al.*, 2010; this study).

Our analysis further indicated that STOMAGEN is involved in the light response of the stomatal pathway, providing a putative link between the photosynthetic activity of internal leaf tissues and the epidermis. Indeed, elevated levels of photosynthetic radiation stimulated stomatal formation and upregulated *STOMAGEN* expression level. Furthermore, STOMAGEN action was rate limiting for the light-induced stomatal formation, especially in the adaxial epidermis. Modulating *STOMAGEN* expression influenced several genes implicated in the stomatal pathway but had little influence on transcripts of the *TMM* receptor and *SPEECHLESS* transcription

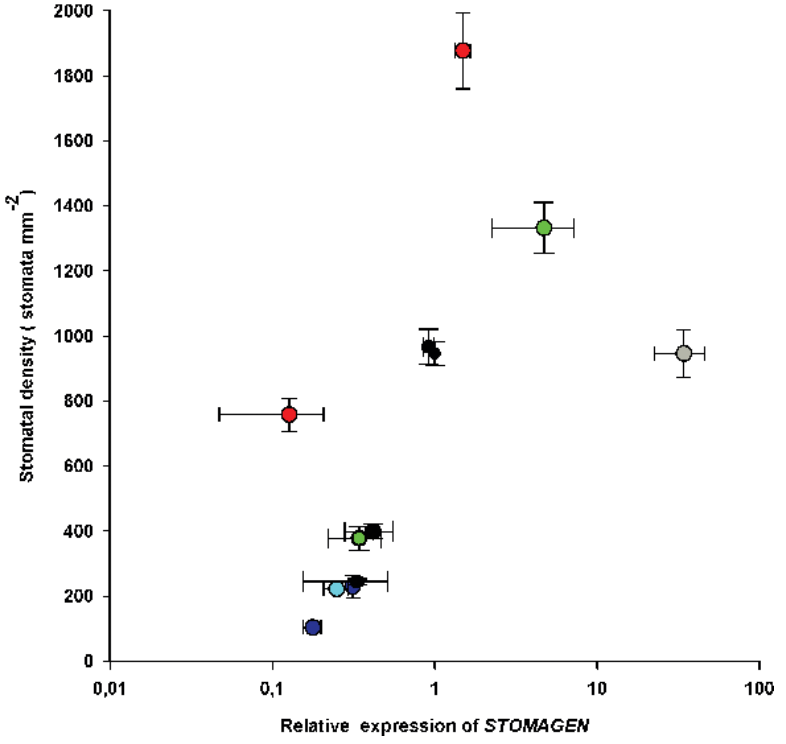

Fig. 6. STOMAGEN expression is positively correlated with SD. The relationship between SD (summed over both leaf sides) and relative mRNA level of *STOMAGEN* is shown. SD=804.15×(relative expression)+205.9, r^2= 0.96, P=0.01 for both WTs (Col-0 and C24) and *tmm1*. WT, black circles; *sdd1*, red circles; and *tmm1*, green circles (for both light intensities); STOMAGEN *ST-RNAi* line, blue circle for LL, cyan circle for HL; and ST-Ox lines, grey circle. Bidirectional error bars represent SEM (n=3–4 for relative expression and 25 for SD). The relationship between SD (on the abaxial epidermis) and *STOMAGEN* relative expression levels for *RNAi*, WT and *ST-Ox* lines under stable light conditions has been published previously (Sugano *et al.*, 2010) and here we included SD (summed over both epidermis sides) of the described genotypes under changing light conditions for direct comparison with the results in *tmm1* and *sdd1* mutants under the same experimental set-up.

factor. This is in line with earlier reports, which suggested that STOMAGEN acts positively on the SPEECHLESS (Lampard and Bergmann, 2007) protein level without influencing the transcript level (Jewaria *et al.*, 2013). Nevertheless, *STOMAGEN* expression followed a similar trend with *MUTE* and *FAMA* involved in the specification and differentiation of GC precursors (Lampard and Bergmann, 2007), in line with its effect on the density and frequency of stomata in the epidermis.

Furthermore, we observed that mutants with elevated SD had elevated levels of *STOMAGEN* expression at elevated photosynthetic irradiation, suggesting that the increased CO_2 uptake and subsequent photosynthetic activity (and CO_2 consumption) triggered *STOMAGEN* expression, which potentially would provide a mechanism for a positive-feedback loop. Alternatively, *STOMAGEN* expression might be transcriptionally downregulated by an operational TMM/SDD pathway, which would provide a negative-feedback loop to control *STOMAGEN* expression.

In conclusion, our observations in loss- and gain-of-function lines support a role for STOMAGEN in inducing stomatal formation. Furthermore, *STOMAGEN* expression is either directly or indirectly regulated by light levels, is affected in mutants with altered SD, and mediates the response of the stomatal pathway to light. Due to its expression pattern, STOMAGEN is a promising candidate for stomatal development-related signalling between the mesophyll and epidermis. These observations suggest that STOMAGEN acts in the link between photosynthetic activity in the leaf mesophyll and epidermal patterning.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Relative *stomagen* expression in the leaves of different insertions.

Supplementary Fig. S2. STOMAGEN-Venus is present in both mesophyll and epidermis.

Supplementary Fig. S3. Imprints of the adaxial leaf surface of HL and LL leaves of Col-0.

Supplementary Fig. S4. Stomatal density (SD) and stomatal index (SI) on the Col-0 leaves grown under three different light conditions.

Supplementary Fig. S5. Stomatal clustering index (SCI) on the mutant *tmm* and *sdd1* leaves.

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