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Regulation of stomatal development by environmental conditions and physiological processes in the leaf

Ph.D. Thesis

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Annotation

Stomatal development and its regulation by environmental conditions (light, CO_2 concentration) and physiological processes in the leaf of higher plants were investigated. The study was based on the assumptions that stomatal development should be regulated by signals coming from both external environment and leaf interior, and that the signal should be transduced from cotyledons to leaves. Transgenerational effect in stomatal development was also studied. Molecular and physiological approaches were applied to reveal the relationship between leaf environment, stomatal development, stomatal function and leaf physiology.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracovala samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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České Budějovice 14. 9. 2017 Martina Vráblová

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"Anyone who stops learning is old, whether at twenty or eighty. Anyone who keeps learning stays young. The greatest thing in life is to keep your mind young."

Henry Ford

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List of papers and author's contribution

The thesis is based on the following papers:

I. Hronková M., Wiesnerová D., Šimková M., Skůpa P., Dewitte W., Vráblová M., Zažímalová E. and Šantrůček J. (2015) Light-induced STOMAGEN-mediated stomatal development in *Arabidopsis* leaves. *Journal of Experimental Botany* 66 (15), 4621 – 4630. (IF = 5.677)
M. Vráblová participated in laboratory experiments and manuscript writing.

II. Šantrůček J., Vráblová M., Šimková M., Hronková M., Drtinová M., Květoň J., Vrábl D., Kubásek J., Macková J., Wiesnerová D., Neuwirthová J. and Schreiber L. (2014) Stomatal and pavement cell density linked to leaf internal CO_2 concentration. *Annals of Botany* 114, 191 – 202. (IF = 3.654)
M. Vráblová participated in laboratory experiments and manuscript writing.

III. Vráblová M., Hronková M., Vrábl D., Kubásek J. and Šantrůček J. Light intensity-regulated stomatal development in three generations of *Lepidium sativum*. (Manuscript)
M. Vráblová participated in laboratory experiments, ^{13}C analyses, data evaluation and manuscript writing.

IV. Vráblová M., Vrábl D., Hronková M., Kubásek J. and Šantrůček J. (2017) Stomatal function, density and pattern, and CO_2 assimilation in *Arabidopsis thaliana tmm1* and *sdd1-1* mutants. Plant Biology 19 (5), 689 -701. (IF = 2.106)
M. Vráblová participated in laboratory experiments, ^{13}C analyses, data evaluation and manuscript writing and revision.

Content

1. General introduction

1.1 Background

Plants are organisms that occupy habitats with fluctuating environmental conditions and respond to heterogeneity in their immediate environment through plasticity. The acquisition of stomata and an impervious leaf cuticle are considered to be key elements in the evolution of advanced terrestrial plants (Raven, 2002), allowing the plants to inhabit a range of different environments but still control water content inside their body. Stomata can adapt to local and global changes on all timescales. These small pores on the plant surfaces first appeared over 400 million years ago and since then have changed markedly in size and density. In today's world stomata exert major controls on both the water and carbon cycles.

Gas exchange (importantly carbon dioxide and water vapour) between the plant and the atmosphere is regulated by controlling the aperture of the stomatal pore and the number of stomata that form on the epidermis. The ratio of CO_2 uptake for photosynthesis (A) to water loss through transpiration (E) determines plant water use efficiency (WUE). Stomata operate to ensure an appropriate balance between these two processes (A/E).

Stomatal morphology, distribution and behaviour respond to a spectrum of signals, from intracellular signalling to global climatic change. Stomatal numbers are tightly controlled by environmental signals including e.g. atmospheric CO_2 partial pressure, light intensity or atmospheric humidity. This requires control of epidermal cell development during the early phase of leaf growth and involves changes in both the density of cells on the leaf surface and the proportion of cells that adopt a stomatal fate.

A stoma consists of a pore, flanked by two epidermal cells (sister guard cells), that lies above an airspace located in the mesophyll. Stomata are separated from one another by intervening epidermal cells, a pattern thought to be a function requirement for efficient gas exchange (Sachs, 1978; Nadeau & Sack, 2002). This pattern is referred to as the one-cell spacing rule

(Nadeau & Sack, 2002). This rule may ensure optimal stomatal function. Guard cell activity requires water and ion exchange with surrounding cells and the subsequent changes in turgor also impose mechanical stresses on the surrounding cells. The one-cell spacing pattern also ensures that there is minimal overlap between gas diffusion shells. Stomatal density (SD; stomata mm^{-2}) and stomatal index (SI; number of stomata per total number of epidermal cells) vary with growth conditions, whereas the spacing pattern does not.

The ability to control stomatal aperture as well as the number and size of stomatal cells together affect the amount of CO_2 assimilated through photosynthesis relative to water loss due to transpiration (Lawson *et al.*, 2014). Although leaf stomatal density significantly affects stomatal conductance (g_s), enhancement of SD is not necessarily associated with enhancement of the leaf's CO_2 assimilation rate and biomass production (Schluter *et al.*, 2003; Tanaka *et al.*, 2013). Changes in pattern of stomatal distribution may also affect the functioning of individual stomata and g_s. Stomatal density, size, pattern and function are products of molecular signalling and ion transport processes triggered by and responding to signals from the leaf mesophyll and/or epidermal cells, mature leaves (Lake *et al.*, 2001; Miyazawa *et al.*, 2006) or seeds, and from the environment. A pivotal role in the long-distance signalling has been attributed to abscisic acid (Lake & Woodward, 2008) or the ^{13}C content in assimilates (Sekiya & Yano, 2008).

Lot of studies investigated environmental stimuli influencing stomatal development – CO_2 concentration (Woodward, 1987; Royer 2001); light quality and quantity (Casson & Gray 2008; Craven *et al.*, 2010; Casson & Hetheringron 2014); water availability (Aasama & Sober, 2011) and others. Cellular and molecular mechanisms for the formation of stomata have been studied intensively over the past two decades (Geisler *et al.*, 1998; Nadeau & Sack, 2002; Nadeau, 2009; Kondo *et al.*, 2010; de Marcos *et al.* 2016). Stomatal function and its influencing by environmental factors and stomatal patterning were also studied recently (Dow *et al.*, 2014; Papanatsiou *et al.*, 2016).

Much attention has been paid to the research of the stomatal development and behavior in last decades, but lot of knowledge is still missing.

Effects of isolated environmental factors on stomatal development have been studied in many cases, and the interconnection between stomatal development and physiology has not been fully explored. Therefore, in my research I was interested more in a combination of factors affecting stomatal development (external and internal), and I also tried to understand how stomatal density and pattern influence leaf physiology and *vice versa*.

1.2 **Stomatal development**

1.2.1 Stomata formation and patterning

In contrast to monocots, where stomata develop serially in cell files (Croxdale, 1998), dicot stomatal formation is dispersed in time and space during the mosaic growth of the leaf. Studies of Arabidopsis leaf cells through time have made it possible to distinguish between rules of stomatal development that are fixed and those that are flexible. All Arabidopsis stomata form through at least one asymmetric and one symmetric division (Fig. 1). The first division takes place in a presumed stem cell that has become committed to the stomatal pathway, the meristemoid mother cell (MCC). This asymmetric division produces a small precursor, a meristemoid (M), which is eventually converted into a guard mother cell (GMC). The symmetric division of the GMC produces the two guard cells that make up the stoma. Thus, the terminal differentiation of a stoma occurs after a progression through the three types of precursor cells (MMC to M to GMC) (Paliwal, 1967; Serna & Fenoll, 1997).

In the C24 accession of *A. thaliana* most stomata are part of clonally related anisocytic stomatal complexes (Serna & Fenoll, 2000). Meristemoids in this accession usually divide asymmetrically three times. Each time a meristemoid divides unequally, the larger epidermal daughter cell is placed on the outside of the forming cluster so that by the end of the division cycle there is a single meristemoid surrounded by three pavement epidermal cells. The elder two pavement cells are much larger than the youngest. At this point the meristemoid differentiates into a guard mother cell. The GMC then

undergoes an equal cell division to produce the two sister guard cells that gate the stomatal pore (Paliwal, 1967) and complete the formation what is known as an anisocytic complex (Fig. 1).

The daughter cells produced from the unequal meristemoidal divisions can also retain their meristemoidal potential before terminally differentiating into a pavement cell. These usually the youngest and smallest pavement cells can go on to form satellite stomatal complexes (Fig. 2) by following the same pattern of several polarised, unequal cell divisions, followed by one equal cell division that the original meristemoid underwent (Serna & Fenoll, 2000).

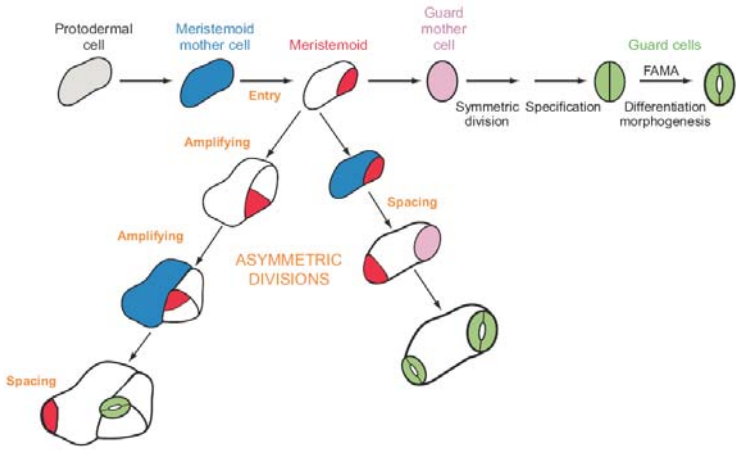


Fig. 1: Key stages and divisions in *Arabidopsis thaliana* stomatal development according to Bergmann & Sack (2007).

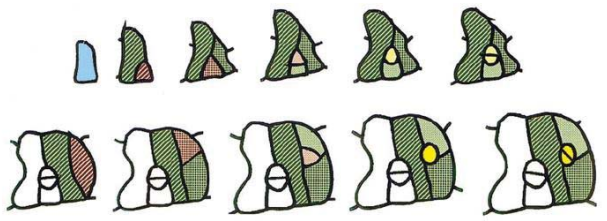


Fig. 2: Stomatal development from primary (A) and satellite (B) meristemoid. Different types of cells are distinguished by colours: Protodermal cell - blue, meristemoids - pink, subsidiary cells - green, guard mother cell and stoma - yellow. In (A) three pavement cells pertain one stoma (ratio 3), whereas in (B) more stomata develop relatively to pavement cells (ratio 2.5). (Serna *et al.*, 2002)

1.2.2 Stomatal clustering

Stomatal clusters are atypical complexes of paired stomata that shared one of the subsidiary cells, or in which the four guard cells are contiguous. Besides twinned stomata, higher-order stomatal clusters of three or more stomata were also found (Serna & Fenoll, 1997). These clusters exhibited a variable geometry, from linear to compact groups of stomata.

Some Arabidopsis mutants, e.g. *too many mouths*, *four lips* and *stomatal density and distribution-1*, develop stomatal clusters (Yang & Sack, 1995; Berger & Altmann, 2000). By using growth conditions which limit gas exchange with open atmosphere, stomatal clusters that look like phenocopies of *flp* and *tmm* have been induced (Serna & Fenoll, 1997), suggesting that stomata spacing is under environmental as well as genetic control in Arabidopsis. The formation of a stoma from a protodermal precursor cell (meristemoid) involves two consecutive events: assuming guard mother cell identity and entering the mitotic cell cycle. The origin of clusters induced by specific growth conditions has been addressed by following promoter activity for genes that are markers for competence for cell division (*cdc2aAt*), mitotic activity (*cyc1aAt*), and guard mother cell and developing guard cell identity (*rha1*). Their different expression patterns in the various cell types during epidermal differentiation and the asynchrony in the development of the various stomata that constitute each cluster suggest that these stomatal clusters derive from a single protodermal cell through a process that involves changes in cell fate in a subset of subsidiary cells (Serna & Fenoll, 1997).

1.3 Control of stomatal development

Light level, atmospheric CO_2 concentration, humidity and drought signals all have an effect on the level of stomatal development. As stomata are intrinsically linked to the rate of CO_2 uptake and fixation, and water loss, it is unsurprising that stomatal numbers should be sensitive to the environmental conditions that affect these central concerns of the plant. This suggests that there are several levels of control over stomatal development: control over

the spacing of individual cells, to ensure that stomata are separated by at least one epidermal pavement cell; control of stomatal numbers as part of tissue patterning during development; and control of stomatal numbers by environmental conditions. These different levels of control must be coordinated to produce the final numbers of stomata found on mature leaves (Bird & Gray, 2003).

1.3.1 Genetic and molecular control of stomatal development

The major factors in determining the pattern of stomata appear to be signals from mature guard cells (or their precursors, GMCs or meristemoids) to their neighbouring cells. Cells that are in contact with a single stoma or precursors are instructed to orient their future division planes such that asymmetric divisions place the smaller cell distal to the pre-existing stoma. Cells that are in contact with two or more stomata are instructed not to divide (Geisler *et al.*, 2000)

One of the first components of stomatal patterning and development to be identified was the *TOO MANY MOUTHS* (*TMM*) gene (Yang & Sack, 1995; Nadeau & Sack, 2002). The *tmm* mutation results in stomatal clustering and increased precursor cell formation in cotyledons and a virtual absence of stomata in the inflorescence stem. The *TMM* gene regulates stomatal production at several developmental levels. It regulates entry into the pathway, that is, meristemoid formation, it also restricts meristemoid activity so that only one guard mother cell is normally produced (and the formation of clusters is prevented) (Yang & Sack, 1995). *TMM* is required for cells to respond appropriately to their position during stomatal development and was found to encode a putative cell-surface leucine-rich repeat (LRR)-containing receptor--like protein and is expressed in cells likely to undergo an entry division (Nadeau & Sack, 2002).

A receptor-based signalling mechanism governing the divisions of the stomatal lineage implies the existence of both ligands and a downstream signalling cascade. Another gene product required to regulate stomatal patterning is the putative serine protease encoded by *SDD1* (*STOMATAL DENSITY AND DISTRIBUTION 1*) (von Groll *et al.*, 2002). The *SDD1* gene is expressed

strongly in stomatal precursor cells (meristemoids and guard mother cells) and is predicted to regulate the number of entry and amplification divisions as well as the orientation of spacing divisions. The *tmm* mutation was completely epistatic to *SDD1* overexpression, indicating that *TMM* and *SDD1* are likely to act in a common pathway. The SDD1 protein appears to be exported to the apoplast where it is localized to the plasma membrane. It is therefore possible that *SDD1* may be involved in processing a peptide precursor of a *TMM* ligand.

Another gene involved in stomatal development is the *FOUR LIPS* gene (Yang & Sack, 1995). The *flp* mutation results in many paired stomata and a small percentage of unpaired guard cells in cotyledons (Fig. 3). *FLP* is expressed in GMC and developing stomata. Loss-of-function mutations in *FLP* result in additional divisions of the GMCs, suggesting that this gene is involved in limiting the cell division competence for these cells.

A mitogen-activated protein kinase (MAPK) kinase kinase (MAPKK) gene, *YODA* (*YDA*), plays a central role in both guard cell identity and pattern formation. *YDA* acts as a cell fate switch; loss of function mutations led to too many cells adopting a guard cell fate, and constitutive activation of *YDA* produces plants that completely lack guard cells (Bergmann *et al.*, 2004). The mutant phenotypes and identities of *TMM*, *SDD1*, and *YDA* suggest that they could act in a common signalling pathway, with *SDD1* cleaving a ligand that is perceived by *TMM* and a coreceptor kinase (Nadeau & Sack, 2003). A signal transduced through this receptor pair could trigger the phosphorylation and activation of the *YDA* MAPKK kinase and its downstream signalling cascade (Bergmann *et al.*, 2004; Gray & Hetherington, 2004).

ERECTA (*ER*) is an LRR-RLK required for diverse processes including growth and development, as well as responses to biotic and abiotic stresses (Lease *et al.*, 2001; Shpak *et al.*, 2004). Many processes affected by loss of *ER* function are united in their requirement for cell proliferation. The *ER*-family has overlapping domains of gene expression with *TMM*. *TMM* and *ER*-family members control asymmetric divisions in stomatal development.

The final cell fate decision, the transition of the GMC to two guard cells, is controlled by *FAMA* (Ohashi-Ito & Bergmann, 2006). Instead of recognizable

stomata, *fama* mutants develop clusters of small, narrow epidermal cells. These cells express markers found in earlier stomatal lineages up to the GMC but not guard cell markers. Ectopic or overexpression of *FAMA* confers guard cell characteristics on a variety of cell types suggesting that *FAMA* not only acts to restrict cell division following the symmetrical division of the GMC but also specifies guard cell identity. Other bHLH proteins closely related to *FAMA* are *SPEECHLESS* (*SPCH*) and *MUTE*. *SPCH* is expressed in a subset of protodermal cells and is necessary and sufficient for asymmetric MMC division (Ohashi-Ito & Bergmann, 2006; Pillitteri *et al.*, 2007). *MUTE* is mainly expressed in meristemoids and protein levels appear to increase with consecutive rounds of asymmetric meristemoid division. Mutations in *MUTE* result in meristemoids that undergo additional rounds of inward-spiraling asymmetric divisions, terminating with an arrested meristemoid at the centre that fails to differentiate into a GMC. These observations indicate that *MUTE* is a candidate asymmetrically segregated intrinsic meristemoid factor that limits asymmetric divisions and induces GMC fate in a concentration-dependent manner. SCREAM (SCRM) and SCRM2 interact with and specify the consecutive action of above-mentioned bHLH factors (Kanaoka *et al.*, 2008).

A candidate extrinsic spacing signal is encoded by the *EPIDERMAL PATTERNING FACTOR* (*EPF1, EPF2*) genes that were identified from a screen in which putative secreted peptides were overexpressed (Hara *et al.*, 2007; 2009). EPFs are synthesized in the epidermis and have been identified as negative regulators of stomatal differentiation. *epf1* mutants display increased numbers of stomata with meristemoids adjacent to guard cells of precursors. *EPF1* is expressed in meristemoids, GMCs and young guard cells, and thus appears to act downstream of *SPCH*, consistent with the apparent absence of signalling between MMCs (Geisler *et al.*, 2000). Another negative regulator, EPFL6 (EPIDERMAL PATTERNING FACTOR LIKE) - CHALLAH (Abrash & Bergmann, 2010) and the positive regulator EPFL9 – STOMAGEN are produced in internal tissue (Hunt *et al.*, 2010; Kondo *et al.*, 2010; Sugano *et al.*, 2010).

Morphogenesis of stomata is further controlled by protein factors regulating asymmetric and symmetric cell division, e.g. FLP and MYB88 (Yang & Sack, 1995; Lee *et al.*, 2013; 2014).

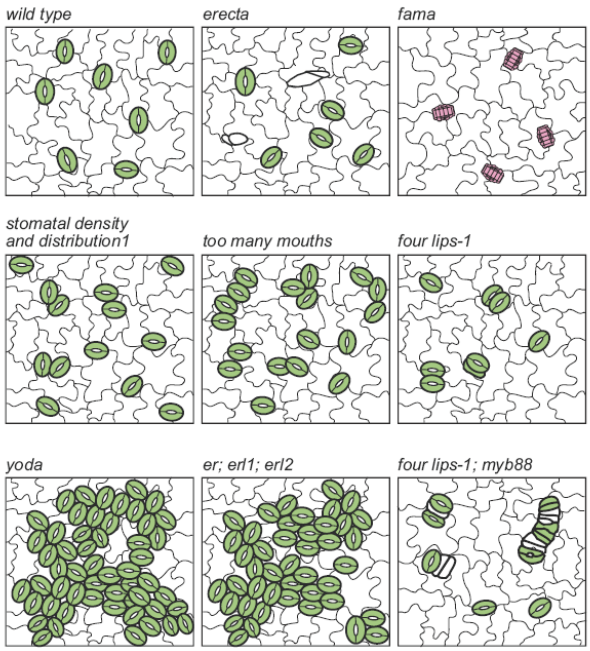


Fig. 3: Diagrams of terminal leaf phenotypes in stomatal mutants indicating the typical number and arrangement of stomata (green) or terminal cell type (pink for guard mother cells). White cells in *erecta* and *flp;myb88* panels represent cell of indeterminate identity (Bergmann & Sack, 2007).

Some of the factors that are required for the development of mature guard cells may be plant hormones. Using the Arabidopsis hypocotyls as a system to study the interactions between hormones in growth control, Saibo *et al.* 2003 found that the application of gibberellins (GAs), especially in combination with ethylene or auxin, resulted in an overproduction of stomata. Moreover, the inhibition of gibberellin signalling eliminated stomata from the hypocotyls but did not affect stomatal production in the leaves. GA-induced production of stomata was confined to the cell files in which stomata are normally found, suggesting that gibberellins act in a normal signalling pathway to promote cell cycling within the hypocotyl. The organ-specific pattern of stomata produced in response to GA is similar to the *tmm* phenotype. *tmm-1* mutants, although they make excess stomata on leaves and cotyledons, make no stomata on hypocotyls, and this phenotype is epistatic to the overproliferation

of hypocotyls stomata in other mutant backgrounds (Geisler *et al.*, 1998). Recently, the effect of brassinosteroids (Kim *et al.*, 2012) and auxin (Le *et al.*, 2014; Zhang *et al.*, 2014) on stomatal development also has been reported.

The effect of abscisic acid (ABA) on stomatal development has been studied in last years. ABA, as the major chemical signal that regulates the physiological response to drought, induces stomatal closure. It was proposed that ABA is also a core regulator of environmentally determined stomatal development (Chater *et al.*, 2014). Pantin *et al.* (2013) have shown that Arabidopsis stomata undergo a developmental priming of ABA sensitivity which is induced by the slight humidity gradient across the plant rosette. Tanaka *et al.* (2013) found out that abscisic acid (ABA) inhibits entry into stomatal-lineage development in Arabidopsis leaves. On the contrary, ABA-treated plants had significantly smaller stomata and higher stomatal density in their lower epidermis in *Tradescantia* virginiana (Franks & Farquhar, 2001). Similarly, in ABA--treated plants of *Lepidium sativum* stomatal aperture decreased and stomatal density increased up to 2.4 times on both leaf sides (Macková *et al.*, 2013). It seems that effect of ABA on stomatal development should be driven by water availability. Under soil water deficit and low atmospheric humidity conditions, ABA appears to slow leaf expansion rates by reducing turgor pressure, cell division rate and cell wall extensibility (Bacon *et al.*, 1998; Tardieu, 2013). This results in smaller leaves with lower stomatal indices. Chater *et al.* (2014) proposed that under optimal water supply ABA acts to promote maximal leaf growth by maintaining high hydraulic conductance and a high density of aquaporins, but that suboptimal water supply primes ABA-mediated stomatal closure in expanding leaves and alters the role of ABA from an indirect growth promoter to a direct growth inhibitor in these (and younger) tissues as well. Connecting external and internal influences, ABA was also proposed to be involved in both CO_2 concentration and light intensity control of stomatal development (for details see Chater *et al.*, 2014).

1.3.2 Environmental control of stomatal development

Stomatal development responds to different environmental stimuli. CO_2 concentration, light quality and quantity, and air and soil humidity are the most important controlling factors. The effect of growth at elevated CO_2 concentration on SD and SI is one of the most intensively studied environmental controls of stomatal development. In 1987, Woodward pointed to evidence in the fossil record that plants have responded to post-industrial increases in atmospheric CO_2 levels with decreases in stomatal density. This response may have been selected during evolution because it allowed reduced water loss, while photosynthesis was maintained due to the increased availability of CO_2. Over a much shorter developmental timescale, some plant species also show an acclimatory response to atmospheric CO_2, with a decrease in stomatal density observed under elevated CO_2 conditions (Woodward & Kelly, 1995). On the contrary, Ainsworth & Rogers (2007) summarized the results from free--air CO_2 enrichment (FACE) experiments via meta-analysis and showed that elevation of CO_2 concentration in FACE experiments reduced stomatal conductance by 22%, yet, this reduction was not associated with a similar change in stomatal density.

Reduction of stomatal frequency results in a declining maximum stomatal conductance. Since this complex parameter is not only determined by stomatal frequency but also by stomatal geometry (i.e. dimensions of stomatal pore) (Parlange & Waggoner, 1970), one might expect that a decreasing stomatal density would be accompanied by decreasing dimensions of stomatal pores. The original studies and meta-analyses show a trend of increasing size rather that the expected decrease, implying that the physiological effects of stomatal frequency reduction are partially offset by a concomitant increase of pore size (Wagner *et al.*, 1996; Franks & Beerling, 2009). In crop species there is some experimental evidence that stomatal density tends to be negatively correlated with pore size (Malone *et al.*, 1993).

Beerling & Woodward (1995) studied stomatal density and stomatal index responses to CO_2 enrichment (350 to 750 μmol mol^{-1}) of ornamental species with variegated leaves. Both these parameters showed a decrease on the green and white portions of variegated leaves, suggesting that

the stomatal-CO_2 responses are unrelated to photosynthetic capacity. The sensitivity of response is related to leaf structure and the responses observed are probably contributing to optimization of plant carbon-water balance at higher CO_2 concentrations.

Stomata develop in a leaf-side specific manner. Partitioning of stomatal conductance between leaf sides is species-specific but can be also strongly modulated by signals from environment (Muir, 2015). A higher degree of amphistomy (adaxial/abaxial ratio) reduces the CO_2 gradient across the leaf and increases whole-leaf photosynthetic rate (Parkhurst & Mott, 1990); however, variation in the distribution of stomata between the two leaf surfaces did not affect water loss in *A. thaliana* (Fanourakis *et al.*, 2015).

Gray *et al.* (2000) demonstrated the expression of the gene *HIC* (*HIGH CARBON DIOXIDE*) in guard cells. In plants in which the *HIC* gene was disrupted, the stomatal index increased up to a 42% upon doubling of experimental CO_2 levels. The *hic* mutants thus exhibited a reversal of the normal inverse correlation between stomatal index and ambient CO_2 concentration. When the expression of *HIC* is reduced, plants respond to increasing CO_2 by a large increase in stomatal density and index. Changes in density brought about by altered growth CO_2 concentration are not coupled with the formation of stomatal clusters. This suggests that the mechanism for the regulation of stomatal density by the ambient CO_2 concentration is mechanistically different from those cell-to--cell mechanisms that determine stomatal spacing. Perturbation of the *HIC* gene also does not result in the formation of stomatal clusters (Gray *et al.*, 2000).

HIC gene encodes a putative 3-ketoacyl coenzymeA synthase – an enzyme involved in the synthesis of very-long-chain fatty acids. In plants, long-chain fatty acids are found in waxes and cutins, which are constituents of the cuticle, as well as in glycerolipids and sphingolipids. Some substance that normally diffuses from pre-existing guard cells under high CO_2 concentrations, and prevent neighboring cells from differentiating into guard cells, may be absent in the *hic* mutants or may be immobilized as a result of alterations in the composition of the cuticular layer (Gray *et al.*, 2000; Serna & Fenoll 2000).

Stomatal patterning also responds to changes in light intensity. In general, an increase in light intensity results in an increase in stomatal index and mature leaves determine the response (Lake *et al.*, 2001; Thomas *et al.*, 2004). The effects of light quality have been studied intensively in last decade. Previously, Schoch *et al.* (1984) suggested that phytochrome controls stomatal development. Blue light or far-red light appeared to decrease stomatal index when provided as a night-treatment at low fluence and compared with darkness or red light in *Vigna sinensis*. Lu *et al.* (1993) showed significant increase of stomatal density in adaxial site of cotton leaves when the blue/red ratio was increased. Alternatively, Rajapakse & Kelly (1993) reported a 10% decrease in stomatal density in *Chrysanthemum* when the blue/red ratio increased. Recently, it was shown that PHYTOCHROME B photoreceptor of red part of the spectrum affects stomatal development (Casson *et al.*, 2009; Casson & Hetherington, 2014). The mechanism involves the downstream transcription factor PHYTOCHROME INTERACTING FACTOR 4 (PIF 4) (Boccalandro *et al.*, 2009; Casson *et al.*, 2009; Kang *et al.*, 2009).

The presence of flavonoids is characteristics of the leaf epidermis. Guard cells are distinguished by their high flavonoid content (Weissenbock *et al.*, 1987). Although many external factors, such as wounding, pathogen invasion and herbivore damage, can induce flavonoid synthesis, light is the most influential factor (Stafford, 1991). Phytochrome, the blue/UV-A receptor and putative UV-B receptor(s) may all participate in induction of flavonoids (Beggs *et al.*, 1987). Liu-Gitz *et al.* (2000) identified lines of soybean that contain a specific flavonol glycoside, kaempferol-3-*O*-2-glycosyl-gentiobioside (K9), and have greatly reduced stomatal density. Morphological studies revealed that the blue light-induced reduction in stomatal density in K9 lines was due to reduced stomatal initiation as well as aborted or abnormal stomatal development. As the phytochrome photostationary state was kept constant, the results indicate that one or more blue receptors are involved in the control of stomatal development.

Water stress is well-known to affect stomatal pore aperture, but very little is known about its effect on stomatal development. Water stress resulting from a reduced soil-watering regime caused a reduction in stomatal index

in wheat (Quarrie & Jones, 1977). Increased humidity resulted in a reduction of the stomatal index of *Scilla nutans* leaves (Salisbury, 1927). *Arabidopsis* grown in closed containers showed increased stomatal density, although the stomatal index was not reported (Serna & Fenoll, 1997). Interestingly, a number of stomata were present as clusters, though it is not possible to determine if this was a result of increased humidity or other variables resulting from growth in the closed conditions. Response of stomatal density of *Pseudoroegneria spicata* to water supply was studied (Fraser *et al.*, 2009). In this case, reducing water supply increased SD, whereas increasing water supply had no effect on SD but reduced leaf area. Water deficit increased the amount of abaxial stomata also in *Vatairea macrocarpa* (Vieira *et al.*, 2017). Authors proposed the strategy of plants under drought stress to compensate the stomatal closure by increasing SD.

Recently, a molecular background of drought tolerance connected to stomatal density has been studied. Arabidopsis thaliana GT-2 LIKE 1 (GTL1) loss-of-function mutations resulted in increased water deficit tolerance and higher integrated WUE by reducing daytime transpiration without a demonstrable reduction in biomass accumulation (Yoo *et al.*, 2010). Lower transpiration was associated with higher SDD1 expression and 25% reduction in abaxial stomatal density. Hughes *et al.* (2017) shown that reduced SD in HvEPF1OE barley lines improved drought tolerance without impact on grain yield. A mutant with enhanced drought tolerance was isolated and named *Arabidopsis drought tolerance mutant 1* (*atdtm1*) (Zhao *et al.*, 2017). *atdtm1* has larger lateral organs, prolonged growth duration, increased relative water content and reduced SD compared to the wild type. The phenotype is caused by the loss of a T-DNA tagged gene encoding CYCLIN-DEPENDENT KINASE C;2 (CDKC;2), which functions in the regulation of transcription by influencing the phosphorylation status of RNA polymerase II (Pol II). CDKC;2 affects the transcription of downstream genes such as cell cycle genes and genes involved in stomatal development, resulting in altered SD and drought tolerance of the plant.

1.4 Stomatal function in leaf gas exchange

Research of stomatal development should not be disconnected from studying their function. Stomatal development is strictly regulated to balance CO_2 uptake and water loss in a long-term scale (days to millennia), whereas the short-term dynamics, derived from changes in the width of the apertures, controls gas exchange at a time scale of minutes. Stomatal conductance depends not only on stomatal density, but also on their shape and size of the aperture. It was shown, that stomatal density and guard cell size should be negatively related (Franks *et al.*, 2009; Doheny-Adams *et al.*, 2012). This makes the relationship between gas exchange and stomatal density more unclear. In addition, changes in stomatal patterning (e.g. formation of clusters) may also affect the functioning of individual stoma and g_s. Dow *et al.* (2014) observed changed leaf conductance for water in *A. thaliana* mutant lines with differing proportions of stomata in clusters. Surprisingly, no remarkable changes in WUE appeared suggesting that stomatal to mesophyll conductance ratio should remain unchanged.

Buessis *et al.* (2006) and Schluter *et al.* (2003) evaluated photosynthesis in *A. thaliana sdd1-1* mutant that differs from wild-type plants only in the number of stomata and their distribution on the leaf surface. The effect of stomatal density enhancement was negligible under low light and increased under moderate light, in which g_s became more limiting for CO_2 assimilation. This illustrates the importance of environmental conditions in stomatal behaviour studies (see also Lawson & Blatt, 2014; Xu *et al.*, 2016).

Studies relating stomatal anatomy and frequency to leaf gas exchange are rare. Most studies are focused on the physiological aspects of stomatal density *per se*, but stomatal patterning and distribution are substantial factors affecting gas exchange. Smaller stomata, typically in high stomatal density leaves, are usually more responsive and faster than larger stomata (Drake *et al.*, 2013; Raven 2014). The kinetics of clustered stomata was studied recently (Lehmann & Or, 2015; Papanatsiou *et al.*, 2016).

Stomatal behaviour (changes in stomatal pore dimensions) is widely affected by environmental conditions. The guard cells control stomatal opening

in response to many abiotic and biotic stimuli such as CO_2 concentration, light, water availability, pathogens etc. Mesophyll photosynthesis and guard cell metabolism also impact stomatal behaviour (Lawson *et al.*, 2014). Stomatal apertures substantially affect total leaf conductance and thereby photosynthesis and water use efficiency (Assmann & Wang, 2001; Schroeder *et al.*, 2001). The effect of atmospheric CO_2 concentration on both SD and stomatal opening was explored (for example Woodward, 1987; Franks & Beerling, 2009). Stomata open in response to low CO_2 and it was recognized, that stomatal guard cells respond to the leaf internal CO_2 concentration rather than to the external CO_2 concentration (Mott 1988; 2009). However, experiments on the relationship of SD to the leaf internal CO_2 were neglected. It is also generally accepted, that stomata open with increasing irradiance (except of CAM plants). Light-induction of stomatal opening is mediated by blue/UV-A photoreceptors phototropins (PHOT1 and PHOT2) (Kasahara *et al.*, 2002). Red light also plays a role in induction of stomatal opening, but requires higher light intensity with lower effect compared to blue light (Shimazaki *et al.*, 2007). Moreover, when leaves were treated under a constant internal CO_2 concentration, red light still stimulated opening of stomata (Messinger *et al.*, 2006) which indicates that the effect of red light is not mediated through CO_2 or photosynthesis of mesophyll cells. On the contrary, stomata close in high CO_2 concentrations, high vapour pressure deficits and in darkness (Assmann, 1999; Outlaw, 2003).

1.5 Transgenerational effect in stomatal development

Stomatal development and function in ontogenesis have been widely studied. Nevertheless, plants are organisms with a little choice in their growth environment and usually limited seed dispersal. The parental environment can have effect on offspring phenotypes and should represent an evolutionary driving force for flexible mechanism of adaptation to heterogeneous environments (Donohue, 2009). This phenomenon known as transgenerational effects (or parental effects), occurs when offspring phenotype is influenced by a parental phenotype independently of the genes that the parents pass

to the offspring. Mechanisms underlying parental effects can be categorized as seed modification (Roach & Wulff, 1987) or epigenetic variation (Rossiter, 1996). Parental effects mediated by seed modification are implemented during early stages of plant development, whereas epigenetic variation can alter phenotypes for whole life of offspring. Transgenerational effects due to maternal environmental conditions were widely studied. Walter *et al.* (2016) showed that variation in the maternal environment (extreme weather) not only affects the number, but also the performance of offspring. Extreme climatic events, terminated before seed set, induced transgenerational effects. Transgenerational plasticity in plant adaptation to light environment was also investigated and it was demonstrated that offspring have greater rates of germination and seedling yield when planted into their maternal environment and that these early life effects influence its fitness (Galloway & Etterson, 2007). Further, the supply of carbohydrate from the maternal plant to the developing fruit at night can have an important influence on oilseed composition and on postgerminative growth in *Arabidopsis thaliana* (Andriotis *et al.*, 2012). Similarly, amount of carbohydrate storage in roots of plants grown at two nutrient levels was affected by nutrient conditions experienced by their mothers (Latzel *et al.*, 2014). On the molecular basis, Tricker *et al.* (2013) examined transgenerational inheritance of low relative humidity-induced DNA methylation for two gene loci in the stomatal developmental pathway (*SPCH* and *FAMA*) in Arabidopsis and the abundance of associated short-interfering RNAs (siRNAs). They found out that transgenerational methylation and a parental low humidity-induced stomatal phenotype were heritable. On the other hand, substantial genetic variation was found for stomatal abundance-related traits in *Arabidopsis thaliana*, which were weakly or not at all affected by laboratory maternal environments (Delgado *et al.*, 2011). Little is known about maternal effect in stomatal development, therefore further investigation is required.

2. Aim and hypotheses

Stomatal development is supposed to be controlled by both genetic heredity and plant environment. The overall aim of the thesis was to reveal the way how stomatal development is influenced by environmental conditions and how stomatal development is projected in leaf physiology. My research was based on the assumptions that stomatal development in leaf epidermis should be regulated by signals coming from:

i) external environment (CO_2 and light effect)
ii) leaf interior (gene expression, internal CO_2)
iii) cotyledon to leaf
iv) mother to offspring (transgenerational effect)

The research was based on (1) molecular approach – the molecular control of the leaf development (*SDD*, *TMM* and *STOMAGEN* genes) and (2) physiological approach – the relationship between stomatal development, stomatal function and leaf physiology (stomatal and mesophyll conductance, photosynthesis).

Specific hypotheses and aims

(h1) **STOMAGEN plays a role in light-regulated stomatal development.**

We explored the function of STOMAGEN which role in the regulatory pathway of stomatal formation has been described before. More specifically, we investigated the influence of light level on STOMAGEN (and some other genes involved in stomata development) expression in control (wt) and STOMAGEN manipulated mutants. In turn, we explored the effect of elevated SD (using mutants with stomata clustering effect – *tmm1* and *sdd1-1*) on STOMAGEN (and selected genes) expression, indicating a putative feedback mechanism. As high light induced decreased level of C_i and STOMAGEN is produced in mesophyll inside the leaf, we proposed its prospective participation on CO_2 signalling but it is still to be confirmed (Paper I).

(h2) **Leaf internal CO_2 concentration regulates stomatal development.**

We tested whether the density of stomata on fully developed true leaves is sensitive to ambient or internal CO_2 concentration. We manipulated C_i by changing various environmental factors affecting photosynthetic rate and/or stomatal aperture (light quantity, abscisic acid treatment and osmotic stress) while keeping ambient CO_2 constant during the plant's growth. Four species were used for testing this question. In two species, we also investigated the C_i response of pavement cells and calculated the SI. Data from the literature for 16 plant species cultivated under different growth conditions were analysed to complement these experiments. Further, we wanted to know whether SD in cotyledons is controlled by CO_2 in a way similar to that in true leaves. (Paper II)

(h3) **Signals from mother to offspring and/or from cotyledons to true leaves regulate stomatal development.**

We tested to what extent stomatal development is controlled by environment in cotyledons and true leaves. We hypothesized that cotyledons, as the first plant assimilation organs, lack the systemic signal. Maternal effect in three generation of plants was explored under different light intensities during plant growth. Relationship between stomatal density and pavement cell density on adaxial and abaxial leaf sides was observed. (Paper II, Manuscript III)

(h4) **Formation of clusters in stomatal mutants changes stomatal efficiency.**

We aimed to investigate how stomatal density and patterning on both adaxial and abaxial epidermes affect diffusional limitation of photosynthesis and, more specifically, if an increase in SD could enhance CO_2 concentration in the leaf tissue and CO_2 assimilation rate (A_N) in Col-0, C24, *sdd1-1*, and *tmm1* genotypes of *A. thaliana*. Furthermore, the dynamic ability of clustered and non--clustered stomata to respond to changes in light intensity was evaluated. (Paper IV)

3. Material and methods

3.1 Plant material

Arabidopsis thaliana was used as a model plant in experiments where methods of molecular biology have been applied (Paper I). The main reason was an availability of wild types differing in phenotype (Fig. 4) and mutants in concrete genes connected to stomatal development (*sdd1-1*, *tmm1*, *ST-RNAi*, *ST-Ox*, *ST-Venus*). Columbia (Col-0) and C24 wild types and their stomatal mutants *tmm1* and *sdd1-1* were selected for stomatal behaviour study (Fig. 5; Paper IV). Disadvantage of Arabidopsis species is a relatively slow growth with a tendency to quickly flourish according to the length of the day. Cotyledons of Arabidopsis are very small and quickly turn into senescence. True leaves usually have trichomes complicating microscopic observation of stomata. Architecture of plants with ground rosettes and smaller leaf area makes gas exchange measurements harder. Therefore, special design of plant cultivation was used in some cases - with plant located on the top of the soil mound for better leaf accessibility (Flexas *et al.* 2007) (Fig. 6) (Paper IV).

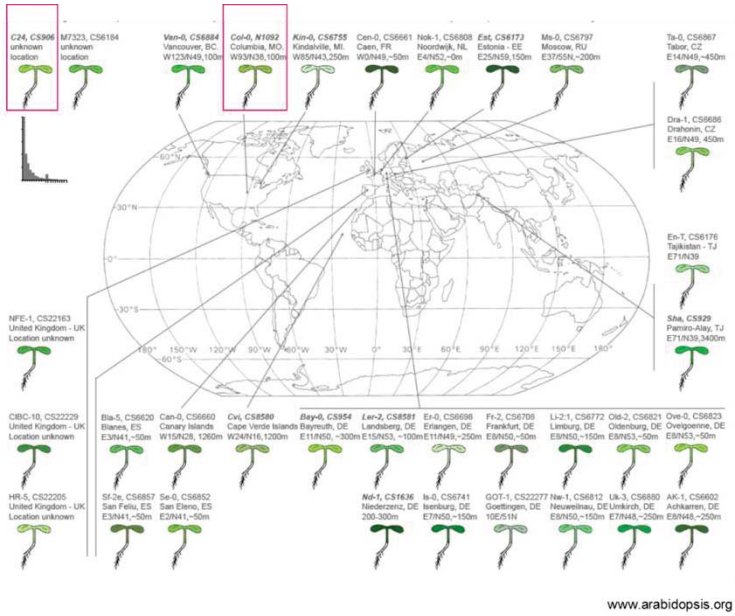
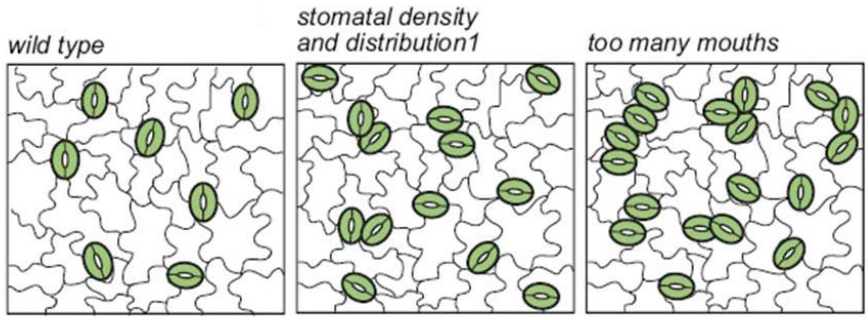


Fig. 4: Ecotypes of *Arabidopsis thaliana* and their worldwide distribution



Nadeau and Sack, 2003

Fig. 5: Stomatal density and pattern in *Arabidopsis thaliana* wild type and selected stomatal mutants (abaxial leaf side).

Fig. 6: Special design of plant cultivation with plant located on the top of the soil mound for better leaf accessibility for gas-exchange measurement. *Tmm1*, C24, Col-0 and *sdd1-1* (from the left) three months old plants are shown.

Other species, *Lepidium sativum* (garden cress), was used in experiments with manipulated environmental conditions (Paper II and III). Garden cress (Fig. 7) was selected for its genetic relationship to Arabidopsis (*Brassicaceae* family), fast growth, relatively large size of cotyledons and easy cultivation. Advantage of garden cress is also the absence of trichomes on leaves that enables easy imprinting of leaves for stomata counting. Disadvantage is small leaf size which makes it almost impossible for gas exchange measurements on a leaf scale.

Fig. 7: Garden cress plantlets. Growth and development in ten days after sowing (A) and the size of cotyledons (B) are shown. Short movie of cress growth in digital and infrared mode is attached to the electronic version of this thesis.

Several plant species were used in the leaf internal CO_2 (C_i)-manipulating experiments: sunflower (*Helianthus annuus*), beech (*Fagus sylvatica*), Arabidopsis (*Arabidopsis thaliana*) and garden cress (*Lepidium sativum*) (Paper II).

Plants were grown *i)* in growth chambers under controlled conditions (humidity, light intensity, temperature, ambient CO_2 concentration) (Paper I - IV) or *ii)* in glass desiccators supplied with artificially mixed atmosphere (synthetic air or helox), controlled gas flow and defined CO_2 concentration, humidity and irradiance (photon flux density) (Fig. 8, Paper II).

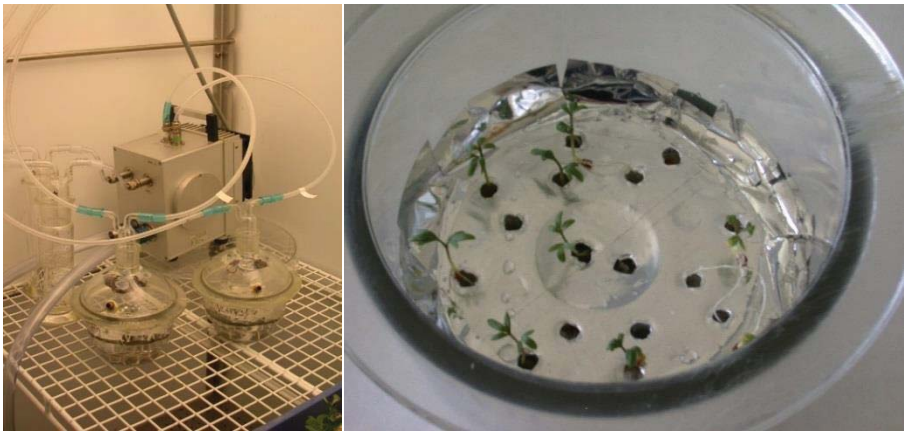


Fig. 8: Glass desiccators with artificial atmosphere placed in the growth chamber (left) and detail of garden cress plants grown at air or helox at variable ambient CO_2 concentration (right).

3.2 Methods

Carbon isotope composition

Isotope ratio mass spectrometry (IRMS) is widely used method for estimation of stable isotope ratio ($^{13}C/^{12}C$) of leaf carbon. Plant CO_2 uptake from atmosphere and assimilation in chloroplasts discriminate ^{12}C against ^{13}C and, thus, the relative leaf ^{13}C content can serve as a marker of both processes. The relative difference in abundance of ^{13}C between plant and CO_2 in ambient atmosphere was shown to be proportional to the ratio of leaf internal to atmospheric CO_2 concentrations (Farquhar *et al.*, 1982; 1989) We used this method for estimation of leaf internal CO_2 concentration in our experiments where environmental conditions (CO_2 concentration, light intensity) have been manipulated and in experiments with stomatal mutants with supposedly increased stomatal density and stomatal conductance. In some cases, carbon isotope composition of seeds was estimated for carbon utilization tracking (experiments with garden cress – Paper II and IV).

Instrumentation: IRMS DeltaXL^{plus}, ThermoFinnigan, Bremen, Germany; EA1110 elemental analyser, ThermoQuest, Milan, Italy.

Light and confocal microscopy

Epidermal imprints were taken from both abaxial and adaxial leaf sides. Light microscopy was used for stomata and pavement cell counting (Paper I - IV) and for stomatal size, leaf thickness and mesophyll airspace assessment (Paper IV). Confocal microscopy was used for the *STOMAGEN*-Venus line observation (Paper I).

Instrumentation and software: Optical microscope BX61, Olympus, Japan, with digital camera EOS 1000D, Canon, Japan; Confocal microscope LSM 5 Duo, Carl Zeiss, Germany; ImageJ – free available software for image analysis (NIH).

Gene expression

We investigated the influence of light level on selected genes expression. Expression of STOMAGEN, TMM, SDD1 and other genes were estimated by Real-time RT-PCR method using Taq-Man assays (Applied Biosystems) (details see in Paper I).

Instrumentation: Step One Real-time PCR system, Applied Biosystems/Life Technologies, Thermo Fisher Scientific, US, plus StepOne software.

Gas exchange measurements

Gas exchange measurements were carried out on leaves of Arabidopsis to obtain physiological parameters connected to CO_2 uptake and H_2O transpiration – stomatal conductance and CO_2 assimilation rate (Paper IV). By simultaneous measurements of gas exchange and ^{13}C isotope discrimination mesophyll conductance (and CO_2 concentration in the chloroplast) was estimated. This measurement enables evaluation of the kinetics of light induction of stomatal opening for better understanding of stomatal function in Arabidopsis mutants.

Instrumentation: Open gas-exchange system LI-6400, LI-COR Biosciences, USA.

Thermal imaging

It is well known that leaf temperature varies with evaporation from leaves and hence is a function of stomatal conductance (Tanner 1963; Jones & Corlett 1992). A number of papers have used formulations of the basic energy balance equation to derive more or less explicit estimates of stomatal conductance from infrared thermography (e.g. Jones, 1999; Leinonen *et al.*, 2006). Thermal methods have the advantage of being more rapid than the conventional gas--exchange measurements of stomatal opening and of not interfering with the stomatal responses through physical contact with the leaves.

During leaf ontogenesis, stomata develop from meristemoids, stomatal conductance for water vapour increases and leaf temperature decreases. When the leaf area is too small for gas exchange measurement, infrared thermography is useful tool for stomatal development monitoring. We used thermal imaging in experiments with Arabidopsis wild types and mutants significantly differing in stomatal density (Fig. 9). The obtained results were published in Paper IV. For interest, short movie of garden cress growth in infrared mode is attached to electronic version of this thesis.

Instrumentation: P660 thermographic camera, FLIR Systems, Sweden.

Fig. 9: *Arabidopsis thaliana* rosette photographed in the visible (left) and infrared (right) spectrum.

4. Overview of My Research

The research on stomatal development focuses on a wide range of factors that could affect stomatal density, pattern and function. These involve environmental conditions, leaf internal factors, genetic and molecular bacground, transgenerational effects etc. A simplied scheme of principal questions leading to hypotheses and my following research is shown in Fig. 10.

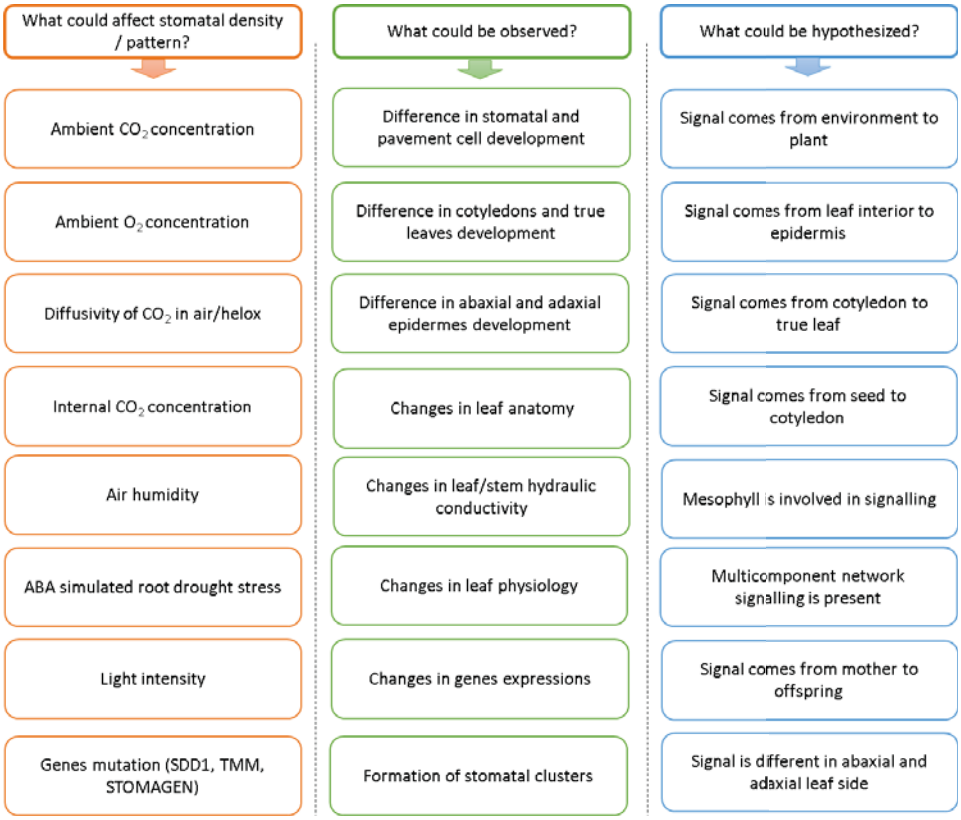


Fig. 10: The main points of my research on stomatal development and its influence by environmental conditions. Each column includes key elements which were studied within my research.

My thesis is based upon three published papers and one manuscript. Paper I evaluates the mechanism of light control of stomatal development

in Arabidopsis (h1). Paper II focuses on leaf internal CO_2 concentration control of stomatal development (h2) and on the systemic signalling from cotyledons (h3) in several plant species. Paper III (unpublished manuscript) is based on a research of a parental effect of light quantity (PPFD) on stomatal development in *Lepidium sativum* (h3). Paper IV deals with stomatal function, density and pattern, and CO_2 assimilation in Arabidopsis wild types and stomatal mutants (h4).

Paper I

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

The initiation of stomata 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.

Arabidopsis wild types and mutants *tmm1*, *sdd1-1*, *ST-RNAi*, *ST-Ox* and *ST--Venus* were grown under two or three levels of PPFD. Stomatal density and gene expression stimulated by light were evaluated.

Our results indicated that elevated 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 with elevated stomatal density and changed stomatal spacing in adaxial/abaxial epidermes such as *stomatal density and distribution* (*sdd1-1*) and *too many mouths* (*tmm1*) displayed elevated *STOMAGEN* expression. As these alleles have repressive effect in pathway regulating stomatal density and pattern, it suggests that STOMAGEN is either under direct control of this pathway or is indirectly affected by stomatal patterning. This is assumption of a feedback mechanism.

Our 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.

Paper II

Stomatal and pavement cell density linked to leaf internal CO_2 concentration.

Stomatal density (SD) generally decreases with rising atmospheric CO_2 concentration, C_a. However, SD is also affected by light, air humidity and drought, all under systemic signalling from older leaves. This makes our understanding of how C_a controls SD incomplete. This study tested the hypotheses that SD is affected by the internal CO_2 concentration of the leaf, C_i, rather than C_a, and that cotyledons, as the first plant assimilation organs, lack the systemic signal.

Sunflower, beech, Arabidopsis and garden cress were grown under contrasting environmental conditions that affected C_i while C_a was kept constant. The SD, pavement cell density (PCD) and stomatal index (SI) responses to C_i in cotyledons and the first leaves of garden cress were compared. ^{13}C abundance in leaf dry matter was used to estimate the effective C_i during leaf development. The SD was estimated from leaf imprints.

SD correlated negatively with C_i in leaves of all four species and under three different treatments (irradiance, abscisic acid and osmotic stress). PCD in Arabidopsis and garden cress responded similarly, so that SI was largely unaffected. However, SD and PCD of cotyledons were insensitive to C_i, indicating an essential role for systemic signalling.

It is proposed that C_i or a C_i-linked factor plays an important role in modulating SD and PCD during epidermis development and leaf expansion. The absence of a C_i–SD relationship in the cotyledons of garden cress indicates the key role of lower-insertion CO_2 assimilation organs in signal perception and its long-distance transport.

Paper III (*manuscript*)

Light intensity- regulated stomatal development in three generations of Lepidium sativum.

A parental effect means that the phenotype of an organism is determined not only by the environment which experiences and its genotype, but also by the environment and genotype of its parent. In our previous work, existence of cotyledon to true leaves signal for stomatal development was proposed. Here we tested the parental effect on stomatal development. We planted three generations of garden cress in two contrasting light environments. Stomatal and pavement cell density, ^{13}C discrimination and leaf area were analysed in cotyledons and first leaves of mothers and offspring. Environmental (light--induced) effect and maternal effect on stomatal development of garden cress were estimated. Our results indicate different environmental effect on adaxial and abaxial epidermes of cotyledons and lower cotyledons' SD sensitivity to high light intensity. Linear relationship between stomatal and pavement cell density was found, causing lower sensitivity of stomatal index in higher SD. Finally, it seems that maternal effect is lower than environmental and can act inversely. Higher SD was found in offspring grown in the same environment that was experienced by its mother.

Paper IV

Stomatal function, density and pattern, and CO_2 assimilation in Arabidopsis thaliana tmm1 and sdd1-1 mutants.

Stomata modulate the exchange of water and CO_2 between plant and atmosphere. Although stomatal density is known to affect CO_2 diffusion into the leaf and thus photosynthetic rate, the effect of stomatal density and patterning on CO_2 assimilation is not fully understood.

We used wild types Col-0 and C24 and stomatal mutants *sdd1-1* and *tmm1* of *Arabidopsis thaliana*, differing in stomatal density and pattern, to study the effects of these variations on both stomatal and mesophyll conductance

and CO_2 assimilation rate. Anatomical parameters of stomata, leaf temperature and carbon isotope discrimination were also assessed.

Our results indicate that increased stomatal density enhanced stomatal conductance in *sdd1-1* plants, with no effect on photosynthesis, due to both unchanged photosynthetic capacity and decreased mesophyll conductance. Clustering (abnormal patterning formed by clusters of two or more stomata) and a highly unequal distribution of stomata between the adaxial and abaxial leaf sides in *tmm1* mutants also had no effect on photosynthesis. Except at very high stomatal densities, stomatal conductance and water loss were proportional to stomatal density. Stomata formation in clusters reduced stomatal dynamics and their operational range as well as the efficiency of CO_2 transport.

5. Conclusions and future prospects

Regulation of stomatal development by environmental factors and stomatal function of genetically manipulated plants were investigated in this thesis. Physiological and molecular approaches were combined in four studies. We manipulated plant growth to obtain a wide spectrum of environmental conditions affecting stomatal density, patterning and function. In particular, CO_2 concentration and light intensity were most important environmental factors entering our experiments. Motivation for this was the fact, that - despite of amount of published papers - mechanisms participating in effects of CO_2 concentration and light intensity on stomatal development was not fully understood. Moreover, to our knowledge, the effect of internal CO_2 on stomatal density had not been studied before apart from the fact that the same effect on stomata function was conclusively demonstrated almost thirty years ago (Mott, 1988). Analyses of stomatal function in Arabidopsis mutants, widely used for research of stomatal development (*sdd1-1* and *tmm1*) on molecular basis, were rare too. Finally, little was known also about the signal transduction within a plant and about transgenerational effects on stomatal development.

Links between molecular biology and plant physiology were lacking in many of published studies. Therefore, a comprehensive view on stomatal development and function issue was my priority. We combined methods of molecular biology and plant physiology. Estimation of genes expressions (*TMM*, *SDD1*, *STOMAGEN*) was followed by molecular insight (^{13}C content in leaf tissue analyses; abscisic acid manipulative experiments) and by anatomical and physiological approaches (cell counting; cell size, leaf size, leaf thickness and mesophyll airspace assessments; gas exchange measurements; thermal imaging of leaves). More plant species and *Arabidopsis* accessions and mutants were used in experiments to increase the credibility of results. Stomata and pavement cells on both adaxial and abaxial leaf epidermes were studied; this is what I feel as really important due to sometimes dissimilar behaviour of the two leaf sides and the two different types of epidermal cells.

In studies dealing with light-induction of stomatal development we have specified the role of STOMAGEN in stomatal development (**I**). Our observations in loss- and gain-of-function lines of Arabidopsis mutants support a role of STOMAGEN in light induced stomatal formation. Furthermore, *STOMAGEN* expression is either directly or indirectly regulated by other members of signalling pathway for stomatal development, as its expression level is affected in mutants with altered SD. STOMAGEN is a promising candidate for stomatal development-related signalling between the mesophyll and the epidermis. These observations suggest that STOMAGEN acts in the link between photosynthetic activity in the leaf mesophyll and epidermal patterning.

In manipulative experiments dealing with leaf internal CO_2 concentration we have deepened our knowledge of the mechanisms of environmental sensing in stomatal development (**II**). We showed, that the SD and PCD of mature leaves co-vary with ^{13}C discrimination caused by altering various environmental factors while keeping ambient CO_2 stable. This translates into an inverse association between SD, PCD and daytime-integrated internal CO_2 concentration of the leaf. There is only a small (if any) change in the relative proportion of stomata to other epidermal cells (SI), with one exception, in a large number of experimental comparisons. Therefore, the results demonstrate overwhelmingly that the reduction in C_i hinders the expansion of leaf area, increases SD and reduces the size of stomata. In contrast, elevated C_i stimulates the expansion, increases the size of stomata and reduces SD. We suggest that the apparent C_i-dependent modulation of SD and PCD could translate the mesophyll demand for CO_2 into a pattern of more numerous and smaller stomata on newly developed leaves. The putative C_i-sensing mechanism could integrate several environmental factors and relies on a signal transported from older, photosynthetically competent organs, including cotyledons in the case of the first true leaves. In contrast, the absence of older photosynthetic organs probably prevented the adjustment of the SD of cotyledons in response to C_i and environmental perturbations.

A study interconnecting light intensity effects on stomatal development, the signal transduction within an individual plant and parental effects involved in stomatal development was performed (**III**). We revealed that light-induced

stomatal development differs between cotyledons and first leaves of garden cress and between adaxial and abaxial epidermes. We proposed cotyledons could sense the light environment and produce relevant signal for stomata development on first leaves. Linear relationship was found between SD and PCD. As a consequence, SI was found less sensitive in leaves with higher SD. In our experiments, environmental (light) effect on stomatal development was stronger than maternal effect, which was present, but acted inversely. It seems that offspring increase SD in the same environment as experienced by its mother compared to offspring of mother grown in contrasting PPFD. In a stable, less variable environment, the offspring should "risk" and enhance the CO_2 uptake through higher stomatal conductance at the expense of water losses. Its fitness then would increase resulting possibly in higher degree of offspring in next generation. Nevertheless, as we have shown in Paper IV, increment in SD is not sufficient *per se* and probably other characteristics (e.g. leaf anatomy and biochemical CO_2 fixation) have to change for better fitness.

The last but not least study was focused more on stomatal function (**IV**). Stomatal mutants (*sdd1-1* and *tmm-1*) and two accessions of Arabidopsis (Col-0 and C24) were used as models for different stomatal patterning. We were looking for the connection between gene mutations, phenotypic expressions and leaf physiology. We demonstrated that the multiplication of stomata *per se* and, presumably, easier access for CO_2 into the leaf is not the only prerequisite for enhanced leaf photosynthesis. Extremely high stomatal density decreases the efficiency of individual stomata for CO_2 and H_2O diffusion, as confirmed by stable carbon isotope discrimination and thermal imaging techniques. Our findings point to the importance of assessing both leaf epidermes when counting the stomata of plants grown under different environmental conditions or when genes are manipulated. According to our measurements, the leaf physiological characteristics reflecting CO_2 diffusion under the growth light regime are affected predominantly by the overall number of stomata on both epidermes rather than by lateral stomatal patterning (abaxial/adaxial ratio). The leaves of *sdd1-1* bear more stomata than those of wild-type plants however the stomata in higher numbers are of similar size but less efficient. This still

enables the leaves to operate at higher C_i, but their rate of carbon assimilation is unchanged likely due to notably reduced g_m for CO_2.

To summarize, the most important conclusions of my research related to the hypotheses h1-h4 include:

(h1) STOMAGEN plays a role in light-regulated stomatal development.
→ We have confirmed a role of STOMAGEN in light induced stomatal formation and proposed that STOMAGEN acts in the link between photosynthetic activity in the leaf mesophyll and epidermal patterning.

(h2) Leaf internal CO_2 concentration regulates stomatal development.
→ We found apparent C_i-dependent modulation of stomatal density that could connect mesophyll demand for CO_2 with stomatal development and integrate several environmental factors.

(h3) Signals from mother to offspring and/or from cotyledons to true leaves regulate stomatal development.
→ We revealed parental effect influencing stomatal development and proposed the role of cotyledons in light intensity sensing.

(h4) Formation of clusters in stomatal mutants changes stomatal efficiency.
→ We demonstrated that the multiplication of stomata *per se* is not the only prerequisite for enhanced leaf photosynthesis, due to decreased efficiency of individual stomata for CO_2 and H_2O diffusion in stomatal mutants with extremely high stomatal density.

My research revealed important traits of environmental control of stomatal development and function. Stomata regulating CO_2 uptake and water loss are crucial epidermal structures with potential for better adaptation of plants to the global climate change. Therefore, understanding of mechanisms underlying their development and regulation is desirable. This thesis is not exhaustive and further research is necessary. Proposed signal

transduction from cotyledons to leaves needs further experiments to found the source and mediators of this type of signalization. Similarly, transgenerational effects in stomatal developments should be explored more thoroughly on other species and environmental factors. Understanding of genetic and molecular control of stomatal development is certainly not completed and further research is required. From physiological point of view, the relationship between stomatal density, stomatal and mesophyll conductance and photosynthesis is also worthy of future study.

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RESEARCH ARTICLES

PAPER I

Light-induced stomagen-mediated stomatal development in Arabidopsis leaves

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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;

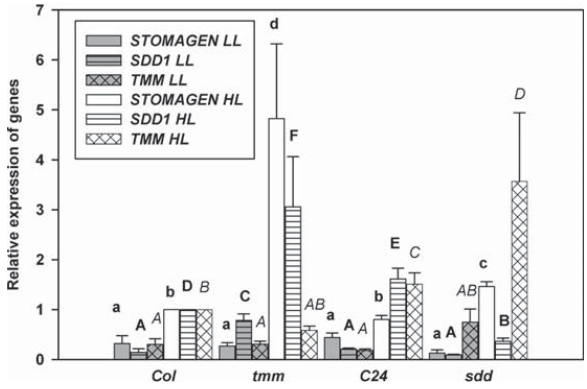

Fig. 1. Expression of *STOMAGEN*, *TMM*, and *SDD1* in WT and *tmm1* and *sdd1* mutants. Expression of *STOMAGEN*, *SDD1*, and *TMM* in young developing leaves of *A. thaliana* WT Col-0 and C24, and mutants *tmm1* and *sdd1* grown under two different light conditions (HL: 250 µmol m^{-2} s^{-1}, and 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).

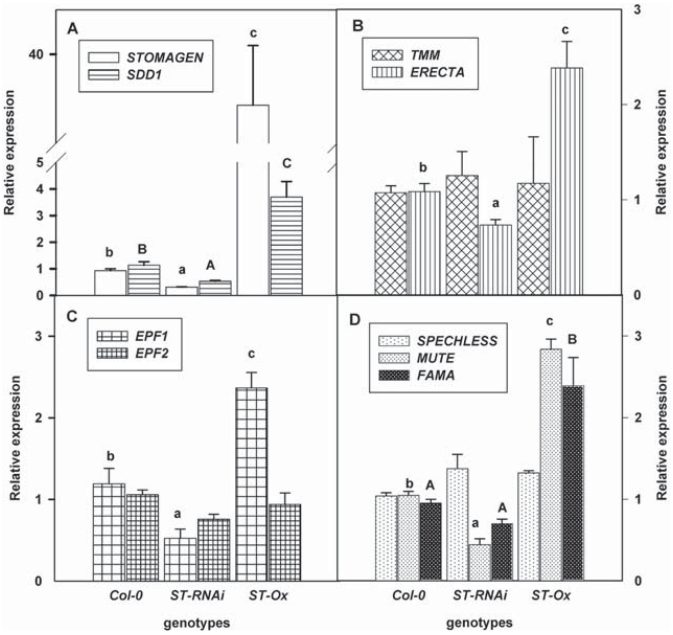

Fig. 2. *STOMAGEN* expression affects expression of a subset of genes involved in stomatal development. The relative expression levels of the selected genes *STOMAGEN* and *SDD1* (A), *TMM* and *ER* (B), *EPF1* and *EPF 2* (C), and *SPEECHLESS*, *MUTE*, and *FAMA* (D) in developing leaves of *STOMAGEN*-overexpressing (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).

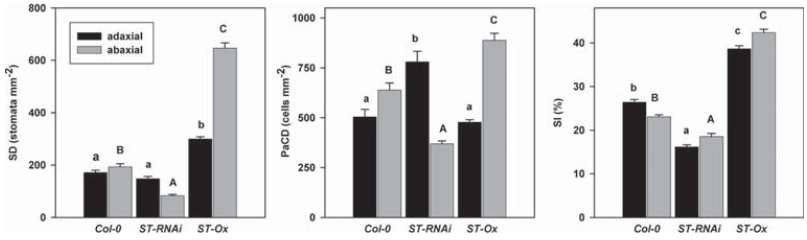

Fig. 3. Expression of *STOMAGEN* affects stomatal and pavement cells density, and the SI. SD (A), density of epidermal pavement cells (B; PaCD), and SI (C) on the adaxial (black columns) and abaxial (grey columns) leaf surfaces of fully developed leaves of *A. thaliana* WT Col-0 and *STOMAGEN*-overexpressing (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.

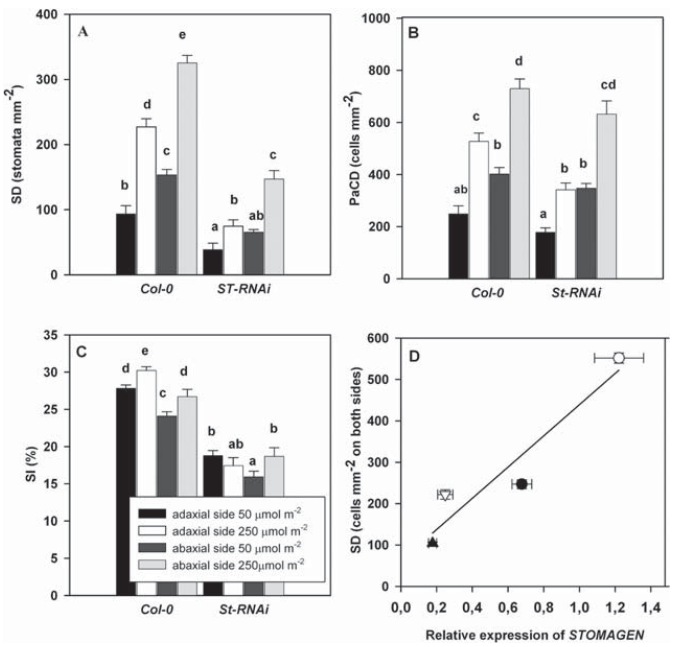

Fig. 4. SD and stomatal frequency in *STOMAGEN* loss-of-function plants under increased light levels. (A–D) SD (A), density of epidermal pavement cells (B; PaCD), and SI (C) on the adaxial (black and white) and abaxial (dark and light grey columns) leaf surfaces of fully developed leaves of *A. thaliana* 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),

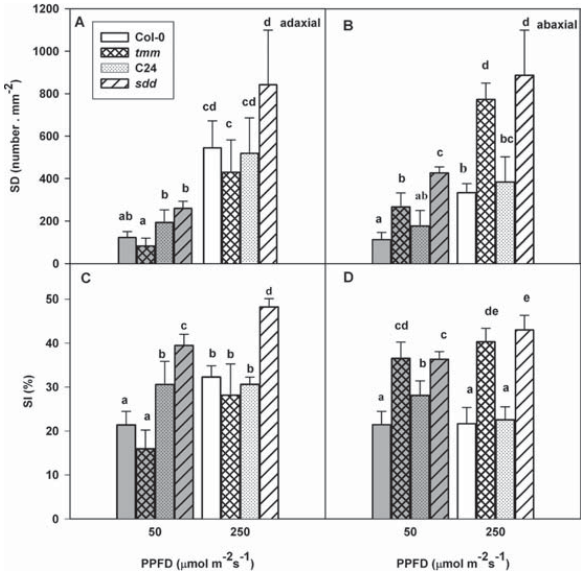

Fig. 5. The effect of photon flux density on SD and SI is modulated in *tmm1* and *sdd1* mutants. SD (A, B) and SI (C, D) on the adaxial (A, C) and abaxial (B, D) sides of the leaf surfaces of fully developed leaves of *A. thaliana* WT Col-0 and C24, and *tmm1* and *sdd1* mutants grown under two different 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

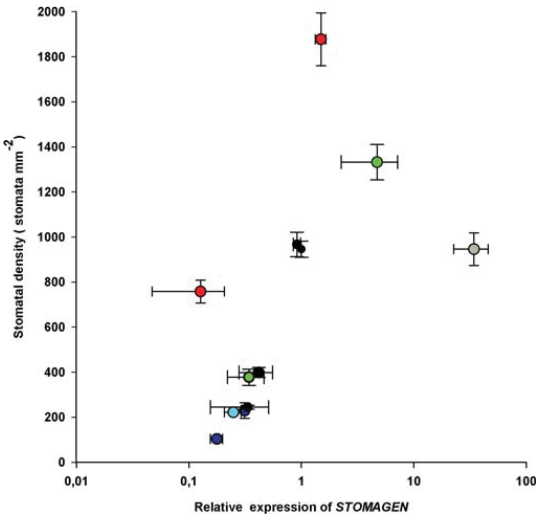

Fig. 6. STOMAGEN expression is positively correlated with SD. The relationship between SD (summed over both leaf sides) and relative mRNA level of *STOMAGEN* is shown. SD=804.15×(relative expression)+205.9, r^2= 0.96, *P*=0.01 for both WTs (Col-0 and C24) and *tmm1*. WT, black circles; *sdd1*, red circles; 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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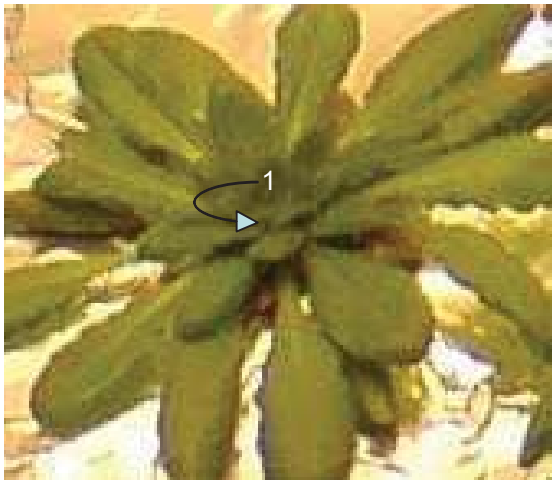
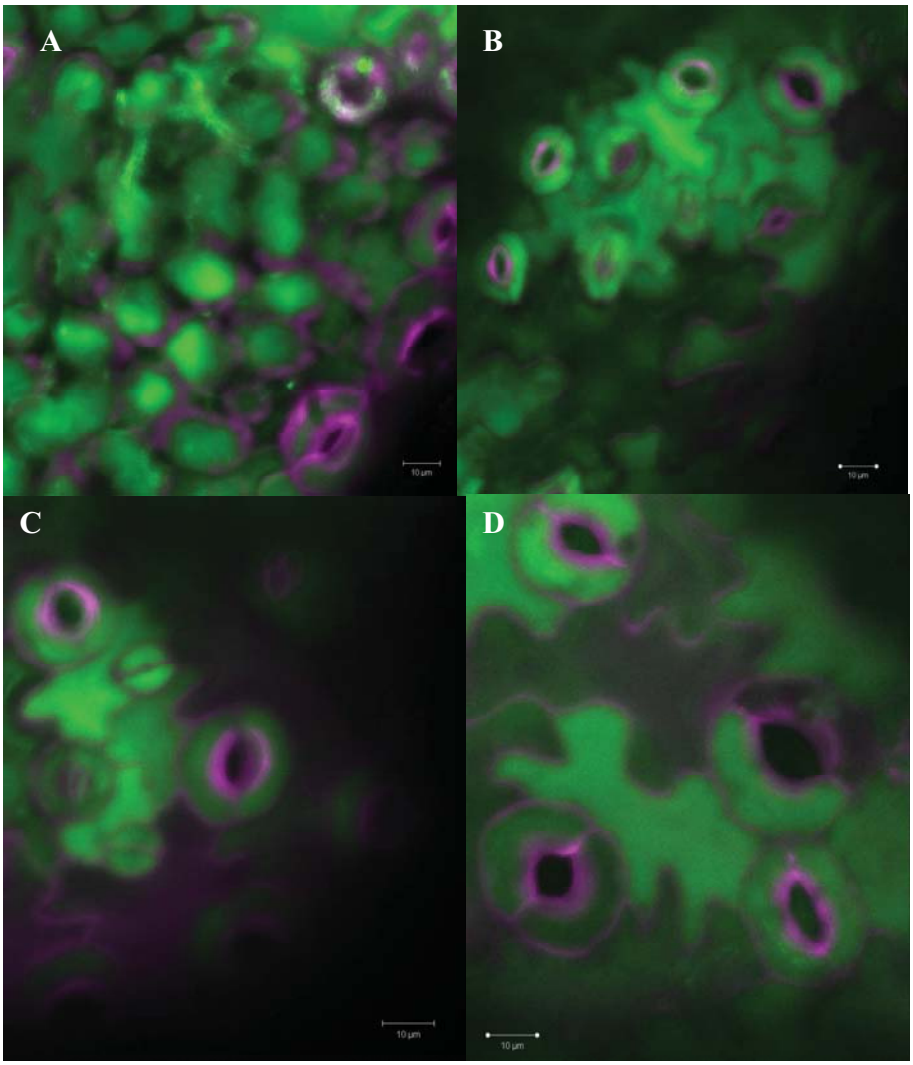
Light induced STOMAGEN-mediated stomatal development in *Arabidopsis* leaves

Marie Hronková, Dana Wiesnerová, Marie Šimková, Petr Skůpa, Walter Dewitte, Martina Vráblová, Eva Zažímalová and Jiří Šantrůček.

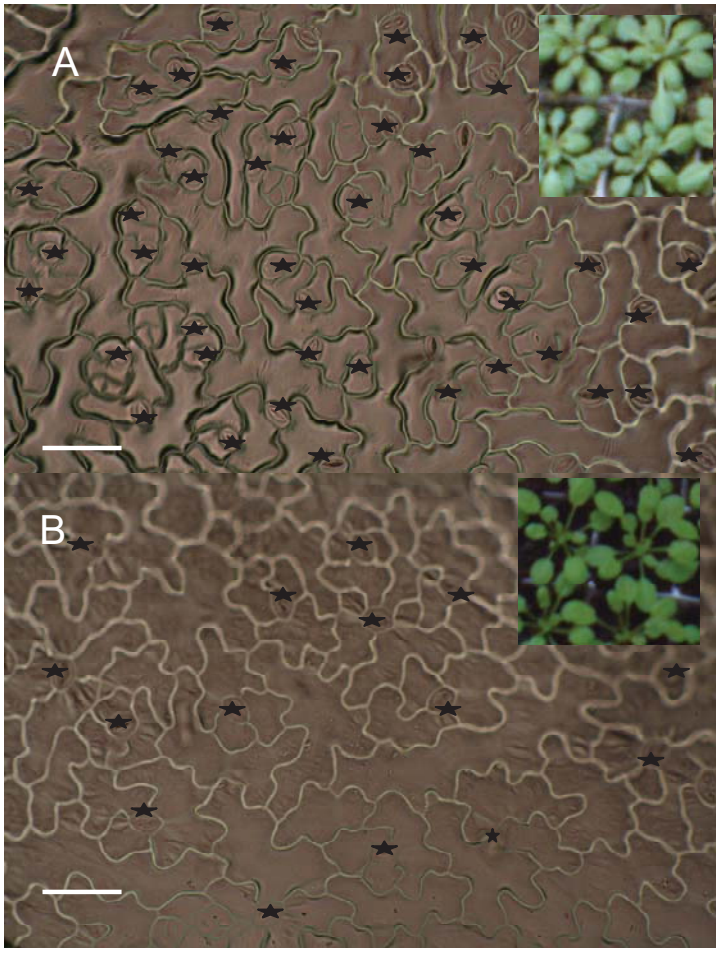


Leaf insertion	Relative *STOMAGEN* expression
1-4	0,99± 0,02 d
5-8	0,60±0,01 c
9-11	0,37±0,05 b
12-14	0,21±0,04 ab
15-	0,08±0,04 a

Supplementary figure S1.
Relative *STOMAGEN* expression (lower panel) in the leaves of different insertions (different ages) marked in the leaf rosette from youngest leaves (upper panel). Values are means of three biological replications ± SE, different letters indicate significant differences, $P<0.05$.

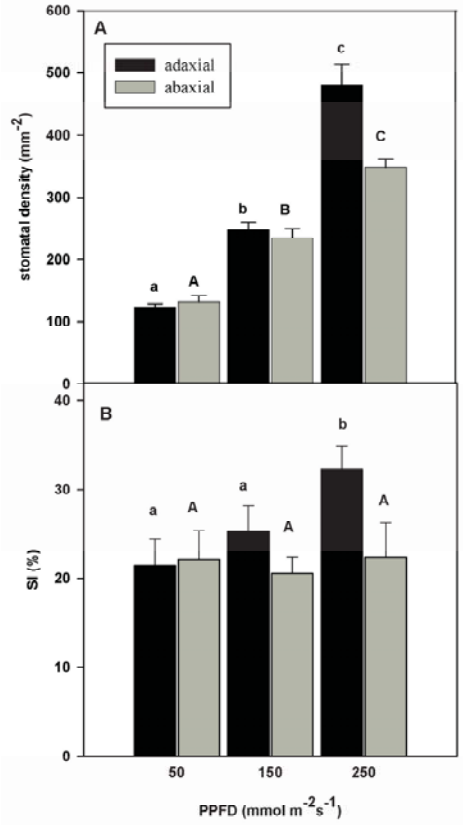


Supplementary figure S2.
Confocal images of the mesophyll (A) and epidermis (B,C,D) of adaxial side of the young rosette leaves in 14-day-old transgenic plants overexpressing the STOMAGEN- VENUS fusion. STOMAGEN-Venus signal is in green, plasma membrane was stained by FM4-64 dye.



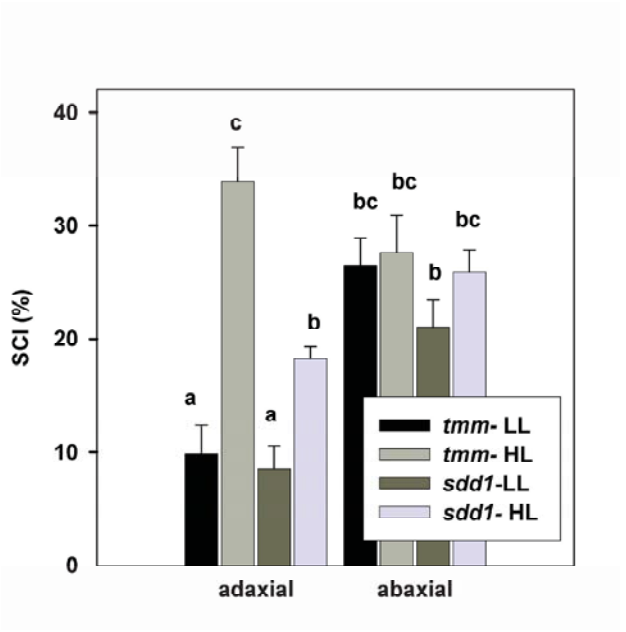
Supplementary figure S3.

Imprints of the adaxial leaf surface of the high light (250 µmol m^{-2} s^{-1}) (A) and the low light (25-50 µmol m^{-2} s^{-1}) leaves of wild type Columbia (Col-0) (B). Stomata are indicated by black asterisks. Scale bars represent 50µm. Inset views show photos of the experimental plants.



Supplementary figure S4.

Stomatal density (SD) (A**)** and stomatal index (SI) (B) on the adaxial (black columns) and abaxial (gray columns) leaf surfaces of fully developed leaves of *Arabidopsis thaliana* wild type Columbia (Col-0) grown under three different light conditions (photosynthetic photon flux density, PPFD): sunny leaves - high light HL (250 µmol m^{-2} s^{-1}) , medium irradiated leaves ML (150 µmol m^{-2} s^{-1}) and shaded leaves - low light-LL (25-50 µmol m^{-2} s^{-1}). Data are means of 25 replicates (5 areas of 0.13 mm^2 chosen randomly on a fully developed leaf, repeated on five different plants), error bars represent SEs. Letters indicate significant differences separately for adaxial (small letters) and abaxial (capitals) leaf sides ($P<0.05$, One way ANOVA, Tukey's test).



Supplementary figure S5.

Stomatal clustering index (SCI) calculated as ratio of clustered stomata to all stomata on the adaxial (left columns) and abaxial (right columns) leaf surfaces of fully developed leaves of *Arabidopsis thaliana* mutants (*tmm* based on Col-0 and *sdd1* based on C24) grown under two light conditions (photosynthetic photon flux density, PPFD): low light-LL (25-50 µmol m^{-2} s^{-1}) and high light HL (250 µmol m^{-2} s^{-1}) . Data are means of 25 repetitions, error bars represent SEs. Letters indicate significant differences over both leaf sides ($P<0.05$, One way ANOVA, Tukey's test).

PAPER II

Stomatal and pavement cell density linked to leaf internal CO_2 concentration

Šantrůček, J, Vráblová, M., Šimková, M.,
Hronková, M., Drtinová, M., Květoň, J., Vrábl, D.,
Kubásek, J., Macková, J., Wiesnerová, D., Neuwirthová, J., Schreiber, L.
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ANNALS OF BOTANY
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Stomatal and pavement cell density linked to leaf internal CO_2 concentration

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• **Background and Aims** Stomatal density (SD) generally decreases with rising atmospheric CO_2 concentration, C_a. However, SD is also affected by light, air humidity and drought, all under systemic signalling from older leaves. This makes our understanding of how C_a controls SD incomplete. This study tested the hypotheses that SD is affected by the internal CO_2 concentration of the leaf, C_i, rather than C_a, and that cotyledons, as the first plant assimilation organs, lack the systemic signal.
• **Methods** Sunflower (*Helianthus annuus*), beech (*Fagus sylvatica*), arabidopsis (*Arabidopsis thaliana*) and garden cress (*Lepidium sativum*) were grown under contrasting environmental conditions that affected C_i while C_a was kept constant. The SD, pavement cell density (PCD) and stomatal index (SI) responses to C_i in cotyledons and the first leaves of garden cress were compared. ^{13}C abundance ($\delta^{13}C$) in leaf dry matter was used to estimate the effective C_i during leaf development. The SD was estimated from leaf imprints.
• **Key Results** SD correlated negatively with C_i in leaves of all four species and under three different treatments (irradiance, abscisic acid and osmotic stress). PCD in arabidopsis and garden cress responded similarly, so that SI was largely unaffected. However, SD and PCD of cotyledons were insensitive to C_i, indicating an essential role for systemic signalling.
• **Conclusions** It is proposed that C_i or a C_i-linked factor plays an important role in modulating SD and PCD during epidermis development and leaf expansion. The absence of a C_i–SD relationship in the cotyledons of garden cress indicates the key role of lower-insertion CO_2 assimilation organs in signal perception and its long-distance transport.

Key words: Stomatal density, stomata development, pavement cells, cotyledons, leaf internal CO_2, ^{13}C discrimination, *Lepidium sativum*, *Helianthus annuus*, *Fagus sylvatica*, *Arabidopsis thaliana*.

INTRODUCTION

Stomata are pores, which can vary in aperture, on plant surfaces exposed to the air: they control water and CO_2 exchange between plants and the atmosphere. Globally, about 17 % of atmospheric CO_2 enters the terrestrial vegetation through stomata and is fixed as gross photosynthesis each year. Water in the earth's atmosphere is replaced more than twice a year by stomata-controlled transpiration (Hetherington and Woodward, 2003). For plants to survive in a changing environment, stomata must be able to respond to environmental stimuli. Their long-term (days to millennia) response manifests itself through variations of stomatal density (SD, number of stomata per unit of leaf area) and/or size, whereas their short-term dynamics derive from changes in the width of the pores' apertures, at a time scale of minutes. The effect of atmospheric CO_2 concentration on both SD and stomatal opening has been recognized (Woodward, 1987; Mott, 1988; Woodward and Bazzaz, 1988; Morison, 1998; Hetherington and Woodward, 2003; Franks and Beerling, 2009), used in reconstruction of paleoclimate (Retallack, 2001; Royer, 2001; Royer *et al*., 2001; Beerling and Royer, 2002; Beerling *et al*., 2002) and subjected to meta-analysis (Ainsworth and Rogers, 2007). It is well known that stomatal guard cells respond to the internal CO_2 concentration (C_i) of the leaf rather than to the external CO_2 concentration: pores open when C_i falls and close when it goes up (Mott, 1988; Willmer, 1988; Mott, 2009). It is tempting to speculate that similar sensing of C_i may also act during the final setting of SD in mature leaves: it seems more plausible that the frequency of the supply 'valves' is controlled by chloroplast-mediated CO_2 demand than by globally modulated availability of CO_2 in the free atmosphere. However, to our knowledge, there are no experiments on the relationship of SD to internal CO_2.

Recently, our knowledge of how stomatal frequency is controlled at the genetic level (Nadeau and Sack, 2003; Coupe *et al*., 2006; Wang *et al*., 2007) and in response to CO_2 (Gray *et al*., 2000; Bergmann, 2006; Casson and Gray, 2008; Hu *et al*., 2010) has increased considerably, but the nature and location of the putative CO_2 sensor remain unresolved. Development of stomata considerably precedes leaf unfolding, and most stomata are already developed before the leaf reaches 10–20 % of its final area, when SD reaches its maximum (Tichá, 1982; Pantin *et al*., 2012). At that stage of ontogeny, stomata and their cell precursors experience a local atmosphere that is probably more humid and enriched in respired CO_2 than ambient

air. Sensing of the free atmospheric environment is thus unlikely. In addition to atmospheric CO_2, other external signals affect SD, among them irradiance, atmospheric and soil water content, temperature, and phosphorus and nitrogen nutrition (Schoch *et al*., 1980; Bakker, 1991; Abrams, 1994; Sun *et al*., 2003; Thomas *et al*., 2004; Lake and Woodward, 2008; Sekiya and Yano, 2008; Xu and Zhou, 2008; Fraser *et al*., 2009; Figueroa *et al*., 2010; Yan *et al*., 2012). It is useful to distinguish between the different steps of stomatal development that may be affected by the external cues, i.e. whether it is, on the one hand, the division of protodermal or meristemoid cells leading toward guard cell and pavement cell formation, or, on the other, the enlargement of already existing guard cells and pavement cells during leaf growth that is affected. Stimulation of the first group of processes usually leads to a change in the fraction of stomata among all epidermal cells [stomatal index (SI)], whereas the proportional enlargement of stomata and pavement cells during leaf expansion reduces SD and pavement cell density (PCD), and increases the maximal stomatal size and aperture. However, both developmental phases overlap (Asl *et al*., 2011).

In young leaves that are still metabolically supported by mature leaves, the signal about conditions in the free atmosphere may come from mature leaves (Lake *et al*., 2001; Miyazawa *et al*., 2006). A pivotal role in the signal has been attributed to abscisic acid (ABA) (Lake and Woodward, 2008) or the ^{13}C content ($\delta^{13}C$) in assimilates (Sekiya and Yano, 2008). (For the sake of simplicity, we omit the '^{13}C' after δ from here on since carbon is the only element whose stable isotopes are considered here. Also, internal CO_2 refers to the CO_2 within the organ.) The degree of carbon isotope discrimination is considered a 'fingerprint' of the ratio of internal CO_2 concentration over ambient CO_2, C_i/C_a, which is negatively proportional to intrinsic water-use efficiency (WUE; Farquhar and Richards, 1984). However, what substitutes for the role of a signal derived from older leaves in the first ever phototrophic organ in a plant's life, the cotyledon? The information on C_i/C_a experienced by the maternal generation could be delivered to cotyledons via assimilates stored in the seed. Alternatively, SD in cotyledons could be controlled by the local environment of primordial cotyledons.

Here, we tested (1) whether the density of stomata on fully developed true leaves is sensitive to ambient or internal CO_2 concentration. We manipulated C_i by changing various environmental factors affecting photosynthetic rate and/or stomatal aperture (light quantity, ABA and osmotic stress) while keeping ambient CO_2 constant during the plant's growth. Four species were used for testing this question. In two species, we also investigated the C_i response of pavement cells and calculated the SI. Data from the literature for 16 plant species cultivated under different growth conditions were analysed to complement these experiments. Further, we wanted to know (2) whether SD in cotyledons is controlled by CO_2 in a way similar to that in true leaves. To manipulate C_i, we grew the plants at various C_a in mixed artificial atmospheres of air or helox [a mixture of gases similar to air but with nitrogen substituted by helium; CO_2 diffuses 2·3 times faster in helox than in air which can increase C_i (Parkhurst and Mott, 1990)] and at reduced total pressure of ambient atmosphere. C_i was estimated from the carbon isotope composition (δ) of leaf dry mass.

MATERIALS AND METHODS

Plants and growth conditions

Experiment 1: mature leaves at invariant C_a. The SD of mature true leaves was estimated in three plant species (sunflower, *Helianthus annuus*; arabidopsis, *Arabidopsis thaliana* ecotypes Columbia and C24; and garden cress, *Lepidium sativum*) grown in a growth chamber (Fitotron, Sanyo, UK). Leaf samples of beech (*Fagus sylvatica*) were collected from a 60-year-old tree growing at a meadow–forest ecotone, from deeply shaded and sun-exposed parts of the crown. Sampling was organized in the course of two seasons (May–October 2007 and 2009) in 2- to 3-week intervals within a single tree in order to eliminate any genetic effects in the seasonal course of C_i. Groups of sunflower and garden cress plants were also treated with ABA or polyethylene glycol (PEG) added to the root medium in order to manipulate C_i via stomatal conductance. All plants were exposed to a free atmospheric CO_2 concentration of about 390 μmol mol^{-1}. Plants cultivated in growth chambers experienced 60 % relative air humidity, day/night temperatures of 25/20 °C and a 16 h photoperiod. The high-light and low-light treatments were species specific. Sunflower was grown at irradiances [photosynthetic photon flux density (PPFD)] of 700 μmol m^{-2} s^{-1} (high light) or 70 μmol m^{-2} s^{-1} (low light), both with the same spectral composition. After 5 weeks, the plants cultivated at each irradiance were divided into three sub-groups, and ABA [10^{-5} M, (+)-abscisic acid, Sigma-Aldrich, Germany] or PEG 6000 [5 % (w/w), Sigma-Aldrich, Germany] was added to the hydroponic solution in two sub-groups, leaving the third as the control. The solutions were renewed once a week. After 3 weeks, newly developed mature leaves were collected for carbon isotope analysis and estimation of SD. Arabidopsis plants were grown for 18 d at a PPFD of 200 or 80 μmol m^{-2} s^{-1} and the first rosette leaf was measured. Garden cress was grown from seeds in 100 mL pots in garden soil for 14 d at a PPFD of 500 μmol m^{-2} s^{-1}. From the third day after germination, half of the plants were watered daily with 10 mL of water (controls) while the second half was given 10 mL of 10^{-4} M ABA solution per day. Beech leaves were collected eight and ten times during the seasons of 2007 and 2009, respectively. The shaded leaves were sampled from a part of the crown exposed to the north and facing the forest; the sun-exposed leaves were collected from the opposite side, facing a meadow. The average PPFD at sampling time (1300–1500 h) was 1301 (±384) and 33 (±19) μmol m^{-2} s^{-1} in the sun-exposed and shaded environment, respectively (PAR sensors and data loggers Minikin R, EMS, Brno, Czech Republic).

Experiment 2: cotyledons and true leaves in air and helox at variable C_a. The SD and PCD on cotyledons and first leaves of garden cress were compared in a set of controlled-atmosphere experiments. We chose garden cress because of its fast growth rate allowing us to reduce costs for compressed gases, mainly helium. The plants were grown for 14 d from seed in an artificial atmosphere in 600 mL glass desiccators, through which a gas flow of 500 mL min^{-1} was maintained. There were 10–30 plants germinated on wet silica sand or perlite in each desiccator. Each harvest on the seventh and 14th day after seed watering (DAW) reduced the number of plants by ten. Plants were watered in 2- to 3-d intervals with tap water or with half-strength

nutrient solution. Desiccators were placed in a growth chamber (Fitotron, Sanyo, UK) and attached to a computer-controlled gas mixing device (Tylan, USA and ProCont, ZAT Easy Control, Czech Republic). Plants were grown at a PPFD of 400 μmol m^{-2} s^{-1}, with a 16 h photoperiod and day and night air temperatures of 23–25 °C. A mixture of He and O_2 at a v/v ratio of 79/21 (helox), or artificial air mixed from N_2 and O_2 at the same v/v ratio (air), with the addition of CO_2 at one of three concentrations (180, 400 and 800 μmol mol^{-1}) was fed into three parallel gas pathways to prepare one line with low humidity (60 ± 5 %; LH) and two separate high humidity (90 ± 5 %; HH) lines (using a two-channel dew point generator; Walz, Germany). The two gas mixtures differing in humidity flowed through two hermetically sealed desiccators with plants, arranged in parallel and having outlets open to the free atmosphere. The third pathway led the humid gas through a reducing valve into the third parallel desiccator with a vacuum pump attached to its outlet. This device allowed us to grow plants in the third desiccator at a total gas pressure reduced to one-half of that in the other two desiccators (hypobaric plants grown at pressure reduced to 450–500 hPa; RP). We included this hypobaric variant since plants grown at reduced total pressure operate at a C_i lower than what would have been expected for the given C_a (Körner *et al.*, 1988). Gas mixtures were prepared from compressed He or N_2 (both with a purity of 4·6), oxygen (3·5) and CO_2 (20 % of CO_2 in N_2; all Messer, Czech Republic). The δ of the source CO_2 was −28·2 ‰. CO_2 and vapour concentrations were measured at the outlets of the desiccators with an IRGA (LiCor 6400; Li-Cor, Lincoln, NE, USA). The fractions of He and O_2 in the outlet atmosphere were measured with an He/O_2 analyser (Divesoft, Prague, Czech Republic) twice a day. Altogether, 18 treatments were applied: two different inert components of the gas mixture (He or N_2), each with three different CO_2 concentrations, and each in dry, humid and hypobaric variants (see Table 1). Different C_a, humidity, He instead of N_2, and reduced pressure in gas mixtures were used to manipulate C_i, the growth-integrated value of which was estimated by ^{13}C discrimination. Seven- and 14-day-old plants were harvested. For plants grown at 180 μmol mol^{-1} of ambient CO_2, we also included harvest at 21 DAW.

Experiment 3: cotyledons and true leaves at different PPFDs and invariant C_a. In the third type of experiment, garden cress was cultivated at eight different PPFDs in glass cuvettes (volume 100 mL) flushed with air pumped from outside of the building (Multi-Cultivator MC 1000, PSI, Brno, Czech Republic). Single plantlets grew in fine perlite in temperature-controlled cuvettes irradiated individually with white light-emitting diodes (LEDs). Plants were grown for 21 d at PPFDs of 100, 170, 240, 310, 380, 450, 520 and 590 μmol (photons) m^{-2} s^{-1}, with a 16 h photoperiod and day/night air temperatures of 24/19 °C. The experiment was repeated six times.

Experiment 4: rosette leaves of arabidopsis at two different PPFDs and invariant C_a. *Arabidopsis thaliana* seeds, ecotypes Columbia Col-0 and C24, were incubated in the dark at 4 °C for 3 d. The plantlets were grown in soil in a growth chamber with a 10 h photoperiod, day and night temperatures of 18 and 15 °C, respectively, and air humidity of 50–70 %. Half of the plants were located on the upper shelf, closer to the fluorescence tubes, and exposed to high light (250 μmol m^{-2} s^{-1}; HL). The other half were kept at the bottom of the same growth chamber and exposed to low light (25 μmol m^{-2} s^{-1}; LL). Two independent repetitions with two different growth chambers (Sanyo, UK and Snijders Scientific, The Netherlands) were carried out. The carbon isotope composition of CO_2 in the chamber atmosphere was spatially homogeneous due to active ventilation (mean over the growth time δ = −11·0 ‰). Stomata were counted on fully expanded leaves of 6-week-old plants which were used for ^{13}C analysis. The designs of all four experiments are summarized in Table 1.

Stomatal density

The SD was estimated by light microscopy (Olympus BX61) on nail varnish imprints obtained directly from leaf surfaces (negatives) of adaxial and abaxial sides of mature leaves (only the abaxial side in beech). Cotyledons and first true leaves of 7-, 14- and 21-day-old garden cress and first rosette leaves of arabidopsis were investigated. Stomata and epidermal cells were counted on three plants per treatment, in each plant on adaxial and abaxial leaf and cotyledon sides. The cells on each side were counted in ten fields of 0·13 mm^2 each, randomly distributed across the leaf (apex, middle part and base). The results were expressed as counts of stomata or pavement cells per mm^2 of projected leaf area (total of adaxial and abaxial leaf sides), SD and PCD, respectively. The SI was calculated, where SD and PCD data were available, as SI(%) = SD/(SD + PCD) × 100.

Carbon isotope composition

The relative abundances of ^{13}C over ^{12}C (δ) were measured in leaf dry matter and, in expt 2 (garden cress in desiccators), also in seeds with testa removed (δ_s) and in the source CO_2 (δ_a) used for mixing the artificial atmospheres. δ_a and δ_s were −28·19 ‰ and −28·13 ‰, respectively. δ_a of growth chamber air was also estimated but was not used in the calculation of the treatment-induced changes of C_i (see later). Leaves were oven-dried at 80 °C, ground to a fine powder, packed in tin capsules and oxidized in a stream of pure oxygen by flash combustion at 950 °C in the reactor of an elemental analyser (EA) (NC 2100 Soil, ThermoQuest CE Instruments, Rodano, Italy). After CO_2 separation, the $^{13}C/^{12}C$ ratio (R) was detected via a continuous flow stable isotope ratio mass spectrometer (IRMS) (Delta plus XL, ThermoFinnigan, Bremen, Germany) connected on-line to the EA. The δ expressed in ‰ was calculated as the relative difference of sample and standard R: $\delta = (R_{sample}/R_{standard} - 1) \times 1000$. VPDB (IAEA, Vienna, Austria) was used as the standard. Cellulose (IAEA-C3) and graphite (USGS 24) were also included to ascertain the reliability of the results. Standard deviations of δ estimated in laboratory standard were < 0·05 ‰.

Leaf intercellular CO_2 *concentration,* C_i

We used the δ of leaf dry mass to evaluate the intercellular CO_2 concentration integrated over the leaf's life time, C_i. The ^{13}C discrimination during photosynthetic CO_2 fixation, Δ, is related to C_i as (Farquhar *et al.*, 1989):

$$\Delta = a + [(b - a)C_i/C_a] \quad (1)$$

TABLE 1. *Overview of the design of the four experiments presented*

Experiment no.	Species (ecotype)	Treatment (PPFD μmol m^{-2} s^{-1})	Sub-treatment	C_a (μmol mol^{-1})	Leaf age at harvest (d)
1	*Helianthus annuus*	HL (700) [↓]	Control	390	56
			+PEG [↓]		
			+ABA [↓]		
		LL (70) [↑]	Control		
			+PEG [↓]		
			+ABA [↓]		
	Arabidopsis thaliana (Columbia, C24)	HL (200) [↓]		390	18
		LL (80) [↑]			
	Lepidium sativum	(500)	Control	390	14
			+ABA [↓]		
	Fagus sylvatica	HL (1301) [↓]	Season, leaf age	390	30–180 (18 harvests)
		LL (33) [↑]			
2	*Lepidium sativum*	Artificial air [↓]	LH [↓]	180 [↓]	7, 14, 21
				400	7, 14
				800 [↑]	7, 14
			HH [↑]	180 [↓]	7, 14, 21
				400	7, 14
				800 [↑]	7, 14
			RP [↓]	180 [↓]	7, 14, 21
				400	7, 14
				800 [↑]	7, 14
		Helox [↑]	LH [↓]	180 [↓]	7, 14, 21
				400	7, 14
				800 [↑]	7, 14
			HH [↑]	180 [↓]	7, 14, 21
				400	7, 14
				800 [↑]	7, 14
			RP [↓]	180 [↓]	7, 14, 21
				400	7, 14
				800 [↑]	7, 14
3	*Lepidium sativum*	(100) [↑]		390	21
		(170) [↑]			
		(240) [↑]			
		(310) [↑]			
		(380) control			
		(450) [↓]			
		(520) [↓]			
		(590) [↓]			
4	*Arabidopsis thaliana* (Columbia, C24)	HL (250) [↓]		400	42
		LL (25) [↑]			

HL, high light; LL, low light; HH, high humidity; LH, low humidity; RP, reduced pressure; PPFD, photosynthetic photon flux density during growth.
Expected effect of the treatment and sub-treatment on C_i: increase [↑] or decrease [↓] with respect to the counterpart treatment or control.

where a and b are $^{13}CO_2$ fractionations due to diffusion in the gas phase [4·4 ‰ in air and 2·0 ‰ in helox; see Farquhar *et al.* (1982) for derivation of a based on reduced molecular masses] and carboxylation by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), excluding CO_2 dissolution and intracellular diffusion (27 ‰), respectively, and C_a is the ambient CO_2 concentration. Contributions of (photo)respiration to Δ were considered to be small and were neglected. Δ is related to ^{13}C abundance in the plant, δ_p, and in air, δ_a, as $\Delta = (\delta_a - \delta_p)/[(\delta_p/1000) + 1]$ where both δ and Δ are expressed in per mil (‰). The value of the denominator $(\delta_p/1000) + 1$ does not deviate much from 1 and can be omitted here; therefore $\Delta \cong (\delta_a - \delta_p)$ and rearrangement of eqn (1) for C_i yields:

$$C_i = C_a\left(\frac{\delta_a - \delta_p - a}{b - a}\right) \quad (2)$$

In calculations of C_i in leaves grown in the free atmosphere and in artificial mixed atmospheres, we used δ_a −8·0 ‰ and −28·2 ‰, respectively. Since the air in growth chambers can be contaminated with the ^{13}C-depleted air exhaled by people handling the plants, we calculated and plotted differences in C_i between the control and treated plants, rather than C_i itself. The advantage of this procedure is that the differences do not depend on δ_a provided that the control and treated plants grew in the same mixed atmosphere, such as in our growth chamber experiments:

$$C_{i,t} - C_{i,c} = C_a\left(\frac{\delta_{p,c} - \delta_{p,t}}{b - a}\right) \quad (3)$$

where the subscripts t and c denote the treatment and control, respectively.

To calculate C_i in semi-autotrophic cotyledons, it was necessary to determine the fraction f of the cotyledons' carbon originating from the heterotrophic source (seed). We grew garden cress in artificial air or in helox with δ significantly different from free air. Our seeds, which were produced in free air conditions at δ_a

close to −8 ‰ and had $\delta_s = -28{\cdot}13$ ‰, grew in glass desiccators supplied with artificial air or helox with $\delta_a = -28{\cdot}19$ ‰. With an increasing proportion of autotrophy and f decreasing below 1, δ_p decreased to values more negative than δ_s, with the sigmoid kinetics approaching a limit in fully autotrophic tissue with $f = 0$. Typically, 14- to 21-day-old true leaves had not yet changed their δ_p value [see Supplementary Data Fig. S3]. The sigmoid regressions of the δ_p time course were converted to the f course, ranging from 1 to 0, and used to determine f of 7- and 14-day-old cotyledons (see Appendix for more details). C_i of cotyledons grown in air or helox was then calculated with eqn (4) (derived in Appendix) as:

$$C_i = \left(\frac{\delta_p - \delta_a}{f - 1} - a\right)\frac{C_a}{b - a} \qquad (4)$$

Statistical evaluation, meta-analysis

Descriptive statistics (mean, s.d.), Pearson correlation and linear regression were calculated using SigmaPlot v. 11.0 (SigmaPlot for Windows, Systat Software, Inc.). Normality was checked using normal probability plots and with Shapiro–Wilk tests. Linear correlations and confidence intervals were analysed using probability thresholds of 5 % and 95 %, respectively. Standard error of the mean of SI (SEM_{SI}) was calculated as the square root of the sum of squares of both partial derivatives of SI, each multiplied by the respective SD and PCD standard errors:

$$SEM_{SI} = \sqrt{\left[\frac{\partial SI}{\partial SD}SEM_{SD}\right]^2 + \left[\frac{\partial SI}{\partial PCD}SEM_{PCD}\right]^2} \qquad (5)$$

After substituting the derivatives this becomes

$$SEM_{SI} = \sqrt{\left[\frac{\overline{PCD}\times 100}{(\overline{SD}+\overline{PCD})^2}SEM_{SD}\right]^2 + \left[\frac{-\overline{SD}\times 100}{(\overline{SD}+\overline{PCD})^2}SEM_{PCD}\right]^2} \qquad (6)$$

where the horizontal lines above SD and PCD denote the mean SD and PCD values. STATISTICA v. 8 (StatSoft Ltd., Tulsa, OK, USA) was used in the meta-analysis. The SD and related δ values were extracted from the available literature. Only those multifactorial studies were included in the meta-analysis where the environmental treatments were applied in a fully factorial design. Also, all studies on monocotyledons (grasses) were excluded from the meta-analysis as their SD can be governed by specific mechanisms.

RESULTS

The C_i response of stomatal density at invariant C_a

All investigated plant species responded to sub-optimal growth conditions with a change in the internal CO_2 concentration of the leaf. Figure 1A shows that reduced irradiance increased C_i in shaded leaves compared with high-light controls in sunflower, beech and arabidopsis, and that there was a concomitant decrease

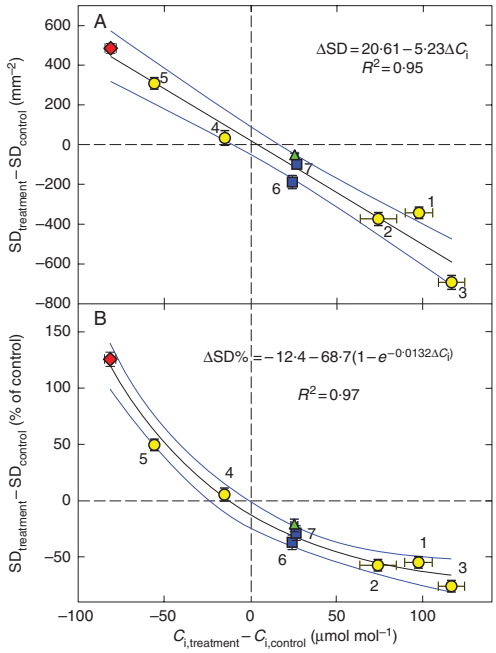

FIG. 1. Effects of shading and water stress treatments on internal CO_2 concentration of the leaf, C_i, as inferred from the $\delta^{13}C$ in leaf biomass, and on stomatal density SD. (A) Circles indicate increased (1, 2, 3) and decreased (4, 5) C_i, and concomitant changes in SD, under low light in sunflower grown in nutrient solution (1), with ABA (2) or PEG (3) added, and under high light in sunflower fed with ABA (4) or PEG (5). Values are differences between treatments and control, i.e. plants grown under high light in plain nutrient solution; $n_{ci} = n_{SD} = 6–8$. The triangle shows the effect of shading on beech leaves (single tree, sampled during the 2007 and 2009 seasons, $n_{ci} = 18$, $n_{SD} = 20$). Squares depict the shading effect in *Arabidopsis thaliana* rosette leaves grown for 18 d at irradiances of 200 or 80 μmol m^{-2} s^{-1}, respectively (ecotypes Columbia and C24, $n_{ci} = 6$, $n_{SD} = 18$). Diamonds indicate the ABA effect in cress plants ($n_{ci} = 5$, $n_{SD} = 48$). All plants experienced an atmospheric CO_2 concentration close to 390 μmol mol^{-1}. (B) The changes in SD from (A) expressed as a percentage of control. The points sharing the same C_i co-ordinate in (A) and (B) represent identical treatments. Standard errors of the mean (bars), regression lines and 95 % confidence intervals are shown.

in SD (see the points in the bottom right quadrant). The additional application of ABA or PEG to low-light-grown sunflower slightly modulated the C_i and SD deviations (points 2 and 3). Application of ABA or PEG to high-light-grown sunflower resulted in the opposite changes of C_i and SD to shading (points 4 and 5 in the upper left quadrant). The slope of the $\Delta SD \sim \Delta C_i$ regression line is 5·23 (stomata) mm^{-2} μmol^{-1} (CO_2) mol. It indicates the sensitivity of the apparent SD response to C_i. Since SD cannot reach zero or infinitely high values in a real plant, the C_i sensitivity applies only to the observed range of SD and should not be extrapolated. In order to overcome this limitation, we normalized the deviation of SD from the control (Fig. 1B). The relative decrement in SD with increasing C_i was attenuated exponentially, reaching an asymptote

of −81·1 % for C_i approaching a theoretical limit 10^6 (pure CO_2) and setting the limit of phenotypic plasticity in lowering SD to about 20 % of the original value.

Comparison of the C_i response in cotyledons and true leaves of garden cress

The results shown above were obtained at stable C_a. In controlled-atmosphere experiments with garden cress, we used ambient (400 μmol mol^{-1} as a control), sub-ambient (180 μmol mol^{-1}) or super-ambient (800 μmol mol^{-1}) CO_2 concentrations to grow cress plants for 14 d (or 21 d for sub-ambient CO_2) in air or in helox atmosphere, each with two different gas humidities and also under reduced total pressure. Variations in C_i were induced primarily by changing C_a, using helox instead of air [CO_2 diffuses 2·3 times faster in helox than in air; see Parkhurst and Mott (1990)] and by reduction of total pressure, and only marginally by reduced air humidity. The time courses of developmental changes in δ and SD in all 18 experimental treatments are shown in Supplementary Data Figs. S1 and S2. The values in the bottom right quadrant of Fig. 2A and B (green triangles) show that the true leaves of super-ambient CO_2 plants had fewer stomata and pavement cells than the control plants. Conversely, in sub-ambient CO_2 plants, the SD and PCD increased (upper left quadrants of the plots). This effect was common for plants grown in air and helox, for plants grown at reduced atmospheric pressure (RP) and for both humidities (HH and LH). Therefore, for the regression analyses we analysed the six treatments (HH, LH and RP, each in helox and air) together and compared the C_i response of true leaves (in 14-day-old plants) and cotyledons (at 7 and 14 DAW). In contrast to the true leaves, cotyledons did not alter the SD and PCD in response to C_i. The slopes of the regression lines approached zero in cotyledons, while in the first leaves they significantly deviated from zero (Fig. 2; Table 2). The pavement cells in true leaves changed their density at 3·2 times the rate of stomata (see the ratio of slopes of the respective regression lines in Fig. 2A, B), which translates to an average reduction of 3·2 pavement cells for each stoma less in response to increasing C_i.

The fraction of stomata among all epidermal cells (the SI) was almost invariant over the whole investigated range of C_i in cotyledons and did not change during their development (SI ± SEM = 25·2 ± 1·4 % and 25·8 ± 2·4 % in 7- and 14-day-old plants, respectively). In true leaves, SI was higher than in cotyledons (29·4 ± 2·0 %) and changed only marginally with C_i (slope = 0·0069 % mol μmol^{-1}, $R^2 = 0·32$; see Table 2). However, standard errors of SI means determined from SD and PCD data were high and the SI changes with C_i non-significant (Fig. 2C). We obtained qualitatively similar C_i responses of SD and PCD in the experiment with arabidopsis true leaves grown at two different irradiances and equal C_a (expt 4, Fig. 3A, B). The SD and PCD increased with C_i reduction in strongly irradiated leaves. The response was similar for both leaf sides and both ecotypes. The SI did not respond to C_i in any systematic way and showed a slightly negative average slope (Fig. 3C).

In order to verify the cotyledons' insensitivity to C_i in a way unbiased by estimation of the seed carbon fractions (see Appendix), we grew garden cress for a longer period (3 weeks)

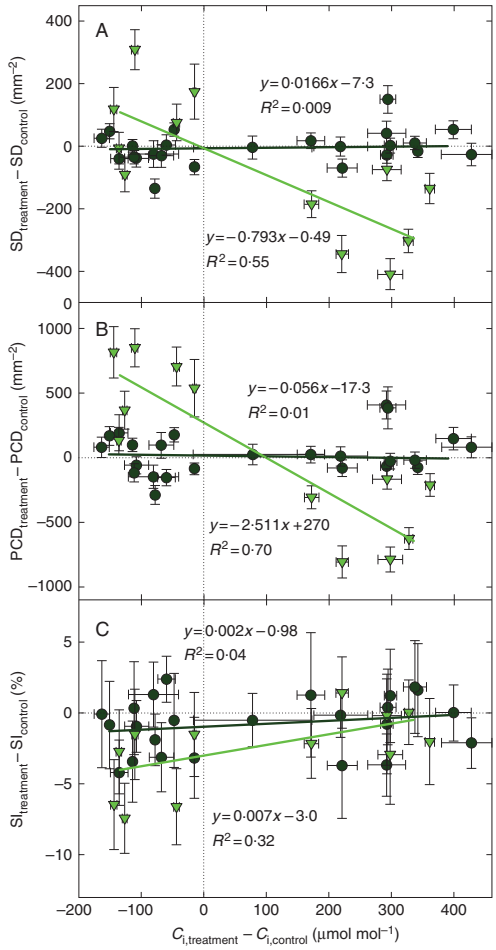

FIG. 2. Changes in internal CO_2 concentration (C_i) of the leaf in *Lepidium sativum* plants grown under sub-ambient or super-ambient CO_2 concentrations, as related to changes in stomatal density (SD; A), pavement cell density (PCD; B) and stomatal index (SI; C). The negative values of the C_i difference show by how much C_i was lowered when C_a was reduced from 400 to 180 μmol mol^{-1}; the positive values indicate the increase of C_i in plants grown at 800 compared with controls kept at 400 μmol mol^{-1}. Stomatal and pavement cell density in the first leaves (light green triangles and light green regression line) was much more sensitive to CO_2 concentration than in cotyledons (dark green circles and dark green regression line). Each point represents a difference between the C_a treatment (180 or 800) and C_a control (400) for one of three sub-treatments (two air humidities and hypobaric plants), each grown either in air or in helox gas mixture. Data for 14-day-old leaves and 7- and 14-day-old cotyledons are shown. The SD and C_i values used in calculation of the differences were means from three independent measurements (three plants per treatment). The C_i values were calculated from $\delta^{13}C$ in plant dry matter (see Appendix). Bars show the standard error of the mean, for SI calculated from the primary SD and PCD data [see eqn (6) in 'Statistical evaluation, meta-analysis'].

TABLE 2. *Comparison of density (number per unit area of leaf) and relative abundance of epidermal cells in cotyledons and the first leaves of garden cress and response of epidermal cell density to variations in the internal CO_2 concentration of the leaf (C_i)*

Age (DAW)	Cotyledons			First leaves		
	SD (mm^{-2})	PCD (mm^{-2})	SI (%)	SD (mm^{-2})	PCD (mm^{-2})	SI (%)
7	439 (25)	1308 (70)	25·2 (1·4)			
14	186 (11)	535 (30)	25·8 (2·4)	535 (84)	1304 (223)	29·4 (2·0)
Slope of C_i response (mol μmol^{-1} mm^{-2} or % mol μmol^{-1} where marked *), (R^2)	0·017 (0·009)	−0·056 (0·011)	0·0019* 0·0407)	−0·793 (0·549)	−2·511 (0·703)	0·0069* (0·317)

Density of stomata (SD) and pavement cells (PCD) per mm^2 of projected leaf area (sum of adaxial and abaxial values) and stomatal index (SI) in cotyledons 7 and 14 d after seed watering (DAW) and in the first leaves 14 DAW are given.

Means and standard error of the means (in parentheses) are shown of sets obtained by pooling data from plants grown in three growth CO_2 concentrations (180, 400 and 800 μmol mol^{-1}), two air humidities (60 and 90 %), two different inert host gases (N_2 and He) or reduced atmospheric pressure ($n = 18$, each n is a mean of three measurements on individual plants). Standard errors of SI means were calculated as means over all 18 treatments each obtained by the formula shown in the Materials and Methods.

simultaneously under eight irradiances and equal C_a. The results confirmed that cotyledons are less sensitive to C_i than the first true leaves or are insensitive (Fig. 4A, B). The SD and PCD on the true leaves increased progressively at reduced C_i. The SI in cotyledons did not respond to C_i. However, in contrast to the previous experiment, SI significantly ($P < 0·001$) decreased with increasing C_i in true leaves (Fig. 4C). The same data as in Fig. 4 but plotted in the form of differences from the 'control' irradiance (310 μmol m^{-2} s^{-1}) and shown separately for abaxial and adaxial leaf sides are presented in Supplementary Data Fig. S4. CO_2 response of SI was steeper for the abaxial (lower) than the adaxial (upper) leaf side.

DISCUSSION

In this study we demonstrate for the first time that the internal CO_2 concentration of the leaf, C_i, correlates strongly with the development of stomatal and pavement cells expressed in terms of their densities. We applied several independent environmental factors to manipulate C_i. Therefore, the experimental results suggest, although they do not prove, that C_i or a C_i-related cue integrates the effects of those environmental parameters and conveys their signal into the machinery controlling SD. Our tests with cotyledons, the first assimilatory organs in a plant's life, support the previous evidence that adult leaves are essential in generating the signal (Lake *et al*., 2001, 2002 Miyazawa *et al*., 2006). The results show that photosynthesis- and stomata-controlled C_i correlates with the number of stomatal and epidermal cells per unit of leaf surface: both insufficient stomatal conductance g_s and/or an enhanced photosynthetic rate reduce C_i, linking these processes directly or indirectly to the development of a new, acclimated leaf having more smaller stomata and pavement cells per mm^2 of the leaf surface. Conversely, elevated C_i, caused for example by shading or elevated ambient CO_2, correlates with fewer stomata and pavement cells per mm^2 of the leaf surface and, consequently, larger epidermal cells develop (for examples of negative relationships between size and density of stomata, see Franks and Beerling, 2009; Franks *et al*., 2012). As this mechanism also operates at invariant C_a, our experiments suggest that the internal rather than ambient CO_2 relates to the changes in SD and putatively exerts its feedback control over both the photosynthetic rate and stomatal conductance by modulating the SD and stomatal size, in analogy to the controls on stomatal aperture that were recognized long ago (Mott, 1988; Morison, 1998).

Lines of evidence for the involvement of a C_i-related factor in stomatal density signalling

We present several pieces of indirect evidence indicating the involvement of C_i or a C_i-linked stimulus in adjustment of SD. First, coefficients of determination indicating the apparent role of C_i in driving SD were fairly high ($R^2 = 0·95$ in our experiments with four different species and several environmental factors; 0·55 and 0·70 for stomata and pavement cells, respectively, in garden cress treated with sub- and super-ambient C_a and a number of environmental factors; 0·73 and 0·61 in garden cress grown at eight different PPFD (see Figs 1, 2 and 4). Secondly, when shifting the axes in such a way that the values of SD and C_i in controls are set to zero as was done in Figs 1 and 2, the points representing the different treatments fall near to each other on a line through the origin. This near [0,0] intercept, shown in Figs 1A, 2A and 5A, suggests to us a dominant role for the C_i-related factor and only relatively minor effects of other factors.

In our estimates, sun-exposed beech leaves showed a 13·7 % increase in SD per 1 ‰ of ^{13}C enrichment, when compared with shaded leaves. Sunflower plants grown under high-light or low-light conditions yielded values of 13·2 (control), 16·3 (PEG-stressed) and 18·9 (ABA-fed) % (SD) ‰$^{-1}$ (δ). A 1 ‰ difference in δ is equivalent to a change in C_i of about 17 and 15 μmol mol^{-1} in air and helox, respectively. Similar values of SD sensitivity to C_i (derived from δ) have been observed (though not analysed) by other authors for a wide spectrum of plants and growth conditions [e.g. leaves of *Vigna sinensis* (Sekiya and Yano, 2008); fossil cuticles of the Cretaceous conifer *Frenelopsis* (Aucour *et al*., 2008); and the complex environmental factors acting along altitudinal gradients (Körner *et al*., 1988)]. Another line of evidence linking C_i with frequency of stomata was obtained by analysing published data from experiments where SD and ^{13}C discrimination in leaf dry matter (δ) were measured concomitantly. We searched for data on δ and SD from controlled, mostly mono-factorial experiments with dicotyledonous plants. Results from 17 studies summarized in

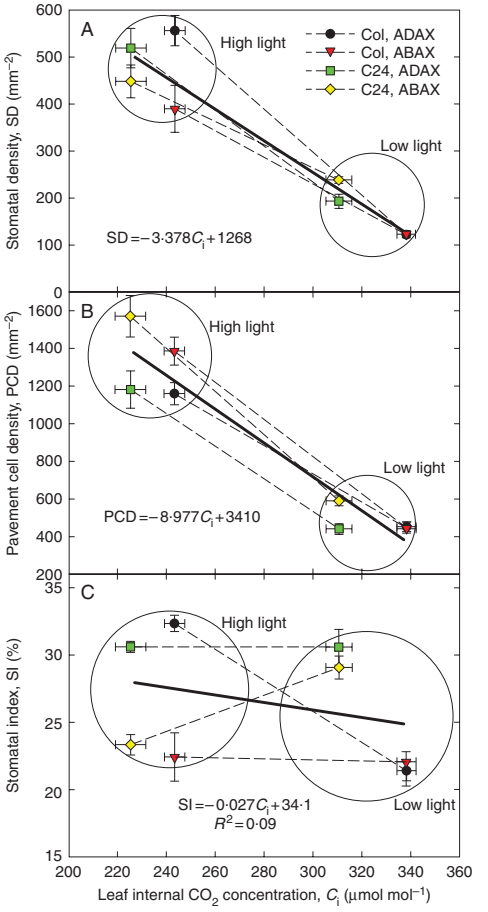

FIG. 3. Response to internal CO_2 (C_i) of stomatal density (SD; A), pavement cell density (PCD; B) and stomatal index (SI; C) of *Arabidopsis thaliana* leaves grown at two irradiances. Two wild ecotypes, Columbia (Col) and C24, were grown under low light (25 μmol m^{-2} s^{-1}) or high light (250 μmol m^{-2} s^{-1}) conditions in growth chambers. Mean values of SD, PCD and SI (symbols) for the adaxial and abaxial sides of mature first rosette leaves are shown as well as the standard error of the mean (bars, $n_{Ci} = 6$ and $n_{SD,PCD,SI} = 16$) and regression lines (thick solid line and equation) for information about leaf-averaged sensitivity (slope) of the relationship. The circles group the low and high light data.

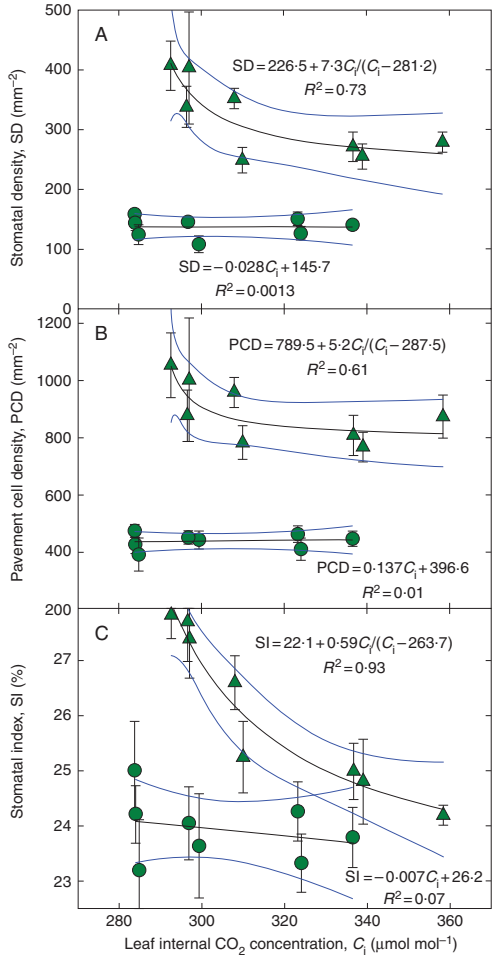

FIG. 4. Response of stomatal density (SD; A), pavement cell density (PCD; B) and stomatal index (SI; C) of *Lepidium sativum* leaves and cotyledons to the internal CO_2 (C_i) of leaves grown at various irradiances. The light green triangles and light green regression lines represent the first leaves; the dark green circles and dark green regression lines show the cotyledons' response. The plants were grown for 21 d under 7–8 photosynthetic photon flux densities (PPFDs), ranging from 100 to 590 μmol m^{-2} s^{-1}, in 100 mL glass cuvettes ventilated with ambient air. The points represent averages from six independent runs of the experiment. Mean values and the standard error of the mean are indicated. Linear or hyperbolic fits with 95 % confidence intervals are shown.

Supplementary Data Table S1 show that the values of SD sensitivity to C_i range between +10 and +20 % (SD) ‰$^{-1}$ (δ). In terms of C_i and at the present ambient CO_2 concentration, SD typically increases by 1 % when C_i drops by 1 μmol mol^{-1}, and vice versa. The differences in δ between treated and control plants, converted to differences in C_i and plotted against the respective differences in SD (Fig. 5), show a similar pattern to those presented in Fig. 1.

SD response to factors other than C_i

Signalling in stomatal differentiation requires membrane-bound receptors, regulatory peptide ligands, a mitogen-activated protein (MAP) kinase module and transcription factors with the

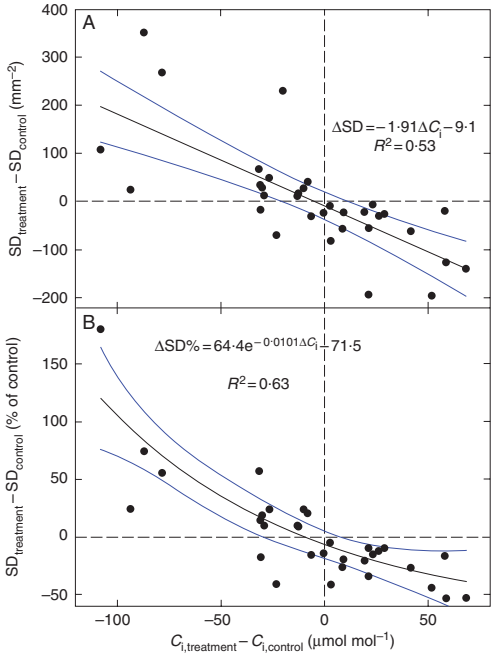

FIG. 5. The effect of various environmental factors on concomitant changes in the internal CO_2 concentration (C_i) and stomatal density (SD) of leaves. The C_i values were calculated from carbon isotope discrimination data extracted together with SD values from 17 publications presenting factorial experiments. The differences between treatment and control plants in SD values (A) and in SD normalized to SD of control (B) are shown together with the best fits and 95 % confidence intervals. The compiled data are shown in Supplementary Data Table S1, and come from the following studies: Bradford *et al.* (1983), Van de Water *et al.* (1994), Beerling (1997), Sun *et al.* (2003), Gitz *et al.* (2005), Takahashi and Mikami (2006), Aucour *et al.* (2008), He *et al.* (2008), Sekiya and Yano (2008), Lake *et al.* (2009), Yan *et al.* (2009), Craven *et al.* (2010), Gorsuch *et al.* (2010), He *et al.* (2012), Sun *et al.* (2012), Yan *et al.* (2012) and Rogiers and Clarke (2013).

respective genes expressed mostly in the epidermis, but also in the mesophyll (Casson and Gray, 2008; Shimada *et al.*, 2011; Pillitteri and Torii, 2012). Co-ordinated and light-dependent development of stomata and chloroplasts is also mediated by brassinosteroids and other phytohormones (Wang *et al.*, 2012). The complex signalling pathway leads to division of protodermal cells, with an increasing proportion of stomata among all epidermal cells (SI), and a rising number of stomata per unit of leaf area (SD) in the early phase of epidermal development. Later on in the phase of intensive leaf area enlargement, presumably all epidermal cells extend in size proportionally, keeping SI stable and reducing SD (Asl *et al.*, 2011). Therefore, the final density and size of stomata on mature leaves is the result of interactions between genomic and environmental factors controlling the entry of cells into the stomatal lineage and their expansion. The idea that such a complex signalling pathway would respond exclusively to only one environmentally modulated factor such as C_i has to be treated with caution. Indeed, a direct response of stomatal development to the red light-activated form of phytochrome B and to blue light signals has recently been observed in arabidopsis (Boccalandro *et al.*, 2009; Casson *et al.*, 2009; Kang *et al.*, 2009). These wavelength-specific light effects on SD often do not conform to the negative SD∼C_i relationship shown here. Thus, it seems that there could be two pathways in the light control of stomatal development: a photomorphogenesis-linked wavelength-specific mechanism and the C_i-mediated, probably CO_2 assimilation-based, control. Due to their specific response to C_i, both pathways probably converge downstream of the putative C_i signalling point. Obviously, the SI sensitivity to C_i, shown in Fig. 4C for true leaves of garden cress, contradicts this scheme. The reasons are not clear and remain to be revealed. Perhaps true leaves of plantlets grown at low irradiances were not fully developed and had a higher proportion of pavement cells and lower SI than at high irradiance. Alternatively, another unknown factor apart from those controlled here affected the proportion of stomata especially on the abaxial leaf side (Supplementary Data Fig. S4C).

Is CO_2 the underlying factor?

In this study, we show that SD correlates with C_i in true leaves. It does not necessarily mean that there is casual relationship between abundance or activity of CO_2 molecules in the leaf interior and stomatal development. One aspect which must be considered is that the C_i values were inferred from discrimination against $^{13}CO_2$, which takes place in the photosynthetically active leaf in light and is recorded in the leaf bulk dry mass (Farquhar *et al.*, 1989). In addition, C_i estimated from ^{13}C is not a simple time average but the photosynthesis-weighted value of C_i averaged over the photoperiod (Farquhar, 1989). Thus, supposing that the C_i-related link to SD exists, we can expect that the putative factor modulating stomatal density and/or size is either photosynthesis-weighted C_i (C_{iP}) or some C_{iP}-linked intermediate averaged over the photoperiod. However, the daylight specificity of C_i as a signal in stomatal size and density remains to be confirmed. Data on the diurnal course of C_i during leaf development and SD in the developed leaf are rare. Recently, Rogiers and Clarke (2013) showed that elevated root-zone temperature increased mid-day and reduced nocturnal C_i (determined via gas exchange) in grapevine. Leaves that emerged during root-zone warming had a lower SD. These results support our finding that higher C_i leads to reduced frequency of stomata and that the daytime, not nocturnal, C_i is the controlling factor. Photorespiration is another powerful internal source of CO_2 in light-exposed leaves which could also be used for testing the C_i effect on SD. Ramonell *et al.* (2001) showed that downregulation of the photorespiratory source of CO_2 by the O_2 content in ambient atmosphere being reduced to 2·5 %, and presumably a reduced C_i, increased both SD and starch content in newly developed leaves of arabidopsis. This indicates that CO_2 or a CO_2-derived cue rather than non-structural assimilates could affect SD and stomatal size.

Systemic vs. local signalling

Whole plants (or whole branches in the case of beech) were subject to fairly homogeneous environmental conditions in our experiments. Therefore, it is not possible to judge whether the

CO_2-derived signal was produced directly in the developing leaf or in a mature, lower insertion leaf and transmitted as a systemic signal (Lake *et al*., 2001, 2002; Coupe *et al.*, 2006, and others). Our experiments in which we compared cotyledons and the first leaves of garden cress support this concept of systemic signalling. Despite the stomatal emergence extending through the period of advanced autotrophy, SD on mature cotyledons remained largely insensitive to C_i, C_a and light (Figs 2 and 4; Supplementary Data Fig. S4). Thus we suggest that, without a pre-existing carbon assimilation organ, the information on availability of CO_2 or a CO_2-related factor is not generated, cannot be conveyed to the developing cotyledons and cannot modulate the genetic programme of development of the stomata and epidermis. Nevertheless, cotyledons are probably competent in production of the systemic signal and transport to the true leaves at 7–9 DAW when they appear.

Conclusions

The SD and PCD of mature leaves co-vary with ^{13}C discrimination caused by altering various environmental factors while keeping ambient CO_2 stable. This translates into an inverse association between SD, PCD and the daytime-integrated internal CO_2 concentration of the leaf. There is only a small (if any) change in the relative proportion of stomata to other epidermal cells (SI), with one exception, in a large number of experimental comparisons. Therefore, the results demonstrate overwhelmingly that the reduction in C_i hinders the expansion of leaf area, increases SD and reduces the size of stomata. In contrast, elevated C_i stimulates the expansion, increases the size of stomata and reduces SD. We suggest that the apparent C_i-dependent modulation of SD and PCD could translate the mesophyll demand for CO_2 into a pattern of more numerous and smaller stomata on newly developed leaves. The putative C_i-sensing mechanism could integrate several environmental factors and relies on a signal transported from older, photosynthetically competent organs, including cotyledons in the case of the first true leaves. In contrast, the absence of older photosynthetic organs probably prevented the adjustment of the SD of cotyledons in response to C_i and environmental perturbations.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Fig. S1: time course of ^{13}C discrimination in garden cress plantlets grown from seed for up to 21 d after watering in air or helox atmosphere at high or low humidity, under total pressure reduced to one-half of normal pressure, and at three different atmospheric CO_2 mixing ratios. Fig. S2: time course of stomatal density in garden cress plantlets grown from seed for up to 21 d after watering in air or helox atmosphere at high or low humidity, under total pressure reduced to one-half of normal pressure, and at three different atmospheric CO_2 mixing ratios. Fig. S3: kinetics of seed-derived carbon in cotyledons of garden cress plants grown at three different ambient CO_2 concentrations from seeds for 14–21 d after the seed watering in an artificially mixed atmosphere. Fig. S4: details of the stomatal density, pavement cell density and stomatal index response of garden cress true leaves and cotyledons to leaf internal CO_2 concentration. Table S1: carbon isotope discrimination and stomatal density data compiled from published controlled factorial experiments with dicotyledonous plants.

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APPENDIX

δ ^{13}C-based determination of the internal CO_2 concentration in mixotrophic tissues of leaves

Stomata develop at an early stage of leaf or cotyledon ontogeny. In *L. sativum,* it was possible to observe the first differentiated stomata on folded cotyledons as early as 48 h after seed soaking (data not shown). Cotyledons are almost entirely heterotrophic and built from seed carbon at that stage of development, which prevents the determination of internal CO_2 concentration in the cotyledon from ^{13}C abundance in its dry mass. A fraction f of the carbon forming the cotyledon is supplied from seed storage and keeps (with presumably minimum change) its ^{13}C abundance (δ_s). The rest of the cotyledon's carbon, $1 - f$, is assimilated by photosynthetic CO_2 fixation and obeys the isotopic fractionation caused mainly by CO_2 diffusion and Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) carboxylation, resulting in ^{13}C depletion of newly formed triose phosphates expressed as δ_t. Only the latter part provides isotopic information on actual growth conditions and can be used for the calculation of C_i in developing cotyledons, provided f is available.The two-source carbon mixing model can be used to express the isotopic composition of the germinating plantlets δ_p:

$$\delta_p = \delta_s f + \delta_t(1 - f) \tag{A1}$$

^{13}C depletion of the newly formed triose phosphates against ambient air, Δ_t, depends on the ratio of the internal CO_2 of the cotyledons and the external CO_2 concentrations, C_i/C_a (Farquhar *et al.*, 1989), as:

$$\Delta_t \approx \delta_a - \delta_t = a + [(b - a)C_i/C_a] \tag{A2}$$

where a and b denote fractionation factors due to diffusion in gas phase and carboxylation by Rubisco. Substitution for δ_t from eqn (A2) into eqn (A1) yields:

$$\delta_p = \delta_a + \{(f - 1)[a + (b - a)C_i/C_a]\} \tag{A3}$$

under the condition that the seeds and atmosphere have the same δ values, $\delta_s \cong \delta_a$, which is not unusual when compressed CO_2 of fossil origin is used as a source for CO_2 in the mixed atmosphere where the seeds germinate. In our case δ_a and δ_s were −28·19 and −28·13 ‰, respectively.

To calculate C_i from this equation, we have to know the fraction of seed carbon f. We derived this value using the time course of isotopic composition δ_p during the plantlets' early development from germination ($f = 1$ at DAW = 0) to the fully autotrophic stage ($f = 0$ at DAW = 14 in our conditions). The typical time course of δ_p had a sigmoid shape, approaching the most negative values between 14 and 21 DAW (Supplementary Data Fig. S3). The δ_p asymptote, δ_{pl}, estimated from sigmoid regression of the δ_p kinetics for true leaves, indicated the fully autotrophic stage. We re-scaled the $\delta_s - \delta_{pl}$ values into the 1–0 range of f and calculated the fraction f in cotyledons of any particular age. The δ_p and f kinetics were specific for various C_a, indicating faster development at higher C_a concentrations (Supplementary Data Fig. S3). The kinetics also varied between plants grown in helox or air, with slightly higher slopes and faster development in helox. With f available, it was possible to calculate C_i in cotyledons and young true leaves as

$$C_i = \left(\frac{\delta_p - \delta_a}{f - 1} - a\right)\frac{C_a}{b - a} \tag{A4}$$

SUPPLEMENTARY DATA

Fig. S1. Time course of ^{13}C discrimination ($\delta^{13}C$) in garden cress (*Lepidium sativum*) plantlets grown from seed for up to 21 days after watering (DAW) in air or helox atmosphere at high (HH; A,D,G) or low humidity (LH; B,E,H), under total pressure reduced to one-half of normal pressure (RP: C,F,I), and at three different atmospheric CO_2 mixing ratios C_a: subambient (180 µmol mol^{-1}; A–C), ambient (400 µmol mol^{-1}; D–F) or superambient (800 µmol mol^{-1}; G–I). Isotopic compositions of CO_2 in the mixed atmosphere (δ_a) and of seed carbon (δ_s) were –28.19 ‰ and -28.13 ‰, respectively. Means and standard deviations (n = 3) are shown. The data indicate that (i) δ of cotyledons at 0, 7, 14 and 21 DAW follows a sigmoid-like time course and approaches the δ value of true leaves after 21 days; (ii) helox-grown plants were almost always depleted in ^{13}C compared to air-grown plants; (iii) ^{13}C discrimination increases (δ becomes more negative) with rising C_a; (iv) the discrimination in hypobaric plants (RP) is remarkably decreased (less negative δ) than in plants grown at normal atmospheric pressure with similar C_a (compare RP at 800/2 with LH at 400 and RP at 400/2 with LH at 180 µmol mol^{-1}).

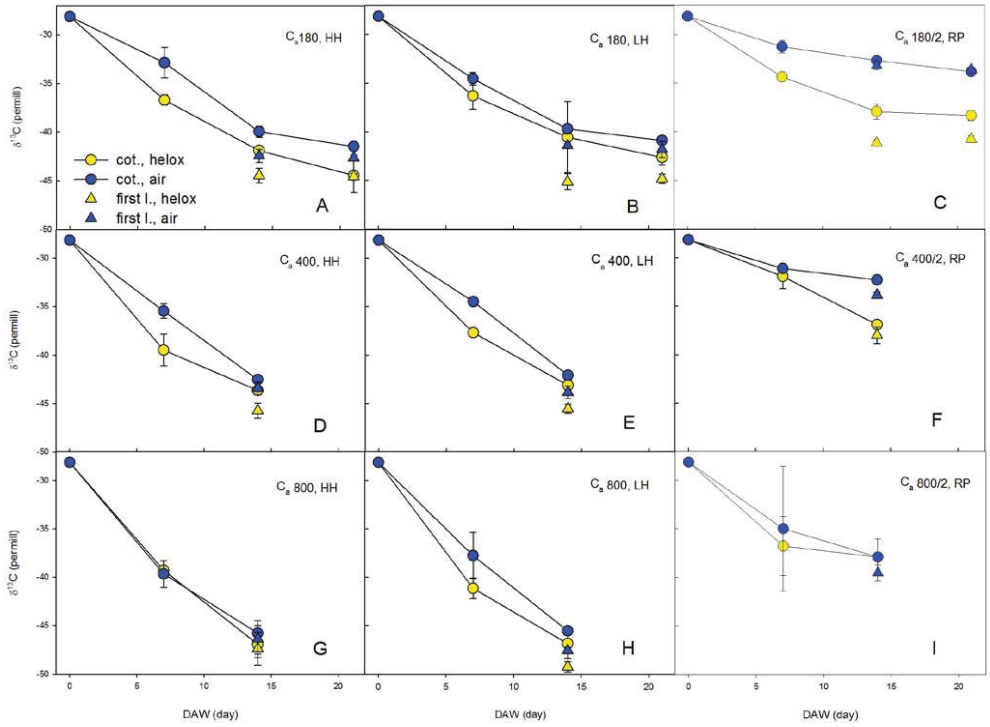

Fig. S2. Time course of stomatal density in garden cress (*Lepidium sativum*) plantlets grown from seed for up to 21 days after watering (DAW) in air or helox atmosphere at high (HH; A,D,G) or low humidity (LH; B,E,H), under total pressure reduced to one-half of normal pressure (RP; C,F,I), and at three different atmospheric CO_2 mixing ratios C_a: subambient (180 µmol mol^{-1}; A–C), ambient (400 µmol mol^{-1}; D–F) or superambient (800 µmol mol^{-1}; G–I). Data points are means of total number of stomata per mm^{-2} of projected leaf area (adaxial plus abaxial side) of counts on 60 areas in samples from three plants. Bars represent standard deviations. The data indicate that (i) stomatal density in cotyledons is insensitive to C_a as well as to atmospheric humidity and to reduced atmospheric pressure; (ii) stomatal density of true leaves decreases with increasing C_a and reduced atmospheric humidity; (iii) stomatal density on hypobaric plant leaves is increased compared to plants grown under normal pressure.

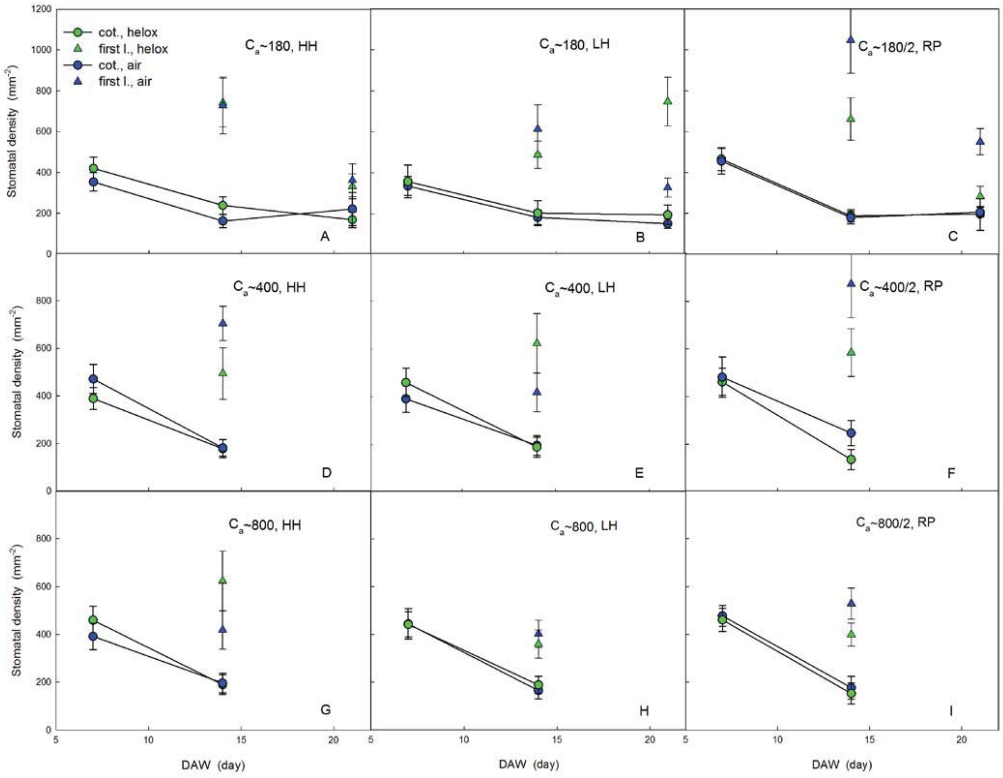

Fig. S3. Kinetics of seed-derived carbon in cotyledons of garden cress plants grown at three different ambient CO_2 concentrations from seeds for 14–21 days after the seed watering (DAW) in artificially mixed atmosphere. Other growth conditions are described in legend of Figs S1 and S2. Sigmoid regression curves are shown. The fraction of seed-derived carbon *f* was calculated from time-course of carbon isotope composition $\delta^{13}C$ in dry matter of the cotyledons and true leaves.

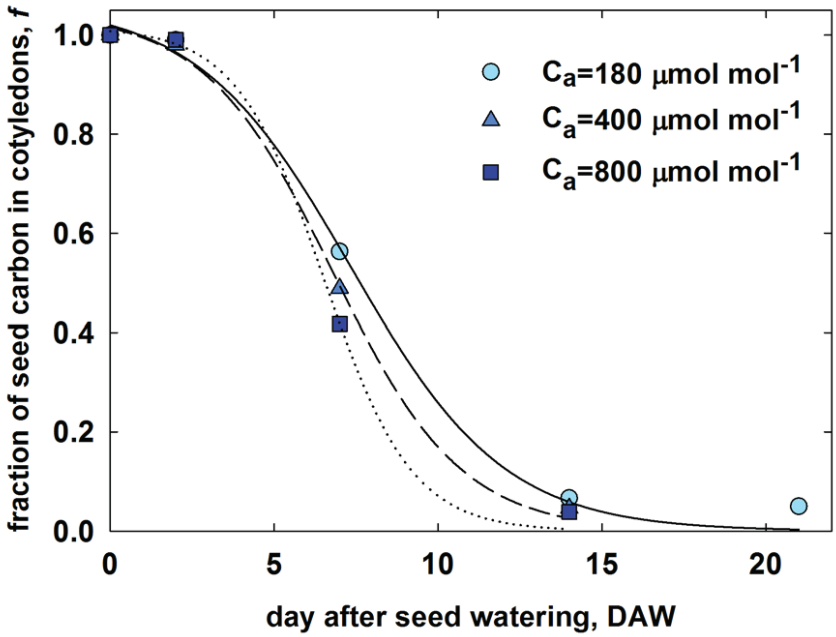

Fig. S4. Details of the stomatal density SD, pavement cell density PCD and stomatal index SI response of garden cress true leaves and cotyledons to leaf internal CO_2 concentration C_i. The plants were grown for 21 days at PPFD of 100, 170, 240, 310, 380, 450, 520, 590 µmol (photons) m^{-2} s^{-1}. The data showing total SD, PCD and SI values summed (SD, PCD) or averaged (SI) over both leaf sides are presented in Fig. 4 of the main text. Here, we present values separately for adaxial and abaxial leaf sides and in the form of differences between treatments (all irradiance levels except 310 µmol m^{-2} s^{-1}) and the "control" arbitrarily set as the optimum irradiance of 310 µmol m^{-2} s^{-1}.

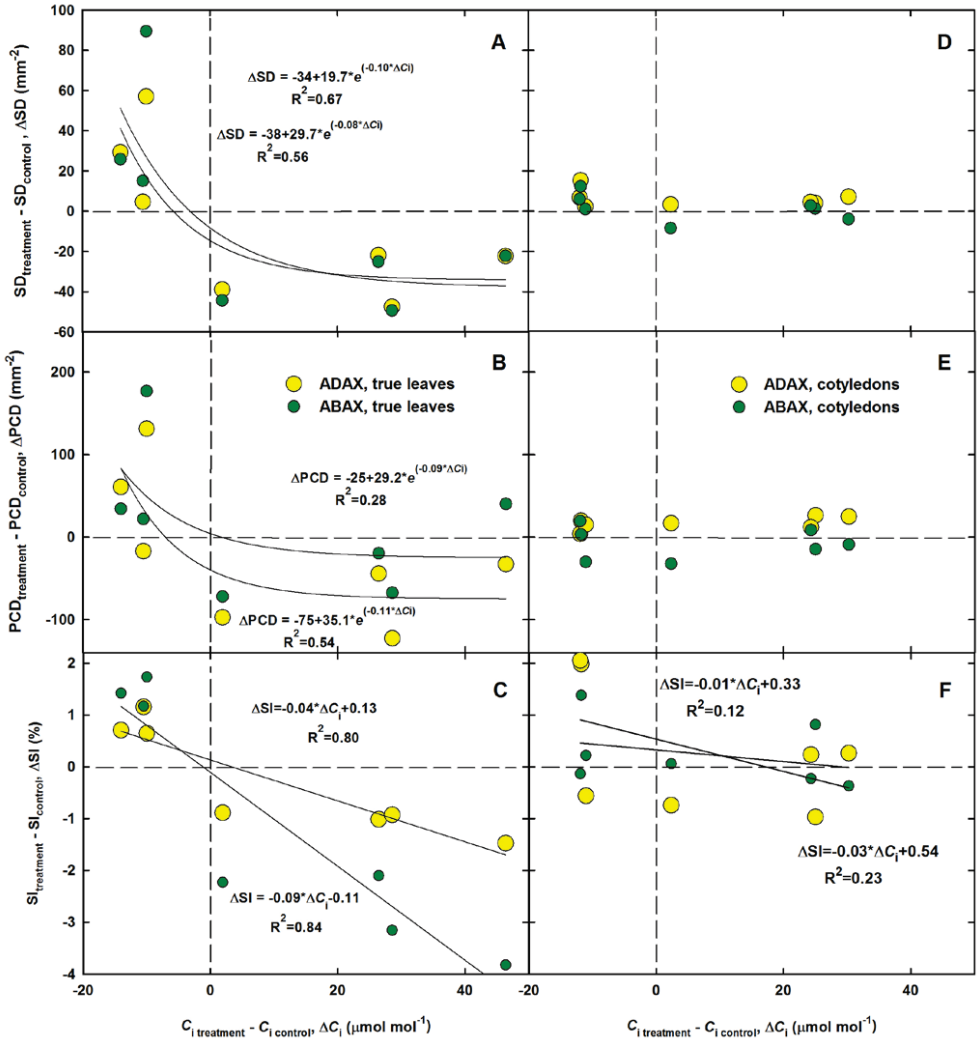

Table S1. Carbon isotope discrimination (δ) and stomatal density (SD) data compiled from published controlled factorial experiments with dicotyledonous plants. The differences of δ and SD between treatments (t) and the respective controls (c) were used in calculation of the treatment effect on leaf internal CO_2 concentration (C_i) using equation 3 and in plotting the C_i response of SD (Fig. 5).

No	species	variant	treatment/level		δ air [‰]	C_a	SD mm^{-2}	Δ or δ	C_i	diff. c-t δ [‰]	difference treat-control SD	C_i	SD [%]	Source
1	*Vigna sinensis*	control c			-8	380	440	16.1	180.0		-			
		treated t	P nutrition, soil water,		-8	380	244	19.4	231.7	3.4	196	51.7	-45	Sekiya & Yano, 2008
2	*Glycine max*	c		-UV	-8	380	170	19.5	233.3					
		t	UV-B	+UV	-8	380	100	18.0	210.1	-1.5	-70	-23.2	-41	Gitz III *et al.*, 2005
3	*Frenelopsis* (3 species)	c			-8	380	60	-27.8	237.9					
		t	salinity		-8	380	168	-20.8	129.8	-7.0	108	-108.1	180	Aucour *et al.*, 2008
4	*Lycopersicon esculentum*	c		-ABA	-8	380	207	-29.6	265.8					
		t	ABA	+ABA	-8	380	248	-29.1	257.7	-0.5	41	-8.2	20	Bradford *et al.*, 1983
5	*Solenites vimineus*	c		high (1896)	-8	380	45	-26.3	214.7					
		t	CO2	low (1512)	-8	380	38	-27.8	237.9	1.5	-7	23.2	-16	Yan *et al.*, 2009
6	*Arabidopsis thal., Col.*	c		-UV	-8	380	563	22.9	285.9		-			
		t	UV-B	+UV	-8	380	369	24.3	307.2	1.4	194	21.3	-34	Lake *et al.*, 2009
7	*Parashorea chinensis*	c		50 m	-8	380	558	-27.8	237.1					
		t	tree height	35 m	-8	380	503	-29.1	258.4	1.4	-55	21.3	-10	He *et al.*, 2008
8	*Oleandra pistillaris*	c		open	-8	380	229	-29.9	270.3					
		t	canopy	understorey	-8	380	167	-32.6	312.0	2.7	-62	41.7	-27	Takahashi & Mikami, 2006
9	*Ginkgo biloba*	c		lit	-8	380	96	-26.6	219.3					
		t		shaded	-8	380	79	-24.6	188.5	-2.0	-17	-30.9	-18	
		c		lit	-8	380	113	-29.3	261.1					
		t	irradiance	shaded	-8	380	91	-29.9	270.3	0.6	-22	9.3	-20	Sun *et al.*, 2003

10	*Acacia koa*	control c		lit	-8	380	237	-28.0	241.1				
		treat. t		med. shaded	-8	380	207	-29.7	267.4	1.7	-30	26.2	-13
		c	irradiance, 100 % soil water capacity	lit	-8	380	237	-28.0	241.1				
		t		shaded	-8	380	111	-31.8	299.8	3.8	-127	58.7	-53
		c	Irradiance, 20 % soil water capacity	lit	-8	380	263	-27.1	227.8				
		t		med. shaded	-8	380	237	-29.0	256.7	1.9	-26	29.0	-10
		c		lit	-8	380	263	-27.1	227.8				
		t		shaded	-8	380	123	-31.6	296.1	4.4	-140	68.3	-53
11	*Pinus sylvestris*	c	CO2	high (560)	-8	560	102	18.9	330.1				
		t		low (~360)	-8	360	127	20.6	236.3	1.7	25	-94	24
		c	irradiance	lit	-8	360	102	18.9	212.0				
		t		shaded	-8	360	81	20.2	231.2	1.3	-21	19.2	-21
12	*Arabidopsis thal., Col.*	c	temperature	warm (25oC)	-8	380	240	-29.7	267.2				
		t		cold (5oC)	-8	380	470	-28.4	247.2	-1.3	230	-20.1	96
13	*Pinus flexilis*	c	CO2	low (glacial)	-5	180	117	-22.3	94.5				
		t		high (holocene)	-6	280	97	-23.8	152.5	1.5	-20	58	-17
14	*Solanum tuberosum*	c	N nutr. DI	medium	-8	380	197	19.8	237.1				
		t		low	-8	380	115	20.0	240.2	0.2	-82	3.1	-42
		c	N nutr. DI	medium	-8	380	197	19.8	237.1				
		t		high	-8	380	214	18.9	224.6	-0.8	17	-12.5	9
		c	N nutr. PRD	medium	-8	380	166	19.3	230.6				
		t		low	-8	380	142	19.3	230.2	0.0	-24	-0.5	-14
		c	N nutr. PRD	medium	-8	380	166	19.3	230.6				
		t		high	-8	380	157	19.5	233.3	0.2	-9	2.6	-5

Craven *et al*., 2010

Beerling 1997

Gorsuch *et al*., 2010

Van de Water *et al*., 1994

Yan *et al*., 2012

15	*Vitis vinifera*	c		warm			117		275.4					Rogiers &Clarke, 2013
		t	root temp.	cool			128		262.3		11	-13	10	
16	*Betula mioluminifera*	c		low	-6	316	256	-29.1	240.6					
		t		high	-6	387	207	-30.9	321.7	1.7	-49	81	-19	
	Carpinus miofangiana	c		low	-6	316	278	-28.3	229.7					
		t	CO2	high	-6	387	244	-30.3	313.1	2.0	-34	83	-12	Sun *et al.*, 2012
17	*Medicago sativa,*	c		75	-8	380	118	-29.6	265.7					
		t		25	-8	380	185	-27.6	234.0	-2.1	67	-31.7	57	
	cv. Algonquin, Longdon, Xinjiangdaye	c		75	-8	380	154	-29.4	263.1					
		t		25	-8	380	182	-27.5	232.9	-2.0	28	-30.1	18	
		c	soil water	75	-8	380	139	-29.6	265.2					
		t	capacity (%)	25	-8	380	152	-27.7	236.0	-1.9	13	-29.2	9	He *et al.*, 2012

PAPER III (Manuscript)

Light intensity-regulated stomatal development in three generations of *Lepidium sativum*

Vráblová, M., Hronková, M., Vrábl, D., Kubásek, J. and Šantrůček, J. (2017)

Light intensity-regulated stomatal development in three generations of *Lepidium sativum.*

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ABSTRACT

Stomata are crucial for CO_2 uptake and water economy in higher plants. Stomatal development is influenced by environmental conditions. During leaf development stomata integrate both leaf-internal and external environmental signals and mature leaves, being fully exposed to the environment, signalize to younger developing leaves. In general, high light (photosynthetic photon flux density, PPFD) actually experienced by the plant increases stomatal density (SD). Moreover, the parental environment can affect offspring's phenotypes. Here we compared actual and transgenerational effects of light on SD. We planted three generations of garden cress in two contrasting light environments. Stomatal and pavement cell density (SD, PCD), ^{13}C abundance as proxy of leaf internal CO_2 concentration, and leaf area were analysed in cotyledons and first leaves of mothers and offspring. Our results indicate that stomata of offspring reflect the light environment of their mother in addition to the actual-light sensitivity of SD. The transgenerational (maternal) effect was much lower than the actual (environmental) one and decreased SD relatively to SD of plants grown in invariable light for generations. Cotyledons' SD was irresponsive to variation of ^{13}C but retain light-sensitivity of SD though reduced compared to true leaves. High light reduced amphistomy in favour of lower (abaxial) leaf side. SD and PCD were linearly proportional irrespective of light level, indicating invariant portions of spacing and amplifying cell divisions and causing lower sensitivity of stomatal index at high SD.

INTRODUCTION

Stomata are epidermal structures enabling gas exchange between a leaf and the atmosphere. The ability to control stomatal aperture as well as the number and size of stomata on leaf epidermis together affect the amount of CO_2 assimilated through photosynthesis and water lost due to transpiration. Leaf stomatal density (number of stomata per unit leaf area; SD) is therefore a significant parameter for plant adaptation to environmental conditions.

There are several levels of control over stomatal development: control over the spacing of individual cells, to ensure that stomata are separated by at least one epidermal pavement cell; control of stomata numbers as part of tissue patterning during development; and control of stomata numbers by environmental conditions. These different levels of control must be coordinated to produce the final numbers of stomata found on mature leaves (Bird and Gray, 2003). Ambient CO_2 concentration, light intensity and spectral composition, and humidity (soil and atmosphere) are the most important environmental cues modulating stomatal density (Woodward *et al.*, 1987; Beerling and Woodward 1995; Serna and Fenoll, 1997; Gray *et al.*, 2000). Molecular mechanisms for the formation of stomata have been intensively studied over the past two decades in Arabidopsis as a model plant (Geisler *et al*., 1998; Nadeau and Sack, 2002; Serna, 2009; Vaten and Bergmann, 2012; Lee *et al.*, 2014). It was found out that the development of stomata is regulated by both positive and negative signals from the epidermis and inner leaf tissues (Shimada *et al.*, 2011; Vaten and Bergmann, 2012).

Stomatal patterning responds to changes in light intensity. In general, an increase in light intensity results in an increase in stomatal index (SI; the ratio of stomata to all epidermal cells). Signal coming from mature leaves determined stomatal density of newly developed leaves in shading experiments with Arabidopsis plants (Lake *et al.*, 2001; Thomas *et al.*, 2004). A pivotal role in leaf-to-leaf signalling has been attributed to abscisic acid (ABA) (Lake and Woodward, 2008) or the ^{13}C content in assimilates (Sekiya & Yano, 2008). Sun leaves have typically higher SD than their shaded counterparts (Schoch *et al.*, 1984; Ticha, 1982; Matos *et al.*, 2009). Molecular control of light-induced stomatal development is attributed to the red part of the spectrum perceptible

by the PHYTOCHROME B photoreceptor (Boccalandro *et al.*, 2009; Casson *et al.*, 2009; Kang *et al.*, 2009). The role of STOMAGEN, the positive regulator EPFL9, in stomatal development response to light, was also proposed (Hronkova *et al.*, 2015).

Plants are organisms with a little choice in their grown environment and usually limited seed dispersal. The maternal environment can have effect on offspring phenotypes and should represent a flexible evolutionary mechanism for adaptation to heterogeneous environments (Donohue, 2009). This phenomenon known as transgenerational effects (or parental effects), occurs when offspring phenotype is influenced by a parental phenotype independently of the genes that the parents pass to the offspring. Mechanisms underlying parental effects can be categorized as seed modification (Roach and Wulff, 1987) or epigenetic variation (Rossiter, 1996). Parental effects mediated by seed modification are implemented during early stages of plant development, whereas epigenetic variation can alter phenotypes for whole life of offspring. Transgenerational effects due to maternal environmental conditions were widely studied. Walter *et al.* 2016 showed that variation in the maternal environment (extreme weather) not only affects the number, but also the performance of offspring. Extreme climatic events, terminated before seed set, induced transgenerational effects. Transgenerational plasticity in plant adaptation to light environment was also investigated and it was demonstrated that offspring have greater rates of germination and early life survival when planted into their maternal environment and that these early life effects influence its fitness (Galloway and Etterson, 2007). Further, the supply of carbohydrate from the maternal plant to the developing fruit at night can have an important influence on oilseed composition and on postgerminative growth in *Arabidopsis thaliana* (Andriotis *et al.*, 2012). Similarly, amount of carbohydrate storage in roots of plants grown at two nutrient levels was affected by nutrient conditions experienced by their mothers (Latzel *et al.*, 2014). In poor nutrient conditions of offspring environment, belowground carbohydrate storage was higher for offspring which mother experienced poor nutrient conditions, and *vice versa* - offspring grown in rich nutrient conditions had higher belowground carbohydrate storage in roots when their mother grew

in rich nutrient conditions. On molecular basis, Tricker *et al.* (2013) examined transgenerational inheritance of low relative humidity-induced DNA methylation for two gene loci in the stomatal developmental pathway in Arabidopsis and the abundance of associated short-interfering RNAs (siRNAs). They found out that transgenerational methylation and a parental low humidity-induced stomatal phenotype were heritable. On the other hand, substantial genetic variation was found for stomatal abundance-related traits in *Arabidopsis thaliana*, which were weakly or not affected by laboratory maternal environments (Delgado *et al.*, 2011).

Here, we explored light-induced stomatal development in garden cress (*Lepidium sativum*) grown under two contrasting light intensities. In addition to evaluating direct influence of light quantity on stomatal development in plants of the same generation, we hypothesized different maternal effect of light on cotyledons and true leaves in offspring. Three generations of garden cress were studied for the maternal effect. Carbon stable isotopes of leaf tissue were used for CO_2 diffusion evaluation. Stomata and pavement cells of adaxial and abaxial epidermes were counted on fully developed cotyledons and first true leaves, thus stomatal density, pavement cell density and stomatal index were calculated. Besides this, estimation of leaf area was used for growth evaluation. Results for *i)* plants grown under low light (LI) and high light intensity (HI) and *ii)* plants grown from seeds from contrasting maternal environments (LI or HI) were compared and environmental (actual) and maternal (transgenerational) effects were distinguished.

MATERIAL AND METHODS

Plant material and growth conditions

Garden cress (*Lepidium sativum*) plants were grown in soil in a growth chamber (Fitotron, Sanyo, UK) equipped with fluorescence tubes (Osram Delux L 55W/830 – warm white) under two levels of irradiance [photosynthetic photon flux density (PPFD)]: 500 μmol m^{-2} s^{-1} (high intensity, HI) or 80 μmol m^{-2} s^{-1} (low intensity, LI). All plants were exposed to a free

atmospheric CO_2 concentration of about 390 ppm, 60 % relative air humidity, day/night temperatures of 23/21 °C and a 16 h photoperiod. From ten to thirty individuals were planted in each group ($n \geq 10$). Plants were grown to seed formation; seeds were collected and used in the follow-up experiment (Fig. 1).

Experiment 1

Commercially available seeds of garden cress (SEMO, Smržice, Czech Republic) were grown in HI or LI conditions.

Experiment 2

Seeds from plants in Experiment 1 (mother plants) were grown in HI or LI conditions. Four variants of first generation of offspring differing in maternal environment and offspring environment were planted (mother – offspring): HI-HI, HI-LI, LI-HI and LI-LI. Seeds from HI-HI and LI-LI plants were collected and used in other experiment.

Experiment3

Seeds form plants in Experiment 2 were grown again in HI or LI conditions. Four variants of second generation of offspring were planted: HI2-HI, HI2-LI, LI2-HI and LI2-LI. In some cases (repeated LI conditions in LI2-LI variant), a worse fitness of seeds led to poor germination, smaller increments of biomass and therefore we didn't have enough plant material for data evaluation ($n < 10$).

Experiment4

Seeds used in Experiments 1 and 2 were planted again at the same time to ensure the same growth conditions. Six groups of plants were grown: mother HI and LI, offspring HI-HI, HI-LI, LI-HI, LI-LI.

Stomatal and pavement cell density

Stomatal and pavement cell numbers per unit leaf area were determined from nail polish imprints of both abaxial and adaxial leaf sides of fully developed cotyledons and first true leaves. A light microscope (Olympus BX61) with 50x magnification and a digital camera (Canon 1000D) were used to photograph a leaf surface area of 0.129 mm^2. Stomatal and pavement cells were counted in five areas from each leaf side and their density expressed as number per mm^2 (stomatal density – SD, pavement cell density – PCD).

Stomatal index (SI) was calculated as the fraction (%) of stomata of all epidermal cells: SI = (number of stomata)/(number of stomata + number of other epidermal cells) x 100 %. Ten cotyledons and ten leaves from each group of plants were evaluated. A pair of cotyledons and a pair of first leaves were used from each plant; always one of the pair was used for abaxial, resp. adaxial epidermis assessment.

Leaf area

Cotyledons and leaves taken for imprinting were fixed on an adhesive tape before a nail polish application. Digital camera (Canon 1000D) was used to photograph cotyledons and leaves together with a scale. ImageJ software (NIH) was used for leaf area calculation based on number of pixels.

Carbon isotope composition

Carbon isotope composition was estimated in cotyledons and leaves and served as a proxy of CO_2 concentration inside the assimilation organs. Ten individuals (cotyledons and leaves) from each group of plants were analysed. Samples were dried, finely milled and wrapped in tin capsules. ^{13}C relative abundance ($\delta^{13}C$) in dry mass was determined using an EA1110 elemental analyser (ThermoQuest, Milan, Italy) linked to an isotope ratio mass spectrometer (IRMS, Delta XL^{Plus}, ThermoFinnigan, Bremen, Germany). The results were calculated versus VPDB standard (IAEA, Vienna, Austria) and expressed as $\delta^{13}C = (R_{sample}/R_{std} - 1) \times 1000$ ‰, where R_{sample} and R_{std} represent the $^{13}C/^{12}C$ ratio in the plant sample and VPDB standard, respectively. The standard deviation of ^{13}C analysis was 0.1 ‰.

Calculation of environmental and maternal effects on SD

SD increment (%) caused by environmental (EnvE) or maternal (MatE) effects was calculated as the difference between HI and LI plants related to LI variant: $SD_i = ((SD_{HI} - SD_{LI}) / SD_{LI}) \times 100$ %. In the environmental effect (EnvE) calculation, HI and LI mean actual growth light conditions, whereas in the maternal effect (MatE) calculation the HI and LI symbols mean maternal environments [e.g. $(SD_{HI\text{-}HI} - SD_{HI\text{-}LI})/(SD_{HI\text{-}LI})$ x 100 % for EnvE,

(SD_{HI-HI} - SD_{LI-HI})/(SD_{LI-HI}) x 100% for MatE]. Data were calculated as total SD and/or adaxial and abaxial SD for cotyledons and for first leaves. In maternal generations, only environmental effect was calculated (history of seeds was unknown).

RESULTS

Stomatal development in different light environments

Light intensity affected stomatal density in cotyledons and first true leaves of garden cress (Fig. 2). In cotyledons, the effect of light was less than in leaves (see also Fig. 7), where HI led to 2.2-fold increase in SD compared to LI. In cotyledons and leaves abaxial epidermis had higher SD (SD_{abax}) than adaxial one (SD_{adax}). The amphistomy ratio (SD_{abax}/SD_{adax}) was smaller (closer to 1) in LI compared to HI in cotyledons (1.3 and 2.0, respectively), and the same for HI and LI in true leaves (1.6). Offspring of HI-mother grown in HI environment had usually more stomata than offspring of LI-mother grown in HI and, *vice versa*, offspring of LI-mother developed more stomata then offspring of HI-mother when both experienced the same LI environment.

The level of coupling in stomata development between opposite leaf sides can be assessed from the separate estimates of SD_{adax} and SD_{abax}. There was no clear relationship between SD_{adax} and SD_{abax} in cotyledons irrespective of PPFD (Fig. 3A). Nevertheless, a tendency in HI-stimulated production of stomata on abaxial relative to adaxial side of cotyledons is remarkable. In contrast, adaxial epidermis of true leaves has less stomata than the abaxial one (69 % in average) in both HI and LI (Fig. 3B).

The difference between SD in leaves and cotyledons ($SD_{leaf} - SD_{cotyledon}$) in plants grown under different PPFD revealed a high-light-induced production/activity of an SD-promoting factor putatively transported from cotyledons into true leaves: HI in the current generation increased ($SD_{leaf} - SD_{cotyledon}$) on both abaxial and adaxial leaf sides compared to LI (Fig. 4).

SD was related linearly to pavement cell density (PCD) in both cotyledons and true leaves (Figs. 5 and S1). The slope of SD to PCD in HI and LI groups of plants was the same (SD = 0.43 x PCD), whereas intercepts differed (- 5.5 in HI, -32.4 in LI). The relation of SD to PCD was similar in cotyledons and leaves. As expected, SI and SD were related non-linearly (Fig. S1). SD was not affected by leaf area in cotyledons (Fig. 6) but a slight effect was visible in first leaves, where larger leaves (HI variant) tended to have more stomata per unit of the leaf area.

Relative abundance of ^{13}C in the leaf tissue was linked to SD but not so in cotyledons (Fig. 7). Cotyledons grown in LI or HI and possessing similar SD differed in $\delta^{13}C$: HI were enriched in ^{13}C (less negative values of $\delta^{13}C$) relative to LI. In the first leaves HI plants with high SD were also ^{13}C enriched which indicates lower leaf internal CO_2 concentration (C_i) values corresponding with expected higher photosynthesis rate. In first leaves, ^{13}C discrimination of LI-grown leaves was similar to that of cotyledons, whereas HI-grown leaves discriminated ^{13}C to less extent compared to its cotyledons.

Maternal effect

SD of first leaves was affected by light intensity more than by environmental history of seeds (Fig. 8, data from Experiment 1 and 3). Large variability was found in absolute values of SD between experiments. Therefore, we calculated SD increment (SD_i in %) caused by both environmental (EnvE) and maternal (MatE) effect in each experiment (Experiment 1 – 4) to obtain a larger dataset (for EnvE and MatE calculations see M&M). The increase of growth light (EnvE) accounted for 35 % increment in parental cotyledons, however EnvE was larger in offspring of HI-mothers (40 %) than in offspring of LI-mothers (10 %) (Fig.9A). MatE was about plus 10 % in offspring currently growing in HI conditions and minus 10 % in offspring experiencing LI conditions. Negative value of MatE means, that offspring of LI-mothers growing again under LI increased SD relatively to offspring of HI-mothers growing under LI. EnvE differed in adaxial and abaxial epidermes and was higher in abaxial leaf side, whereas MatE was almost not influenced by the leaf side (Fig. S2). In first leaves, EnvE was much higher (maximum 120 %) than in cotyledons (Fig.9B).

MatE was almost zero in first leaves of offspring from HI environment and negative in offspring grown in LI environment.

Difference in stomatal density (calculated as SD_{HI} minus SD_{LI}) between offspring lines which originated from HI- or from LI- mothers revealed systematic shift according to the maternal light environment (Fig. 10). In low PPFD, HI-lines (offspring from HI-mothers) tended to have less stomata than LI-lines (offspring from LI-mothers) thus the shift is toward negative values, and *vice versa*, in high PPFD, HI-lines tended to have more stomata than LI-lines thus the shift is toward positive values.

DISCUSSION

As expected, stomatal density was affected by actual light intensity, generally with increasing SD in higher PPFD. The effect of light on formation of stomata was more evident in first leaves than in cotyledons. From previous experiments with garden cress, we hypothesized that cotyledons are not so tightly coupled to external environment (Santrucek *et al.*, 2014). Stomata form on epidermis of garden cress leaves early after the seed hydration (Fig. 11). We found out that stomata in cotyledons developed rapidly during first three days after seed watering; then SD decreased due to predominant leaf area expansion and limited ongoing cell division. Similar time course was described and reviewed several times (e.g. Ticha 1982). Recently, lot of studies have showed factors that positively or negatively regulated cell proliferation and/or expansion and determined the final size of organs (e.g. Hepworth and Lenhard, 2014; Zhao *et al.*, 2017). Nevertheless, the exact mechanism matching stomatal development with leaf area (LA) enlargement is still unknown. In cotyledons of garden cress, SD was not influenced by LA, whereas in first true leaves in average higher SD was accompanied by higher LA when both light environments were pooled. In HI group, SD tended to negatively correlate with LA (Fig. 6). Delgado *et al.* (2011) found negative correlation between cotyledon size and SD in 62 accessories of *Arabidopsis thaliana*. We also observed prolonged presence of meristemoids in HI leaves when day-to-day stomatal development was estimated suggesting longer cell-division phase than

in LI leaves. This is in agreement with previously published results (Hronkova *et al.*, 2015).

Light intensity affected both adaxial and abaxial leaf epidermes; the effect of light intensity on SD was leaf-type and also leaf-side dependent (Fig. 3). Omitting abaxial or adaxial epidermis from evaluation is common in physiological studies (e.g. above mentioned Delgado *et al.*, 2011). It is based on the assumption that stomata develop equally on abaxial and adaxial epidermis, which can be incorrect. In our study, in cotyledons HI caused increment in SD on abaxial relative to adaxial epidermis, whereas in first leaves light intensity affected both leaf sides in the same manner. In general, SD_{abax} is higher than SD_{adax} independently on light intensity and this is also our case with garden cress.

We found a linear relationship between SD and PCD suggesting common regulation of these types of cell divisions (Fig. 5). We observed similar trends in Arabidopsis plants (Hronkova *et al.*, 2015; Vrablova *et al.*, 2017; and also Delgado *et al.*, 2011). In garden cress, the intercepts of linear regression in high PPFD and low PPFD were -5.5 and -32.4, respectively. Thus for formation of "the first stoma", minimum of 5 - 6 (HI) and 32 – 33 (LI) pavement cells is required on leaf epidermis. Slope of the SD vs. PCD regression line was 0.43 in both PPFD, thus for each new stoma 2.3 pavement cells has to be developed. The proportion between SD and PCD (Figs. 5 and S1) is often evaluated by stomatal index. However, this parameter should be used with caution especially at high SD values, when SI changes "saturate" with respect to SD (Fig. S1).

Analysis of ^{13}C discrimination is an effective tool for CO_2 uptake assessment (Evans *et al.*, 1986). Leaves grown under HI have low ^{13}C discrimination ($\delta^{13}C$ values are less negative) (Fig. 7). This is caused by high CO_2 assimilation rate leading to lower leaf internal CO_2 concentration. In cotyledons, maternal ^{13}C is still present in its tissue and during growth is mixed with newly assimilated ^{13}C (see Supplementary information in Santrucek *et al.* 2014 for garden cress). If all external conditions are the same, differences in ^{13}C content in the leaf tissue are caused by leaf anatomy and physiology. This should comprise stomatal density, stomatal

conductance, mesophyll conductance, photosynthetic processes etc. (Evans *et al.*, 1986; Flexas *et al.*, 2007). Therefore, when carbon isotope signal differed within one group of plants (e. g. leaves grown in HI), some anatomical or physiological adjustment leading to different C_i had to be present.

Effect of growth environment seems to be much more significant than maternal effect in both cotyledons and leaves of the 1st generation of offspring (Fig. 9). Lower EnvE in cotyledons than in leaves supports our hypothesis that cotyledons are more independent on external environment partly due to their development within the seed. Cotyledons are supposed to be antenna-like organs enabling plants sensing of its environment (Santrucek *et al.*, 2014). The possibility of signal transduction from older to younger leaves was demonstrated in several experiments (Lake *et al.*, 2001; Miyazawa *et al.*, 2006). Cotyledon-to-leaf signal transduction is therefore presumable and the signal could be a "mother-deposited" information about the prevailing ancestor's environment. To what extent is this maternal information involved in the light--induced stomatal development is shown in Fig. 10. An offspring of HI-mother grown again in HI environment tends to increase SD and similarly an offspring of LI-mother grown again in LI environment increased SD. If we expect that higher SD leads to higher stomatal conductance and CO_2 assimilation rate (see Wong *et al.*, 1979; Buessis *et al.*, 2006; Drake *et al.*, 2013), it seems that offspring thrives better in the same environment as experienced by their mothers. This is in agreement with Latzel *et al.* (2014) who found similar trend in nutrient conditions experiments.

Maternal effect could at least partly depend on epigenetic modification of genes involved in stomata development cascade e.g. SPECHLESS and FAMA (bHLH transcripption factors). Tricker *et al.* (2013) found that these factors became *de novo* cytosine methylated and transcriptionally repressed under low relative humidity conditions. Transgenerational methylation and parental low humidity-induced stomatal phenotype were heritable, but this was reversed in the progeny under repeated treatment in a previously unsuspected manner.

We suppose that in a stable, less variable environment, the offspring should "risk" and enhance the CO_2 uptake through higher stomatal conductance at the expense of water losses. Its fitness then would increase

resulting into higher degree of offspring in next generation. Nevertheless, increment in SD is not enough in itself (Vrablova *et al.*, 2017) and probably other characteristics (e.g. leaf anatomy and biochemical CO_2 fixation) have to change for better fitness.

CONCLUSIONS

Light-induced stomatal development differed between cotyledons and first leaves of garden cress. Cotyledons reflected actual light conditions substantially less than first leaves likely due to their advanced phase of development inside the seed and absence of any preexisting photosynthetically competent organ in the early ontogeny. Yet, cotyledons could sense the light environment and produce relevant signal for stomata development on first leaves.

Carbon isotope ratio ($\delta^{13}C$) was linked to frequency of stomata in true leaves but not in cotyledons. Despite, $\delta^{13}C$ and likely C_i as well varied in cotyledons with growth light. It indicates that stomata on true leaves develop in response to leaf internal CO_2 while the C_i sensitivity is absent in cotyledons.

Leaf sides seem partly autonomous in light-modulated stomatal development. The amphistomy ratio (SD_{abax}/SD_{adax}) departed to higher values (>1) under high light. Surprisingly, this effect was more pronounced in cotyledons than in true leaves which can reflect the differences in anatomy and optical properties between thick cotyledons and thin first leaves.

Stomata and pavement cells were produced proportionally in the 1:2.3 ratio in both cotyledons and true leaves and irrespectively of growth light. It indicates conservative stoichiometry of spacing and amplifying divisions during epidermis development. Consequently, SI is less sensitive to changes in PPFD in leaves with higher SD.

Environmental (light) effect on stomatal density was stronger than maternal (transgenerational) effect and these effects could act in opposite directions. Nevertheless, we confirmed that light environment experienced by the mother plant is imprinted in seeds and expressed in SD of offspring. It seems that the tentative information transmitted by seeds from contrasting

light environment reduce SD compared to generations grown in homogeneous light. Sensing of actual conditions by cotyledons combined with preservation and transfer of ancestor's information to offspring should help plant to enhance its fitness in changing environment.

FIGURES

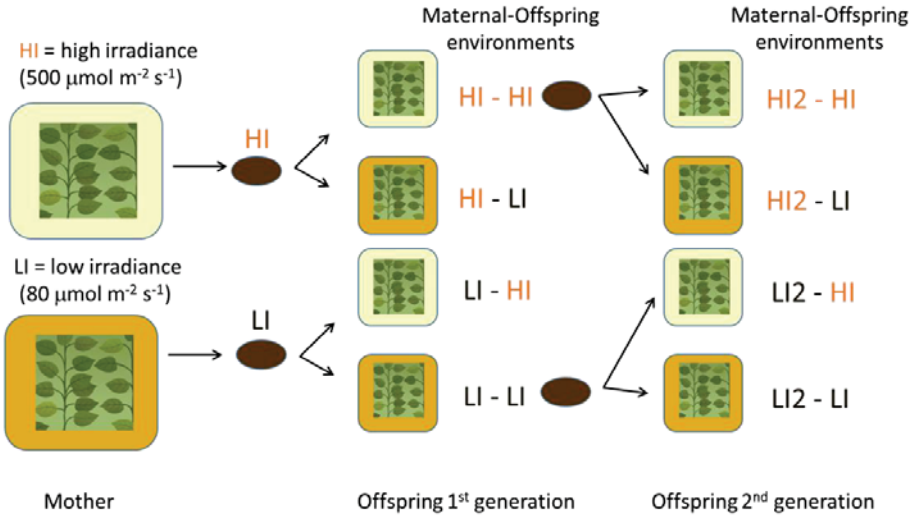

Fig. 1: A scheme of experiments with garden cress. Plants were grown in high PPFD (HI) or low PPFD (LI). Seeds were collected and planted again in HI or LI. This was repeated with seeds from HI-HI and LI-LI variants, thus two generations of offspring and eight variants of maternal-offspring environments were obtained.

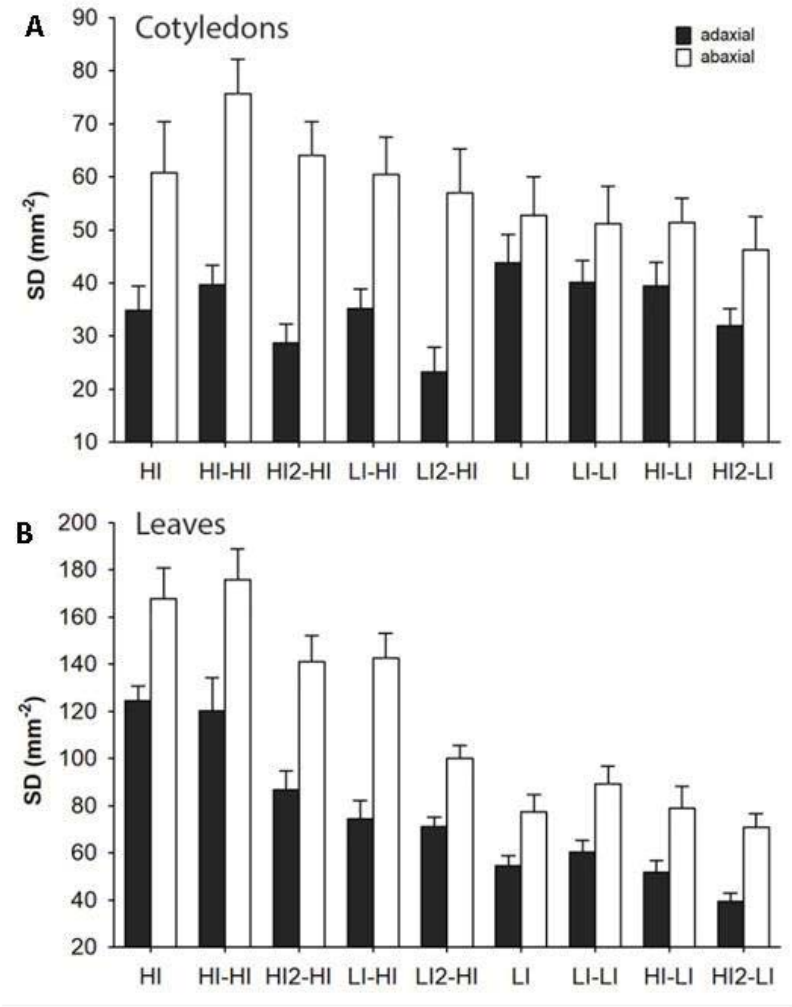

Fig. 2: Stomatal density of maternal (HI, LI), 1st (HI-HI, HI-LI, LI-HI, LI-LI) and 2nd (HI2-HI, HI2-LI, LI2-HI) generation of offspring cotyledons (A) and first true leaves (B) grown under high (HI) and low (LI) PPFD. Adaxial and abaxial leaf sides are shown separately. The columns are sorted by decreasing sum of SD (adax + abax). Columns and bars show mean values and standard errors, respectively ($n \geq 10$).

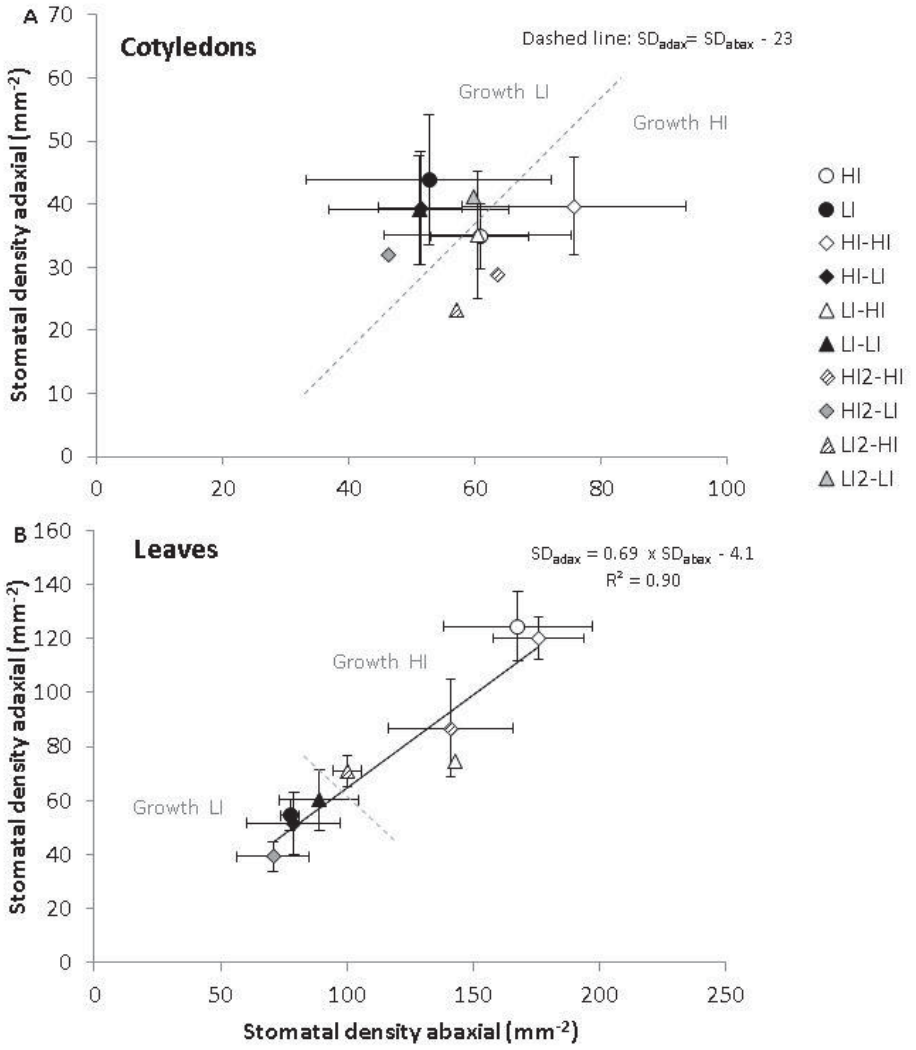

Fig. 3: Stomatal density on adaxial vs. abaxial epidermis in cotyledons (A) and first leaves (B) of garden cress. Maternal generation (HI, LI), 1st generation of offspring (HI-HI, HI-LI, LI-HI, LI-LI) and 2nd generation of offspring (HI2-HI, HI2-LI, LI2-HI, LI2-LI) are shown. Mothers are represented by circles, offspring of HI-mothers by diamonds, offspring of LI-mothers by triangles. Plants were grown in HI (white or dashed symbols) or LI (grey or black symbols) environments. Mean values, standard deviations ($n \geq 10$) and regression line (black) are shown. The broken grey lines separates two growth PPFD (HI = 500 µmol m^{-2} s^{-1}, LI = 80 µmol m^{-2} s^{-1}).

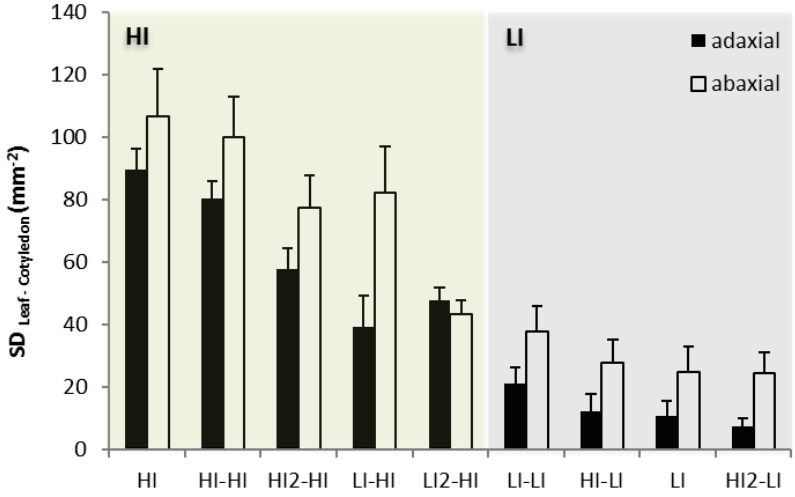

Fig. 4: An increase in SD of garden cress leaves compared to cotyledons in different variants of growth conditions and maternal background. Adaxial (black columns) and abaxial (empty columns) epidermes are shown. Parental line (HI and LI), 1st generation (HI-HI, HI-LI, LI-HI, LI-LI) and 2nd generation (HI2-HI, LI2-HI, HI2-LI) of offspring were estimated. The columns are ordered in descending (adax + abax) SD sum. Columns show mean values; bars indicate standard deviations (n ≥ 10).

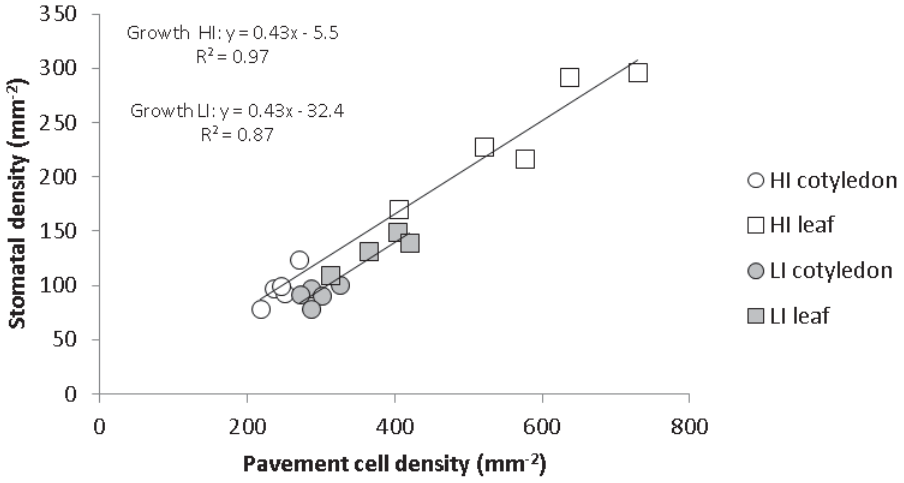

Fig. 5: Relationship between stomatal density and pavement cell density in cotyledons and first leaves of garden cress. Plants were grown in HI (white symbols) or LI (grey symbols) conditions. Mean values are shown (n ≥ 10).

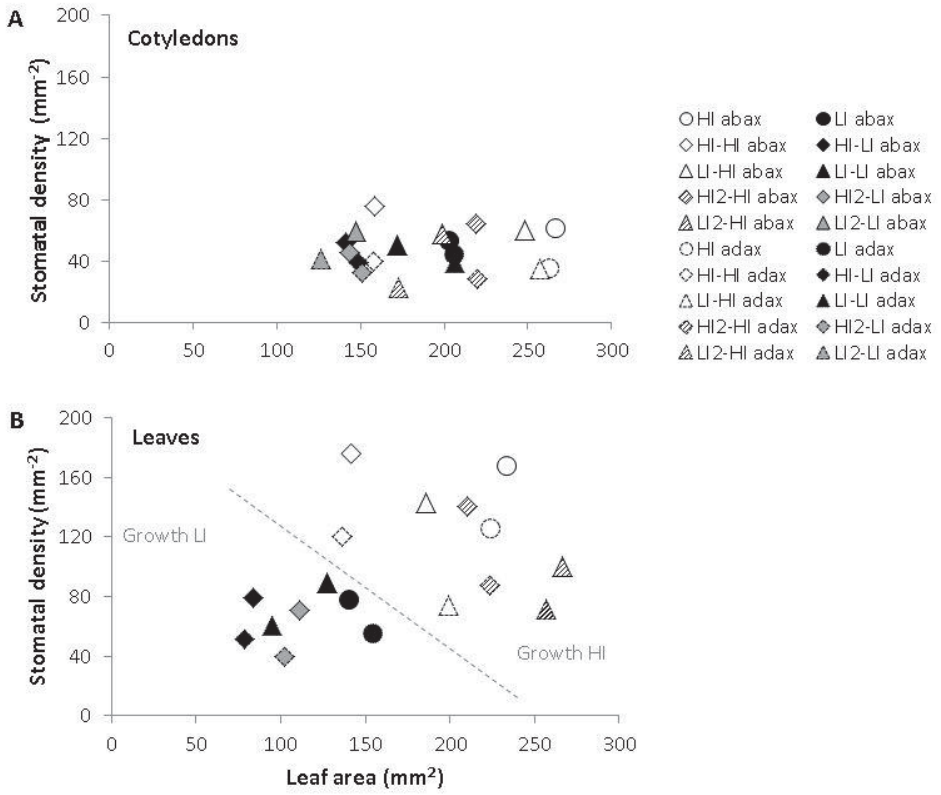

Fig. 6: Stomatal density related to leaf area of cotyledons (A) and first leaves (B) of garden cress. Mothers, 1st and 2nd generation of plants grown in HI (white or dashed symbols) or LI (grey or black symbols) environments were studied. Mean values are shown ($n \geq 10$). The broken line separates two growth PPFD (HI = 500 µmol m^{-2} s^{-1}, LI = 80 µmol m^{-2} s^{-1}).

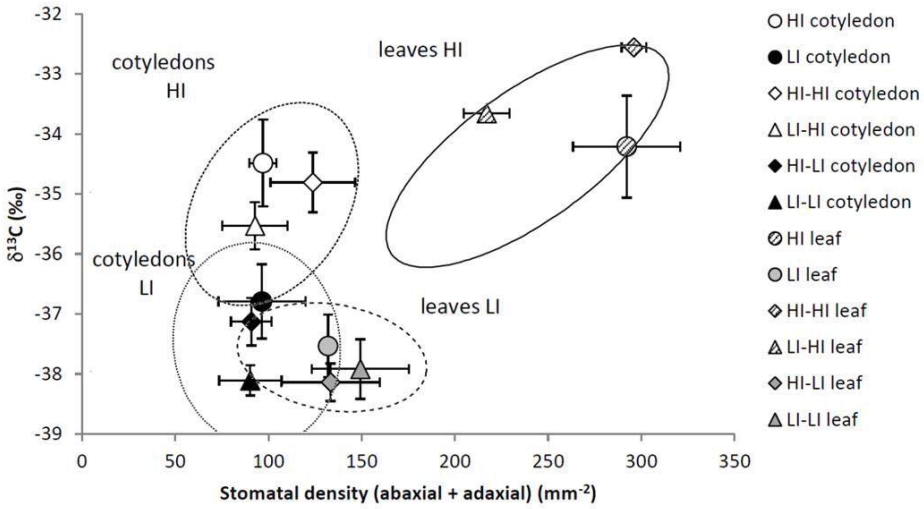

Fig. 7: ^{13}C discrimination and SD relationship in mother plants (HI, LI - circles) and 1st generation of offspring (offspring of HI-mothers: diamonds, LI – mothers: triangles) planted in HI (white and dashed symbols) or LI (grey or black symbols) conditions. Cotyledons (white and black symbols) and first leaves (dashed and grey symbols) were estimated independently. Mean values, standard deviations (bars) and 95% confidence ellipses are shown (n ≥ 10).

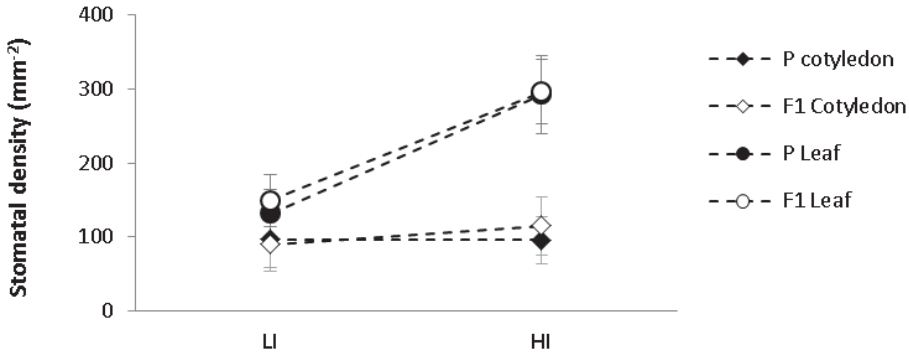

Fig. 8: Stomatal density in parental and offspring generations of garden cress grown in LI or HI conditions (Experiments 1 and 3). P - mothers, F1 - 1st offspring generation. Cotyledons – diamonds, true leaves – circles. Dashed lines mean shifts in SD from one light environment to another. Mean values and standard deviations are shown (n ≥ 10).

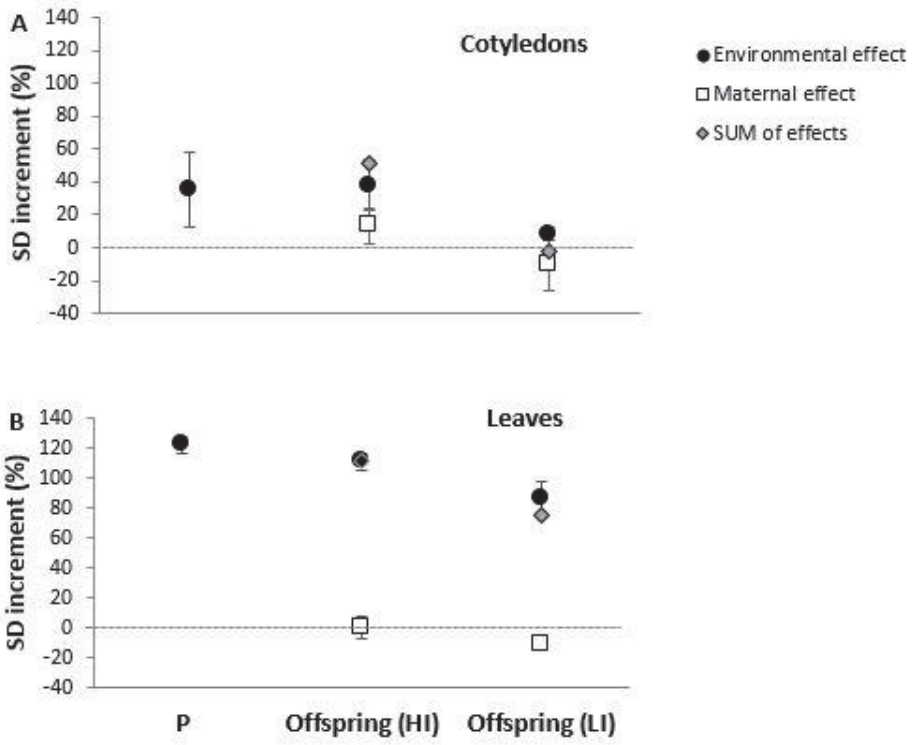

Fig. 9: Effects of current growth light environment (Environmental effect, EnvE) and of the light environment experienced by the mother plants (Maternal effect, MatE) on relative increment in stomatal density (SD increment, SD_i) in parents (P) and offspring of garden cress grown under high light (HI) or low light (LI). (A) Cotyledons and the sum of SD_i on both leaf sides. (B) First leaves and the sum of SD_i on both leaf sides. SD increment was calculated as $SD_i = ((SD_{HI} – SD_{LI}) / SD_{LI}) \times 100$ %. In calculation of EnvE, HI and LI represent different growth conditions of offspring from the same mother, whereas in MatE HI and LI represent different light experienced by mother plant the offspring grew in stable light environment (HI or LI) and were SD-evaluated. On x-axis, in case of EnvE HI and LI represent maternal experience (we calculated EnvE for offspring of HI-mothers and LI-mothers separately), whereas in case of MatE HI and LI represent offspring experience (we calculated MatE for offspring grown in HI or LI separately). Mean values and standard deviations are shown. Dashed lines represent zero values (no effect is present).

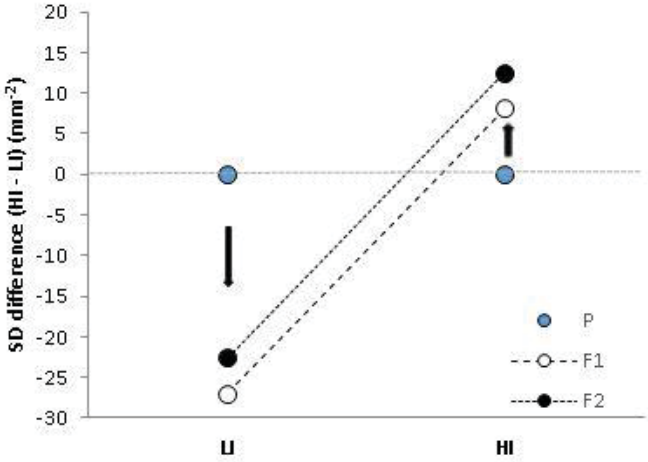

Fig. 10: SD differences between offspring lines from HI and LI mothers grown in LI or HI environments (x-axis). Means are shown for cotyledons of garden cress. Parental (P) lines (HI, LI) were set arbitrary to zero values, 1st (F1) and 2nd (F2) generation of its offspring are shown. Positive values of SD difference mean that under the same PPFD offspring of HI-mother had higher SD than offspring of LI-mother and vice versa, negative values of SD difference mean that offspring of HI-mothers had lower SD than offspring of LI-mothers. Black arrows represent tendency to shift in SD due to maternal light experience.

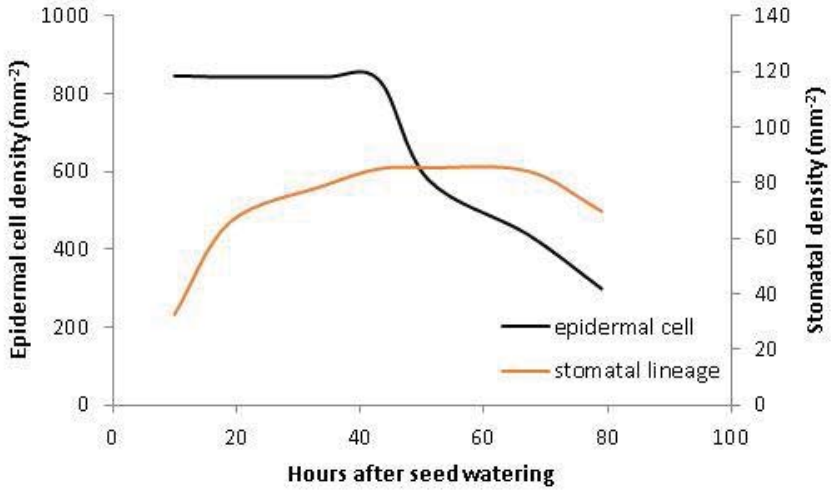

Fig. 11: Stomatal and epidermal cell density in garden cress cotyledons in several days after seed watering. Stomatal density was calculated as the sum of all stomatal lineage cells (meristemoids, guard mother cells, stomata) per mm^2 of leaf area.

Supporting Information

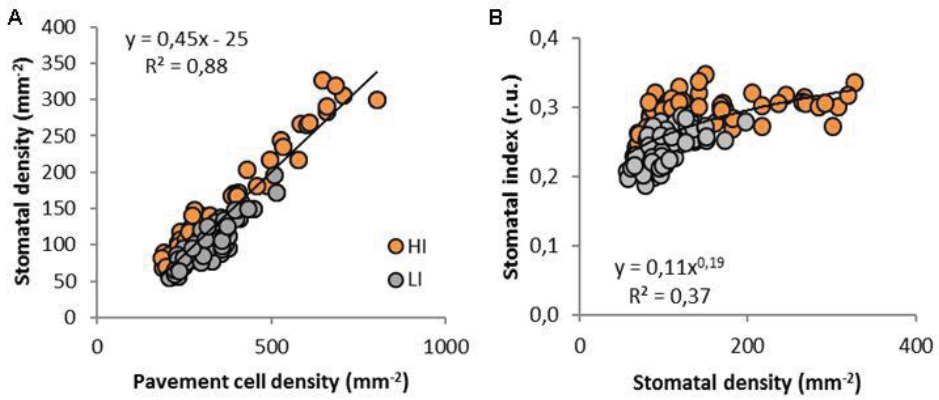

Fig. S1: Relationship of stomatal to pavement cell density (A) and stomatal index to stomatal density (B) in cotyledons and true leaves of garden cress grown in HI (orange symbols) and LI (grey symbols).

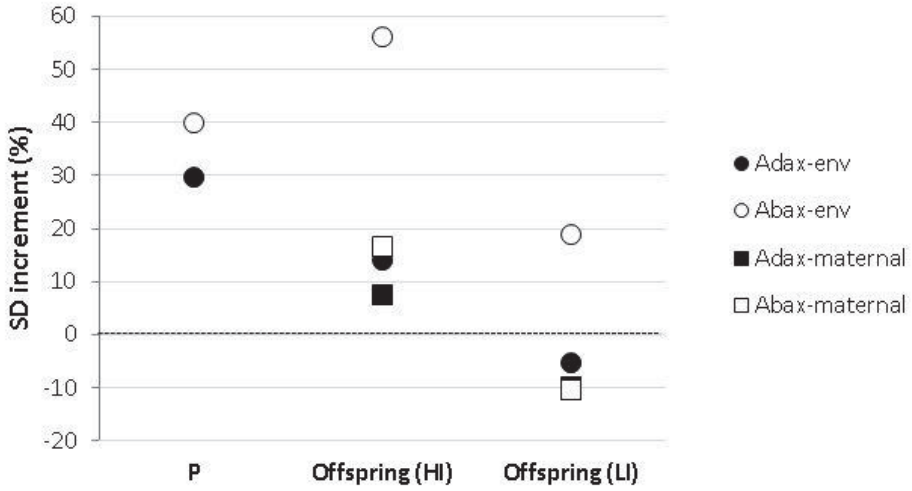

Fig. S2: Effects of elevation in current light environment (LI→HI, -env) and elevation of the light environment experienced by the mother plants (-maternal) on relative increment in stomatal density (SD increment) in parents (P) and offspring of garden cress grown under high light (HI) or low light (LI). Abaxial and adaxial leaf sides of cotyledons are shown separately.

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PAPER IV

Stomatal function, density and pattern, and CO_2 assimilation in *Arabidopsis thaliana tmm1* and *sdd1-1* mutants.

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RESEARCH PAPER

Stomatal function, density and pattern, and CO_2 assimilation in *Arabidopsis thaliana tmm1* and *sdd1-1* mutants

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Keywords
^{13}C discrimination; *Arabidopsis thaliana*; CO_2 assimilation; leaf internal CO_2; stomatal conductance; stomatal density; stomatal function.

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ABSTRACT

- Stomata modulate the exchange of water and CO_2 between plant and atmosphere. Although stomatal density is known to affect CO_2 diffusion into the leaf and thus photosynthetic rate, the effect of stomatal density and patterning on CO_2 assimilation is not fully understood.
- We used wild types Col-0 and C24 and stomatal mutants *sdd1-1* and *tmm1* of *Arabidopsis thaliana*, differing in stomatal density and pattern, to study the effects of these variations on both stomatal and mesophyll conductance and CO_2 assimilation rate. Anatomical parameters of stomata, leaf temperature and carbon isotope discrimination were also assessed.
- Our results indicate that increased stomatal density enhanced stomatal conductance in *sdd1-1* plants, with no effect on photosynthesis, due to both unchanged photosynthetic capacity and decreased mesophyll conductance. Clustering (abnormal patterning formed by clusters of two or more stomata) and a highly unequal distribution of stomata between the adaxial and abaxial leaf sides in *tmm1* mutants also had no effect on photosynthesis.
- Except at very high stomatal densities, stomatal conductance and water loss were proportional to stomatal density. Stomatal formation in clusters reduced stomatal dynamics and their operational range as well as the efficiency of CO_2 transport.

INTRODUCTION

Stomata are crucial epidermal components, modulating gas exchange between the leaf and atmosphere. The ability to control stomatal aperture as well as the number and size of stomatal cells together affect the amount of CO_2 assimilated through photosynthesis relative to water loss due to transpiration. Although leaf stomatal density (number of stomata per unit leaf area) significantly affects stomatal conductance (g_s), enhancement of g_s is not necessarily associated with enhancement of the leaf's CO_2 assimilation rate. Moreover, the fact that stomatal density and guard cell size should be negatively related (*e.g. Eucalyptus globulus*, Franks *et al.* 2009; *A. thaliana* EPF family mutants, Doheny-Adams *et al.* 2012) makes the relationship between gas exchange and stomatal density more ambiguous. In addition, changes in the pattern of stomatal distribution (*e.g.* formation of clusters of two or more stomata) may also affect the functioning of individual stomata and g_s. All of these parameters – stomatal density, size, pattern and function – are products of molecular signalling and ion transport processes triggered by and responding to signals from the leaf mesophyll and/or epidermal cells as well as from the environment.

Cellular and molecular mechanisms for the formation of stomata have been studied intensively over the past two decades in the model plant *Arabidopsis thaliana* (L.) Heynh. (Geisler *et al.* 1998; Nadeau & Sack 2002; Nadeau 2009; Serna 2009). Stomatal development is controlled by a complex network of negative regulators from the epidermis, *e.g.* epidermal patterning factors such as EPF1, EPF2 (Hara *et al.* 2007, 2009; Hunt & Gray 2009; Shimada *et al.* 2011; Vaten & Bergmann 2012; Torii 2015) and the only known positively acting factor produced in mesophyll, called STOMAGEN or EPFL9 (Kondo *et al.* 2010; Sugano *et al.* 2010). Interaction of these peptide ligands with membrane receptors (*e.g.* TOO MANY MOUTHS (TMM); Yang & Sack 1995; Geisler *et al.* 1997; de Marcos *et al.* 2016), with other negative regulators like the subtilisin-like protease STOMATAL DENSITY AND DISTRIBUTION 1 (SDD1; Berger & Altmann 2000) and linkage with several transcription factors (SPEECHLESS, MUTE, FAMA) determine the final pattern, size and number of stomata (Lau & Bergmann 2012; Pillitteri & Torii 2012; Simmons & Bergmann 2016) in response to internal and external environmental conditions (Woodward 1987; Coupe *et al.* 2006; Casson & Gray 2008; Lake & Woodward 2008; Santrucek *et al.* 2014). A central paradigm in stomatal development is the one-cell spacing rule, which ensures that a stoma is separated from any surrounding stomata by at least one epidermal cell (Geisler *et al.* 2000; Serna *et al.* 2002). Spatial separation by at least one pavement (subsidiary) cell seems to be a precondition for correct stomatal function in terms of CO_2 response and with respect to the range of stomatal opening (Papanatsiou *et al.* 2016) but,

surprisingly, not regarding leaf water use efficiency (WUE; Dow *et al.* 2014b). Mature rosette leaves of *A. thaliana* mutant lines with different proportions of stomata in clusters did not show any remarkable changes in WUE, although their leaf conductances for water did (cf. figs 8 and 3 in Dow *et al.* 2014b). This indicates that mesophyll conductance for CO_2 changes in proportion to stomatal conductance in leaves with clustered stomata and, thus, the ratio of stomatal to mesophyll conductances (g_s/g_m) should remain unchanged with an increasing proportion of stomata in clusters. One of the objectives of our work was to test this hypothesis.

Stomata develop in a leaf-side specific manner. Partitioning of stomatal conductance between leaf sides is species-specific but can be strongly modulated by light and other signals from the environment (*e.g.* Mott & Michaelson 1991; Muir 2015). A higher degree of amphistomy (adaxial/abaxial g_s ratio approaching 1) reduces the CO_2 gradient across the leaf and increases whole-leaf photosynthetic rate (Parkhurst & Mott 1990); however, variation in the distribution of stomata between the two leaf surfaces did not affect water loss among various *A. thaliana* genotypes (Fanourakis *et al.* 2015). It is well established that leaves exposed to higher photosynthetic active radiation (PAR) develop more stomata per unit of their area (*e.g.* Schluter *et al.* 2003). Moreover, increasing light intensity promotes stomatal development (both stomatal density and stomatal index) in the adaxial epidermis of *A. thaliana* genotypes more strongly than on the abaxial side (Hronkova *et al.* 2015) and, surprisingly, amphistomy is more common among plants from low precipitation environments (Muir 2015). Putatively, one might suggest that leaf WUE increases with the fraction of stomata that are located on the adaxial leaf surface. Studying both leaf epidermes – adaxial and abaxial – in genotypes that differ in the level of amphistomy should help us connect aspects of leaf physiology and stomatal development.

Earlier papers have shown that CO_2 assimilation rates were correlated with g_s over a broad range of other environmental factors (*e.g.* Wong *et al.* 1979, 1985a,b,c). Therefore, stomatal limitation seems to constitute a rather small fraction (<20%) of the total photosynthetic limitation in well-adapted plants cultivated under conditions of high light (Jones 1998). Both Buessis *et al.* (2006) and Schluter *et al.* (2003) evaluated photosynthesis in an *A. thaliana* mutant (*sdd1-1*) that differs from wild-type plants only in the number of stomata and their distribution on the leaf surface. Although the stomatal density of *sdd1-1* leaves was found to be elevated 2.5-fold, this translated into only a slightly higher CO_2 assimilation rate. The effect of stomatal density enhancement was negligible under low light and increased under moderate light, in which g_s became more limiting for CO_2 assimilation. This illustrates the importance of environmental conditions in stomatal behaviour and the significance of examining g_s limitation on CO_2 assimilation rate (A_N) at appropriate irradiance and CO_2 concentration (see also Lawson & Blatt 2014; Xu *et al.* 2016). For this reason, we applied three different PAR levels when steady-state values of g_s in different *A. thaliana* lineages were compared.

Studies relating stomatal anatomy and frequency to leaf gas exchange are rare. Most of the research is focused on the physiological aspects of stomatal density *per se*, but stomatal patterning and distribution, too, are substantial factors affecting CO_2 uptake and water loss. Manipulation of specific genes controlling stomatal distribution and patterning enables breaking the one-cell spacing rule and producing stomata in clusters. How does clustering affect the ability of stomata to control gas exchange? The sensitivity of clustered stomatal pores to external environmental factors was investigated by Dow *et al.* (2014b). Their results indicate that stomata in clusters are active, that they control gas exchange, but that their conductance is significantly reduced by tight contact with other stomata. Reduction of gas exchange due to clustering and patchy patterning of stomata was modelled by Lehmann & Or (2015). These authors also found that genotypes of *A. thaliana* with clustered stomata exhibit reduced sensitivity of g_s to CO_2 concentration in comparison to non-clustered stomatal genotypes. Smaller stomata, typically in high stomatal density leaves, are usually more responsive and faster than larger stomata (Drake *et al.* 2013; Raven 2014; Lawson & Blatt 2014; but see Elliott-Kingston *et al.* 2016). The kinetics of clustered stomata, with limited ionic exchange with neighbouring cells (Outlaw 1983) and loss of mechanical support from surrounding epidermal cells (Edwards *et al.* 1986; Franks & Farquhar 2007) have been studied recently (Papanatsiou *et al.* 2016).

In the present study, we aimed to investigate how stomatal density and patterning on both adaxial and abaxial epidermes affect diffusional limitation of photosynthesis and, more specifically, if an increase in stomatal density could enhance CO_2 concentration in the leaf tissue and CO_2 assimilation rate (A_N) in Col-0, C24, *sdd1-1* and *tmm1* genotypes of *A. thaliana*. Furthermore, the dynamic ability of clustered and non-clustered stomata to respond to changes in light intensity was evaluated. We combined several methods of evaluating stomatal plasticity. Together with gas exchange measurements, thermal imaging was used to assess short-term changes in g_s. Because CO_2 concentration in leaf tissue (C_i) and the associated discrimination of the heavy isotope of carbon, ^{13}C, respond to varying g_s, isotope ratio mass spectrometry was employed for time-integrated and A_N-weighted assessment of C_i. Stomatal and mesophyll development are closely connected, as each stoma lies above the intercellular cavity in the mesophyll and therefore mesophyll structure should reflect the structure of the epidermis. Moreover, signalling from the mesophyll is thought to play an important role in stomatal development. Simultaneously with g_s, therefore, we also assessed mesophyll conductance (g_m) and related leaf anatomical features (thickness and fraction of airspace in the mesophyll) for all *A. thaliana* genotypes.

MATERIAL AND METHODS

Plant material and growth conditions

Arabidopsis thaliana wild-types Columbia (Col-0) and C24, and mutants *tmm1* (based on Col-0) and *sdd1-1* (based on C24) were grown together in a controlled environment chamber in standard soil with 8 h/16 h day/night photoperiod; photon flux density [PPFD] was 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ or 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$, day/night temperature 20/18 °C and relative humidity 60%. Seeds were stratified for 3 days at 4 °C in darkness. After 2 weeks, plants were re-potted into small pots (7-cm diameter) with mounds of soil elevated above the pot edges (according to Flexas *et al.* 2007). Plants grew from the tops of the mounds, with the rosettes spreading downwards, to facilitate access to basal rosette leaves for the LI-6400 chamber head (Li-Cor, Lincoln, NE, USA). Individual plants were positioned in a

randomised manner. Gas exchange measurements were carried out 8 weeks after germination on mature, fully developed leaves (no. 7 and higher from the top). Preliminary results showed similar stomatal and diffusional characteristics of Col-0 and C24 (see Results and Supporting Information), and thus only Col-0 plants will be presented as the control for most experiments.

Stomatal and pavement cell density

Stomatal and pavement cell numbers per unit leaf area were determined from dental putty impressions (Stomaflex; Spofa Dental, Czech Republic) of both abaxial and adaxial leaf sides of fully developed leaves. Nail polish was applied to the dental imprints to obtain positive leaf surface replicas. A light microscope (Olympus BX61) with 50× magnification and a digital camera (Canon) were used to photograph a leaf surface area of 0.129 mm^2. Stomatal and pavement cells were counted in five areas from each leaf side and their density expressed as number per mm^2. Stomatal index (SI) was calculated as the fraction (%) of stomata of all epidermal cells: SI = (number of stomata)/(number of stomata + number of other epidermal cells) × 100%. Imprints were taken from fully expanded leaves used for gas exchange measurements (one leaf from each of five plants) or thermal imaging (one leaf from each of five plants).

Stomatal size and calculation of anatomical g_s

Stomatal size was assessed as the total area of guard cells and the pore from leaf imprints (30 stomata for each genotype and leaf side) in ImageJ (NIH, Bethesda, MD, USA). Geometry of stomatal pores and density of stomata determine the anatomical maximum of stomatal conductance, g_{smax}. We estimated stomatal geometry from imprints of leaves acclimated to growth conditions, where stomata were not fully open. Therefore we calculated g_s in analogy with g_{smax} according to Franks & Farquhar (2001) and Dow *et al.* (2014a):

$$g_s = (d \times D \times a)/(v(l + \pi/2) \times \sqrt{(a/\pi)}) \quad (1)$$

where d is water diffusivity in air, v is the molar volume of air, D is stomatal density (abaxial or adaxial), a is the area of a stomatal pore (area of an ellipse with its major axis equal to pore length and minor axis equal to pore width), and l is pore depth (taken as equal to guard cell width). Anatomical g_s was calculated separately for adaxial and abaxial epidermis and whole leaf $g_s = g_s(\text{adax}) + g_s(\text{abax})$. Both clustered and separated stomata were evaluated separately.

Leaf thickness and airspace fraction of mesophyll

Pieces of leaf tissue (approximately 2 mm × 2 mm) were fixed in 0.1 $mol \cdot l^{-1}$ phosphate buffer (pH 7.3) with 5% glutaraldehyde and 4% sucrose for at least 48 h. The samples were post-fixed in 1% osmium tetroxide, dehydrated by washing in solutions with increasing ethanol content and embedded in Spurr resin.

Semi-thin sections (thickness 400 nm) were transferred to glass slides, stained for 1 min with toluidine blue, and examined under an Olympus light microscope. Leaf thickness and the (two-dimensional) proportion of airspace in the mesophyll (%) were determined with ImageJ (NIH).

Thermal imaging

The experiments were performed under laboratory conditions, where PPFD, temperature and relative humidity were controlled and identical to growth conditions. The temperature distribution on the leaf surface was measured using an FLIR P660 thermographic camera (FLIR Systems, Sweden; spectral range 7.5–13.0 μm; 640 × 480 pixels; resolution 0.06 °C). The camera was operated by remote control. The internal software requires setting the object emissivity (0.96), the object distance, the ambient temperature and the relative humidity. ThermaCAM Reporter 8.2 software (FLIR Systems) was used for data processing. One leaf per plant, of the same size but on the opposite side of the rosette as that of the leaf measured with the gas exchange system, was scanned using the infrared camera. Ten pictures were taken from each leaf at a frequency of one picture per 3 min, and the mean temperature was calculated from these ten images. Only the leaf's middle part (without tip and base) was used for temperature analysis, *i.e.* approximately the same part of the leaf that was examined with the gas exchange system.

Carbon isotope composition

Carbon isotope composition was estimated in leaves with the same leaf size and a similar position in the rosette as those of leaves used for gas exchange and infrared measurements. Two leaves from each plant were analysed. ^{13}C relative abundance ($\delta^{13}C$) in leaf dry mass was determined using an EA1110 elemental analyser (ThermoQuest, Milan, Italy) linked to an isotope ratio mass spectrometer (IRMS, DeltaXL^{plus}; ThermoFinnigan, Bremen, Germany). The results were calculated *versus* VPDB standard (IAEA, Vienna, Austria) and expressed as $\delta^{13}C = (R_{sample}/R_{std} - 1) \times 1000‰$, where R_{sample} and R_{std} represent the $^{13}C/^{12}C$ ratio in the plant sample and VPDB standard, respectively. The standard deviation of ^{13}C analysis was 0.1‰. ^{13}C discrimination (Δ) was calculated according to Farquhar *et al.* (1982): $\Delta = a + (b - a) \times C_i/C_a$, where a is the fractionation occurring due to diffusion in air (4.4‰), b is the net fractionation by Rubisco and phosphoenolpyruvate carboxylase (27‰), and C_i and C_a are the intercellular and ambient CO_2 concentrations, respectively.

Gas exchange measurements

All gas exchange measurements were carried out on fully expanded leaves with an LI-6400 open gas-exchange system (Li-Cor) equipped with an integrated light source (LI-6400-40; Li-Cor). A specific measurement protocol was established (Fig. 4). The protocol was initiated by estimating gas exchange parameters under growth conditions. This was followed by reducing PPFD to 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$. When steady states of A_N and g_s were reached, PPFD was increased to 800 $\mu mol \cdot m^{-2} \cdot s^{-1}$ to evaluate the induction of photosynthesis and g_s. A_N did not increase further at higher light levels. The rates of the light induction of stomatal opening and

photosynthesis, dg_s and dA_N, respectively, were estimated as slopes of the linear time courses recorded 200–900 s after PPFD was increased and are expressed as changes of g_s and A_N per second. This time interval represents the slow phase of light induction, in which stomatal opening is assumed to dominate changes in g_s and A_N. More details on the light induction rates are shown in Fig. S3.

Estimation of mesophyll conductance

Mesophyll conductance (g_m) was estimated analogously to Evans *et al.* (1986) by comparing predicted and actual discrimination of $^{13}CO_2$ during photosynthesis. Predicted discrimination (Δ_i) was calculated according to Farquhar *et al.* (1982):

$$\Delta_i = a + (b - a) \times C_i/C_a \quad (2)$$

where a is the fractionation occurring due to diffusion in air (4.4‰), b is the net fractionation by Rubisco and phosphoenolpyruvate carboxylase (27‰), and C_i and C_a are the intercellular and ambient CO_2 concentrations, respectively, estimated from gas exchange data. Actual discrimination of ^{13}C, Δ_{act}, was inferred from IRMS measurements as $\Delta_{act} = \delta_a - \delta_p/(1 + \delta_p/1000)$, where δ_p is the $\delta^{13}C$ value of leaf dry matter and δ_a, the isotopic composition of CO_2 in the growth chamber air, was 11‰. Finally, g_m was calculated using equation 7 from Evans & von Caemmerer (1996):

$$\Delta_i - \Delta_{act} = (27 - 1.8) \times (A_N/g_m)/C_a \quad (3)$$

where 1.8‰ is the discrimination due to dissolution and diffusion of CO_2 in water. Fractionation resulting from respiration and photorespiration was assumed to be negligible (Warren *et al.* 2003; Flexas *et al.* 2007).

Statistical evaluation

Data were analysed using a *t*-test or one-way ANOVA with unequal N HSD *post-hoc* test and/or its non-parametric equivalent, the Kruskal–Wallis test with multiple comparisons of mean ranks of all groups. Statistical differences (Fig. 3) were tested by hierarchical three-way ANOVA and *post-hoc* Tukey HSD test. The significance level was set at $P = 0.05$. Statistical evaluation was performed using Statistica (Dell Inc., Tulsa, OK, USA).

RESULTS

As expected, wild-types Col-0 and C24 had stomatal patterns that were consistent with the one-cell spacing rule, and almost uniform distributions of stomata between adaxial and abaxial epidermes. In contrast, *sdd1-1* and *tmm1* mutants had stomata in clusters, higher stomatal density and different adaxial/abaxial ratios (Fig. 1). C24 had lower stomatal density than Col-0 and its stomatal conductance was proportionally lower; on the other hand, C24 closely matched Col-0 in stomatal patterning, abaxial/adaxial stomatal density ratio, induction of stomatal opening by light, $^{13}CO_2$ isotope discrimination and CO_2 assimilation rate. Therefore, only data for Col-0 are shown in most figures. The complete set of data for the C24 genotype is shown in Fig. S1.

Stomatal density and pattern

Wild types and mutants *tmm1* and *sdd1-1* differed in stomatal density on the adaxial and/or abaxial leaf side (Fig. 2). Stomatal density was higher on the abaxial leaf side regardless of plant genotype. The most balanced adaxial/abaxial stomatal density ratio was found in Col-0, while both mutants formed stomata preferentially on the abaxial leaf side. Surprisingly, *tmm1* showed the lowest stomatal density on the adaxial epidermis. The sum of stomata on both leaf sides was the same for Col-0 and *tmm1* (Fig. 2), whereas the mutation in the *SDD* gene led to multiplication of stomata on both abaxial and adaxial epidermes by a factor of up to 2.5.

During stomatal development, Col-0 followed the one-cell spacing rule, *i.e.* each stoma was surrounded by at least one epidermal cell and no stoma touched another. Mutations in *SDD* and *TMM* genes led to disruptions of this rule, and stomata were very often found in clusters of two or more (Fig. 2). Interestingly, even in *sdd1-1* the majority of stomata were separated, and the ratio of clustered to separated stomata was similar on both leaf sides (Fig. 2). Clusters were usually formed by two stomata per cluster, only rarely by more, and never by more than four. On the other hand, the *tmm1* formed very few stomata on the adaxial epidermis, with a ratio of clustered to separated stomata close to that of *sdd1-1*, whereas on the abaxial side there were four times more stomata, most of which in multiple stomata clusters (formed by three on average, although clusters of four to seven stomata were frequent), although the sum of stomata on both sides was comparable with Col-0 (Fig. 2).

The highest density of meristemoids (precursors of guard cells) was found on the leaves of *sdd1-1*, but their proportion was similar to that for Col-0 (2% and 3% of all stomatal lineage cells and stomata, respectively). Meristemoids were found solely on the abaxial epidermis. Pavement cell density mirrored the trends in stomatal density, with the highest value found in *sdd1-1* plants (Fig. 1). Stomatal indices were slightly higher for adaxial epidermes, despite their lower stomatal density, except in the case of *tmm1*, which had a very high stomatal index in the abaxial epidermis caused by a very high number of clustered stomata (Fig. 2).

Stomata occurring in clusters were smaller than separated stomata on *sdd1-1* and *tmm1* epidermes on both leaf sides (Fig. 3). The total epidermis area covered by stomata was therefore slightly lower when more clustered stomata were present than would be expected from the numbers of stomata. Surprisingly, the *sdd1-1* mutation led to a very high overall area of stomata on the epidermis, with a relatively low area covered by stomata in clusters, whereas in *tmm1* the area of epidermis covered by stomata was approximately the same as that of Col-0 (Fig. 3). All characteristics of stomatal anatomy and patterns are summarised in Fig. 1.

Stomatal and mesophyll conductance

Stomatal density and size provide only limited information about the anatomical disposition for CO_2 diffusion inside leaves. Stomatal function was estimated by gas exchange and fluorescence measurements using a LI-COR LI-6400XT portable measuring system. The measurement procedure was initiated with gas exchange measurements under growth

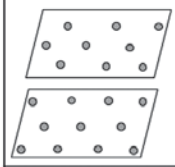
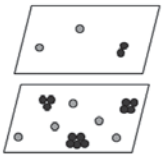
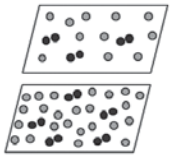
	***wild type* Col-0** 	***tmm1*** 	***sdd1-1*** 
	Mean ± SD	*Mean ± SD*	*Mean ± SD*
Stomatal density *(mm^{-2})*			
•*adaxial*	223 ± 63	101 ± 46	465 ± 98
•*abaxial*	322 ± 65	428 ± 140	1046 ± 106
Pavement cell density *(mm^{-2})*			
•*adaxial*	425 ± 103	275 ± 76	455 ± 95
•*abaxial*	716 ± 167	431 ± 66	1151 ± 126
Stomatal index			
•*adaxial*	34 ± 3	26 ± 5	51 ± 3
•*abaxial*	31 ± 2	48 ± 6	48 ± 2
Stomatal Ratio *abax/adax*	1.4	4.2	2.2
Number of clusters *(mm^{-2})*	–	17 ± 8	66 ± 22
% of stomata in clusters			
•*adaxial*	–	40 ± 14	30 ± 5
•*abaxial*	–	70 ± 7	22 ± 4
Size of stomata (μm^2)			
•*Separated adaxial / abaxial*	222 ± 36/234 ± 46	298 ± 23/310 ± 74	266 ± 26/234 ± 33
•*Clustered adaxial / abaxial*	–/–	221 ± 25/192 ± 33	202 ± 29/179 ± 24
Stomatal conductance *(mol m^{-2} s^{-1})*			
•*Growth light (300 μmol m^{-2} s^{-1})*	0.19 ± 0.05	0.19 ± 0.05	0.32 ± 0.05
•*Saturating light (800 μmol m^{-2} s^{-1})*	0.55 ± 0.19	0.36 ± 0.08	0.50 ± 0.17
Mesophyll conductance *(mol m^{-2} s^{-1})*			
•*Growth light (300 μmol m^{-2} s^{-1})*	0.46 ± 0.26	0.13 ± 0.01	0.07 ± 0.01
•*Saturating light (800 μmol m^{-2} s^{-1})*	0.06 ± 0.02	0.06 ± 0.01	0.06 ± 0.01

Fig. 1. Summary of stomatal anatomy and patterns of *A. thaliana* genotypes. The first row contains schematic representations of the adaxial and abaxial leaf surfaces of each genotype with stomata occurring separately (light grey) or in clusters (dark grey). Mean values ± SD (n = 5) are shown.

conditions (Fig. 4). Stomatal conductance under growth conditions was significantly higher in *sdd1-1*, while no differences between Col-0 and *tmm1* were observed (Fig. 5). This pattern corresponds to that in stomatal density. On the other hand, stomatal density enhancement in *sdd1-1* had no significant influence on its CO_2 assimilation rate under growth conditions (Fig. 5). Similarly, a very slight or non-significant difference in the ratio of leaf intercellular to ambient CO_2 concentration (C_i/C_a) was observed in *sdd1-1* (data not shown). In contrast, g_m was highest in wild-type plants and significantly reduced in both mutants (Fig. 5). Under high light conditions, most distinctions between the mutants and wild-type plants disappeared (Fig. 5). Likewise, no differences in the patterns of A/C_i curves measured under high light were observed (Fig. S2). This indicates that the carboxylation capacity was not affected by mutations affecting stomatal density and distribution.

The dynamics of stomatal regulation were deduced from the induction rate of stomatal opening in response to high light intensity in leaves acclimated to very low light (Fig. 4). At very

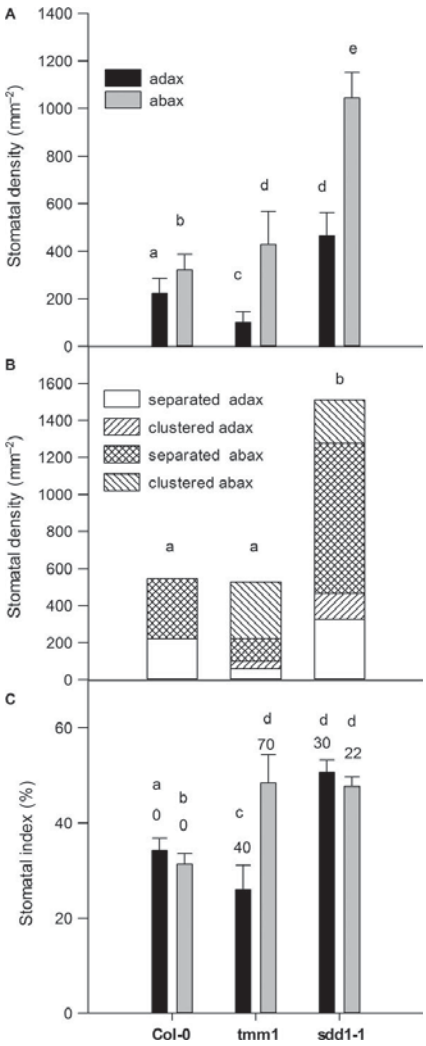


Fig. 2. Stomatal density and index of *A. thaliana* Col-0, *tmm1* and *sdd1-1* genotypes. (A) Stomatal density of adaxial *versus* abaxial epidermis in fully expanded leaves of plants grown in a controlled environment chamber in standard soil with 8 h/16 h day/night photoperiod (PPFD 300 µmol·m^{-2}·s^{-1}; 20/18 °C; 60% relative humidity). (B) Stomatal density of separated and clustered stomata on adaxial and abaxial leaf sides. Total height of the column represents the overall stomatal density for each genotype. (C) Stomatal indices of adaxial and abaxial epidermes. The proportion of clustered stomata (%) is shown as the number above each column. Note that both stomatal index and stomatal density are lowest in *tmm1* adaxial epidermis. Error bars show ±SD (n = 5). Different letters indicate statistically significant differences at $P < 0.05$.

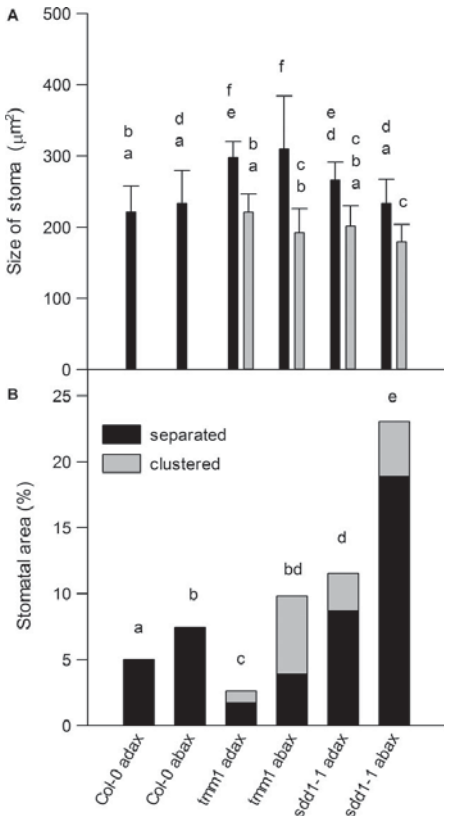


Fig. 3. Stoma size and total stomatal area on *A. thaliana* genotypes Col-0, *tmm1* and *sdd1-1*. (A) Size of individual stoma occurring separately (separated) or in clusters of two or more stomata (clustered). Clustered stomata are significantly smaller than separated ones. (B) Total stomatal area as percentage of the relevant leaf side area. Adaxial and abaxial epidermes were assessed separately. The highest proportion of stomatal area was found in the abaxial epidermis of *sdd1-1*. Error bars denote ±SD (n = 5). Different letters indicate statistically significant differences at $P < 0.05$.

low photon flux density (PPFD; 30 µmol·m^{-2}·s^{-1}), g_s of *tmm1* was the same as that of Col-0, whereas g_s of *sdd1-1* was significantly higher. The residual leaf water loss, *i.e.* water escaping mainly due to incomplete stomatal closure and cuticular transpiration, was controlled less effectively in *sdd1-1* (Fig. 6). Clear differences were observed between Col-0 and both mutants (*tmm1* and *sdd1-1*) in the dynamics of g_s and A_N induction. The response of Col-0 to high light induction was more than twice as strong in A_N and three times as strong in g_s than that of *sdd1-1* and *tmm1* (Fig. 6).

A mutation in the *TMM* or *SDD* gene affected stomatal density and distribution of stomata between adaxial and

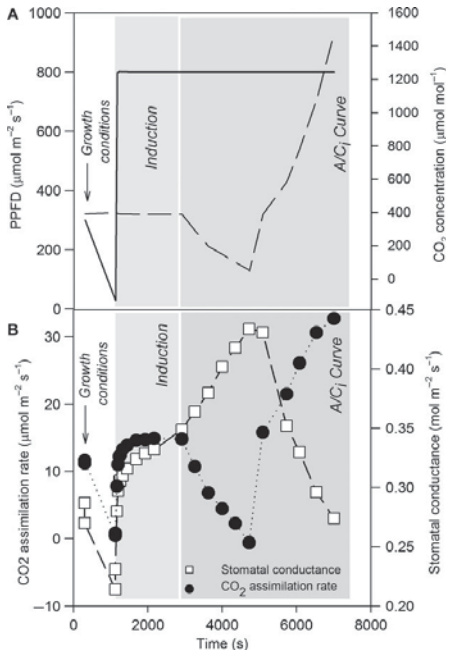


Fig. 4. Protocol for gas exchange measurements. (A) Light intensity (solid curve) and CO_2 concentration (dashed curve) settings. (B) The first point represents A_N (circles) and g_s (squares) under growth conditions (PPFD = 300 µmol·m^{-2}·s^{-1}, [CO_2] = 400 µmol·mol^{-1}). Subsequently, PPFD was set to a very low level (30 µmol·m^{-2}·s^{-1}). When steady states of A_N and g_s were reached, PPFD was increased to saturation level (800 µmol·m^{-2}·s^{-1}) and A_N and g_s were recorded during the induction. Finally, a CO_2 response curve was determined by step-wise variation of the ambient CO_2 concentration.

abaxial epidermis. Discrepancies between g_s calculated from guard cell anatomy (anatomical g_s), g_s measured at growth irradiance and g_s measured at saturating light intensity were enhanced with rising stomatal density (Fig. 7). Stomatal conductance observed in *sdd1-1* was only slightly higher than in Col-0, whereas stomatal density of *sdd1-1* was more than twice as high.

Thermal imaging showed a clear correlation between leaf temperature and g_s in both wild-type plants and mutants (Fig. 8), while leaf temperature responded to stomatal density only up to stomatal density of *ca*. 600 stomata·mm^{-2}. The leaf temperatures of *sdd1-1*, with stomatal density near 1000 stomata·mm^{-2}, and *tmm1* plants were similar (Fig. 8).

Carbon isotope discrimination, C_i and stomatal density

As expected, leaves showing higher g_s were more depleted in ^{13}C, and so *sdd1-1* exhibited higher discrimination of heavy carbon in comparison to *tmm1* and Col-0, both of which had lower g_s values. Mean values of $\delta^{13}C$ in leaf dry mass were $-30.8 \pm 0.5‰$ (*sdd1-1*), $-28.8 \pm 0.4‰$ (*tmm1*) and $-29.4 \pm 0.3‰$ (Col-0). C_i, estimated from gas exchange data in short-term measurements, was correlated with $\delta^{13}C$ of leaf dry mass accumulated during leaf growth (Fig. S4). The slope of this relationship showed that a $-1‰$ difference in $\delta^{13}C$ was equivalent to C_i changes slightly above 17 µmol·mol^{-1} (slope k = 17.3). Stomatal density, due to its equivocal relationship with g_s and C_i, was not correlated with $\delta^{13}C$ values. The C_i/C_a values measured under growth light and saturating light conditions and those calculated from anatomical g_s are plotted in Fig. 9. Leaves with very high stomatal density (near 1000 stomata·mm^{-2}) apparently exceeded the maximum theoretical values of the C_i/C_a ratio ($C_i/C_a = 1$) and the maximum values of $\Delta^{13}C$ ($\Delta^{13}C = 27‰$) calculated from C_i/C_a for the situation where CO_2 diffusion is unlimited by stomata (Fig. 9). Due to the high stomatal density, the contribution per stoma to C_i and thus to ^{13}C discrimination was lower in *sdd1-1* than in Col-0 and *tmm1*plants (Fig. 9).

Leaf thickness and airspace fraction of mesophyll

Leaf thickness assessed from semi-thin sections differed between Col-0 and *sdd1-1* plants; *sdd1-1* leaves were 46% thicker than leaves of Col-0 (Fig. S5). *sdd1-1* also had the highest fraction of airspace in mesophyll (27.7% *verus* 17.0% in C24, which had the lowest value among genotypes).

DISCUSSION

Mutation in the *SDD1* and *TMM* genes caused alterations in stomatal density and/or distribution. Enhanced g_s under growth conditions was observed only in *sdd1-1* mutants, due to higher stomatal density. Regardless of this improvement in CO_2 access into the leaf, increased g_s was not linked to an enhanced CO_2 assimilation rate. This is in agreement with the results of Schluter *et al.* (2003) and Buessis *et al.* (2006). On the other hand, Lawson *et al.* (2014) found a higher CO_2 assimilation rate in *sdd1* than in Col-0 and a weak correlation ($R^2 = 0.32$) between g_s and A_N across eight stomatal mutants. Nevertheless, our data confirmed that CO_2 assimilation capacity and carboxylation activity are not affected in *sdd1-1*. Mutations in both genes resulted in the formation of stomatal clusters, although the extent of clustering differed notably: 55% of stomata were found in clusters in *tmm1* but only 26% in *sdd1-1*. Comparing g_s under saturating and low light indicates that the operational ranges of g_s (differences between the maximum and minimum values) in *sdd1-1* and *tmm1* are about half of that in wild-type plants (0.24 and 0.20 *versus* 0.41 mol·m^{-2}·s^{-1}, respectively). Similarly, the rate of stomatal opening (change in g_s over time) at a step-wise increase in PPFD from 30 to 800 µmol·m^{-2}·s^{-1} was reduced in both mutants in comparison to wild-type plants. These down-regulated dynamics of stomatal light response also significantly affected the dynamics of CO_2 assimilation rate. We can, therefore, conclude that clustering limits stomatal opening (hindering complete closure as well as full opening) and slows down the stomatal and photosynthetic responses to light.

In plants grown under different environmental conditions, stomatal density of the leaf epidermis reflects acclimation to these conditions (*e.g.* PPFD and humidity affect stomatal frequency positively while CO_2 concentration does so

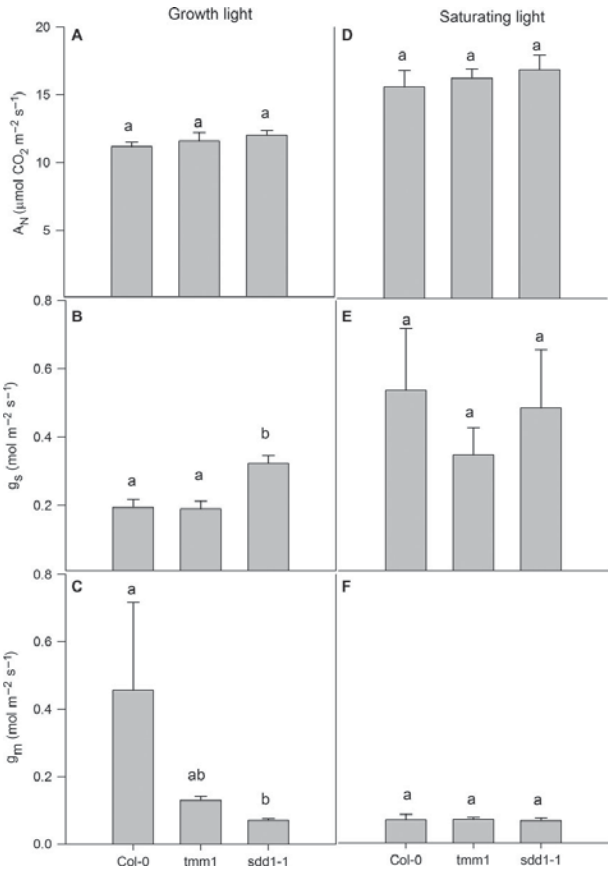


Fig. 5. CO_2 assimilation rate and diffusional characteristics of *A. thaliana* Col-0, *tmm1* and *sdd1-1* genotypes. (A–C) CO_2 assimilation rate (A_N), stomatal conductance (g_s) and mesophyll conductance (g_m) in leaves examined under growth conditions (PPFD = 300 µmol·m^{-2}·s^{-1}, [CO_2] = 400 µmol mol^{-1}) using a LI-6400 gas exchange system. (D–F) A_N, g_s and g_m measured under saturating PPFD (800 µmol·m^{-2}·s^{-1}). Differences were found between light conditions in both g_s and g_m; g_s increased and g_m decreased under saturating PPFD. The *sdd1-1*, with its very high stomatal density, exhibited the highest g_s and lowest g_m. Different letters (within individual panels) indicate statistically significant differences between genotypes, and error bars show ±SD.

negatively) and leads to adjustments in g_s, C_i and photosynthesis (Woodward 1987; Royer 2001; Bunce 2007; Casson *et al.* 2009; Casson & Hetherington 2010, 2014; Craven *et al.* 2010; Aasamaa & Sober 2011; Engineer *et al.* 2014; Santrucek *et al.* 2014). Stomata usually develop according to the one-cell spacing rule (Sachs 1991; Serna *et al.* 2002). Following this pattern, the size and density of stomatal pores (which determine g_s) are large enough for adequate CO_2 diffusion inside the leaf (Parkhurst 1994) without the stomata needing to 'compete' with one another due to overlapping diffusion shells (Lehmann & Or 2015). Mutations can cause stomatal density to be excessively high. In our case, *sdd1-1* had the highest stomatal density and leaves with the highest fraction of airspace in the mesophyll, whereas its mesophyll conductance was lower than that of Col-0 (Figs 5, S5). Rhizopoulou & Psaras (2003) found opposite trends in the intercellular space of the mesophyll (ICS, in %) and stomatal density in developing leaves of *Capparis spinosa* L. – while ICS increased, stomatal density decreased during leaf development. Morison & Lawson (2007) summarised results on leaf porosity (% of intercellular airspace in the mesophyll) obtained from sun and shade leaves to point out that sun leaves are usually less porous than shade leaves. Following this rule, higher stomatal density in sun leaves (for *A. thaliana* see *e.g.* Hronkova *et al.* 2015) should be accompanied by increased thickness and density of the mesophyll. An increase in mesophyll thickness causes a decrease in the diffusional conductance in the intercellular spaces (Terashima *et al.* 2001). Leaves of *sdd1-1*, with higher stomatal density, higher leaf thickness and lower mesophyll conductance, resemble sun leaves; in contrast, leaf porosity of *sdd1-1* (higher than in Col-0) and CO_2 assimilation rate (the same as in Col-0 with lower stomatal density) point to different adjustments in leaf anatomy than in sun leaves.

We confirmed that the *sdd1-1* is 'over-saturated' by stomata such that g_s estimated with the gas exchange technique is lower

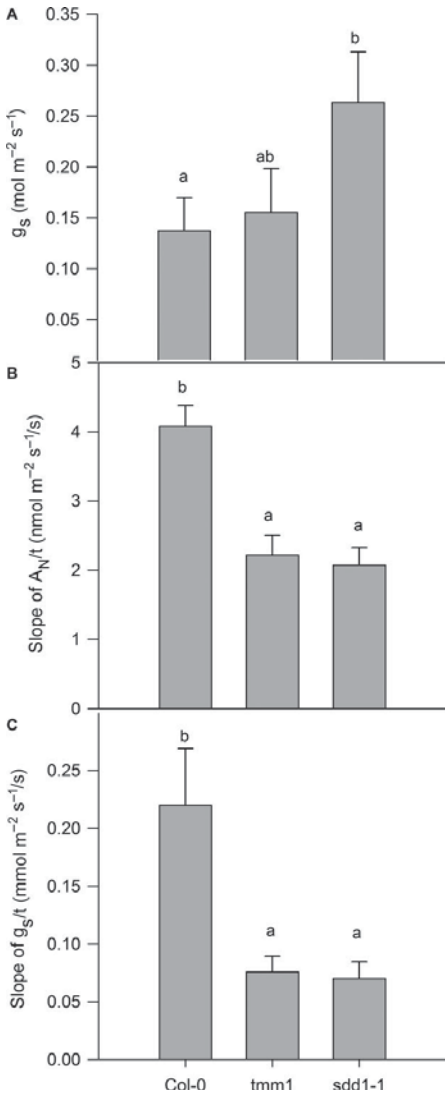


Fig. 6. Light induction of stomatal conductance (g_s) and CO_2 assimilation rate (A_N) of *A. thaliana* genotypes. (A) g_s of *A. thaliana* leaves at PPFD 30 μmol·m^{-2}·s^{-1}. The mutant *sdd1-1* with very high stomatal density exhibited the highest g_s in low light conditions. (B, C) Slope of A_N and g_s light induction curves after an increase in PPFD from 30 to 800 μmol·m^{-2}·s^{-1}. Both stomatal mutants showed significantly slower induction of both g_s and A_N than wild-type Col-0. Different letters indicate statistically significant differences between genotypes, and error bars show ±SD.

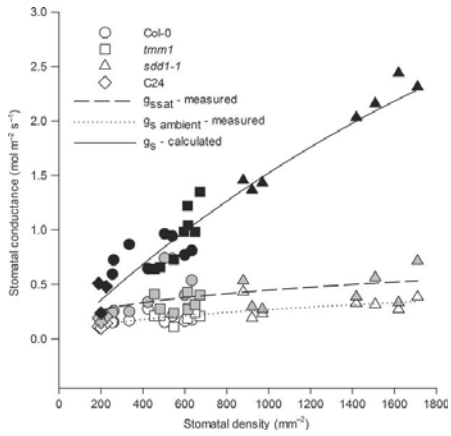


Fig. 7. Relationships between stomatal conductance (g_s) of Col-0, C24, *sdd1-1* and *tmm1* genotypes of *A. thaliana* and their stomatal density. g_s was measured using a portable gas exchange instrument (LI-6400XT) under growth irradiance ($g_{s\ ambient}$, white symbols) or saturating irradiance (g_{ssat}, grey symbols). Stomatal pore size was used to calculate anatomical g_s (g_s, black symbols). Anatomical g_s was calculated according to Farquhar *et al.* (2001) and Dow *et al.* (2014a) as $g_s = (d \times D \times a)/(v(l + \pi/2)\times\sqrt{(a/\pi)})$, where d is water diffusivity in air, v is the molar volume of air, D is stomatal density (abaxial or adaxial), a is stomatal pore area, and l is pore depth (taken as equal to guard cell width). The lines represent regression functions.

than would be expected from anatomical measurements (Fig. 7). This may be due both to the overlapping diffusional areas above or below neighbouring stomata, as proposed in Franks & Casson (2014) and modelled by Lehmann & Or (2015), and to a loss of function in some clustered stomata (due to the absence of subsidiary cells or a space effect when stomata open against each other). Differences between anatomical g_s and experimental g_s (under growth and saturating light) rise more strongly with total stomatal density than with the fraction of clustering: *sdd1-1* plants with only 26% of stomata clustered but very high stomatal density (up to 1700 stomata·mm^{-2} on both sides) showed a larger difference between measured diffusive g_s and calculated anatomical g_s than *tmm1* plants with 55% of stomata in clusters and stomatal density of up to 700 mm^{-2}. In contrast to our results, Dow *et al.* (2014b) showed that genotypes with correct spacing (<5% stomata in clusters) achieved g_{smax} values estimated from gas exchange measurements that were comparable to anatomical g_{smax} values across a ten-fold increase in stomatal density, while genotypes with patterning defects (>19% stomata in clusters) did not. In that experiment, stomatal density of *A. thaliana* genotypes with correct spacing or clustering reached maxima of 442 mm^{-2} and 387 mm^{-2}, respectively, much lower than those obtained in our experiment. Moreover, we showed that increasing stomatal density caused by gene mutation was not accompanied by decreasing stomatal size of separated stomata (Fig. 3), which should be expected when stomatal density is influenced by the

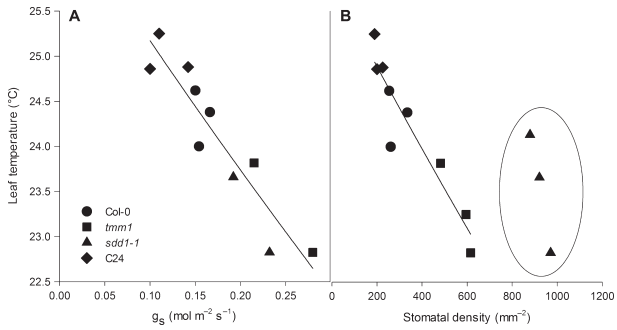


Fig. 8. Thermal imaging of *A. thaliana* genotypes. (A) Correlation between leaf temperature and stomatal conductance (g_s) and (B) correlation between leaf temperature and stomatal density of both leaf sides in wild-type (Col-0, C24) and mutant (*sdd1-1*, *tmm1*) plants. Leaf temperature was measured in growth conditions (PPFD 200 μmol·m^{-2}·s^{-1}) with an infrared camera (ThermaCam P660) together with dry and wet surfaces (paper) to balance fluctuations in external conditions. Stomatal conductance was estimated by gas exchange (LI-6400) and stomatal density was calculated from leaf imprints (n = 3). *sdd1-1* mutants (black triangles in the ellipse in panel (B) were excluded from the regression. Regression equations: (A) $y = 26.7 \cdot e^{-0.6x}$, $R^2 = 0.87$, (B) $y = 25.8 \cdot e^{-0.0002x}$, $R^2 = 0.89$.

environment (Franks & Beerling 2009; Franks *et al.* 2009; but cf. Haworth *et al.* 2013). Our data show that the excessively high stomatal density induced by gene mutation, which is not accompanied by reduced size of individual stomata, impairs gas exchange to a comparable or higher extent than stomatal clustering.

We observed no effect of stomatal density on photosynthetic rate measured under growth light conditions (Fig. 5). That is in agreement with Tanaka *et al.* (2013), who modulated stomatal density by over-expressing *STOMAGEN* (ST-OX). Nevertheless, under saturating light intensity they found a positive effect of higher g_s on A_N, which is inconsistent with our findings. Tanaka *et al.* showed that despite the higher influx of CO_2 through stomata, C_i remained unchanged compared to controls. This could be caused by alteration in (i) diffusional characteristics of the mesophyll (g_m), or (ii) carboxylation rate (V_{cmax}). In our study, mesophyll conductance to CO_2 transport was inversely related to g_s as well as to stomatal density. The g_s/g_m ratio in *sdd1-1* was ten times as high as in Col-0 (g_s/g_m = 0.46, 1.46, 4.57 in Col-0, *tmm1* and *sdd1-1*, respectively). However, this translated to only a 1.4-fold decrease of intrinsic WUE in *sdd1-1* under growth light. The dominant role of g_s over g_m in both mutants and the apparently independent behaviour of both conductances are surprising. However, some studies have shown that the proportional response of g_s and g_m to different variables can be disrupted. For example, the significant decline of g_s induced by the application of abscisic acid (ABA) was not accompanied by any reduction of g_m (Vrabl *et al.* 2009). A few studies even found g_s and g_m responding in opposite directions to any given treatment: Pons & Welschen (2003) reported that during midday depression of photosynthesis at 28–33 °C, an initial increase of g_m was accompanied by a simultaneous decrease of g_s, and more recently Scafaro *et al.* (2011) have shown opposing temperature response patterns of g_s and g_m in the range of 18–42 °C in three *Oryza* species. To the best of our knowledge, the relationship between g_m and stomatal density has not previously been evaluated.

Stomatal conductance affects leaf temperature due to its control over water evaporation from the leaf, and so thermal imaging allows estimation of g_s (Leinonen *et al.* 2006). Thermal imaging was used for screening stomatal mutants in *A. thaliana* that varied considerably in g_s, due to defects in stomatal regulation (*e.g. ost1*, *ost2*, *aba2*; Merlot *et al.* 2002; Wang *et al.* 2016). Infrared imaging has less frequently been applied for detection of differences in stomatal density or environmental effects on g_s (Crawford *et al.* 2012; Doheny-Adams *et al.* 2012; Takahashi *et al.* 2015). We found that leaf temperature correlated with g_s in all genotypes (wild-types Col-0 and C24 and mutants *tmm1* and *sdd1-1*) independently of stomatal pattern. Leaf temperature was correlated with stomatal density only up to a value of 600–700 stomata·mm^{-2}, and so *sdd1-1* with stomatal density close to or more than 1000 mm^{-2} exhibited leaf temperature higher than expected (Fig. 8). Except for leaves with stomatal density >600 mm^{-2}, thermal imaging may be used as a good predictor of the actual diffusional leaf–atmosphere coupling. When gas exchange is not measurable (*e.g.* due to small leaf area), it is a better proxy than calculated anatomical g_s. A comparison of the responses of leaf temperature to g_s and stomatal density (Fig. 8) supports the conclusion stated above that the surplus of stomata in *sdd1-1* did not contribute to water loss, and a similar conclusion holds true for CO_2 uptake (Fig. 5).

The ^{13}C discrimination in *sdd1-1* leaf biomass was higher than in *tmm1* and Col-0. This was caused by the higher C_i/C_a ratio in *sdd1-1* leaves. Although more stomata permit higher g_s, when stomatal density rises due to mutation, CO_2 assimilation rate is maintained, and the potential of stomata for CO_2 diffusion remains unutilised. Calculating the mean ^{13}C discrimination gained per operating stoma reveals the low efficiency of *sdd1-1* stomata compared to those of Col-0 and *tmm1* (Fig. 9). The high stomatal density acquired by gene mutation thus has only a limited effect on photosynthesis.

Leaf internal CO_2 is consumed by assimilation and the CO_2 concentration is balanced by diffusion of CO_2 from the

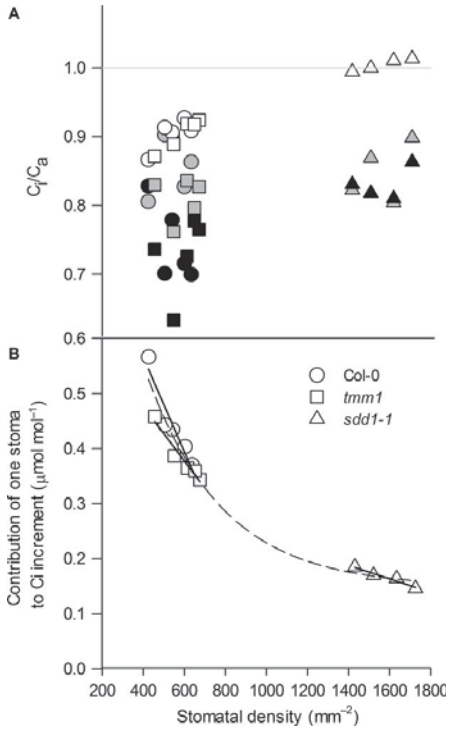


Fig. 9. Leaf internal CO_2 concentration (C_i) in relation to stomatal density of *A. thaliana* genotypes. (A) Ratio of C_i to ambient CO_2 concentration (C_a), measured by gas exchange under growth light and saturating light conditions (growth light – black symbols, saturating light – grey symbols) and calculated with anatomical g_s (white symbols), as related to stomatal density in Col-0 (circles), *tmm1* (squares) and *sdd1-1* (triangles) genotypes. The grey line equal to 1 represents the maximum theoretical value for infinite stomatal conductance. (B) Contribution of one extra stoma to an increment in leaf C_i at various stomatal densities in Col-0, *tmm1* and *sdd1-1*. The contribution values were assessed as the ratio of mean C_i, calculated from leaf biomass $\delta^{13}C$, and stomatal number (abaxial + adaxial). Lines represent trends for individual genotypes that are close to linear in the narrow range of stomatal density but exponential across the entire span of stomatal density values.

ambient atmosphere into the leaf through stomata on both leaf sides in *A. thaliana* and other amphistomatous plants. It seems that stomatal development differs between the two leaf surfaces. Hronkova *et al.* (2015) found that the light-dependent development of abaxial and adaxial epidermes proceeds differently in wild-type plants and *sdd1-1* and *tmm1* mutants. Here, we confirmed that the abaxial–adaxial differences in stomatal density are notably larger in *tmm1* and *sdd1-1* than in Col-0 and C24 (with abaxial/adaxial ratios of 4.3, 2.3, 1.4 and 1.6, respectively). The leaf-side heterogeneity induced by mutations can partly explain the invariance in g_s and photosynthetic rate compared to wild-type plants, probably due to uneven diffusion of CO_2 inside the leaf.

In conclusion, multiplication of stomata *per se* and, presumably, easier access for CO_2 into the leaf is not a prerequisite for enhanced leaf photosynthesis. Extremely high stomatal density decreases the efficiency of individual stomata for CO_2 and H_2O diffusion, as confirmed by stable carbon isotope discrimination and thermal imaging techniques. Our findings point to the importance of assessing both leaf epidermes when counting the stomata of plants grown under different environmental conditions or when genes are manipulated. According to our measurements, overall leaf physiological characteristics, reflecting CO_2 diffusion under the growth light regime, are affected predominantly by the overall number of stomata on both epidermes rather than by lateral stomatal patterning (abaxial/adaxial ratio). The leaves of *sdd1-1* bear more stomata than those of wild-type plants. Those stomata in higher numbers are of similar size but less efficient. This still enables the leaves to operate at higher C_i, but their rate of carbon assimilation is unchanged due to notably reduced g_m for CO_2. The relationship between genetically controlled stomatal density and mesophyll conductance is worthy of future study.

ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Variations in stomatal and gas exchange characteristics between Col-0 and C24 wild types of *A. thaliana* grown at a PPFD of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$. (A) Leaf side specific stomatal density, (B) stomatal conductance g_s at growth and saturating PPFD, (C) net CO_2 assimilation rate A_N, (D) ratio of leaf internal to ambient CO_2 concentration C_i/C_a, (E) relative abundance of ^{13}C carbon isotope $\delta^{13}C$, (F) rates of photosynthetic (dA_N) and stomatal (dg_s) responses to an abrupt change in PPFD from 30 to 800 $\mu mol \cdot m^{-2} \cdot s^{-1}$

Fig. S2. CO_2 response of photosynthetic rate in Col-0 wild-type and *tmm1* and *sdd1-1* mutants of *A. thaliana* grown at PPFD of 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Fig. S3 The time course of A_N and g_s induction after a stepwise increase in PPFD from 30 to 800 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and values from which the A_N and g_s slopes were calculated. The slope was calculated from the slow part of the induction curve, which reflects stomatal opening. At least six values were used for the calculation of the slopes.

Fig. S4 Leaf internal CO_2 concentration (C_i) in relation to relative abundance of ^{13}C ($\delta^{13}C$) in leaf biomass of *A. thaliana* genotypes. C_i was estimated by gas exchange (n = 5) at growth conditions; $\delta^{13}C$ was measured by IRMS in mature leaves of Col-0, *tmm1* and *sdd1-1* (n = 5). Linear regression ($y = -17.3x - 220.8$) shows that a $-1‰$ difference in $\delta^{13}C$ was equivalent to C_i changes slightly above 17 $\mu mol \cdot mol^{-1}$.

Fig. S5 Leaf thickness and proportion of airspace in the mesophyll of *A. thaliana* genotypes. (A) Leaf thickness estimated from semi-thin sections of leaves examined by light microscopy and (B) proportion of airspace in mesophyll (two-dimensional) determined by pixel counting in ImageJ software. Different letters indicate statistically significant differences between genotypes, and error bars show stomatal density (n = 5).

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SUPPORTING INFORMATION

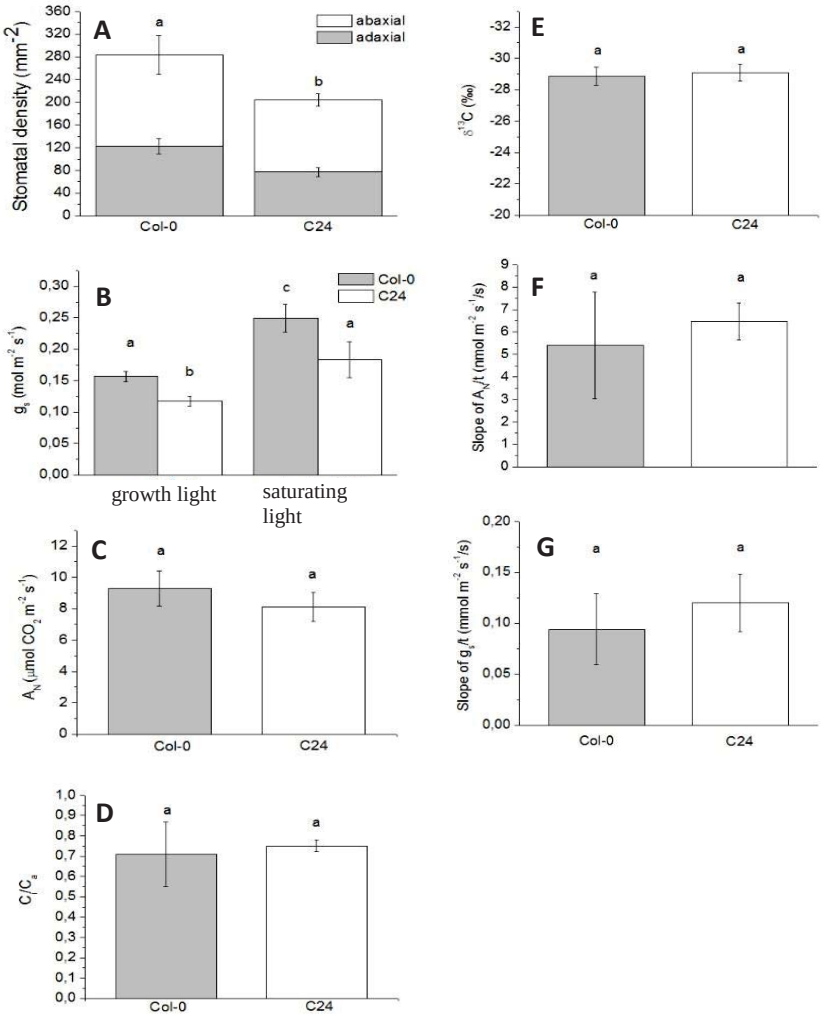

Fig. S1 Variations in stomatal and gas exchange characteristics between Col-0 and C24 wild-types of *A. thaliana* grown at a photosynthetic photon flux density (PPFD) of 200 µmol m^{-2} s^{-1}. (A) Leaf side specific stomatal density, (B) stomatal conductance g_s at growth and saturating PPFD, (C) net CO_2 assimilation rate A_N, (D) ratio of leaf internal to ambient CO_2 concentration C_i/C_a, (E) relative abundance of ^{13}C carbon isotope $\delta^{13}C$, (F) rates of photosynthetic (dA_N) and stomatal (dg_s) responses to an abrupt change in PPFD from 30 to 800 µmol m^{-2} s^{-1}.

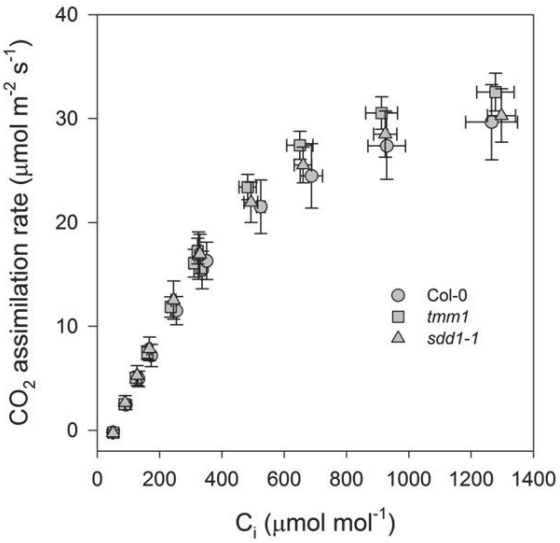

Fig. S2 CO_2 response of photosynthetic rate in Col-0 wild-type and *tmm1* and *sdd1-1* mutants of *A. thaliana* grown at a photosynthetic photon flux density (PPFD) of 300 µmol m^{-2} s^{-1}.

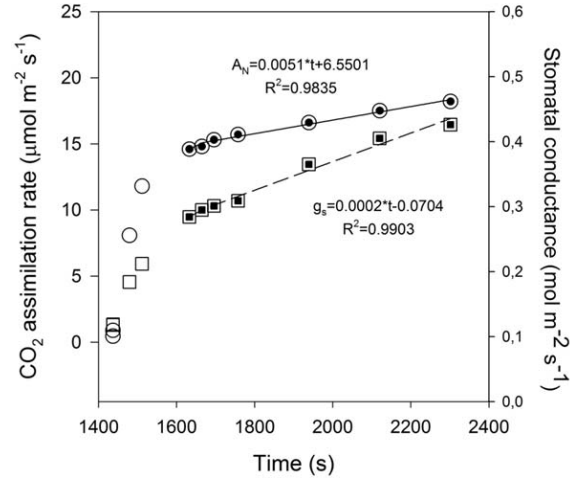

Fig. S3 The time course of A_N and g_s induction after a step-wise increase in PPFD from 30 to 800 µmol m^{-2} s^{-1} and the values from which the A_N and g_s slopes were calculated. The slope was calculatedfrom the slow part of the induction curve, which reflects stomatal opening. At least 6 values were used for the calculation of the slopes.

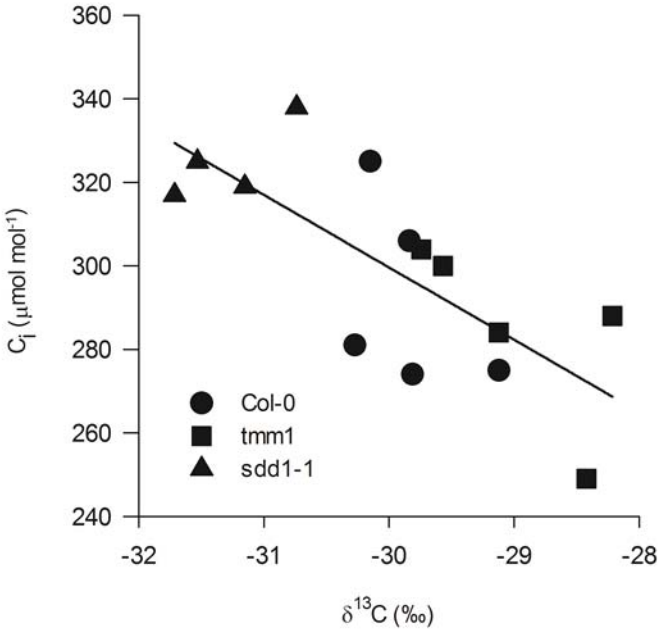

Fig. S4 Leaf internal CO_2 concentration (C_i) in relation to relative abundance of ^{13}C ($\delta^{13}C$) in leaf biomass of *A. thaliana* genotypes. C_i was estimated by gas exchange (n=5) at growth conditions; $\delta^{13}C$ was measured by IRMS in mature leaves of Col-0, *tmm1*, and *sdd1-1* (n=5). Linear regression (y = -17.3x – 220.8) shows that a −1‰ difference in $\delta^{13}C$ was equivalent to C_i changes slightly above 17 µmol mol^{-1}.

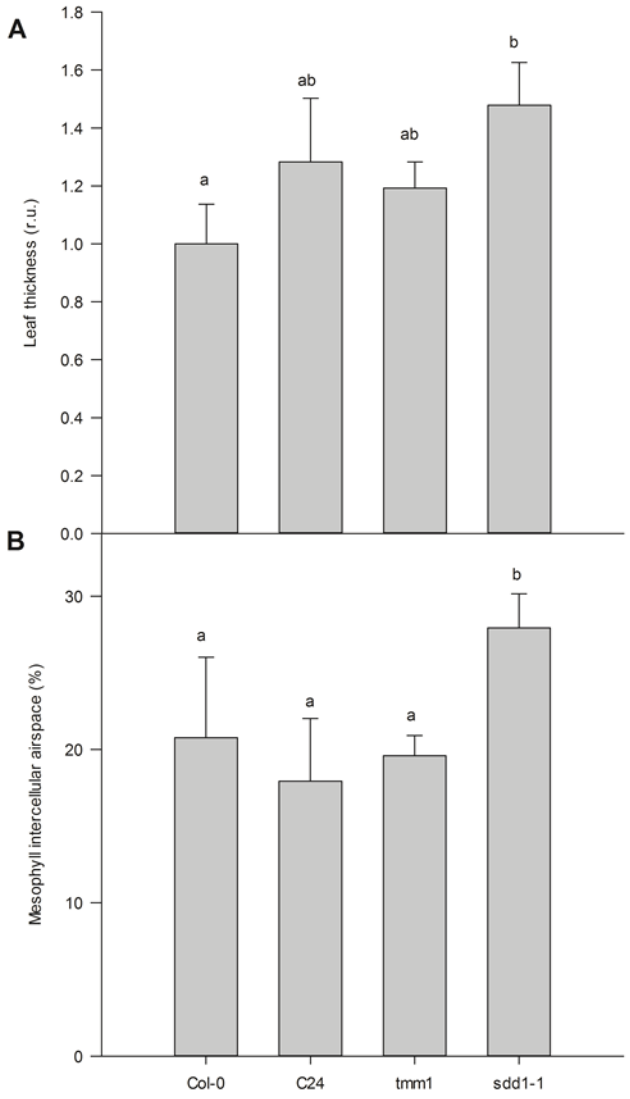

Fig. S5 Leaf thickness and proportion of airspace in the mesophyll of *A. thaliana* genotypes. (A) Leaf thickness estimated from semi-thin sections of leaves examined by light microscopy and (B) proportion of airspace in mesophyll (two-dimensional) determined by pixel counting in ImageJ software (NIH, Bethesda, MD, USA). Different letters indicate statistically significant differences between genotypes, and error bars show standard deviations (n=5).

Curriculum Vitae

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Academic achievements

2007 – 2017	Ph.D. at Department of Experimental Plant Biology, Faculty of Science, University of South Bohemia in České Budějovice; *interrupted in 2011 – 2015 (maternity leave)*
2001 – 2006	M.Sc. at Department of Physics, Faculty of Science, University of Ostrava, Ostrava

Professional positions

2015 – up to now	Analyst, Institute of Environmental Technology, Technical University of Ostrava, Ostrava
2007 – 2011	Part time researcher, Department of Experimental Plant Biology, Faculty of Science, University of South Bohemia in České Budějovice
2006 – 2007	Technician, Laboratory of Isotope Ratio Mass Spectrometry, University of South Bohemia in České Budějovice

Professional experience

2010	Short research fellowship, University of the Balearic Islands, Department of Biology, Spain (hosted by J. Flexas)
2009	Training Courses on Pressure Probes Technics and Basics Water relations, The French national institute for agricultural research (INRA), Nancy, France
2007	Short research fellowship, Institute for Cellular & Molecular Botany, University of Bonn, Germany (hosted by L. Schreiber)
2007	SIBAE Spring School Stable Isotopes in Ecology, University of Vienna, Austria

Participation in research projects

2008 – 2011 Regulation of stomatal morphogenesis by the conditions of the environment and physiological processes in the leaf (GACR 206/08/0787)

2009 – 2011 Diffusion of CO_2 within the leaf mesophyll: The role of aquaporin proteins (GAAV KJB601410917, 2009-2011)

2009 – 2011 Comparison of methods of plant physiology and molecular biology for evaluation of wheat and barley sensitivity to drought and selection of water stress tolerant genotypes (MZE QH91192)

2006 – 2008 Patterns of isotopic composition of plant organs – a marker of activity, environmental conditions and leaf anatomy (GAAV IAA601410505)

Publications

Vráblová M., Vrábl D., Hronková M., Kubásek J. and Šantrůček J. (2017) Stomatal function, density and pattern, and CO_2 assimilation in *Arabidopsis thaliana tmm1* and *sdd1-1* mutants. *Plant Biology*, 19 (5), 689 -701.

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