J. Plant Biol. (2014) 57:162-173
DOI 10.1007/s12374-014-0017-1

ORIGINAL ARTICLE

Comparison of Arabidopsis Stomatal Density Mutants Indicates Variation in Water Stress Responses and Potential Epistatic Effects

Shaneka S. Lawson[1,2]*, Paula M. Pijut[1,2] and Charles H. Michler[1,2]

[1]*USDA Forest Service, Northern Research Station, Hardwood Tree Improvement and Regeneration Center (HTIRC), 195 Marstellar Street, West Lafayette, Indiana USA 47907*
[2]*Purdue University, Department of Forestry and Natural Resources, HTIRC, 715 West State Street, West Lafayette, Indiana USA 47907*

Received: January 10, 2014 / Accepted: March 18, 2014

Abstract Recent physiological analysis of Arabidopsis stomatal density (SD) mutants indicated that SD was not the major factor controlling aboveground biomass accumulation. Despite the general theory that plants with fewer stomata have limited biomass acquisition capabilities, *epf1* and several other Arabidopsis mutants varied significantly in leaf fresh weight despite having similar stomatal numbers. The in-depth mechanisms controlling increased or decreased leaf area biomass remain undetermined. This work used calculations of SD, overnight water-loss, and LI6400XT measurements to reject the premise that SD is a primary factor controlling leaf biomass accumulation in Arabidopsis. With respect to our data, SD is not the primary factor influencing biomass accumulation in Arabidopsis *epf1* mutants as it did not positively correlate to any of the physiological parameters examined. Further observation of morphological differences between the mutants hinted that additional pathways were interrupted when these mutants were generated. Each mutant examined showed a variation in physiological measurements despite SD. Many SD mutants also showed morphological abnormalities in addition to altered stomatal numbers. These phenotypes may indicate epistatic effects related to the mutation of SD genes in the studied mutants.

Keywords: Morphology, Stomatal conductance, Stomatal density, Transpiration

*Corresponding author; Shaneka S. Lawson
Tel : +1-765-412-6119
E-mail : sslawson@purdue.edu

Introduction

Stomatal density (SD) is known to vary with respect to a number of physiological and environmental factors. This study of Arabidopsis mutants included positive and negative SD regulators. The EPIDERMAL PATTERNING FACTOR (EPF)-LIKE (EPFL) family of secreted cysteine-rich peptides is composed of three homologous negative SD regulators, where overexpression leads to reduced stomatal number and increased pavement cell formation [EPF1/2 and EPFL6 or CHALLAH (CHAL)], and one positive regulator [(EPFL9 or STOMAGEN (STOM)] (Sugano et al. 2010). Mutations in TOO MANY MOUTHS (*TMM*) or *EPF1* result in increased SD and a stomatal bunching phenotype (Hoover 1986; Hara et al. 2009) rather than adequate one-cell spacing (Yang and Sack 1995). Overexpression of *EPF1* (extremely high levels led to total ablation of stomata and infertility) resulted in a decreased stomatal index (SI), and overexpression of *TMM* led to increased stomatal numbers. Dependent upon TMM, overexpression of the STOMATAL DENSITY AND DISTRIBUTION1 (SDD1) gene also led to decreased stomatal numbers and formation of arrested stomata (von Groll et al. 2002; Yoo et al. 2010). Since *sdd1* mutants cannot alleviate *EPFL6* overexpression phenotypes, SDD1 seemed to be divergent from the ER and TMM pathways. However, mutations in *sdd1*, *epf1*, and *tmm* led to increased SI and occasional bunching (Lampard et al. 2008; Hara et al. 2009; Abrash and Bergmann 2010). Mutations in ER family members *er*, *erl1*, and *erl2*, and *yda* (YODA) resulted in almost exclusive production of guard cells and few pavement cells (Bergmann et al. 2004; Shpak et al. 2004). Required for stomatal pore formation, CYCLIN-DEPENDENT KINASE B1;1 (CDKB1;1), a cyclin dependent kinase responsible for arresting cell cycle proliferation (Porter 2008; Boudolf et al.

Table 1. Gene identification information, stomatal density, and water loss data over a 36 h period for a selection of *Arabidopsis* mutants

Arabidopsis	Full Name	GenBank ID	TAIR ID	SD[1] (mm^{-2})	WL[2]* (g) 36h
cdc6	Cell Division Control6	NM_179806	AT2G29680	210.6 ± 18.3	2.33
cdkb1;1	Cyclin-Dependent KinaseB1;1	NM_115278	AT3G54180	544.3 ± 27.8	2.36
edt1	Enhanced Drought Tolerance1	NP_177479	AT1G73360	626.8 ± 22.9	1.13
epf1	Epidermal Patterning Factor1	NM_127657	AT1G34245	441.5 ± 24.1	9.43
er	Erecta	NM_128190	AT2G26330	253.1 ± 12.4	2.12
erl1	Erecta-like1	NM_125617	AT5G62230	211.4 ± 41.3	1.26
flp	Four Lips	NM_001084065	AT1G14350	252.6 ± 15.7	5.60
mkk4	Map Kinase Kinase4	NM_104044	AT1G51660	189.2 ± 26.9	1.07
mkk5	Map Kinase Kinase5	NM_113017	AT3G21220	204.7 ± 14.5	5.61
mpk3	Mitogen-Activated Protein Kinase3	NM_114433	AT3G45640	192.3 ± 31.9	6.27
mpk6	Mitogen-Activated Protein Kinase6	NM_129941	AT2G43790	224.9 ± 7.7	6.79
sdd1	Stomatal Density and Distribution1	NM_100292	AT1G04110	462.7 ± 36.0	2.99
tmm	Too Many Mouths	NM_106657	AT1G80080	428.3 ± 20.7	1.75
yda	Yoda	NM_105047	AT1G63700	290.4 ± 25.3	1.11
Col-0	Columbia-0	-	-	168.8 ± 17.6	3.86

[1]SD, Stomatal density; [2]WL, water loss; *Total WL data were obtained from a total of three mutant *Arabidopsis* plants and was not an average.

2009), is involved in symmetric cell division. This last cellular division leads to guard cell formation and is required for the proper functioning of FOUR LIPS (FLP), a developmental regulator that targets CDKB1;1 and coordinates cell cycle exit before and after asymmetric division (Xie et al. 2010a). The FLP (MYB124) gene and its paralog MYB88, two proteins which share an amino acid substitution specific to members of the MYB family involved in development of stomata, were not required for stomatal fate (Lai et al. 2005). These proteins functioned to limit GMCs to one symmetric division, and thus prevented additional "daughter-cell" divisions as well as coerced guard cell formation.

During periods of water stress it was expected that stomatal numbers and pore apertures would be decreased, a common occurrence in Arabidopsis and grass species (Nawazish et al. 2006; Liu et al. 2009) however in some plant species research data showed that decreased water availability resulted in increased SD (Fraser et al. 2009). It was suggested that fluctuations in temperature also influenced SD (Xu and Zhou 2008). In Arabidopsis, a general decrease in SD has been shown in response to elevated temperatures, light, and CO_2 levels (Berger and Altmann 2000; Pospisilova 2003). Arabidopsis SD varies with genotype and species. However, analysis of the variations in three primary physiological factors such as *E*, *A*, and g_s between a subset of mutants involved in stomatal development has not been conducted. Stomata are found on nearly all aerial plant parts, but this study focused only on the leaf. Pore aperture controls the amount of water leaving the leaf during *E*. In response to environmental influences, stomatal densities have been known to increase or decrease over long periods of time (Chen et al. 2001) however genetic mutations can also influence density and distribution of stomata (Alonso et al. 2003). Evidence is presented here to indicate relative differences in *A*, *E*, and g_s between a subset of Arabidopsis SD mutants.

Stomatal development and density in Arabidopsis has been described and characterized and the reference data concerning the various mutant phenotypes has increased. By examining the developmental cues for stomatal patterning, a greater degree of comprehension can be achieved when evaluating developmental signaling pathways. Studies that address stomatal size, density, and aperture have been described and a number of physiological variations as a result of changes in water availability have been recorded amongst various species including Arabidopsis (Serna 2009; Peterson et al. 2010). A number of SD mutants have been characterized, however this study incorporated some of those most widely described in the literature and provided background and expression data for several others (see Supplemental Table 1).

It is hypothesized that analysis of SD in addition to several physiological parameters would result in significant correlations between CO_2 assimilation rate (*A*) and SD. Decreased SD could be presumed to coincide with decreased leaf transpiration (*E*) or provide data to support assumptions that increased SD does not greatly influence growth and development. Despite our knowledge that a variety of endogenous signals could be at work to override SD effects, we are using these data to demonstrate variation in physiological parameters of mutants with varied SD. The objective was to examine the density of leaf stomata in a selection of Arabidopsis SD mutants and examine the

physiological parameters involved in growth under identical experimental conditions.

Results

Arabidopsis SD Mutants Varied in Water Loss

Stomatal pore apertures decreased as guard cell lengths increased in Arabidopsis control plants exposed to water-stress (Fig. 1). Stomatal apertures of well-watered plants were 2.55±0.29 μm however, apertures of mildly water-stressed and severely water-stressed plants were significantly decreased (1.75±0.16 μm) and (1.23±0.21 μm) respectively (Fig. 1A). The observed increase in guard cell length was also statistically significant (Fig. 1B). These data indicated that care needed to be taken when examining SD results to ensure that the mutant Arabidopsis plants being used for analysis had not suffered from water stress. Measurements of water loss in Arabidopsis SD mutants indicated that mutants with the greatest SD were not necessarily associated with the greatest water loss. The data collected were the result of a

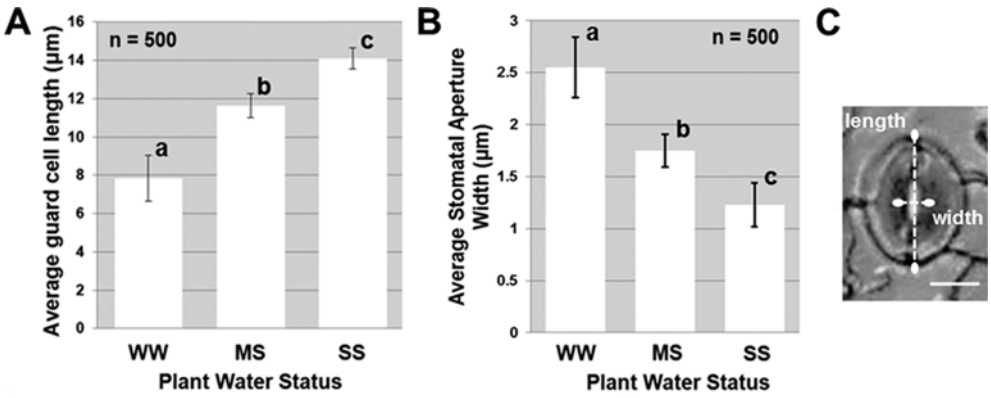

Fig. 1. Changes in stomatal pore aperture and guard cell length in water-stressed Arabidopsis. (A) Bar graph of differences in guard cell length clearly indicated a decreased stomatal aperture in response to water stress. (B) Bar graph of stomatal apertures indicated the expected corresponding variation in pore width. (C) Illustration of a mildly-stressed plant stoma with demarcations as to where measurements were taken. Well-watered (WW), Mildly-stressed (MS) and severely stressed (SS) plants were indicated. Means superscripted with the same letter were not significantly different at $p < 0.05$. Bar = 2.5 μm Error bars (±SEM)

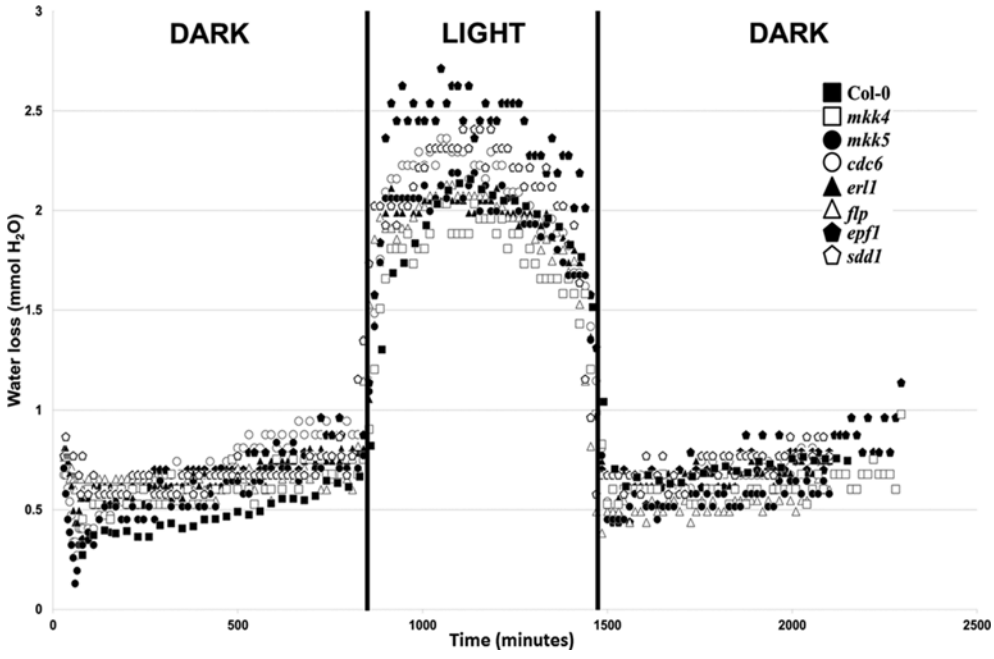

Fig. 2. Arabidopsis mutant gravimetric water loss data. (A) Transpiration data of mutants randomly selected for additional physiological analysis and (B) those mutants not further analyzed. (C) Compilation of data from all mutants in the study indicated *epf1* displayed the greatest water loss. Dark and light periods were clearly marked.

single measurement of three plants with no replication (Table 1). Gravimetric analysis of individual mutants over the course of 36 h showed that SD mutants varied widely in degree of water loss under continued water-withdrawal conditions (Fig. 2, Table 1). The *epf1* mutant line, over the course of 36 h, showed the greatest degree of water loss. Excessive water loss from these lines was initially attributed to the increased stomatal numbers found in the mutant plants. The SD variation between the mutants in this study ranged from 168.8 mm^{-2} to 626.8 mm^{-2} (Table 1). An in-depth analysis of published Arabidopsis SD mutant data was used to help formulate theories to explain this phenomenon. Suppositions to explain this occurrence were: incomplete stomatal closure at night, premature stomatal opening, or abscisic acid (ABA) insensitivity. Theories of incomplete stomatal closure were illustrated by examination of overnight gravimetric water loss for each mutant when compared to wild-type plants while ABA insensitivity was not tested in this study. Although Arabidopsis plants typically exhibit a small degree of nighttime respiration, *epf1* mutants represented the greatest volume of overall water loss over 36 h when compared to controls and the other mutant lines (Fig. 2). Closer inspection of the gravimetric water loss data showed that the mutant lines with the highest water loss over 36 h were *epf1*, *mpk3*, and *mpk6* while the *tmm*, *mkk4*, and *yoda* mutants had lower amounts of overnight water loss (Fig. 2). Transpiration analysis data also indicated *epf1* mutant stomata did not open or close prematurely to bolster water loss rates.

Table 2. Stomatal density and physiological parameters for a selection of Arabidopsis mutants

Mutant	*A* (μmol m^{-2} s^{-1})	g_s (mol m^{-2} s^{-1})	*E* (mmol m^{-2} s^{-1})	SD (mm^{-2})	WUE_i (μmol $mmol^{-1}$)
cdc6	12.40 ± 0.23^{e}	0.50 ± 0.16^{d}	7.07 ± 1.69^{f}	210.6 ± 18.3^{e}	1.75 ± 0.11^{e}
epf1	19.95 ± 0.26^{c}	0.49 ± 0.10^{d}	7.08 ± 0.92^{f}	441.5 ± 24.1^{b}	$2.82 \pm 0.18^{b,c,d}$
erl1	22.70 ± 1.99^{b}	$0.61 \pm 0.14^{c,d}$	$8.66 \pm 1.75^{c,d,e}$	211.4 ± 41.3^{e}	2.62 ± 0.14^{b}
flp	23.36 ± 3.46^{b}	0.77 ± 0.07^{b}	$9.77 \pm 0.16^{b,c}$	252.6 ± 15.7^{d}	$2.39 \pm 0.29^{b,c,d}$
mkk4	22.79 ± 0.20^{b}	0.85 ± 0.08^{b}	9.96 ± 0.21^{b}	189.2 ± 26.9^{f}	$2.29 \pm 0.64^{c,d}$
mkk5	25.91 ± 1.32^{a}	$0.59 \pm 0 .08^{b}$	$7.67 \pm 0.78^{e,f}$	$204.7 \pm 14.5^{e,f}$	3.38 ± 0.91^{a}
sdd1	21.95 ± 0.52^{b}	0.65 ± 0.05^{c}	$8.30 \pm 0.75^{d,e}$	462.7 ± 36.0^{a}	$2.65 \pm 0.62^{b,c}$
Col-0	17.46 ± 1.91^{d}	0.26 ± 0.03^{e}	4.56 ± 0.13^{g}	168.8 ± 17.6^{g}	3.83 ± 0.46^{a}

A, Net CO_2 assimilation; g_s, Stomatal conductance; *E*, Transpiration; SD, Stomatal density; WUE_i, Instantaneous water-use efficiency. All statistical data were obtained from comparisons within each physiological parameter. Means superscripted with the same letter were not significantly different at $p < 0.05$.

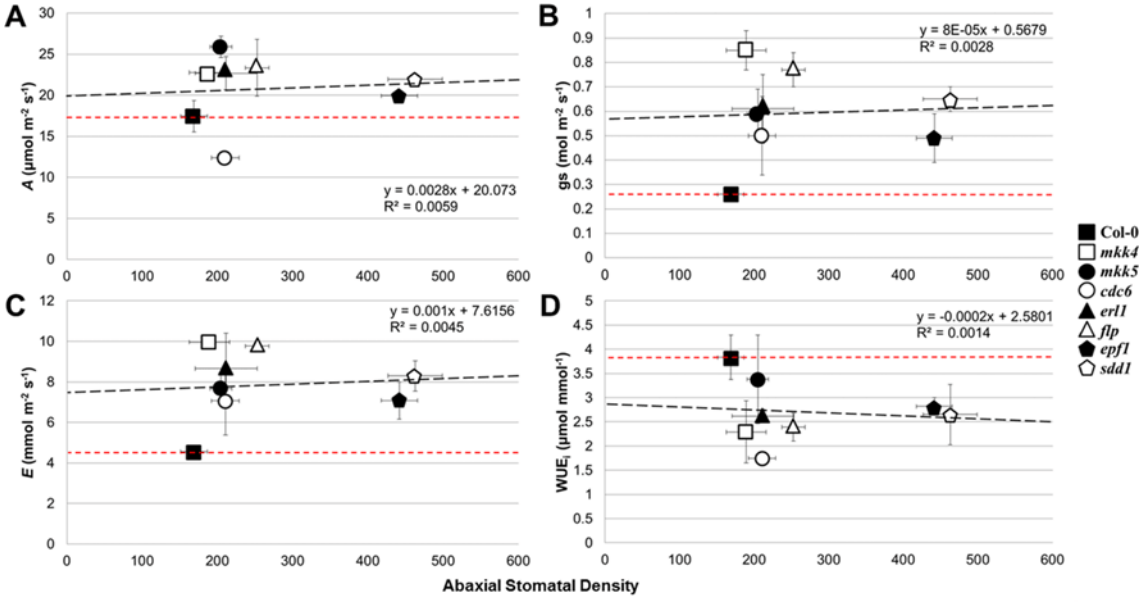

Fig. 3. Comparison of stomatal density and physiological parameters. (A) Net CO_2 assimilation (*A*) data from the Arabidopsis mutants indicated no correlation existed, with the square of the correlation coefficient (R^2) value of 0.0117. (B) Stomatal conductance (g_s) data with a value of 0.0067, (C) transpiration (*E*) data with a value of 0.0006, and (d) instantaneous water-use efficiency (WUE_i) data with a value of 0.0014 all showed no correlation. Control plants were indicated by the dashed black line while the trendline was represented by the dashed grey line. Error bars (±SEM)

Arabidopsis Mutant SD and Physiological Parameters

Individual Arabidopsis SD mutant lines grown under identical conditions demonstrated varied rates of g_s, net *A*, and *E* (Table 2). Graphical analysis of *A* and average SD indicated that a number of mutant lines demonstrated greater rates of *A* than Col-0 plants (Fig. 3A, Table 2). These data indicated that previous findings where decreased SD led to decreased *A* were likely true in one situation, but not another. The variation in water loss among lines led to the theory that stomatal aperture and not density may be a contributing factor to irregularities in assimilation. The *mkk5*, *flp*, *mkk4*, and *erl1* mutant lines displayed the higher *A* and the lower assimilation rates were recorded for *cdc6* and the wild-type Col-0 (Fig. 3A, Table 2). These high and low assimilation rates varied across a wide range (from 12.4 to 25.9 μmol m^{-2} s^{-1}) and could have contributed to the slower initial growth for those mutants with lower assimilation rates when observed in the greenhouse (Table 2, data not shown). Although *cdc6* plants generated the lowest assimilation values, total leaf fresh weight data indicated that this line did not have the lowest fresh weight recorded (Table 3). Upon bolting, these mutants were indistinguishable from the other lines with the exception of *yda*. Several mutants with lower stomatal densities had greater *A* rates than those Arabidopsis mutants with higher SD. No observable patterns were found for *A* among the selected mutants and SD in this study.

It has been speculated that reduced SD leads to reduced g_s, because with fewer available stomata a more limited amount of CO_2 would be obtained and in response, less water can be transpired. There was no correlation observed between g_s data and SD for the mutant lines examined in this study (R^2= 0.0028) (Fig. 3B). A common pattern emerged between g_s and *A* in *cdc6* and several other mutant lines. In addition to having the lowest overall *A* rates (12.40±0.23 μmol m^{-2} s^{-1}), *cdc6* lines also displayed one of the lower g_s rates (0.50± 0.16 mol m^{-2} s^{-1}) and *E* rates (7.07±1.69 mmol m^{-2} s^{-1}) (Fig. 3A, B). The greatest conductance rates were seen in *mkk4* (0.85±0.08 mol m^{-2} s^{-1}) and *flp* (0.77±0.07 mol m^{-2} s^{-1}) mutant plants. Lines that displayed the lower conductance readings were Col-0, *epf1*, and *cdc6*. With the exception of Col-0, g_s data from the remaining mutants were all within 0.36 mol m^{-2} s^{-1} from highest to the lowest.

Analysis of the Arabidopsis SD mutant transpiration rates indicated that *E* varied independently of SD (Fig. 3C). Yoo et al. (2010) reported that decreased SD led to decreased *E* in *gtl1*, a SD mutant with a close association with the *sdd1* mutant examined in this study, and increased WUE_i. No correlation existed (R^2=0.0045) among mutants in this study despite SD levels for some mutants being greater than control plants. Those lines with greater SD than wild-type did not also exhibit higher rates of *E* (Fig. 3C). The greater transpiration rates were seen in *mkk4* plants (9.96±0.21 mmol m^{-2} s^{-1}) and *flp* (9.77±0.16 mmol m^{-2} s^{-1}) while the lower transpiration rates were seen in wild-type (9.96±0.21 mmol m^{-2} s^{-1}), *cdc6* (7.07±0.69 mmol m^{-2} s^{-1}), and *epf1* (7.08±0.92 mmol m^{-2} s^{-1}) lines (Table 2). The WUE_i data from the individual mutant lines demonstrated no correlation (R^2=0.0014) to SD in the Arabidopsis mutant lines examined (Fig. 3D). Calculation of WUE_i among all of the mutants showed a two-fold difference between the lowest and highest efficiency lines (Fig. 3D). The lowest WUE_i was observed in *cdc6* (1.75±0.11 μmol $mmol^{-1}$) while the highest efficiencies were recorded, as expected, in Col-0 (Table 2). The Arabidopsis *mkk5* mutants had a WUE_i that was closer to Col-0 than any of the other mutant lines. When all physiological data were compared to SD, no clear patterns emerged (Fig. 3A-E, 4, Table 2). There existed a number of differences between SD, g_s, *A*, and *E* between each mutant line (Table 3). None of the mutants studied were similar to Col-0 based on any observed physiological parameter with the exception of *A* and WUE_i. Col-0 and *mkk5* had similar WUE values, however the majority

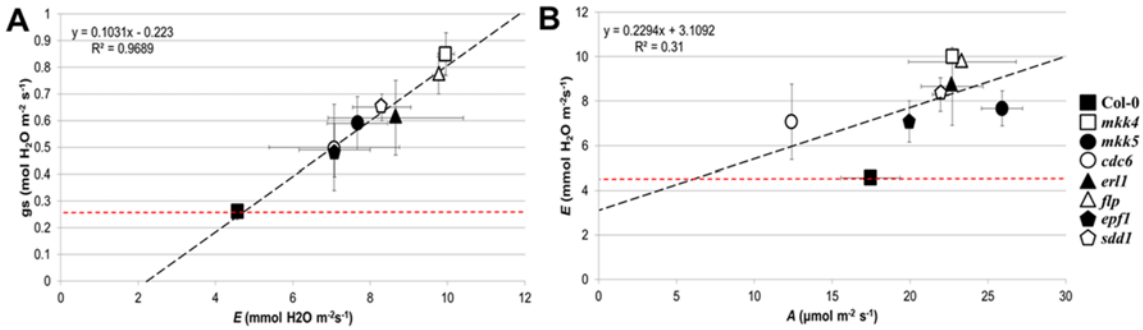

Fig. 4. Comparisons of stomatal conductance (g_s) and transpiration (*E*) and *E* with assimilation (*A*). The g_s and *E* as well as *E* and *A* data from the Arabidopsis mutant lines were graphed to determine if a correlations existed between the two parameters. (A) The square of the correlation coefficient (R^2) indicated a strong positive correlation with a value of 0.9078 existed between the Arabidopsis mutants when g_s versus *E* were evaluated while (B) *E* versus *A* data showed no correlation with a (R^2) value of 0.0062. The control plants were indicated by the dashed black line while the trendline was represented by the dashed grey line. Error bars (±SEM)

all other parameters for the two lines were significantly different (Table 2). Comparisons of SD with the physiological parameters resulted in no correlations being seen between SD and *A* (R^2=0.0059), g_s (R^2=0.0028), *E* (R^2=0.0045), or WUE_i (R^2=0.0014). A strong positive correlation (R^2=0.9689) was seen between g_s and *E* as expected (Fig. 4A). There was a slight correlation between *E* and *A* (R^2=0.31) (Fig. 4B).

Arabidopsis Leaf Fresh Weight (LFW) and Physiological Parameters

Visual observation of above-ground biomass at the start of the study indicated no difference in the size of these mutant plants with the exception of the *yda* mutants, however statistical analysis of leaf fresh weight (LFW) indicated significant differences that may help to explain why more correlations were not seen within the generated data (Fig. 5, Table 3). Measurements of LFW were necessary to verify that undulations in water loss were not attributed to differences in initial plant size. A mutant that displays a dwarf phenotype, it was not a surprise to note the resultant diminutive growth of plants in the *yda* line. Attempts to find relationships

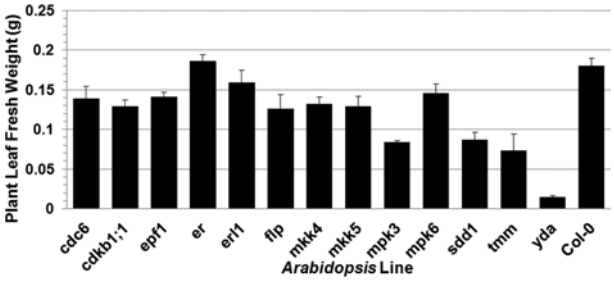

Fig. 5. Arabidopsis mutant average leaf fresh weight. All leaves from three plants of each mutant line were removed and immediately weighed to obtain fresh weight. Numerical data and statistical analysis for these lines can be found in Table 3. Error bars (±SEM)

Table 3. Summary of *Arabidopsis* mutant leaf fresh weights[1]

Arabidopsis Mutant Line	*Arabidopsis* Leaf Fresh Weight (mg)
cdc6	$138.6 \pm 15.8^{a,b,c}$
cdkb1;1	$129.0 \pm 9.2^{b,c,d}$
epf1	$140.9 \pm 8.0^{a,b,c}$
er	186.6 ± 9.1^{a}
erl1	$159.2 \pm 30.9^{a,b}$
flp	$162.1 \pm 27.8^{a,b}$
mkk4	$131.8 \pm 6.7^{b,c,d}$
mkk5	$129.3 \pm 25.5^{b,c,d}$
mpk3	$84.0 \pm 4.4^{d,e}$
mpk6	$145.7 \pm 28.2^{a,b}$
sdd1	$86.8 \pm 13.2^{d,e}$
tmm	73.8 ± 4.7^{e}
yda	14.4 ± 0.87^{f}
Col-0	$180.0 \pm 11.2^{a,b}$

[1]Weights represent the average of all *Arabidopsis* leaves from three mutant plants. Means (±SEM) superscripted with the same letter were not significantly different at $p < 0.05$.

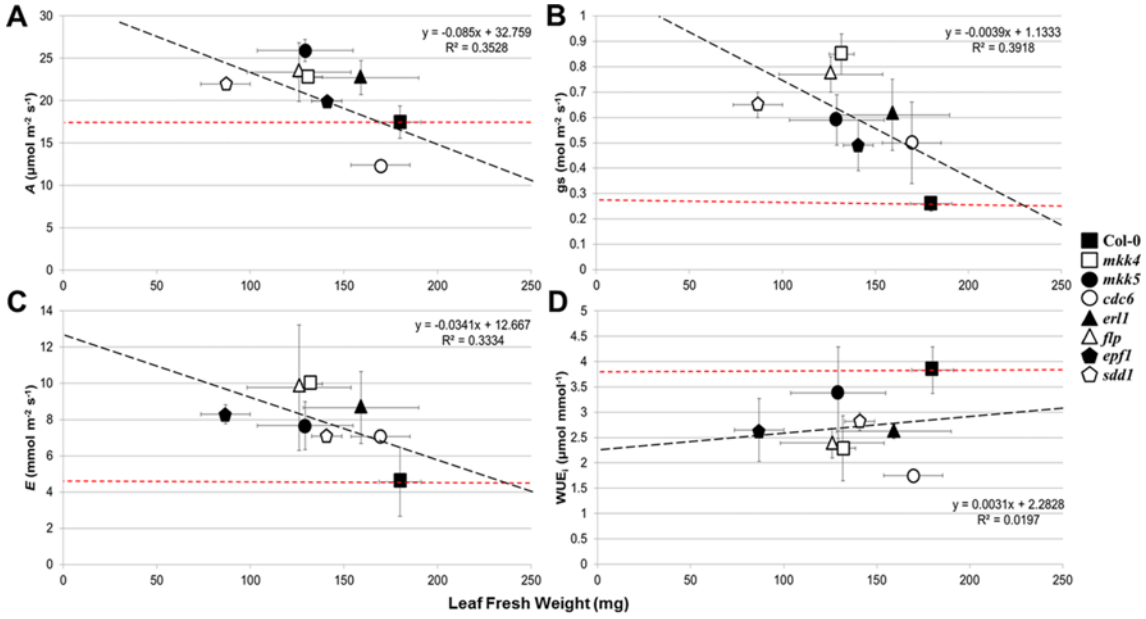

Fig. 6. Arabidopsis mutant physiological parameters compared with average leaf fresh weight (LFW_{avg}). Negative correlations existed between LFW_{avg} and (A) Net CO_2 assimilation (*A*) (R^2=0.0444), (B) stomatal conductance (g_s) (R^2=0.5026), and (C) transpiration (*E*) (R^2=0.516), and both g_s and *E* showed greater negative correlations than *A*. (D) A weak positive correlation (R^2=0.2227) was seen between LFW_{avg} and WUE_i. The control plants were indicated by the dashed black line while the trendline was represented by the dashed grey line. Error bars (±SEM)

between LFW and physiological parameters resulted in negative correlation being drawn between g_s, net *A*, and *E.* No correlation was observed between leaf area and WUE_i. Associations between fresh weight and WUE_i were the poorest (R^2=0.0197) followed by *E* (R^2=0.3334), net *A* (R^2= 0.3528), and g_s (R^2=0.3918) (Fig. 6).

Abnormal Mutant Arabidopsis Phenotypes

In addition to the bunching phenotype often found in some of these SD mutants, a number of other morphological alterations were observed in these plants. Exceedingly long pavement cells in two of the mutated protein kinase lines *mpk3* and *mpk6* were observed (Fig. 7A, B). These significantly lengthened pavement cells were found on both leaf surfaces and clearly indicated a break-down in cell division signaling when compared to WT (Fig. 7C). Statistical analysis indicated that pavement cells of *mpk3* plants were longer while those of *mpk6* were wider than pavement cells in WT plants (Fig. 7C-E). The characteristic abnormalities observed were joined and clustered stomata. Many lines demonstrated clustered stomata, but consistent pairing phenotypes were observed in *epf1*, *erl1*, *mpk3*, *mkk5*, and *sdd1* (Fig. 8). All of the SD mutants have different mechanisms of action, yet many of these displayed the same or similar paired phenotypes. This pairing tendency found in a number of the studied mutants emphasized the ablation of the one-cell spacing rule that EPF1 and other genes control.

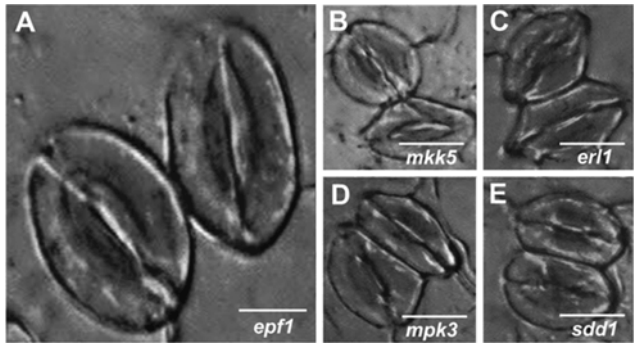

Fig. 8. Paired stomata were found in a number of mutant lines. A number of joining morphologies were observed from (A) barely touching parallel joins in *epf1* to (B) barely touching perpendicular joining in *mkk5*. (C) A greater degree of parallel joining was seen in *erl1*, (D) *mpk3*, and (E) *sdd1*. (A) Bar = 5 μm, (B–E) Bar = 10 μm

An unusual phenotype discovered that has not previously been described was variation in trichome morphology between mutant lines (Fig. 9A-D). Although abnormal trichomes were not highly prevalent, these were present in noticeable numbers. Rather than the typical trident-shaped trichomes, *flp*, *mpk3*, *mpk6*, and *sdd1* lines displayed abnormal formations of trichomes in addition to the described stomatal mutations and morphologies (Fig. 9A-D). When trichomes were removed, the supportive pavement cells surrounding the base were revealed (Fig. 9E).

Examination of these cells led to the hypothesis that these particular pavement cells provided a foundation for the

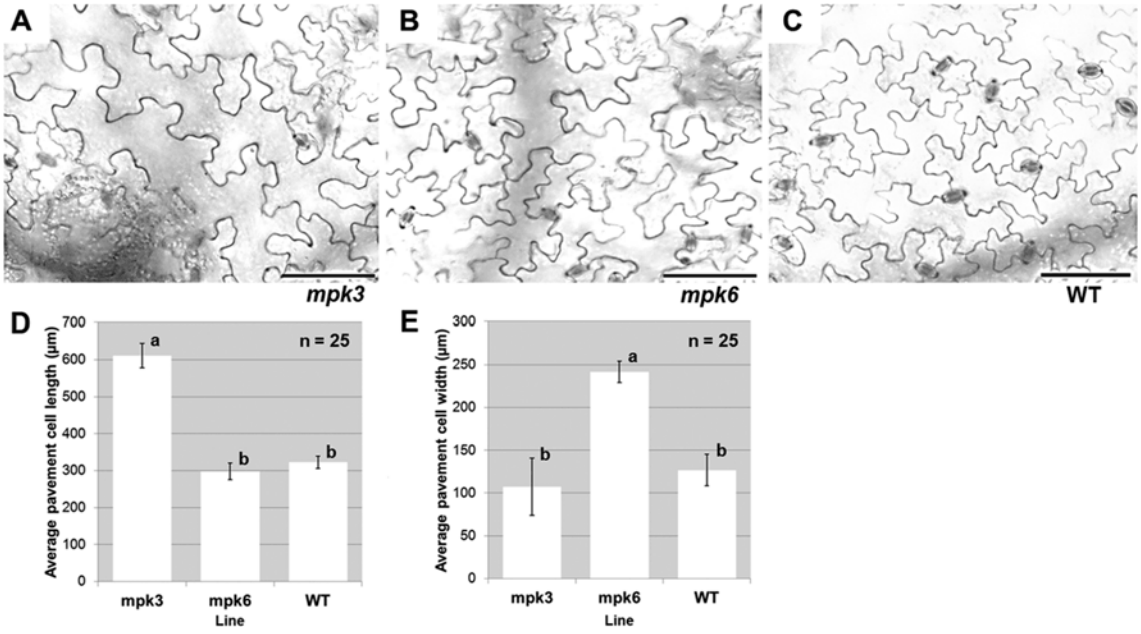

Fig. 7. Abnormally shaped pavement cells. (A) The pavement cells in *mpk3* were greater than four times longer than adjacent cells while the pavement cells in *mpk6* (B) were elongated both lengthwise and widthwise when compared to (C) wild-type. Statistical analysis confirmed observations for (D) length and (E) width. Means superscripted with the same letter were not significantly different at $p < 0.05$. Bar = 200 μm Error bars (±SEM)

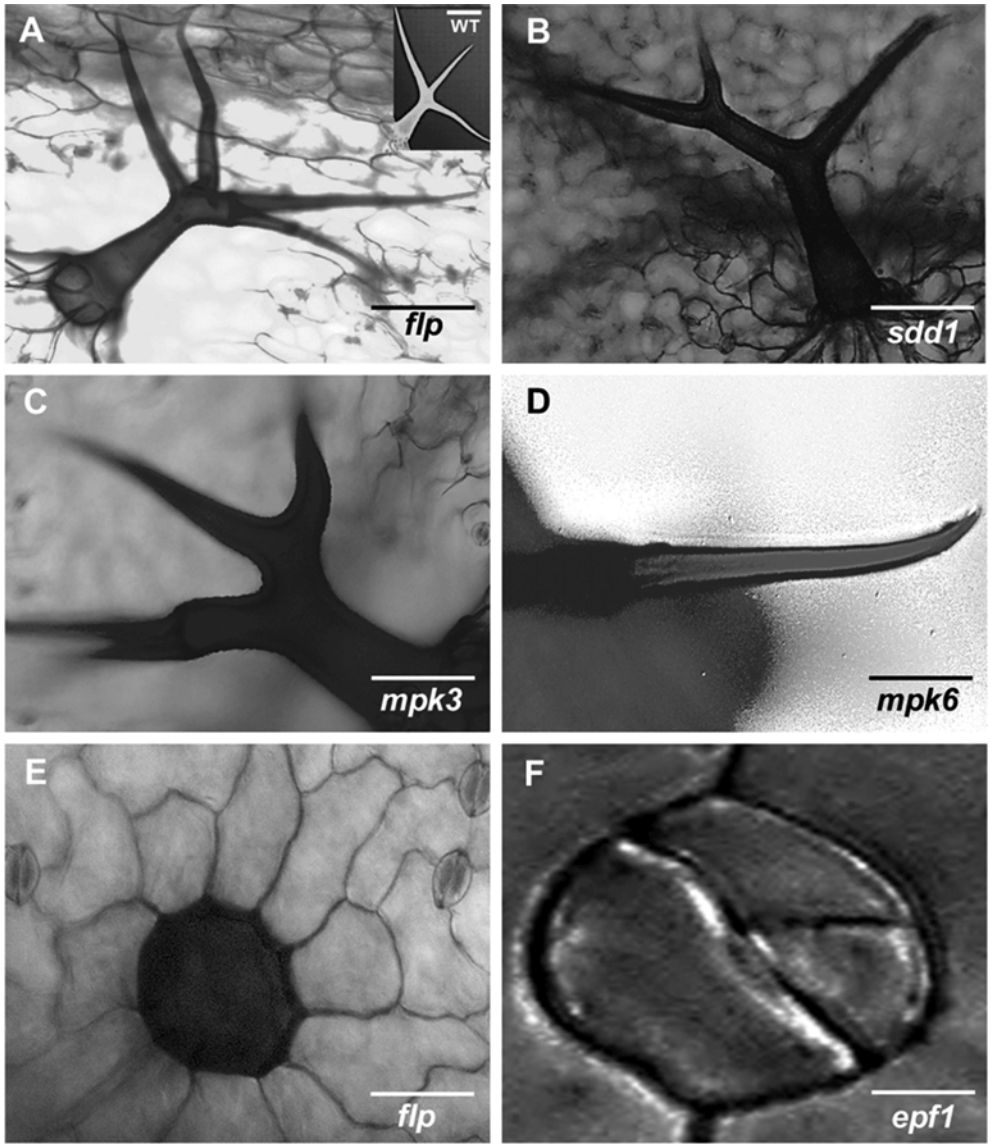

Fig. 9. Unusual trichome phenotypes. Rather than (A, inset) three equilateral branches found per trichome in wild-type (WT), (A) *flp* mutants were often found with an additional branch point when compared to WT. (B) Instead of the formation of an additional branch point *sdd1* mutants exhibited a single main fork with an additional branch point at the tip of one side of the fork. (C) Additional branch points were found on both tips of a single fork in *mpk3*. (D) A small subset of trichomes in *mpk6* showed a distinctive lack of branching phenotype in a number of leaves examined. (E) In this Arabidopsis *flp* leaf, removal of a trichome emphasized the number of supportive pavement cells associated with each individual trichome. These pavement cells also seem to act to ensure stomata were not formed in close proximity to trichomes. (F) Although only one mutant line is pictured, several mutant lines (*epf1*, *fama*, *scream1*, and *sdd1*) also exhibited an arrested guard cell phenotype such as the one seen in *epf1* (Table 1). (A–E) Bar = 100 μm (F) Bar = 5 μm

trichome, and a boundary against the formation of adjacent stomata in addition to preventing the establishment of stomata in close proximity to the trichome. None of the mutant lines examined displayed phenotypes that were an exception to this rule. As trichomes can serve as an additional water reservoir, examination of their presence, absence, or abnormal morphology could explain the increased transpiration of particular mutant lines.

Despite all of the leaf samples being harvested when the plants were fully mature, the presence of immature guard cells was a frequent occurrence in several mutant lines including *epf1* (Fig. 9F). These immature cells indicated an ablation of the signal required for proliferation and differentiation into mature guard cells.

Conclusions

Stomata are well known for their plasticity in response to varied abiotic conditions. Data presented here show that

decreased leaf SD does not prevent uptake of additional CO_2 or hinder leaf biomass accumulation. Measurements of total leaf area indicated that mutants with higher stomatal densities than Col-0 plants did not exhibit the expected increase in leaf area. Therefore, additional studies with SD mutants or studies that involve the highest biomass producing Arabidopsis mutant plants are needed to more convincingly state whether or not leaf area would be significantly affected in plants with decreased stomatal densities. Destructive sampling of each mutant line indicated significant differences in fresh weights for many of the examined mutant lines with the greatest difference being seen in *yda*, a mutant with a dwarf phenotype and delayed growth. Further studies that characterize mutants with progressively fewer stomata may uncover a threshold where normal growth and development begins to decrease, however this study did not reach that physiological limit. The results obtained here indicated that present levels of CO_2 were sufficient to satisfy growth requirements for these Arabidopsis mutants even in the absence of uniform stomatal numbers. The fact that the majority of the plants measured in this study were mutant lines could be a factor that contributed to the variations in physiological parameters. It was also possible that additional epistatic or downstream effects may have influenced resultant observations. The effect that each mutation has on growth has not been overlooked. Thorough examination of previously published research indicated an absence of data regarding large numbers of Arabidopsis SD mutants examined for these parameters. Future studies should focus either on two or three mutant lines that are closely related in SD to observe growth and biomass variation as well as morphological abnormalities throughout the lifetime of the plant, or a single mutant line and its growth in response to a host of environmental conditions. These milieus may be responsible for variation seen both here and in the literature regarding correlations between A, g_s, E, and WUE_i. It was not the objective of this work to thoroughly exhaust all possible causes for biomass variation in these mutants. These data uncover and highlight variation in SD mutant biomass, morphology, and physiological characteristics and provide plausible justifications worthy of further study.

Materials and Methods

Plant Materials and Growth Conditions

Arabidopsis plants were grown in the greenhouse under controlled conditions. Average greenhouse temperatures ranged between 23.2°–23.7°C each day with the highest and lowest recorded temperatures being 18.4° and 30.7°C on one day of the growth period. Relative humidity remained steady at 60% under a long-day photoperiod (16/8-h) and light levels ranged from 200–400 μmol $m^{-2}s^{-1}$ based on natural light. All plants were grown in individual 10 cm pots. Pots were placed in watering trays (28 cm × 43 cm) that were filled to the brim with water every 3 d. All pots were removed from the trays after 1.5 h and placed on greenhouse benches. The water regime was only altered at the beginning of the water withdrawal experiment. No additional water was provided once the experiment started. The soil mix was a 3:1 mixture of superfine germinating mix (Farfand) composed of 55% Canadian sphagnum peat, perlite and vermiculite to turface (MVP).

Arabidopsis Seed Disinfestation

Seeds of Arabidopsis (Col-0) were rinsed with distilled water and surface-disinfested with 15% commercial bleach solution (5.25% sodium hypochlorite) and 0.1% sodium dodecyl sulfate (SDS) for 10 min. Seeds were then rinsed three times with sterile, distilled water. Aseptic seeds were stratified at 4°C in a 0.1% agar solution for 4 days before being germinated on half-strength Murashige and Skoog (MS; [Sigma M519]) medium (Murashige and Skoog 1962).

Arabidopsis Mutant Material and Seed Screening

Mutant lines used for this experiment were: *cdc6* (SALK_093678), *cdkB1;1* (SALK_073457), *epf1* (SALK_137549), *er* (CS20), *erl1* (SALK_019571), *flp* (SALK_033970C), *mkk4* (SALK_058307), *mkk5* (SALK_050700), *mpk3* (SALK_100651), *mpk6* (SALK_073907), *sdd1* (SALK_035560), *tmm* (SALK_017816), and *yoda* (CS85662; Torii et al. 1996; Till et al. 2003). Lines were grown to the T3 generation after continuous screening for kanamycin resistance based on the protocol by Harrison et al. 2006. PCR confirmed the presence of kanamycin (data not shown). All mutant seeds were obtained from the Arabidopsis Biological Research Center (ABRC) found at The Arabidopsis Resource Center (TAIR) website (www.arabidopsis.org). Each mutant was generated and reported by Alonso et al. (2003) unless otherwise noted.

Gravimetric Analysis

Gravimetric transpiration data were obtained from 5-week-old Arabidopsis mutants grown in inverted 50 ml conical tubes in a growth chamber. The solid end cap was replaced by a wire mesh and a punctured end cap during pre-analysis growth to allow for adequate watering. At the start of the analysis period the mesh and punctured end caps on the 50 ml conical tubes were replaced with a solid end cap for all mutants. Soil evaporation was eliminated by sealing the bottom and top of the tube. No additional water was provided once the experiment began and only the aerial parts of the plant protruded. Whole plant transpiration data (mmol H_2O^{-1}) was obtained by placing a total of three individually grown plants onto a balance that recorded weight measurements in 5 min intervals over the course of 36 h. The graph depicts data from every 30 min for improved clarity. Only one plant was placed on each balance in this study. Data represent transpirational water loss from each mutant. Each "icon" represents the average of 3 trials performed within the growth chamber. Statistical error bars (± SEM) are not visualized for clarity.

Leaf Fresh Weight

Arabidopsis mutant average leaf total fresh weight data were obtained by carefully removing all of the leaves from three plants from each mutant line. All of the individual leaves were immediately weighed to obtain fresh weight data. No stems or petioles were included.

Stomatal Imaging by Microscopy

Three fully-expanded rosette leaves were collected from healthy

mature Arabidopsis plants several weeks after bolting and silique growth, cleared with 70% ethanol, and stained with Safranin-O (1 μg mL^{-1}). Differential Interference Microscopy (DIC) images of the abaxial surface were captured with a Zeiss LSM710 microscope (Carl Zeiss, Inc.). Images of four mid-leaf sections (0.5 mm^2) from each of three leaf rosettes were obtained for each mutant. Counts of stomatal number were performed by outlining an image to include a single 1 mm^2 area. Photos were then printed and stomata were counted by hand and average densities were calculated through the examination of three photos per rosette leaf per Arabidopsis mutant.

Guard Cell Length and Pore Aperture Measurements

Two fully-expanded rosette leaves were collected from healthy mature Arabidopsis plants several weeks after bolting and silique growth, immediately coated with a thin layer of clear nail polish, and placed on a cover slip. After 30 seconds, polish was peeled from the leaves and placed on a labeled cover slip. Images of each leaf were captured using DIC with a Zeiss LSM710 microscope (Carl Zeiss, Inc.). Two hundred fifty stomata were measured per leaf. This study was completed in triplicate with 3 blocks of plants (well-watered, mildly water-stressed, severely water-stressed).

Physiological Measurement Collection

A Li-Cor LI6400XT (LICOR Biosciences) was used to obtain transpiration, photosynthesis, and stomatal conductance data. All data were collected from plants distributed in a complete random block design. Measurements were taken in triplicate on the same day for 3 days in a row. Final calculations were based on three consecutive days of data collection from three leaves obtained from three individual plants. Instantaneous water-use efficiency [WUEi; (μmol $mmol^{-1}$)] was calculated from *A* divided by *E*.

Statistical Analysis

Calculation of standard error of the mean (SEM) and balanced analysis of variance were performed on SD data. Results of $p < 0.001$ were deemed significant. Analysis was conducted on data gathered from abaxial leaf surfaces only. All analyses were performed using SAS software programs (SAS Institute Inc. 2008).

Acknowledgements

The authors thank Drs. Yiwei Jiang and Jaemo Yang for their thoughtful comments on a previous version of this manuscript. This work was supported by the Fred M. van Eck Foundation [grant number FVE51020058] and the Alliance for Graduate Education and Professoriate (AGEP) [grant number NSF0450373] at Purdue University. Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the US Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that also may be suitable.

Author's Contributions

SSL designed the experimental plans, acquired and screened the Arabidopsis seeds, grew and maintained the plants, collected and analyzed the data, and wrote the manuscript. PMP and CHM reviewed a first draft of the manuscript. All the authors agreed on the contents of the paper and post no conflicting interest.

Supporting Information

Table S1. Summary of some of the most studied Arabidopsis stomatal density and development genes.

References

Abrash E, Bergmann DC (2010) Regional specification of stomatal production by the putative ligand CHALLAH. Development 137:447–455. DOI: 10.1242/dev.040931

Abrash E, Lampard GR (2010) A view from the top: new ligands controlling stomatal development in Arabidopsis. New Phytol 186:561–564. DOI: 10.1111/j.1469-8137.2010.03265.x

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, Gadrinab C, Heller C, Jeske A, Koesema E, Meyers CC, Parker H, Prednis L, Ansari Y, Choy N, Deen H, Geralt M, Hazari N, Hom E, Karnes M, Mulholland C, Ndubaku R, Schmidt I, Guzman P, Aguilar-Henonin L, Schmid M, Wrigel D, Carter DE, Marchand T, Risseeuw E, Brogden D, Zeko A, Crosby WL, Berry CC, Ecker JR (2003) Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science 301:653–657. DOI: 10.1126/science.1086391

Alwerdt JL, Gibson DJ, Ebbs SD, Wood AJ (2006) Intraspecific interactions in *Arabidopsis thaliana* and the stomatal mutants *tmm1-1* and *sdd1-2*. Biol Plantarun 50:205–209. DOI: 10.1007/s10535-006-0008-2

Berger D, Altmann T (2000) A subtilisin-like serine protease involved in the regulation of stomatal density and distribution in *Arabidopsis thaliana*. Gene Dev 14:1119–1131. DOI: 10.1101/gad.14.9.1119

Bergmann DC, Lukowitz W, Somerville CR (2004) Stomatal development and pattern controlled by a MAPKK kinase. Science 304:1494–1497. DOI: 10.1126/science.1096014

Bertoni G (2009) Integration of signaling pathways in stomatal development. Plant Cell 21:2542. DOI: 10.1105/tpc.109.210910

Bhave NS, Veley KM, Nadeau JA, Lucas JR, Bhave SL, Sack FD (2009) TOO MANY MOUTHS promotes cell fate progression in stomatal development of Arabidopsis stems. Planta 229:357–367. DOI: 10.1007/s00425-008-0835-9

Boudolf V, Lammens T, Boruc J, van Leene J, van Den Daele H, Maes S, van Isterdael G, Russinova E, Kondorski E, Witters E, De Jeager G (2009) CDKB1;1 forms a functional complex with CYCA2;3 to suppress endocycle onset. Plant Physiol 150:1482–1493. DOI: 10.1104/pp.109.140269

Brownlee C (2001) The long and the short of stomatal density signals. Trends Plant Sci 6:441442. DOI: 10.1016/S1360-1385(01)02095-7

Büssis D, von Groll U, Fisahn J, Altmann T (2006) Stomatal aperture can compensate altered stomatal density in *Arabidopsis thaliana* at growth light conditions. Funct Plant Biol 33:1037–1043. DOI: 10.1071/FP06078

Chen LQ, Cheng-Sen L, Chaloner WG, Beerling DJ, Sun Q-G, Collinson ME, Mitchell PL (2001) Assessing the potential for the stomatal characters of extant and fossil Ginkgo leaves to signal atmospheric CO2 change. Am J Bot 88:1309–1315. DOI: 10.2307/3558342

Chinnusamy V, Ohta M, Kanrar S, Lee BH, Hong X, Agrawal M, Zhu JK (2003) ICE1: a regulator of cold-induced transcriptome and freezing tolerance in Arabidopsis. Gene Dev 17:1043–1054. DOI: 10.1101/gad.1077503

Dong J, MacAlister CA, Bergmann DC (2009) BASL controls asymmetric cell division in Arabidopsis. Cell 137:1320–1330. DOI: 10.1016/j.cell.2009.04.018

Fraser LH, Carlyle C, Turkington R, Friedman CR (2009) Adaptive phenotypic plasticity of *Pseudoroegneria spicata*: response of

stomatal density, leaf area and biomass to changes in water supply and increased temperature. Ann Bot- Lon 103:769–775. DOI: 10.1093/aob/mcn252

Gao T, Wu Y, Zhang Y, Liu L, Ning Y, Wang D, Tong H, Chen S, Chu C, Xie Q (2011) *Os*SDIR1 overexpression greatly improves drought tolerance in transgenic rice. Plant Mol Biol 76:145–156. DOI: 10.1007/s11103-011-9775-z

Geisler M, Nadeau J, Sack FD (2000) Oriented asymmetric divisions that generate the stomatal spacing pattern in Arabidopsis are disrupted by the *too many mouths* mutation. Plant Cell 12:2075–2086. DOI: 10.2307/3871106

Guseman JM, Lee JS, Bogenschutz NL, Peterson KM, Virata RE, Xie B, Kanaoka MM, Hong Z, Torii KU (2010) Dysregulation of cell-to-cell connectivity and stomatal patterning by loss-of-function mutation in Arabidopsis *CHORUS* (*GLUCAN SYNTHASE-LIKE 8*). Development 137:1731–1741. DOI: 10.1242/dev.049197

Hara K, Kajita R, Torii KU, Bergmann DC Kakimoto T (2007) The secretory peptide gene EPF1 enforces the stomatal one-cell-spacing rule. Gene Dev 21:1720–1725. DOI: 10.1101/gad.1550707

Hara K, Yokoo T, Kajita R, Onishi T, Yahata S, Peterson KM, Torii KU, Kakimoto T (2009) Epidermal cell density is autoregulated via a secretory peptide, EPIDERMAL PATTERNING FACTOR2 in Arabidopsis leaves. Plant Cell Physiol 50:1019–1031. DOI: 10.1093/pcp/pcp068

Harrison SJ, Mott EK, Parsley K, Aspinall S, Gray JC, Cottage A (2006) A rapid and robust method of identifying transformed *Arabidopsis thaliana* seedlings following floral dip transformation. Plant Methods 2:19. DOI: 10.1186/1746-4811-2-19

Hoover WS (1986) Stomata and stomatal clusters in *Begonia*: Ecological response in 2 Mexican species. Biotropica 18:16–21. DOI: 10.2307/2388356

Hord CLH, Sun Y-J, Pillitteri LJ, Wang H, Zhang S, Ma H (2008) Regulation of Arabidopsis early anther development by the mitogen-activated protein kinases, MPK3and MPK6, and the ERECTA and related receptor-like kinases. Mol Plant 1:645–658. DOI: 10.1093/mp/ssn029

Hunt L, Gray JE (2009) The signaling peptide EPF2 controls asymmetric cell divisions during stomatal development. Curr Biol 19:864–869. DOI: 10.1016/j.cub.2009.03.069

Hunt L, Gray JE (2010) BASL and EPF2 act independently to regulate asymmetric divisions during stomatal development. Plant Signal Behav 5:278–280. PMCID: PMC2881277

Kanaoka MM, Pillitteri LJ, Fujii H, Yoshida Y, Bogenschutz NL, Takabayashi J, Zhu J-K, Torii KU (2008) SCREAM/ICE1 and SCREAM2 specify three cell-state transitional steps leading to Arabidopsis stomatal differentiation. Plant Cell 20:1775–1785. DOI: 10.1105/tpc.108.060848

Kondo T, Kajita R, Miyazaki A, Hokoyama M, Nakamura-Miura T, Mizuno S, Masuda Y, Irie K, Tanaka Y, Takada S, Kakimoto T, Sakagami Y (2010) Stomatal density is controlled by a mesophyll-derived signaling molecule. Plant Cell Physiol 51:1–8. DOI: 10.1093/pcp/pcp180

Korn RW (2009) A new hypothesis for stomatal placement in Arabidopsis. J Theor Biol 260:172–174. DOI: 10.1016/j.jtbi.2009.05.012

Lai LB, Nadeau JA, Lucas J, Lee E-K, Nakagawa T, Zhao L, Geisler M, Sack FD (2005) The Arabidopsis R2R3 MYB proteins FOUR LIPS and MYB88 restrict divisions late in the stomatal lineage. Plant Cell 17:2754–2767. DOI: 10.1105/tpc.105.034116

Lammertsma EI, de Boer HJ, Dekker SC, Dilcherc DL, Lotter AF, Wagner-Cremer F (2011) Global CO_2 rise leads to reduced maximum stomatal conductance in Florida vegetation. Proc Natl Acad Sci USA 108:4035–4040. DOI: 10.1073/pnas.1100371108

Lampard GR, Lukowitz W, Ellis BE, Bergmann DC (2009) Novel and expanded roles for MAPK signaling in Arabidopsis stomatal cell fate revealed by cell type–specific manipulations. Plant Cell 21:3506–3517. DOI: 10.1105/tpc.109.070110

Lampard GR, MacAlister CA, Bergmann DC (2008) Arabidopsis stomatal initiation is controlled by MAPK-mediated regulation of the bHLH SPEECHLESS. Science 322:1113–1116. DOI: 10.1126/science.1162263

Liu T, Ohashi-Ito K, Bergmann DC (2009) Orthologs of *Arabidopsis thaliana* stomatal bHLH genes and regulation of stomatal development in grasses. Development 136:2265–2276. DOI: 10.1242/dev.032938

Lukowitz W, Roeder A, Parmenter D, Somerville C (2004) A MAPKK kinase gene regulates extra-embryonic cell fate in Arabidopsis. Cell 116:109–119. DOI: 10.1016/S0092-8674(03)01067-5

Lumbreras V, Vilela B, Irar S, Sole M, Capellades M, Valls M, Coca M, Pages M (2010) MAPK phosphatase MKP2 mediates disease responses in Arabidopsis and functionally interacts with MPK3 and MPK6. Plant J 63:1017–1030. DOI: 10.1111/j.1365-313X.2010.04297.x

MacAlister CA, Ohashi-Ito K, Bergmann DC (2007) Transcription factor control of asymmetric cell divisions that establish the stomatal lineage. Nature 445:537–540. DOI: 10.1038/nature05491

Metzinger CA, Bergmann DC. (2010) Plant asymmetric cell division regulators: pinch-hitting for PARs? F1000 Biol Reports. 2:25. DOI:10.3410/B2-25

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plantarum 15:473–497. DOI: 10.1111/j.1399-3054.1962.tb08052.x

Nawazish S, Hameed M, Naurin S (2006) Leaf anatomical adaptations of *Cenchrus cilliaris* L., from the salt range, Pakistan against drought stress. Pakistan J Bot 38:1723–1730. http://www.pakbs.org/pjbot/PDFs/38(5)/PJB38(5)1723.pdf

Nadeau JA, Sack FD (2002) Control of stomatal distribution on the Arabidopsis leaf surface. Science 296:1697–1700. DOI: 10.1126/science.1069596

Ohashi-Ito K, Bergmann DC (2006) Arabidopsis FAMA controls the final proliferation/differentiation switch during stomatal development. Plant Cell 18:2493–2505. DOI: 10.1105/tpc.106.046136

Peterson KM, Rychel AL, Torii KU (2010) Out of the mouths of plants: The molecular basis of the evolution and diversity of stomatal development. Plant Cell 22:296–306. DOI: 10.1105/tpc.109.072777

Pillitteri LJ, Bogenschutz NL, Torii KU (2008) The bHLH protein, MUTE, controls differentiation of stomata and the hydathode pore in Arabidopsis. Plant Cell Physiol 49:934–943. DOI: 10.1093/pcp/pcn067

Pillitteri LJ, Sloan DB, Bogenschutz NL, Torii KU (2007) Termination of asymmetric cell division and differentiation of stomata. Nature 445:501–505. DOI: 10.1038/nature05467

Porter ACG (2008) Preventing DNA over-replication: a Cdk perspective. Cell Division 3:3. DOI:10.1186/1747–1028-3-3.

Pospíšilová J (2003) Participation of phytohormones in the stomatal regulation of gas exchange during water stress. Biol Plantarum 46:491–506. DOI: 10.1023/A:1024894923865

Rodriguez MCS, Peterson M, Mundy J (2010) Mitogen-activated protein kinase signaling in plants. Annu Rev Plant Biol 61:621–649. DOI: 10.1146/annurev-arplant-042809-112252

Rychel AL, Peterson KM, Torii KU (2010) Plant twitter: ligands under 140 amino acids enforcing stomatal patterning. J Plant Res 123:275–280. DOI: 10.1007/s10265-010-0330-9

SAS Institute Inc (2008) Available at www.sas.com/, Accessed January 5, 2014.

Serna L (2009) Cell fate transitions during stomatal development. BioEssays 31:865–873. DOI: 10.1002/bies.200800231

Shpak ED, Berthiaume CT, Hill EJ, Torii KU (2004) Synergistic interaction of three ERECTA-family receptor-like kinases controls Arabidopsis organ growth and flower development by

promoting cell proliferation. Development 131:1491–1501. DOI: 10.1242/dev.01028

Shpak ED, McAbee JM, Pillitteri LJ, Torii KU (2005) Stomatal patterning and differentiation by synergistic interactions of receptor kinases. Science 309:290–293. DOI: 10.1126/science.1109710

Sugano SS, Shimada T, Imai Y, Okawa K, Tamai A, Mori M, Hara-Nishimura I (2010) Stomagen positively regulates stomatal density in Arabidopsis. Nature 463:241–244. DOI: 10.1038/nature08682

The Arabidopsis Information Resource (TAIR) (2008) Available at http://www.arabidopsis.org, Accessed January 1, 2014.

Till BJ, Reynolds SH, Greene EA, Codomo CA, Enns LC, Johnson JE, Burtner C, Odden AR, Young K, Taylor NE, Henikoff JG (2003) Large-scale discovery of induced point mutations with high-throughput tilling. Genome Res 13:524–530. DOI:10.1101/gr.977903

Torii KU, Mitsukawa N, Oosumi T, Matsuura Y, Yokoyama R, Whittier RF, Komeda Y (1996) The Arabidopsis ERECTA gene encodes a putative receptor protein kinase with extracellular leucine-rich repeats. Plant Cell 8:735–746. DOI: http://dx.doi.org/10.1105/tpc.8.4.735

Umbrasaite J, Schweighofer A, Kazanaviciute V, Magyar Z, Ayatollahi Z, Unterwurzacher V, Choopayak C, Boniecka J, Murray JAH, Bogre L, Meskiene I (2010) MAPK phosphatase AP2C3 induces ectopic proliferation of epidermal cells leading to stomata development in Arabidopsis. PLOS One 5:e15357. DOI: 10.1371/journal.pone.0015357

von Groll U, Berger D, Altmann T (2002) The subtilisin-like serine protease SDD1 mediates cell-to-cell signaling during Arabidopsis stomatal development. Plant Cell 14:1527–1539. DOI: 10.1105/tpc.001016

Wang H, Liu Y, Bruffett K, Lee J, Hause G, Walker JC, Zhang S (2008) Haplo-insufficiency of MPK3 in MPK6 mutant background uncovers a novel function of these two MAPKs in Arabidopsis ovule development. Plant Cell 20:602–613. DOI: 10.1105/tpc.108.058032

Wang H, Ngwenyama N, Liu Y, Walker JC, Zhang S (2007) Stomatal development and patterning are regulated by environmentally responsive mitogen-activated protein kinases in Arabidopsis. Plant Cell 19:63–73. DOI: 10.1105/tpc.106.048298

Xie Z, Lee E-K, Lucas JR, Morohashi K, Li D, Murray JAH, Sack FD, Grotewold E (2010b) Regulation of cell proliferation in the stomatal lineage by the Arabidopsis MYB FOUR LIPS via direct targeting of core cell cycle genes. Plant Cell 22:2306–2321. DOI: 10.1105/tpc.110.074609

Xie Z, Li D, Wang L, Sack FD, Grotewold E (2010a) Role of the stomatal development regulators FLP/MYB88 in abiotic stress responses. Plant J 64:731–739. DOI: 10.1111/j.1365-313X.2010.04364.x

Xu Z, and Zhou G (2008) Responses of leaf stomatal density to water status and its relationship with photosynthesis in a grass. J Exp Bot 59:3317–3325. DOI: 10.1093/jxb/ern185

Yang M, Sack FD (1995) The *too many mouths* and *four lips* mutations affect stomatal production in Arabidopsis. Plant Cell 7:2227–2239. DOI: 10.2307/3870164

Yoo CY, Pence HE, Jin JB, Miura K, Gosney MJ, Hasegawa PM, Michelbart MV (2010) The Arabidopsis GTL1 transcription factor regulates water use efficiency and drought tolerance by modulating stomatal density via transrepression of *SDD1*. Plant Cell 22:4128–4141. DOI: 10.1105/tpc.110.078691