Enhanced Stomatal Conductance by a Spontaneous Arabidopsis Tetraploid, Me-0, Results from Increased Stomatal Size and Greater Stomatal Aperture

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The rate of gas exchange in plants is regulated mainly by stomatal size and density. Generally, higher densities of smaller stomata are advantageous for gas exchange; however, it is unclear what the effect of an extraordinary change in stomatal size might have on a plant's gas-exchange capacity. We investigated the stomatal responses to CO₂ concentration changes among 374 Arabidopsis (Arabidopsis thaliana) ecotypes and discovered that Mechtshausen (Me-0), a natural tetraploid ecotype, has significantly larger stomata and can achieve a high stomatal conductance. We surmised that the cause of the increased stomatal conductance is tetraploidization; however, the stomatal conductance of another tetraploid accession, tetraploid Columbia (Col), was not as high as that in Me-0. One difference between these two accessions was the size of their stomatal apertures. Analyses of abscisic acid sensitivity, ion balance, and gene expression profiles suggested that physiological or genetic factors restrict the stomatal opening in tetraploid Col but not in Me-0. Our results show that Me-0 overcomes the handicap of stomatal opening that is typical for tetraploids and achieves higher stomatal conductance compared with the closely related tetraploid Col on account of larger stomatal apertures. This study provides evidence for whether larger stomatal size in tetraploids of higher plants can improve stomatal conductance.

Gas exchange is a vital activity for higher plants that take up atmospheric CO₂ and release oxygen and water vapor through epidermal stomatal pores. Gas exchange affects CO₂ uptake, photosynthesis, and biomass production (Horie et al., 2006; Evans et al., 2009; Tanaka et al., 2014). Stomatal conductance (gs) is used as an indicator of gas-exchange capacity (Franks and Farquhar, 2007). Maximum stomatal conductance (gsmax) is controlled mainly by stomatal size and density, two parameters that change with environmental conditions and are negatively correlated with each other (Franks et al., 2009).

Given a constant total stomatal pore area, large stomata are generally disadvantageous for gas exchange compared with smaller stomata, because the greater pore depth in larger stomata increases the distance that gas molecules diffuse through. This increased distance is inversely proportional to gsmax (Franks and Beerling, 2009). The fossil record indicates that ancient plants had small numbers of large stomata when atmospheric CO₂ levels were high, and falling atmospheric [CO₂] induced a decrease in stomatal size and an increase in stomatal density to increase gs for maximum carbon gain (Franks and Beerling, 2009). The positive relationship between a high gs and numerous small stomata also holds true among plants living today under various environmental conditions (Woodward et al., 2002; Galmés et al., 2007; Franks et al., 2009). Additionally, the large stomata of several plant species (e.g. Vicia faba and Arabidopsis [Arabidopsis thaliana]) are often not

1 This work was supported by Grants-in-Aid for Scientific Research on Priority Areas (grant nos. 21114002 and 26221103 to K.I., grant no. 15K18556 to K.M., and grant no. 243688 to K.M.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and by Core Research for Evolutional Science and Technology from Japan Science and Technology Agency.
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1 Articles can be viewed without a subscription. www.plantphysiol.org/cgi/doi/10.1104/pp.15.01450
effective for achieving rapid changes in $g_s$, due to slower solute transport to drive movement caused by their lower membrane surface area-to-volume ratios (Lawson and Blatt, 2014).

Stomatal size is strongly and positively correlated with genome size (Beaulieu et al., 2008; Franks et al., 2012; Lomax et al., 2014). Notably, polyploidy causes dramatic increases in nucleus size and stomatal size (Masterson, 1994; Kondorosi et al., 2000). In addition to the negative effects of large stomata on gas exchange (Franks et al., 2009), polyploids may have another disadvantage; del Pozo and Ramirez-Parra (2014) showed that artificially induced tetraploids of Arabidopsis have a reduced stomatal density (stomatal number per unit of leaf area) and a lower stomatal index (stomatal number per epidermal cell number). Moreover, tetraploids of Rangpur lime ($Citrus limonia$) and Arabidopsis have lower transpiration rates and changes in the expression of genes involved in abscisic acid (ABA), a phytohormone that induces stomatal closure (Allario et al., 2011; del Pozo and Ramirez-Parra, 2014). On the other hand, an increase in the ploidy level of $Festuca arundinacea$ results in an increase in the CO$_2$-exchange rate (Byrne et al., 1981); hence, polyploids may not necessarily have a reduced gas-exchange capacity.

Natural accessions provide a wide range of information about mechanisms for adaptation, regulation, and responses to various environmental conditions (Bouchabke et al., 2008; Brosché et al., 2010). Arabidopsis, which is distributed widely throughout the Northern Hemisphere, has great natural variation in stomatal anatomy (Woodward et al., 2002; Delgado et al., 2011). Recently, we investigated leaf temperature changes in response to [CO$_2$] in a large number of Arabidopsis ecotypes (374 ecotypes; Takahashi et al., 2015) and identified the Mechthausen (Me-0) ecotype among ecotypes with low CO$_2$ responsiveness; Me-0 had a comparatively low leaf temperature, implying a high transpiration rate. In this study, we revealed that Me-0 had a higher $g_s$ than the standard ecotype Columbia (Col), despite having tetraploid-dependent larger stomata. Notably, the $g_s$ of Me-0 was also higher than that of tetraploid Col, which has stomata as large as those of Me-0. This finding resulted from Me-0 having a higher $g_s$-to-$g_{smax}$ ratio due to more opened stomata than tetraploid Col. In addition, there were differences in ABA responsiveness, ion homeostasis, and gene expression profiles in guard cells between Me-0 and tetraploid Col, which may influence their stomatal opening. Despite the common trend of smaller stomata with higher gas-exchange capacity, the results...
with Me-0 confirm the theoretical possibility that larger stomata can also achieve higher stomatal conductance if pore area increases sufficiently.

RESULTS

Identification of an Ecotype with Unusually Large Stomata, Arabidopsis Me-0

Leaf temperature is a convenient indicator of transpiration and can quickly reveal phenotypes with altered stomatal behavior (Negi et al., 2014). We used infrared thermal imaging to investigate stomatal responsiveness to changes in [CO₂] in 374 Arabidopsis ecotypes (Takahashi et al., 2015). In this study, the Me-0 ecotype was identified as an ecotype with a lower leaf temperature than Col regardless of the [CO₂] levels (Fig. 1A).

To investigate the reason for the low leaf temperature in Me-0, we imaged and measured the morphology of stomata within the leaf epidermal tissue (Fig. 1, B–D). Microscopic imaging revealed that Me-0 had much larger stomata than Col. We then measured several stomatal morphological traits, such as guard cell length, stomatal area, stomatal density (number of stomata per unit of leaf area), and stomatal index (number of stomata per total number of epidermal cells; Fig. 1, E and F). Me-0 had approximately 1.5-fold greater guard cell length, 1.9-fold greater stomatal area, 57% of the stomatal density, and 75% of the stomatal index compared with Col.

Next, we investigated the probable cause for large stomata in Me-0. One of the known causes for cell enlargement is an increase in ploidy level (Kondorosi et al., 2000). We compared the stomatal characteristics between Me-0 and an autotetraploid Col line. Tetraploid Col had a higher leaf temperature than Me-0 but had small numbers of large stomata, as found for Me-0 (Fig. 1, A–F). This similarity in stomatal morphological characteristics indicated that Me-0 might be a tetraploid. Therefore, we stained epidermis tissue with 4′,6-diamidino-2-phenylindole (DAPI) and microscopically observed the nuclei of guard cells (Fig. 1, G–L). The nuclei of Me-0 were much larger than those of diploid Col, and the nuclear size was very similar to that of tetraploid Col. We investigated the fluorescence intensity of stained nuclei in guard cells using fluorescence microscopy (Fig. 1M). Me-0 had a higher level of nuclear fluorescence intensity, and the intensity was at the same level as for tetraploid Col and twice that for diploid Col. We also performed flow cytometry analysis (Fig. 1, N–P). The result was consistent with Figure 1M. Not only the nuclei of guard cells, but also nuclei in leaf tissue of Me-0, showed a similar tendency of increased fluorescence intensity (Supplemental Fig. S1). In order to confirm the accuracy of the flow cytometry analysis, we measured the fluorescence intensity of rice (Oryza sativa) as a control (Fig. 1Q). The DNA amount of rice is 0.91 pg 2C⁻¹ (Uozu et al., 1997), and that of Arabidopsis Col is 0.32 pg 2C⁻¹ (Bennett et al., 2003); hence, the ratio of DNA amount between rice and Arabidopsis Col is 2.84:1. This ratio matches that of the measured peaks of fluorescence intensities. We interpreted these results to mean that Me-0 guard cells are tetraploid.

Figure 2. Measurements of gs. A and B, Time courses of gs in response to [CO₂] changes (A) and to light and dark (B). CO₂ and light intensity were changed as indicated. C and D, Time courses of gs-to-anatomical gsmax ratio in response to [CO₂] changes (C) and to light and dark (D). The values of gs were from A and B, and the values of anatomical gsmax were from Table 1. Data presented are means ± se (n > 10). Col (2x), Diploid Col; Col (4x), tetraploid Col; PAR, photosynthetically active radiation.

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Me-0 Has a High $g_s$ But Tetraploid Col Does Not

Me-0 has large stomata and a lower leaf temperature than diploid Col and tetraploid Col (Fig. 1A). The lower leaf temperature indicates a high transpiration rate, suggesting that Me-0 has a high $g_s$. There were few differences in the morphological traits of Me-0 and tetraploid Col stomata (Fig. 1, E and F). If the lower leaf temperature in Me-0 is due to an increase in stomatal size, the $g_s$ of Me-0 and tetraploid Col should be similar. In order to investigate this possibility, we compared the $g_s$ of all three accessions measured with a gas-exchange system. First, we measured the $g_s$ in response to [CO$_2$] (Fig. 2A). At 0 $\mu$L L$^{-1}$ [CO$_2$], Me-0 had a 1.2-fold higher $g_s$ than diploid Col. Increasing the [CO$_2$] resulted in lower $g_s$ in both ecotypes, but Me-0 also had a comparatively high $g_s$. Interestingly, tetraploid Col did not have as high a $g_s$ as Me-0; the $g_s$ was similar to that of diploid Col, especially at a low [CO$_2$]. These $g_s$ values were measured from whole aerial plant parts. We also measured the $g_s$ of abaxial and adaxial leaf surfaces in response to [CO$_2$] (Supplemental Fig. S2). In all three accessions, the absolute $g_s$ values of the abaxial surfaces were higher than those of the adaxial surfaces; however, both $g_s$ values for the abaxial and adaxial leaf surfaces showed a similar tendency, with Me-0 $g_s$ being higher than that of the other two accessions. Next, we measured the $g_s$ in response to light and dark (Fig. 2B). The $g_s$ of Me-0 was higher than that of the other two accessions, similar to the CO$_2$ response result. This $g_s$ tendency was not changed when the light intensity was increased gradually (Supplemental Fig. S3). The transpiration rates in response to [CO$_2$], light, and dark were also very similar to the results of the $g_s$ response (Supplemental Fig. S4). These data demonstrate that, in spite of their similar stomatal morphological features, Me-0 has a high $g_s$ but tetraploid Col does not.

The Difference in Stomatal Aperture Causes the Difference in $g_s$ between Me-0 and Tetraploid Col

We compared the anatomical $g_{\text{smax}}$, which is the calculated maximum rate of $g_s$ to water vapor as determined by stomatal size and density in a diffusion-based equation (Eq. 1). The anatomical $g_{\text{smax}}$ values of Me-0 and tetraploid Col were both 1.3- to 1.4-fold higher than that of diploid Col (Table I). This result indicates that large stomata certainly contribute to high $g_s$ if the stomata are opened to their widest possible apertures. We then compared the measured $g_s$-to-anatomical $g_{\text{smax}}$ ratio among the three accessions in response to [CO$_2$] and light and dark conditions (Fig. 2, C and D). The $g_s$-to-anatomical $g_{\text{smax}}$ ratio of Me-0 was lower than that of diploid Col but higher than that for tetraploid Col under the conditions inducing stomatal opening (low [CO$_2$] from 0 to 200 $\mu$L L$^{-1}$ or light conditions). Under the conditions inducing stomatal closing (high [CO$_2$] or dark conditions), Me-0 had the highest $g_s$-to-anatomical $g_{\text{smax}}$ ratio among the three accessions. On the other hand, tetraploid Col had a lower $g_s$-to-anatomical $g_{\text{smax}}$ ratio than Me-0 throughout the measurement in response to [CO$_2$] and light and dark conditions. These results suggest that Me-0 stomata allow for higher gas-exchange rates than tetraploid Col.

We hypothesized that the differences of measured $g_s$ and $g_s$-to-anatomical $g_{\text{smax}}$ ratio between Me-0 and tetraploid Col are derived from the stomatal aperture, which is the most fluctuating parameter among factors determining anatomical $g_s$. In order to test this hypothesis, we compared the change of stomatal aperture (width/length) in response to [CO$_2$], humidity, and light among the three accessions (Fig. 3). Our data showed that stomatal aperture was significantly larger in diploid Col than in both Me-0 and tetraploid Col.
suggesting that the large stomata of tetraploids are more difficult to open than the small stomata of diploids; however, Me-0 had larger stomatal apertures than tetraploid Col. The difference in stomatal opening between Me-0 and tetraploid Col may be indicative of why Me-0 had higher \( g_s \) and \( g_s \)-to-anatomical \( g_{smax} \) ratio than tetraploid Col, although Me-0 and tetraploid Col have similar stomatal size and density.

**Physiological Analyses to Investigate the Reasons for the High Stomatal Opening Ability of Me-0**

Me-0 can open its giant stomata more completely than tetraploid Col (Fig. 3). Our next objective was to examine the cause for the high stomatal opening ability in Me-0. First, we focused on hormonal regulation. We compared ABA responsiveness among the three accessions (Fig. 4A). Tetraploid Col showed higher ABA responsiveness than diploid Col; on the other hand, the responsiveness of Me-0 was similar to that of diploid Col. These differences in ABA responsiveness may cause the differences in stomatal apertures shown in Figure 3. We also quantified the ABA content of leaves under high or low relative humidity (RH) conditions (80% or 40% RH, respectively). Figure 4B shows that the ABA contents were very similar among the three accessions in low or high RH conditions; this finding suggests that both Arabidopsis tetraploids have a normal ability to synthesize ABA.

We also focused on ion balance in guard cells. An influx of \( K^+ \) ions triggers stomatal opening by increasing the osmotic pressure within the guard cells, and \( Cl^- \) and \( malate^{2-} \) are the primary anions that counterbalance the influx of \( K^+ \) during stomatal opening (Schroeder and Hagiwara, 1989; MacRobbie, 1998). We compared the \( K^+ \), \( Cl^- \), and \( malate^{2-} \) contents of the guard cell protoplasts (GCPs) from the three accessions. Both the \( K^+ \) and \( Cl^- \) contents of Me-0 and tetraploid Col were higher than those of diploid Col; however, Me-0 had the highest levels of \( malate^{2-} \), whereas tetraploid Col had the lowest levels of \( malate^{2-} \) among the three accessions (Fig. 5). It is unclear whether the difference in \( malate^{2-} \) level is related to the difference in stomatal opening between Me-0 and tetraploid Col, but higher \( malate^{2-} \) generally induces stomatal opening. The higher \( K^+ \) content of Me-0 and tetraploid Col GCPs is possibly connected with the report that tetraploid Arabidopsis leaves accumulate a higher [\( K^+ \)] (Chao et al., 2013).

**The Genetic Difference That May Be Involved in the High Stomatal Opening Ability of Me-0**

Our data showed that Me-0 has a greater capacity for stomatal movement compared with tetraploid Col. We performed microarray analysis to investigate whether gene expression differences between Me-0 and tetraploid Col on GCPs are related or not to the regulation of stomatal aperture. We identified 1,777 probes as up-regulated and 1,706 probes as down-regulated differentially expressed genes (DEGs) in Me-0 compared with tetraploid Col (adjusted \( P < 0.001 \) and \( \log_2 \) fold change greater than 1 or less than \( -1 \); Fig. 6A). Using gene set analysis to functionally characterize DEGs, there were eight significant gene sets (\( P < 0.01 \)) in the up-regulated genes and seven significant gene sets among the down-regulated genes (Fig. 6, B and C; Supplemental Tables S1 and S2). The most significant gene sets, which were enriched in up-regulated genes, were related to plant-type cell wall loosening, an essential step in guard cell swelling (Wei et al., 2011). In down-regulated genes, there were several significant
gene sets related to the defense responses for immunity, hypoxia, and ozone, which also influence stomatal responses or gas exchange (Heath, 1994; Melotto et al., 2006; Gil et al., 2009). Microarray analysis demonstrated that these gene expression differences may be related to the differences in stomatal aperture between Me-0 and tetraploid Col evaluated in Figure 3.

DISCUSSION

This study identified Me-0 as a unique Arabidopsis ecotype having large stomata and a high \( g_s \). Investigating mutants provides abundant knowledge about the genes involved in the stomatal opening or closure response (Negi et al., 2014) and development (Pillitteri and Torii, 2012) and relationships among these genes. On the other hand, natural accessions like Me-0 have desirable traits for studies of stomatal movement and anatomy. For example, we reported previously that ecotype Cape Verde Islands-0 is insensitive to ABA and has a low stomatal response to environmental signals (Monda et al., 2011). Additionally, we found three ecotypes of Arabidopsis, Köln-4, Gabelstein-0, and Chisdra-1, that have particularly low CO₂ responses among 374 ecotypes and seem to be regulated by a common response mechanism (Takahashi et al., 2015). Moreover, there are natural variations in stomatal response to environmental stimuli (Bouchabke et al., 2008; Brosché et al., 2010; Imai et al., 2015) and in stomatal anatomy (Woodward et al., 2002; Russo et al., 2010; Delgado et al., 2011). These reports suggest that natural accessions provide valuable information for understanding actual stomatal adaptation mechanisms that cannot be obtained from only the study of mutants or transformants.

The cause of the high \( g_s \) in Me-0 is giant stomata due to tetraploidization; however, plants tend to have a smaller stomatal size in order to enhance \( g_s \) (Galmés et al., 2007; Franks et al., 2009). This is because larger stomata have a proportionally longer stomatal pore depth that results in a longer diffusion path length and, thus, increased stomatal diffusive resistance (Franks and Beerling, 2009). Hence, smaller stomata are advantageous for enhancing \( g_s \) if the total pore area is not changed. Total stomatal pore area tends to be maintained at the same level regardless of changes in

![Graph showing ion contents](image)

**Figure 5.** K⁺, Cl⁻, and malate²⁻ levels in guard cell protoplasts. Ion contents were measured in GCPs from each accession. Ion contents were corrected for the Na⁺ content. The data presented are means ± se (n = 4–6 GCP samples). Values marked with different letters are significantly different among accessions (P < 0.05, Steel-Dwass test). Col (2x), Diploid Col; Col (4x), tetraploid Col.

![Microarray analysis](image)

**Figure 6.** Microarray analysis. The GCPs of Me-0 and tetraploid Col were used for these measurements. A, DEGs identified in a microarray experiment. A volcano plot displays 32,920 probes. The x axis is log₂ fold change, and the y axis is the −log₁₀ (adjusted P-value). Red and blue points are up and down DEGs, respectively, in Me-0 compared with tetraploid Col (adjusted P < 0.001 and log₂ fold change of greater than 1 or less than −1). B and C, Functional classification of DEGs. Bar plots represent the statistical significance of the enrichment in either up DEGs (B) or down DEGs (C) in Me-0 compared with tetraploid Col. The vertical axis indicates Gene Ontology (GO) terms, and the horizontal axis indicates the enrichment −log₁₀ (P-value) for each GO term. All GO terms listed on the bar plots had enrichment P < 0.01.
stomatal size and density (Miskin et al., 1972; Jones, 1987); however, Me-0 and tetraploid Col have high anatomical $g_{\text{sm}}$ due to a significantly increased total stomatal pore area per unit of leaf area (Table I). This result indicates that tetraploidization is useful for improving $g_{\text{sm}}$; however, the observed increase in $g_{s}$ is small (about 20%) compared with what might be expected from a doubling of stomatal size (Figs. 1, B–E, and 2).

We calculated the anatomical $g_{\text{sm}}$ using the value measured from mixed samples of all rosette leaves in order to archive consistency with the $g_{s}$ data measured from whole individual plants; however, the differences in stomatal properties between abaxial and adaxial sides may affect the calculation of anatomical $g_{\text{sm}}$. Nevertheless, we measured the $g_{s}$ of abaxial and adaxial leaf surfaces in response to $[\text{CO}_2]$ and confirmed that the relationships of the relative magnitudes of $g_{s}$ among the three accessions were similar between abaxial and adaxial sides despite the differences in absolute $g_{s}$ values (Supplemental Fig. S2). Therefore, the difference between abaxial and adaxial sides does not seem to cause a serious problem in considering the differences of $g_{s}$ and anatomical $g_{\text{sm}}$ among the three accessions.

Although both Me-0 and tetraploid Col have the same level of anatomical $g_{\text{sm}}$ (Table I), Me-0 has a greater $g_{s}$ than tetraploid Col (Fig. 2); tetraploid Col and diploid Col have similar $g_{s}$ values. The difference is due to stomatal aperture (Fig. 3). Anatomical $g_{\text{sm}}$ is calculated on the assumption that stomatal pore area is maximal (Franks and Beerling, 2009); however, it is rare that stomata are opened maximally in response to actual environmental conditions (Desikan et al., 2004; Franks et al., 2009; Dow et al., 2014a; McElwain et al., 2016). The difference in $g_{s}$ between Me-0 and tetraploid Col shows that $g_{s}$ is determined not only by stomatal anatomical traits but also by stomatal aperture; however, we must not forget that stomatal movement may be affected to some extent by anatomical traits, as will be discussed later.

Dow et al. (2014b) reported that irregular stomatal spacing, such as stomatal clustering, significantly affects $g_{s}$ value. From our observations, the three accessions had regular stomatal spacing and their stomata were not clustered (Fig. 1, B–D), but Me-0 and tetraploid Col had lower stomatal indices compared with diploid Col (Fig. 1F). The differences in stomatal index may affect stomatal density and size, thereby influencing anatomical $g_{\text{sm}}$.

We investigated several potential causes for the differences in stomatal aperture between Me-0 and tetraploid Col (Figs. 4–6). One factor is ABA responsiveness. Our data show that tetraploid Col has greater ABA responsiveness than Me-0 and diploid Col (Fig. 4A). Moreover, gene expression profiles show that tetraploid Col has a higher expression of genes involved in defense responses that are involved with ABA responsiveness in comparison with Me-0 (Fig. 6C). These differences related to ABA responsiveness seem to affect stomatal aperture between Me-0 and tetraploid Col. del Pozo and Ramirez-Parra (2014) reported that not only tetraploid Arabidopsis Col but also the Landsberg erecta ecotype have smaller stomatal apertures and exhibit differential expression of genes involved in ABA metabolism, compared with their diploid relatives. Tetraploids of another plant species, Rangpur lime, also alter the expression of ABA-related genes and accumulate excessive ABA, thereby decreasing $g_{s}$ (Allario et al., 2011, 2013). These reports support the possibility that increasing the ploidy level in some ecotypes and plant species changes ABA responsiveness or biosynthesis; however, tetraploidization does not always affect ABA responsiveness or induce an equal effect on ABA responsiveness, as in Me-0 and tetraploid Col.

Increasing stomatal size may also change the stomatal opening ability due to a structural reason. Previous studies suggested that larger stomata open or close more slowly than smaller stomata because of surface-to-volume ratios and the requirement for solute transport to drive movement (Hetherington and Woodward, 2003; Drake et al., 2013). Moreover, in most cell types, cell wall thickness increases in proportion to cell size (John et al., 2013). If larger stomata have thicker cell walls, they may not open as far (relative to full aperture) as smaller stomata for the same turgor pressure. Our data show that the ion contents in Me-0 and tetraploid Col were mostly higher than in diploid Col (Fig. 5) despite the reduced ability for stomatal opening (Fig. 3). In Figure 6 and in a previous study (del Pozo and Ramirez-Parra, 2014), the expression of genes involved in cell wall formation was different between tetraploid Col and Me-0 and between tetraploid Col and diploid Col. The difference may be related to the cell wall thickness among these accessions. Our study proposes that stomatal aperture is an important factor when determining whether large stomata are advantageous for gas exchange. In order to elucidate stomatal adaptation mechanisms for optimizing gas exchange, further analyses of the interactive effects among stomatal size, density, and aperture will be needed.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**

*Arabidopsis (Arabidopsis thaliana)* ecotype Col was from our laboratory stocks. Me-0 (CS28491) and the tetraploid line of Col (CS3151) were obtained from the Arabidopsis Biological Resource Center. Seeds were sown on solid Murashige and Skoog medium (Murashige and Skoog, 1962). After vernalization at 4°C for 3 d, the plants were grown for 16 d in a growth chamber (LH-1P-NC8; Nippon Medical & Chemical Instruments) under conditions of constant white light of 80 $\mu$mol m$^{-2}$ s$^{-1}$, 22°C, and 60% RH and then transplanted into vermiculite pots supplemented with mineral nutrients. Unless noted otherwise, plants used for this study were 23 to 26 d old.

**Thermal Imaging**

Twenty-three-day-old plants in a growth chamber were transferred to a custom-made growth cabinet equipped with an automatic CO$_2$ control unit (TMC-LW1208A/K; TM Systems) and incubated under the conditions of constant white light of 100 $\mu$mol m$^{-2}$ s$^{-1}$ at 22°C, 40% RH, and CO$_2$ free for 2 h.
After the incubation, plants were exposed to a high [CO2] (700 μL L⁻¹) for 1 h. Thermal images were captured using a thermography camera (InfReC Thermography R300; NEC Avio Infrared Technologies) and an InfReC Analyzer NS9500 Standard (NEC Avio Infrared Technologies).

Stomatal Trait Analysis

Stomatal morphological features (guard cell length, stomatal area, stomatal density, stomatal index, etc.) were measured for all leaves within rosettes. Leaves were blended for 25 s and filtered through a 100-μm nylon mesh. The epidermal peels from the blended leaf tissues were placed in a drop of distilled water on a glass slide and observed. Stomatal area was measured from closed stomata.

Stomatal aperture measurements were performed as described previously (Monda et al., 2011). Plants used to measure the [CO₂] response were incubated in a growth cabinet under a high [CO₂] (700 μL L⁻¹) for 1 h after having been incubated in CO₂-free air for 2 h. In the light response measurements, plants were incubated in the light (22°C, 40% RH) for 3 h. Unless noted otherwise, the environmental conditions in the dark for 3 h. Plants used for measuring low-humidity responses were incubated under low humidity (40% RH) for 1 h after incubation under high humidity (80% RH) for 3 h. Unless noted otherwise, the environmental conditions in the growth cabinet were constant white light of 100 μmol m⁻² s⁻¹ at 22°C, 40% RH, and 350 μL L⁻¹ [CO₂]. The abaxial epidermis of rosette leaves was peeled using Scotch tape and photographed. The measurements were done within 15 min after peeling in order to avoid changing the stomatal apertures. For the ABA response measurements, slices of epidermal peels were floated on a test medium containing 30 mM KCl, 5 mM MES-KOH, pH 6.15, and 1 mM CaCl₂, and were incubated in the growth chamber (constant white light of 80 μmol m⁻² s⁻¹, 22°C, and 60% RH) for 1 h, and then a solution of ABA in dimethyl sulfoxide (DMSO) was added to the test medium or DMSO only was added as a control. The final concentrations of ABA and DMSO were 2 μM and 0.002% (v/v), respectively. After 3 h of incubation, stomatal apertures were measured. All measurements of stomatal aperture (CO₂ light and dark conditions, RH, and ABA response) were analyzed on the abaxial surface of the third pair of rosette leaves. All stomatal traits were analyzed from photographs of peels taken with a digital camera attached to a microscope (IX71; Olympus).

Staining Nuclei

Rosette leaves from the fifth or sixth position of 27-d-old plants were stained with DAPI to observe nuclei and to measure the relative fluorescence intensity of nuclei from the three accessions. Peeled leaves were fixed in a solution of 70% ethanol for 1 h and then in McIlvaine buffer, pH 4.1 (Coleman et al., 1981). The tissues were placed in a drop of DAPI at a concentration of 0.005 mg mL⁻¹ in the McIlvaine buffer on a glass slide. A BZ-9000 All-in-One Fluorescence Microscope (Keyence) was used to observe the tissue, and BZ-II Analyzer version 1.32 (Keyence) was used to analyze the fluorescence intensity from microscopic images. The filter set used was DAPI-BP (exciter, BP340-380; emitter, BP455-485).

Flow Cytometry

Flow cytometry was conducted to estimate DNA ploidy levels of Arabidopsis accessions as described previously (Ishigaki et al., 2010) with minor modifications. To stain nuclei completely, 50 μL of propidium iodide was added into the suspensions of leaf tissues or GCPs (isolated as described previously; Negi et al., 2008), and the samples were kept at room temperature for at least 5 min. Fluorescence intensities of the samples were measured with an EPICS XL. Flow Cytometer equipped with a 488-nm argon laser and long-path filter (Beckman Coulter). The fluorescence intensities of rice (Oryza sativa) nuclei were also measured as a control.

Measurement of gs

The gs of abaxial/adaxial leaf surfaces to water vapor was measured using a portable gas-exchange fluorescence system (GFS-3000; Heinz Walz). The stomata of opposite surfaces were covered by petroleum. Leaf temperature was measured by thermocouple in a measuring head. The gs of whole plants to water vapor was measured as described previously (Monda et al., 2011) using GFS-3000 equipped with a 3010-A Arabidopsis chamber. In order to avoid leakage of air, a plastic cover was inserted between the aerial plant parts and the pot, and the hole in the cover was filled with a nontoxic paste (SAE-755S; Nihon Cima). Before measuring the gs of samples, we measured the gs without a sample and confirmed that the gs was approximately zero throughout the measurement. The leaf temperature throughout the measurements was regarded as very close to the cuvette temperature. The cuvette temperature (22°C), flow rate (750 μmol s⁻¹), and RH (40%) were kept constant throughout the gas-exchange experiments. The gs measured by GFS-3000 was per unit of leaf area.

Calculating Anatomical gs max

The anatomical gs max to water vapor (mol m⁻² s⁻¹) was estimated using the double end-corrected version of the equation by Franks and Farquhar (2001):

\[
\text{anatomical } gs_{\text{max}} = \frac{D \cdot n \cdot s_{\text{max}}}{\sqrt{\left(\frac{1}{l} + \frac{2}{\sqrt{s_{\text{max}}}}\right)}}
\]

where D is the diffusivity of water in air (2.5(1 - 1.1 × 10⁻⁵ m² s⁻¹ at 22°C), n is stomatal density (m⁻²), s max is the maximum pore area (m²); defined as an ellipse with the major axis equal to the pore length and the minor axis equal to one-half the pore length (Dow et al., 2014b) and calculated by the equation \(\pi / 8 \times \text{ pore length}^2\), l is the pore depth (mm, taken to be equal to the guard cell width), and \(s_{\text{max}}\) is the mathematical constant. Anatomical gs max was calculated using empirical values of n, l, and s max for stomata on a mixture of adaxial and abaxial epidermal tissues.

Quantitative Analysis of ABA

About 100 mg of fresh leaves per sample was used to quantify ABA levels. Plants used for measurements of low-humidity responses were incubated in a growth cabinet under low-humidity conditions (40% RH) for more than 1 h after incubation under high-humidity conditions (80% RH) for 3 h. The environmental conditions in the growth cabinet were constant white light of 100 μmol m⁻² s⁻¹ at 22°C and 350 μL L⁻¹ [CO₂].

Measurement of Organic and Inorganic Ions in GCPs

Isolation of GCPs was performed as described previously (Negi et al., 2008). Malate⁻² and Cl⁻ in GCPs were separated using a suppressed ion chromatography system (HSC-SF; Shimadzu) equipped with a Shim-pack IC-SA3 high-separation column (250 mm × 4.0 mm) and Shim-pack IC-SA3 (G) guard column (10 mm × 4.6 mm) with a mobile phase of 3.6 msi sodium carbonate and a flow rate of 0.8 mL min⁻¹ at 45°C. The amounts of malate⁻² and Cl⁻ were determined using an electroconductivity detector (CDD-10A VP; Shimadzu). K⁺ and Na⁺ in GCPs were analyzed using a nonsuppressed ion chromatography system (HIC-NB; Shimadzu). A Shim-pack IC-C3 cation-exchange column (100 mm × 4.6 mm) was used with a mobile phase of 2.5 msi oxalic acid and a flow rate of 1.2 mL min⁻¹ at 40°C. The amounts of K⁺ and Na⁺ were determined using electroconductivity detectors (CDD-6A; Shimadzu).

Microarray Experiments and Analysis

Total RNA was isolated from GCPs using RNeasy Plus (Takara Bio). RNAs were amplified, labeled, and hybridized to an Agilent Arabidopsis (V4) Gene Expression Microarray, 4x44K (product no. G2519F), based on the manufacturer's instructions. Microarray raw data were exported from the Agilent Feature Extraction software. In a preprocessing procedure, raw data were normalized using quintile normalization followed by median scaling and log₂ transformed. Probes expressed at low levels were filtered out because such probes might be insufficiently reliable. In this study, we removed probes with more than one absent call in tetraploid Col or more than two absent calls in Me-0 on the basis of GeneSpring flag calls. Accordingly, 32,920 out of 43,603 probes remained and were analyzed subsequently. DEGs were extracted by Limma (Smyth, 2004) with a significance level of adjusted P < 0.001 and a log₂ fold change of greater...
than 1 or less than –1. We also performed GOstats (Falcon and Gentleman, 2007) to functionally characterize DEGs using Biological Process data sets without GO terms annotating more than 500 or fewer than 10 genes. \( P = 0.01 \) was set to be the significance level for gene sets, with the additional requirement of at least five genes with annotated biological functions. All bioinformatics analyses were performed by R/Bioconductor software.

Microarray data were deposited at the National Center for Biotechnology Information’s Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) with series accession number GSE71914.

### Supplemental Data

The following supplemental materials are available.

**Supplemental Figure S1.** Flow cytometric analysis of leaf tissues.

**Supplemental Figure S2.** \( g_s \) measurements of abaxial and adaxial sides.

**Supplemental Figure S3.** Measurement of \( g_s \) in response to increased light intensity.

**Supplemental Figure S4.** Measurement of transpiration rate.

**Supplemental Table S1.** Functional classification of up-regulated genes in Me-0 compared with tetraploid Col.

**Supplemental Table S2.** Functional classification of down-regulated genes in Me-0 compared with tetraploid Col.

### ACKNOWLEDGMENTS

We thank Ichiro Terashima (University of Tokyo) for critical reading of the article, Kazuhiko Nishitani (Tohoku University) for discussions about this study, Naomi Kawahara for skillful assistance in sample preparation, and Shinobu Ishikawa for identifying the Me-0 ecotype using thermography.

Received October 26, 2015; accepted January 8, 2016; published January 11, 2016.

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