Overexpression of plasma membrane H$^+$-ATPase in guard cells promotes light-induced stomatal opening and enhances plant growth

Yin Wang[a], Ko Noguchi[b], Natsuko Ono[a], Shin-ichiro Inoue[a], Ichiro Terashima[b], and Toshinori Kinoshita[a,c,1]

[a]Division of Biological Science, Graduate School of Science, Nagoya University, Chikusa, Nagoya 464-8602, Japan; [b]Department of Biological Sciences, Graduate School of Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan; and [c]Institute of Transformative Bio-Molecules (WPI-ITbM), Nagoya University, Chikusa, Nagoya 464-8602, Japan

Stomatal pores surrounded by a pair of guard cells in the plant epidermis control gas exchange between plants and the atmosphere in response to light, CO$_2$, and the plant hormone abscisic acid. Light-induced stomatal opening is mediated by at least three key components: the blue light receptor phototropin (phot1 and phot2), plasma membrane H$^+$-ATPase, and plasma membrane inward-rectifying K$^+$ channels. Very few attempts have been made to enhance stomatal opening with the goal of increasing photosynthesis and plant growth, even though stomatal resistance is thought to be the major limiting factor for CO$_2$ uptake by plants. Here, we show that transgenic *Arabidopsis* plants overexpressing H$^+$-ATPase using the strong guard cell promoter *GC1* showed enhanced light-induced stomatal opening, photosynthesis, and plant growth. The transgenic plants produced larger and increased numbers of rosette leaves, with ~42–63% greater fresh and dry weights than the wild type in the first 25 d of growth. The dry weights of total flowering stems of 45-d-old transgenic plants, including seeds, siliques, and flowers, were ~36–41% greater than those of the wild type. In addition, stomata in the transgenic plants closed normally in response to darkness and abscisic acid. In contrast, the overexpression of phototropin or inward-rectifying K$^+$ channels in guard cells had no effect on these phenotypes. These results demonstrate that stomatal aperture is a limiting factor in photosynthesis and plant growth, and that manipulation of stomatal opening by overexpressing H$^+$-ATPase in guard cells is useful for the promotion of plant growth.

Arabidopsis thaliana | stomatal conductance | photosynthetic rate | biomass

In the present era of global climate changes and the threat of food insufficiency, finding ways to improve the uptake of CO$_2$ by terrestrial plants is an increasingly important problem. Stomata, key organs in the uptake of CO$_2$, are microscopic pores surrounded by two specialized cells in the epidermis (named guard cells) and are mainly found on the leaf surface in terrestrial plants. Because the leaf surface is nearly impermeable to air and water, stomata provide the major pathway for the diffusion of CO$_2$, O$_2$, and water vapor between the ambient atmosphere and the interior of the leaf. This facilitation of gas exchange by stomatal opening is one of the most essential processes in plant photosynthesis and transpiration (1, 2). A recent study indicated that stomatal transpiration limited photosynthesis in rice (3). Therefore, increased stomatal opening/transpiration is expected to promote photosynthesis and thereby increase plant growth. Condon et al. (4) examined diverse wheat genotypes and showed that increased stomatal conductance, especially abaxial stomatal conductance, may have a positive effect on crop biomass. However, to our knowledge, no previous studies have determined stomatal effects on plant growth by manipulating stomatal aperture via gene regulation in guard cells, perhaps because of the difficulty in balancing the counteracting effects of taking up CO$_2$ while losing water vapor through the stomata (5).

Light is one of the principal factors that stimulates stomatal opening, and various mechanisms underlie stomatal opening in response to different light wavelengths (6–8). Red light is thought to induce stomatal opening via photosynthesis in the mesophyll and guard cell chloroplasts, as well as the reduction of the intercellular CO$_2$ concentration (Ci) (5, 9, 10). However, the detailed mechanisms of stomatal responses to red light are under debate (11, 12). In contrast, blue light acts as a signal and exerts the most pronounced effect on stomatal opening. The blue light receptors phototropins (phot1 and phot2) activate plasma membrane H$^+$-ATPase through the phosphorylation of the penultimate threonine and subsequent binding of the 14-3-3 protein to the phosphorylated threonine (13–15). Blue light-activated H$^+$-ATPase induces hyperpolarization of the plasma membrane, which allows K$^+$ uptake through inward-rectifying K$^+$ (K$^+_{in}$) channels (16–21). Accumulation of K$^+$ induces the swelling of guard cells and stomatal opening. Thus, these three components (phototropins, plasma membrane H$^+$-ATPase, and K$^+_{in}$ channels) have important roles in blue light-induced stomatal opening. In addition to these components, *FLOWERING LOCUS T* (*FT*) is suggested to be a positive regulator for stomatal opening via its effect on the activation status of the plasma membrane H$^+$-ATPase (22).

Significance

Stomata are microscopic pores surrounded by two guard cells and play an important role in the uptake of CO$_2$ for photosynthesis. Recent researches revealed that light-induced stomatal opening is mediated by at least three key components: blue light receptor phototropin, plasma membrane H$^+$-ATPase, and plasma membrane inward-rectifying K$^+$ channels. Here, we showed that only increasing the amount of H$^+$-ATPase in guard cells had a significant effect on light-induced stomatal opening. Transgenic *Arabidopsis* plants by overexpressing H$^+$-ATPase in guard cells exhibited enhanced photosynthesis activity and plant growth. Our results demonstrate that stomatal aperture is a limiting factor in photosynthesis and plant growth, and that overexpression of the H$^+$-ATPase in guard cells is useful for promotion of plant growth.

PLANT BIOLOGY

In this study, we produced transgenic plants expressing key components active in stomatal opening under the control of the strong guard cell promoter *GC1* to promote stomatal opening in *Arabidopsis thaliana* (23). We showed that transgenic *Arabidopsis* plants overexpressing H^+-ATPase in guard cells exhibited enhanced light-induced stomatal opening, photosynthesis, and plant growth, and that stomatal aperture is a limiting factor in photosynthesis and plant growth.

Results

Overexpression of H^+-ATPase in Guard Cells Promotes Light-Induced Stomatal Opening.
To produce transgenic plants showing enhanced stomatal opening in response to light, we attempted to express three key components of stomatal opening: *PHOTOTROPIN 2* (*PHOT2*), an isoform of the blue light receptor phototropin (13); *ARABIDOPSIS H^+-ATPASE 2* (*AHA2*), a typical isoform of the plasma membrane H^+-ATPase (24); and *POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 1* (*KAT1*) and *ARABIDOPSIS K^+ TRANSPORTER 1* (*AKT1*), isoforms of plasma membrane K^+_{in} channels (19, 20, 25–27). We used the strong guard cell promoter *GC1* (22, 23) because these proteins function exclusively within guard cells. Immunohistochemical and Western blotting analyses demonstrated that the levels of *AHA2* expressed under the control of the *GC1* promoter (*GC1::AHA2*) in guard cells of *AHA2*-transgenic plants were ~1.5-fold higher than in wild-type (WT) guard cells (Fig. 1*A* and Fig. S1*D*). Quantitative (q) PCR demonstrated that *AHA2* transcripts in the epidermal tissue of *AHA2*-transgenic plants, including guard cells, were present at levels 3.4- to 5.2-fold higher than in WT plants (Fig. 1*B*). We did not detect differences in the levels of *AHA2* transcripts in leaves or roots (Fig. S1). Thus, we examined light-induced stomatal opening in these plants. The *AHA2*-transgenic plants showed ~25% wider stomatal apertures than WT plants exposed to light illumination for 2.5 h, but these stomata closed in darkness just as in the WT (Fig. 1 *C* and *E*). We also demonstrated that stomata of the *AHA2*-transgenic plants opened more quickly than WT stomata over a period of 30 min of illumination (Fig. 1 *D* and *E* and Fig. S2). In contrast, the overexpression of *PHOT2-GFP* (*GC1::PHOT2-GFP*), which has been shown to restore light-induced stomatal opening in *phot1 phot2*, *AKT1* (*GC1::AKT1*), and *KAT1* (*GC1::KAT1*), had no effect on stomatal opening under these conditions, even though these components were present at increased levels in guard cells or the epidermis (Fig. S3). These results indicated that H^+-ATPase, not phototropins or K^+_{in} channels, is the limiting factor in light-induced stomatal opening and that increasing the amount of H^+-ATPase in guard cells increased the magnitude of stomatal opening.

Note that the light-induced stomatal opening in *AHA2*-transgenic plants was inhibited by the plant hormone abscisic acid (ABA) to the same extent as in the WT (Fig. 1*C*). The weight decrease of detached leaves in *AHA2*-transgenic plants was also similar to that in the WT (Fig. 1*F*). Thus, the drought response of *AHA2*-transgenic plants was normal. Importantly, WT and *AHA2*-transgenic plants withered in the same way in response to drought stress (Fig. S4), suggesting that the drought sensitivity of *AHA2*-transgenic plants was similar to that of the WT.

Overexpression of H^+-ATPase in Guard Cells Enhances both Stomatal Conductance and Photosynthetic Activity.
We examined stomatal conductance and photosynthetic activity (CO_2 assimilation rate) in detail in intact leaves of *AHA2*-transgenic plants using a gas-exchange system. Stomatal conductance and the CO_2 assimilation rate were significantly higher in *AHA2*-transgenic plants than in WT plants at photon flux densities ≥ 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ ($P < 0.05$) (Fig. 2 *A* and *B* and Table S1). The increments of stomatal conductance of the *AHA2*-transgenic plants to that of the WT were getting higher with the increasing of light intensities, resulting in decreased water-use efficiency (the ratio
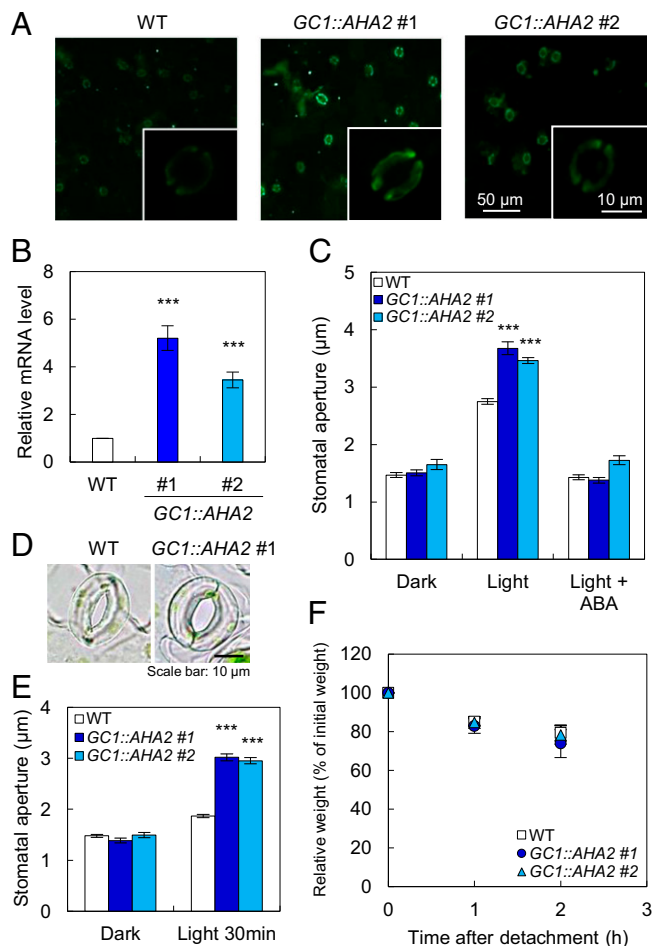

Fig. 1. Overexpression of *AHA2* using the *GC1* promoter promotes stomatal opening. (*A*) Typical fluorescence images of immunohistochemical detection of the guard cell H^+-ATPase in the *Arabidopsis* epidermis. (For details regarding the immunohistochemical conditions, see *Materials and Methods*.) (*B*) qPCR analysis of *AHA2* expression. Error bars represent the SEM ($n \geq 6$). Significant differences were detected by Student *t* test (***$P < 0.001$). (*C*) Stomatal apertures under 2.5 h of darkness, light (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ red light and 10 $\mu mol \cdot m^{-2} \cdot s^{-1}$ blue light), or light in the presence of 20 μM ABA. (*D*) Typical stomata in the epidermis illuminated with light for 30 min. Light conditions were the same as in *C*. (*E*) Stomatal apertures in darkness or after 30 min of light treatment. The light conditions were the same as in *C*. The stomatal aperture values in *C* and *E* are the means of measurements of 25 stomata; error bars represent the SEM. Differences in stomatal aperture were detected using Student *t* test (***$P < 0.001$). (*F*) Kinetics of the change in fresh weight of detached rosette leaves from 4-wk-old WT and *AHA2*-transgenic plants. The relative weights of the leaves are presented as a percentage of the initial weight, which was the weight of each rosette leaf immediately after detachment from the plant. Data are the mean values for seven leaves; error bars represent the SD.

between the photosynthetic rate and the transpiration rate) in the *AHA2*-transgenic plants compared with WT plants (Table S1). The gas-exchange parameters of *AHA2*-transgenic plants of line #2 were somewhat variable, probably because of the relatively low overexpression of *AHA2* (Fig. 1*B* and Fig. S1*B*). To determine whether the higher photosynthetic rate of the *AHA2*-transgenic plants was attributable to the increased stomatal opening, we examined the response curve between the CO_2 assimilation rate and the leaf intercellular CO_2 concentration using a gas-exchange system under saturating-light conditions (Fig. 2*C*). The two curves were almost coincident with each other. According to Farquhar et al. (28), these results indicate that
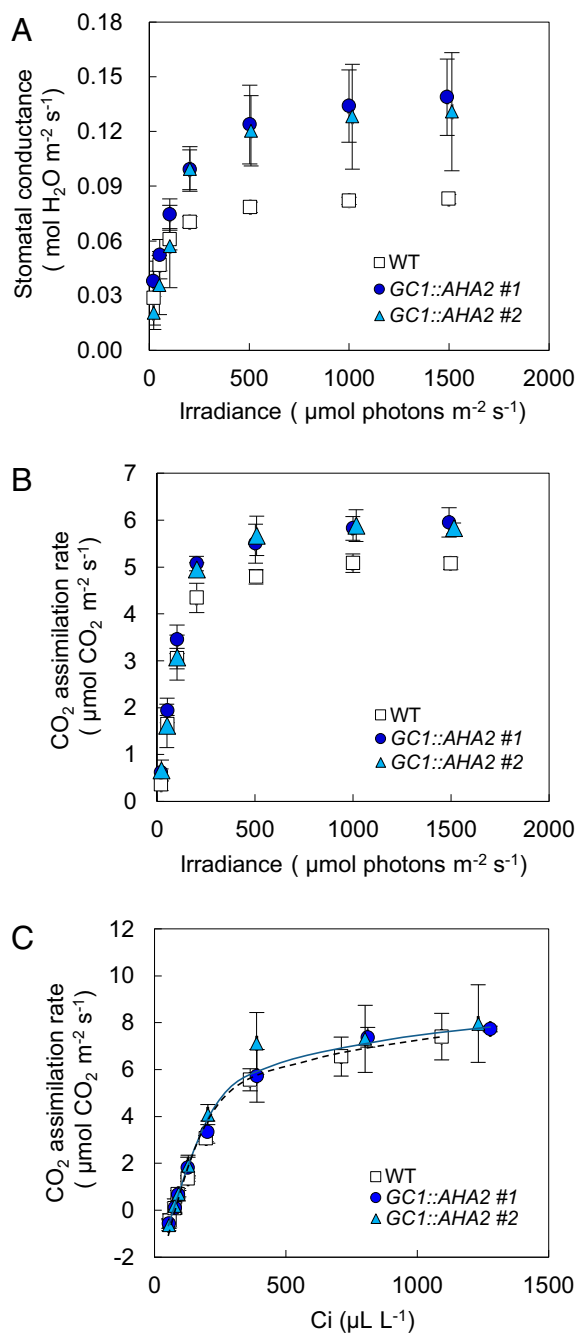
Fig. 2. Gas-exchange properties of *AHA2*-transgenic plants. (*A* and *B*) Light responses of stomatal conductance (*A*) and the CO_2 assimilation rate (*B*) in WT and *AHA2*-transgenic plants. Measurements were conducted at 380 $\mu L \cdot L^{-1}$ CO_2; the leaf temperature and leaf chamber relative humidity were maintained at 24 °C and 40–50% (Pa/Pa), respectively. (*C*) Relationship between the CO_2 assimilation rate and the intercellular CO_2 concentration in WT and *AHA2*-transgenic plants. Measurements were conducted in white light of ~750 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The leaf temperature and relative humidity in the leaf chamber were maintained at 24 °C and 40–50% (Pa/Pa), respectively. Data are the means of measurements on three different plants; error bars represent the SD and are not shown if smaller than the symbols. White squares, WT plants; dark blue circles and light blue triangles, *AHA2*-transgenic plants.

both the Rubisco carboxylation capacity and the electron transport capacity were similar in the WT and transgenic plants, but only the stomatal conductance was greater in the *AHA2*-transgenic plants (Fig. 2*C*). These results indicate that the enhanced stomatal

opening in *AHA2*-transgenic plants contributes to the increased photosynthetic rate.

Overexpression of H⁺-ATPase in Guard Cells Promotes Plant Productivity Under Conditions of Sufficient Light. We examined the growth characteristics of *AHA2*-transgenic plants (Fig. 3). Notably, the *AHA2*-transgenic plants, which exhibited wider stomatal aperture, produced larger and increased numbers of rosette leaves with ~42–63% greater fresh and dry weights than WT plants when the plants were grown under a light intensity of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 25 d. Moreover, the dry weights of total flowering stems in 45-d-old *AHA2*-transgenic plants, including seeds, siliques, and flowers, were ~36–41% greater than those of the WT under the same growth conditions. The number of siliques per *AHA2*-transgenic plant was much greater than for WT plants, although the dry weights of individual siliques from *AHA2*-transgenic plants were comparable to those from WT plants (Fig. S5). In contrast, the stomatal density and index and the size of *AHA2*-transgenic plants were comparable to those of WT plants (Table 1). These results indicate that the increased productivity was not attributable to differences in stomatal morphology or differentiation and that the increased light-induced stomatal opening promoted plant growth.

Plants with higher stomatal conductance fix more ¹²C than ¹³C, making the ratio of ¹³C/¹²C (δ¹³C) less than in plants with lower stomatal conductance (29). To confirm that the wider stomatal aperture of the *AHA2*-transgenic plants contributed to plant growth, we examined δ¹³C in WT and *AHA2*-transgenic plants. The *AHA2*-transgenic plants had a lower δ¹³C than WT plants under well-watered conditions (Fig. 3*H*), although the δ¹³C values for *AHA2*-transgenic line #2 plants seemed to vary widely over a 25-d period. In contrast, no difference in stomatal aperture was observed between WT and *AHA2*-transgenic plants under mild drought conditions, and no observed changes in weight or δ¹³C were detected between WT and *AHA2*-transgenic plants (Fig. S6). These results also suggest that increases in the biomass of *AHA2*-transgenic plants are dependent primarily on stomatal aperture.

Note that when *AHA2*-transgenic plants were grown under a light intensity of 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$, the differences in plant growth between *AHA2*-transgenic and WT plants were small (Table 2). Intercellular CO_2 concentration may still affect the photosynthetic rate because the limiting electron transport is partitioned between carboxylation-related and oxygenation-related reactions (28). However, under a relatively low experimental light intensity, the photosynthetic electron-transport rate, rather than stomatal aperture size, was most likely the limiting factor for photosynthesis (30). Therefore, *AHA2*-transgenic plants showed superior growth under relatively high light intensity (200 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Further investigations are needed to examine the light dependency of plant growth in *AHA2*-transgenic plants. In contrast, the growth of *PHOT2*-, *KAT1*-, and *AKT1*-transgenic plants was similar to that of the WT, even under a light intensity of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Table 2).

Constitutive Open Stomata Phenotype Has No Effect on Plant Growth. In this study, WT *AHA2* was overexpressed in guard cells. The stomata of the *AHA2*-transgenic plants showed increased stomatal opening in response to light, but closed normally in darkness and in response to ABA (Fig. 1). Thus, we examined stomatal phenotype and plant growth in the transgenic plants, which exhibited a constitutive open stomata phenotype. We produced *AHA2-P68S*-transgenic plants (*GC1::AHA2-P68S*), which were presumed to have constitutive high levels of active H⁺-ATPase as a result of a Pro68-to-Ser point mutation in the first transmembrane domain (31). Indeed, the stomata of the *AHA2-P68S*-transgenic plants were constantly open with a wide aperture under both light and dark conditions and even in the
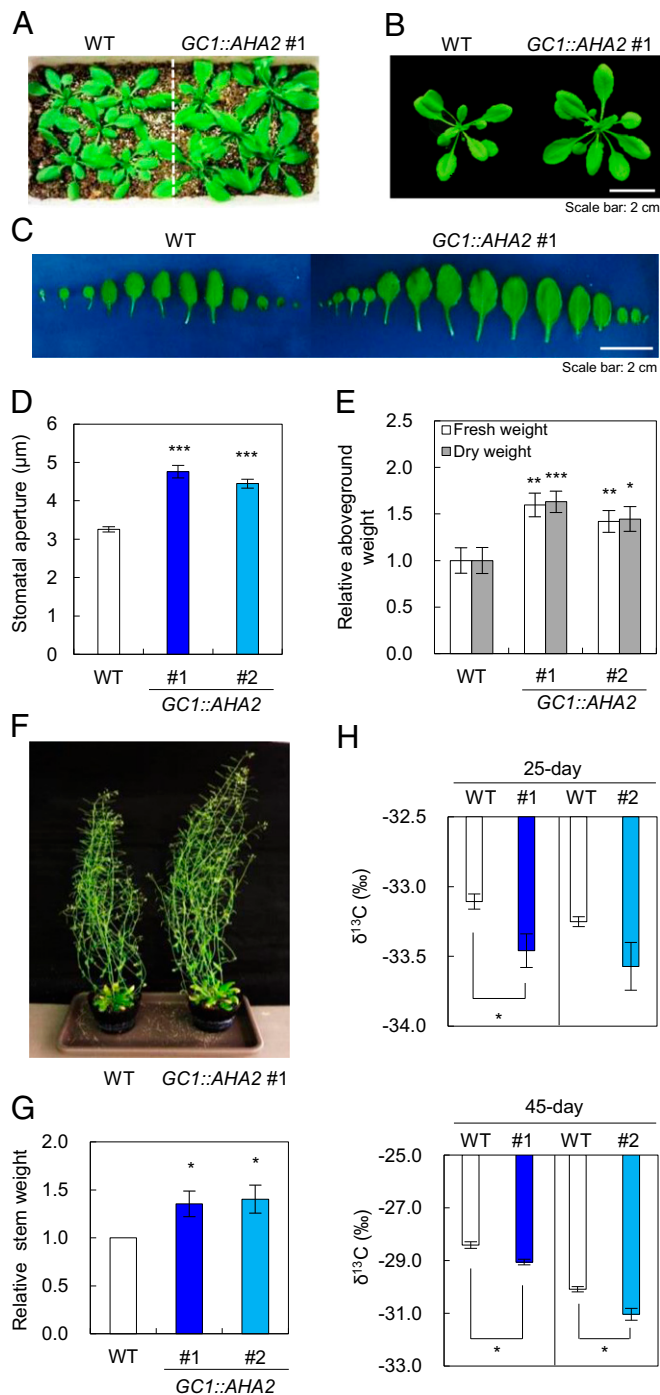
Fig. 3. Phenotypic characterization of *AHA2*-transgenic plants. (*A* and *B*) Phenotypes of WT and *AHA2*-transgenic plants grown under high light conditions (200 μmol·m^{-2}·s^{-1}) for 25 d. (*C*) Rosette and juvenile leaves of WT and *AHA2*-transgenic plants grown under high light conditions. (*D*) Stomatal aperture under high light conditions (200 μmol·m^{-2}·s^{-1}) in 25-d-old plants. Error bars represent the SEM ($n = 25$). Significant differences in stomatal aperture were detected using Student t test (***$P < 0.001$). (*E*) Relative aboveground fresh and dry weights of 25-d-old plants. (*F*) Phenotypes of WT and *AHA2*-transgenic plants grown under high light conditions for 50 d. (*G*) Relative dry stem weights of 45-d-old plants. Fresh and dry weights are the means of measurements of more than five plants; error bars represent the SEM. (*H*) Carbon isotope ratio (δ^{13}C) of WT and *AHA2*-transgenic plants at 25 d (*Upper*) and 45 d (*Lower*); #1 and #2 represent the *AHA2*-transgenic lines *GC1::AHA2* #1 and #2, respectively. Error bars represent the SEM ($n = 3$–4 plants). Differences were detected by Student t test (*$P < 0.05$; **$P < 0.005$; ***$P < 0.001$).

presence of ABA (Fig. S7*A*). However, no enhanced plant growth was observed in the *AHA2-P68S*-transgenic plants (Fig. S7 *B* and *C*). We also examined *FT*-transgenic plants (*GC1::FT-GFP*) because *FT* is a positive regulator of stomatal opening (22). Stomata of *FT*-transgenic plants were constantly open with a larger aperture than the stomata of WT plants under light and dark conditions but closed in response to ABA (22). However, the growth of *FT*-transgenic plants was comparable to that of WT plants (Table 2). These results indicate that a constitutive open stomata phenotype has no effect on plant growth.

Discussion

In this study, we produced *A. thaliana* transgenic plants overexpressing the important components in light-induced stomatal opening, such as the plasma membrane H$^+$-ATPase, phot2, and K$^+_{in}$ channels, using the strong guard cell promoter *GC1*. Only increasing the amount of plasma membrane H$^+$-ATPase in guard cells had a significant effect on light-induced stomatal opening (Figs. 1 and 3*D*), indicating that the plasma membrane H$^+$-ATPase is the limiting factor in the stomatal opening process. We suspect that this effect is attributable to the electrical properties of plasma membrane K$^+_{in}$ channels. The voltage–current relationship of the plasma membrane K$^+_{in}$ channels shows that the activity of K$^+_{in}$ channels is hyperpolarization-dependent (18). Therefore, increasing the levels of guard cell H$^+$-ATPase, which promotes hyperpolarization of the plasma membrane in response to blue light (16), effectively induces K$^+$ uptake into guard cells and stomatal opening. In addition, *AHA2*-transgenic plants showed enhanced photosynthetic activity and increased growth in leaves, seeds, siliques, and flowers under relatively high light intensity (200 μmol·m^{-2}·s^{-1}) (Figs. 2 and 3, Table 2, and Table S1). In their examination of diverse wheat varieties using carbon or oxygen isotopes, Farquhar and coworkers demonstrated that stomatal conductance may have positive effects on crop biomass (4, 32). In this study, we manipulated stomatal opening by means of gene regulation in guard cells to promote plant growth. Our results show that manipulation of the plasma membrane H$^+$-ATPase is very useful not only for promoting stomatal opening but also for enhancing photosynthesis and plant growth. In support of our results, control of H$^+$-ATPase translocation to the plasma membrane by *PATROL1*, which is involved in intercellular membrane traffic, was recently reported to affect stomatal opening, photosynthesis, and plant growth (33).

Note that other key components of light-induced stomatal opening investigated here (phototropins and K$^+_{in}$ channels) had no effect on stomatal opening, photosynthesis, or plant growth under the conditions of this study (Fig. S3 and Table 2). These results suggest that the amounts of endogenous phototropins and K$^+_{in}$ channels in guard cells are sufficient and are not limiting factors in light-induced stomatal opening. This is consistent with

Table 1. Stomatal density, index, and size of WT and *GC1::AHA2* transgenic plants

Adaxial / Abaxial	WT	GC1::AHA2 #1	GC1::AHA2 #2
Stomatal density, mm^{-2}			
Adaxial	88.5 ± 13.8	84.8 ± 12.8	87.0 ± 12.2
Abaxial	130.2 ± 18.8	129.5 ± 25.4	122.0 ± 15.6
Stomatal index			
Adaxial	0.36 ± 0.05	0.37 ± 0.05	0.37 ± 0.05
Abaxial	0.30 ± 0.06	0.29 ± 0.03	0.30 ± 0.03
Stomatal size, μm			
Adaxial	24.94 ± 1.94	24.83 ± 2.34	24.59 ± 1.97
Abaxial	23.28 ± 2.16	23.54 ± 2.66	23.20 ± 1.76

Stomatal density, index, and size were calculated according to a previous method (12).

Table 2. Plant growth of WT and transgenic plants

Plants	Fresh weight, %	Dry weight, %
High light (25-d)		
WT	100	100
GC1::AHA2 #1	159.7 ± 12.7[†]	163.1 ± 11.6[†]
GC1::AHA2 #2	142.1 ± 11.7[‡]	144.6 ± 13.3*
GC1::PHOT2-GFP #3	99.3 ± 7.3	95.6 ± 6.5
GC1::AKT1 #1	78.4 ± 7.5	78.0 ± 8.0
GC1::KAT1 #2	101.5 ± 26.2	106.5 ± 27.6
GC1::FT-GFP #1	94.5 ± 13.5	102.0 ± 13.7
GC1::AHA2-P68S #1	99.7 ± 6.4	100.0 ± 6.3
High light (45-d)		
WT		100
GC1::AHA2 #1	not detected	135.6 ± 13.3*
GC1::AHA2 #2	not detected	140.6 ± 14.6*
Low light (25-d)		
WT	100	100
GC1::AHA2 #1	107.4 ± 26.2	112.8 ± 15.4

Differences were detected by Student t test (*$P < 0.05$; [†]$P < 0.005$; [‡]$P < 0.001$). The dry weights of high light (45-d) plants refer to the final stem weight presented in Fig. 3. High light, photon flux density at 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$; low light, photon flux density at 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

data suggesting that native K^+_{in} channel activity levels are high and predicted not to be rate-limiting for stomatal opening (18, 19, 34). Overexpression of *PHOT2* in guard cells may enhance blue light sensitivity because blue light sensitivity in etiolated seedlings is suggested to depend on the expression level of phot2 (35). Thus, *PHOT2*-transgenic plants may perform better under relatively low light intensity than under relatively high light intensity. Further investigations are needed to clarify this point.

Recently, the rice mutant *slac1*, a drought-sensitive open-stomata mutant, has been shown to have increased rates leaf photosynthesis (3). However, the *slac1* mutation had no effect on plant growth because of an increased sensitivity to drought stress. Consistent with this finding, *AHA2-P68S* transgenic plants, which have constitutive open-stomata and ABA-insensitive phenotypes, did not show enhanced plant growth (Fig. S7). In addition, the stomata of *FT*-transgenic plants (*GC1::FT-GFP*), which have a normal ABA response, were constantly open with a wide aperture under both light and dark conditions (22). However, the growth of *FT*-transgenic plants was comparable to that of WT plants (Table 2). These results indicate that a lack of sensitivity to drought or increased water loss at night may negatively impact biomass accumulation. Further studies on the mechanisms underlying the relationship between nighttime water loss and plant growth are required. Therefore, the stomata of *AHA2*-transgenic plants, which exhibit increased stomatal opening in light but also close normally in darkness and in response to ABA (Fig. 1C), contribute to enhanced photosynthesis and plant growth. Application of this strategy in crops and fuel plants is expected to contribute greatly to the promotion of plant production and a sustainable low-carbon society.

Materials and Methods

Plant Materials and Growth Conditions. *Arabidopsis thaliana gl1* [Columbia (Col), carrying the homozygous recessive *gl1*], used here as the WT, was the background ecotype of all transgenic plants. Plants used for experiments in gene expression, stomatal aperture, and gas exchange were grown in soil under a 16-h fluorescent light (80 $\mu mol \cdot m^{-2} \cdot s^{-1}$)/8-h dark cycle at 24 °C in 55–70% (Pa/Pa) humidity in a growth room. Plants used for biomass productivity measurements were grown under the same environmental conditions, except the light intensity was ~80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (low light conditions) and 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (high light conditions).

Construction of Transformation Vectors and Transformation of Plants. The plasmid vectors used for plant transformation were constructed according to

a previous method (22). In brief, a genomic DNA fragment spanning 1,702 to 1 bp upstream of the start codon of *GC1* (At1g22690) flanked by *Hind*III and *Xba*I sites was used to replace the corresponding region of *CaMV35S* in pPZP211 (pPZP211-GC1). Before *Hind*III digestion, a single-nucleotide substitution (1,453 C to G) was introduced into the *GC1* fragment by site-directed mutagenesis. cDNA fragments of *AHA2* (At4g30190), *PHOT2* (At5g58140), *KAT1* (At5g46240), and *AKT1* (At2g26650) were amplified with the following oligonucleotide primers: 5′-CGGGATCCGAGATGTCGAGTCTCGAAGATATCAA-GAAC-3′ and 5′-CGGGATCCCTCACACAGTGTAGTGACTGGG-3′ for *AHA2*; 5′-GCC-TCTAGAGTTATGGGGATGGAGAGGCCAAGAGCCC-3′ and 5′-CATGCCATGGCGA-AGAGGTCAATCTCCAAGTCCG-3′ for *PHOT2*; 5′-GCCTCTAGAAAAGATGTCGATCT-CTTGGACTCG-3′ and 5′-GCCTCTAGATCAATTTGATGAAAAATACAAATGATCAC-C-3′ for *KAT1*; and 5′-GCCTCTAGAGTGATGAGAGGAGGGGCTTTGTTATGC-3′ and 5′-GCCTCTAGATTAAGAATCAGTTGCAAAGATGAGATGATC-3′ for *AKT1*. The amplified DNA fragments were inserted into pPZP211-GC1 using *Bam*HI, *Xba*I, or *Nco*I. The stop codon of the *PHOT2* coding sequence in pPZP211-GC1::PHOT2 was replaced with a gene encoding synthetic green fluorescent protein with an S65T mutation (GFP). A single amino acid substitution (P68S) was introduced into pPZP211-GC1::AHA2 by site-directed mutagenesis using the following primers: 5′-GGGGTTTATGTGGAATTCACTTTCATGGGTCATGG-3′ and 5′-CCATGACCCATGAAAGTGAATTCCACATAAACCCC-3′. The construction of GC1::FT-GFP was described previously (22). All of the plant transformation plasmid vectors were introduced into *Agrobacterium tumefaciens* (GV3101), which was then used to transform plants using a standard method (22). F3 homozygous plants were used for the experiments.

Reverse transcription–PCR and qPCR. Total RNA was extracted from epidermal fragments using the RNeasy Plant Mini Kit (Qiagen). Epidermal fragments from fully expanded rosette leaves, whole leaves, and roots were isolated from 4- to 6-wk-old plants as described previously (36). First-strand cDNAs were synthesized from total RNA using the PrimeScript II First Strand cDNA Synthesis Kit using oligo(dT) primers (Takara). cDNA fragments of *AHA2* and *TUB2* were amplified with the following oligonucleotide primers: 5′-GGGGAATTCATGTCGAGTCTCGAAG-3′ and 5′-GGGGAATTCTACACAGTG-TAGTGAC-3′ for *AHA2* and 5′-CATTGTTGATCTCTAAGATCCGTG-3′ and 5′-TACTGCTGAGAACCTCTTGAG-3′ for *TUB2*. All PCRs were performed for 30 cycles.

qPCR was performed using Power SYBR Green PCR Master Mix and the StepOne Real-Time PCR system (Applied Biosystems) as described previously (37). For gene-specific amplification of the *AHA2* transcripts, the following primer sets were used: 5′-TTGTTGAACGTCCTGGAGCA-3′ and 5′-AATTCC CAGTTGGCGTAAACC-3′. Relative quantification was performed using the comparative cycle threshold method, and the relative amount of PCR product amplified using the above primer sets was normalized to the *TUB2* gene fragment as an internal control amplified using the primers 5′-AAACTCAC-TACCCCCAGCTTTG-3′ and 5′-CACCAGACATAGTAGCAGAAATCAAGT-3′.

Immunohistochemical Detection of Plasma Membrane H^+-ATPase in Guard Cells. Immunohistochemical detection was performed according to a previous method (36) with modifications. Fully expanded rosette leaves of WT and *AHA2*-transgenic plants were harvested and then cut into small pieces. The pieces were fixed with 4% (wt/vol) formaldehyde freshly prepared from paraformaldehyde in fixation buffer [50 mM Pipes·NaOH (pH 7.0), 5 mM MgSO$_4$, 5 mM EGTA] for 1 h at room temperature. The fixed samples were washed with PBS (137 mM NaCl, 8.1 mM Na$_2$HPO$_4$, 2.68 mM KCl, 1.47 mM KH$_2$PO$_4$), destained with 100% methanol at 37 °C, and then washed with Milli-Q water (Millipore) at room temperature. The destained samples were attached to slide glasses, sealed with cover glasses, and then frozen and thawed five times with liquid nitrogen. After removing the cover glasses, the samples were completely dried overnight. On the second day, the samples were digested on the slide glasses with 4% (wt/vol) Cellulase Onozuka R-10 (Yakult) and 0.5% (wt/vol) Macerozyme R-10 (Yakult) in PBS for 1 h at 37 °C. After digestion, the samples were washed with PBS and permeabilized with 3% (vol/vol) Nonidet P-40 (Nonidet P-40; MP Biomedicals) and 10% (vol/vol) dimethyl sulfoxide (Wako) for 30 min at room temperature. The samples were then washed with PBS and blocked in 3% (wt/vol) BSA Fraction V (Sigma) in PBS for 1 h at room temperature. Thereafter, the samples were incubated with anti–H^+-ATPase antibody at a dilution of 1:5,000 in PBS with 3% (wt/vol) BSA for 6 h at 37 °C or overnight at 4 °C. The samples were washed with PBS and incubated with Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen) at a dilution of 1:500 in PBS with 3% (wt/vol) BSA at 37 °C for 3 h in darkness. After a wash with PBS, each specimen was sealed under a cover glass with 50% (vol/vol) glycerol. The specimens were observed under a fluorescence microscope (BX50; Olympus) with a narrow excitation band-pass filter set for Alexa Fluor 488, model nos. BP460–480HQ BA495–540HQ

(U-MGFPHQ; Olympus), using a Hg arc lamp as a source of excitation light. Fluorescent images were collected using a CCD camera system (DP72; Olympus) and processed using DP2-BSW software (Olympus). To estimate fluorescence intensities, all images were taken at identical exposure times. The fluorescence intensities of stomata were quantified using ImageJ (National Institutes of Health).

Measurement of Stomatal Aperture. Stomatal apertures were measured according to a previous method (38) with some modifications. Epidermal tissues isolated from overnight dark-adapted 3- to 4-wk-old plants were incubated in basal buffer [5 mM MES·bis-Tris propane (pH 6.5), 50 mM KCl, and 0.1 mM CaCl$_2$]. For inhibition of light-induced stomatal opening by ABA, epidermal tissues were incubated under blue/red light [blue light (Stick-B-32; EYELA) at 10 μmol·m^{-2}·s^{-1} superimposed on background red light (LED-R; EYELA) at 50 μmol·m^{-2}·s^{-1}] for 2.5 h at 24 °C in the presence or absence of 20 μM ABA. Stomatal apertures in the abaxial epidermis were measured microscopically. Stomatal apertures are presented as the mean of 25 stomata with SEM. The results were confirmed by blind reassessment by another researcher.

Gas-Exchange Measurements. Gas-exchange measurements were performed using the LI-6400 system (Li-Cor), and parameters were calculated with the software supplied by the manufacturer. White light was provided by a fiberoptic illuminator with a halogen projector lamp (15 V/150 W; Moritex) as a light source. The power supply (MHAB-150W; Moritex) was used to power the lamp. The light was attenuated with a series of optical crown glass metallic neutral density filters (Newport Japan, Hakuto). The flow rate, leaf temperature, and relative humidity were kept constant at 500 μmol·s^{-1}, 24 °C, and 40–50% (Pa/Pa), respectively. For light response curves, the conditions were the same, and after the initial 30 min of dark adaptation, the intensity of the white light (20, 50, 100, 200, 500, 1,000, and 1,500 μmol·m^{-2}·s^{-1}) was increased in steps of more than 20-min duration (stable statue). For CO$_2$ response curves, leaves were first acclimated at 400 μL·L^{-1} CO$_2$ under saturating white-light conditions (~750 μmol·m^{-2}·s^{-1}) for 40 min, and then the CO$_2$ concentration was increased stepwise from 50 to 1,500 μL·L^{-1} CO$_2$ at intervals of ~20 min.

Carbon Isotope Ratio Analyses. Carbon isotopes were measured with a system combining an elemental analyzer (Vario Micro; Elementar) and a stable isotope ratio mass spectrometer (IsoPrime) (39). Dried tissue from 25-d or 45-d rosette leaves was used for the carbon isotope analysis. Different transgenic plant lines and WT plants (control) were grown in different planters or in the different period. Carbon isotope ratio (δ^{13}C, ‰) was obtained in δ-notation, where $\delta = R_{sample}/R_{standard} - 1$ and R_{sample} and $R_{standard}$ are the isotope ratios of the plant sample and the PeeDee belemnite, respectively (39, 40).

Statistical Analysis. Significance was determined according to Student t test using Microsoft Excel. Two-sided tests were performed for homoscedastic matrices.

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