GOLDEN 2-LIKE transcription factors for chloroplast development affect ozone tolerance through the regulation of stomatal movement

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Stomatal movements regulate gas exchange, thus directly affecting the efficiency of photosynthesis and the sensitivity of plants to air pollutants such as ozone. The GARP family transcription factors GOLDEN 2-LIKE1 (GLK1) and GLK2 have known functions in chloroplast development. Here, we show that *Arabidopsis thaliana* (*A. thaliana*) plants expressing the chimeric repressors for GLK1 and -2 (GLK1/2-SRDX) exhibited a closed-stomata phenotype and strong tolerance to ozone. By contrast, plants that overexpress GLK1/2 exhibited an open-stomata phenotype and higher sensitivity to ozone. The plants expressing *GLK1-SRDX* had reduced expression of the genes for inwardly rectifying K^+ (K^+_{in}) channels and reduced K^+_{in} channel activity. Abscisic acid treatment did not affect the stomatal phenotype of *35S:GLK1/2-SRDX* plants or the transcriptional activity for K^+_{in} channel gene, indicating that GLK1/2 act independently of abscisic acid signaling. Our results indicate that GLK1/2 positively regulate the expression of genes for K^+_{in} channels and promote stomatal opening. Because the chimeric GLK1-SRDX repressor driven by a guard cell-specific promoter induced a closed-stomata phenotype without affecting chloroplast development in mesophyll cells, modulating GLK1/2 activity may provide an effective tool to control stomatal movements and thus to confer resistance to air pollutants.

ozone | stomatal movement | transcription factor | repressor | K^+_{in} channel

Tropospheric ozone (O_3) is a major photochemical oxidant and one of the most phytotoxic air pollutants (1). High concentrations of ozone induce oxidative stress, which activates programmed cell death and significantly inhibits plant growth, causing crop losses estimated to be in the billions of dollars (2, 3). To study the mechanisms of ozone damage in plants, researchers have isolated *Arabidopsis thaliana* mutants that exhibit hypersensitivity to ozone exposure (4), including several mutants with higher stomatal conductance than wild type (WT), such as *radical-induced cell death1* (*rcd1*), *rcd2*, and *ozone sensitive1/slow anion channel-associated1* (*ozs1/slac1*) (5–8). In addition, the *Arabidopsis* ecotype *Cvi-0*, which has higher stomatal conductance than other ecotypes, also exhibits hypersensitivity to ozone compared with other ecotypes (9, 10). These observations indicate that stomatal movement has a strong relationship to ozone sensitivity, likely because stomata regulate the first step of ozone absorption into plant cells.

Stomatal movements are controlled by turgor pressure in guard cells in response to environmental stimuli such as CO_2 concentration, light intensity, humidity, and air pollutants (11). Hyperpolarization of the plasma membrane caused by the H^+-ATPases promotes stomatal opening through activation of the voltage-gated K^+ inward-rectifying channels (K^+_{in}), encoded by *K⁺ CHANNEL IN ARABIDOPSIS THALIANA1* (*KAT1*), *KAT2*, and *ARABIDOPSIS*

K TRANSPORTER 1 (*AKT1*), which induce water entry into guard cells (11, 12). By contrast, depolarization of the plasma membrane activates outward-rectifying K^+ channels (6–8), which induce water efflux from guard cells, resulting in stomatal closure (11, 12).

Recent studies have further elucidated the signaling pathways that regulate guard cell movement; these pathways include second messengers, plant hormones, and transcription factors (11–13). The MYB60 and MYB61 transcription factors regulate light-induced stomatal opening and dark-induced stomatal closure, respectively (14–16). MYB44, MYB15, ERF7, and NFYA5 also participate in stomatal movement in *A. thaliana* (17–20). The bHLH transcription factors ABA-RESPONSIVE KINASE SUBSTRATES1 (AKS1), AKS2, and AKS3 function as positive regulators of stomatal opening (21). They facilitate K^+ uptake through positive regulation of *KAT1*, but abscisic acid (ABA) represses their transcriptional activation activity through phosphorylation (21). The *aks1 aks2* mutants exhibit a weak closed-stomata phenotype and weak down-regulation of *KAT1* (21). These phenotypes suggest that other, functionally redundant transcription factor(s) may regulate the expression of the genes for K^+_{in} channels and stomatal opening. Modulation of stomatal movement using transcription factors may provide a useful strategy to confer ozone

Significance

Stomata regulate the efficiency of photosynthesis and affect plants' resistance to air pollutants. However, the transcriptional regulation of the genes modulating stomatal movement has not been well characterized. Using chimeric repressor gene-silencing technology (CRES-T), we identified another function of the previously studied regulators of chloroplast development GOLDEN 2-LIKE1 (GLK1) and GLK2, as positive regulators of stomatal movement and K^+_{in} channel genes. The chimeric GLK repressors induce closed stomata and tolerance to ozone exposure and reduced gene expression and activity of K^+_{in} channels in guard cells. Guard cell-specific expression of the chimeric GLK repressor may be a useful tool to confer resistance to air pollutants.

tolerance, because a number of known transcription factors act as master regulators of various cellular processes. However, screens for changes in the response to ozone have yet to identify mutant lines or transcription factors that confer tolerance to ozone, possibly because of redundant functions of these key genes.

In this study, we screened a set of transgenic *Arabidopsis* lines expressing chimeric repressors for *Arabidopsis* transcription factors and found that lines expressing the chimeric repressors for GOLDEN 2-LIKE1 (GLK1) and GLK2, which regulate chloroplast development (22–26) and exhibit remarkable ozone tolerance and a closed-stomata phenotype. These transgenic plants also show down-regulation of gene expression and activity of K^+_{in} channels and of other genes involved in stomatal movement. We propose here that GLKs act as positive regulators of stomatal movement and that the guard cell-specific expression of the chimeric GLK repressor can confer tolerance to air pollutants, possibly providing a useful tool for crop protection.

Results

Plants Expressing the Chimeric Repressors for GLKs Exhibit Remarkable Tolerance to Ozone. The chimeric repressor gene silencing technology (CRES-T) system converts a transcriptional activator into a strong repressor by fusion to the SRDX repression domain; this process induces a phenotype similar to loss-of-function alleles (27) and can also confer tolerance to various abiotic stresses (28). To identify a chimeric repressor that confers tolerance to ozone stress, we screened a set of transgenic *Arabidopsis*-expressing chimeric repressors for *Arabidopsis* transcription factors, termed CRES-T lines (27), exposing the plants to 0.3 ppm ozone for 7 h. We found that the CRES-T lines for the two redundant GARP family transcription factors, GOLDEN 2-LIKE1 (GLK1) and GLK2 (*35S:GLK1/2-SRDX*), which regulate chloroplast development (22–26), exhibited tolerance to ozone exposure, showing 12-fold less ion leakage than the WT plants (Fig. 1 *A* and *B*). By contrast, plants overexpressing GLK1 or GLK2 (*35S:GLK1/2*) exhibited hypersensitivity to ozone, with higher ion leakage than WT (Fig. 1 *C* and *D*).

In addition to ozone, *35S:GLK1/2-SRDX* seedlings also exhibited tolerance to sulfur dioxide, an oxidative stress reagent similar to ozone. However, the *35S:GLK1/2-SRDX* seedlings showed no resistance to aqueous paraquat (methyl viologen) in the medium, as measured by the inhibition of root elongation (Fig. S1). These results suggest that the tolerance of *35S:GLK1/2-SRDX* plants to ozone and sulfur dioxide was due to an alteration of gas absorption through stomata, not to an alteration of sensitivity to oxidative stress. The *35S:GLK1/2-SRDX* plants showed a similar stomatal density as the WT plants (Fig. S2), indicating that their differing sensitivity to ozone may be attributable to an alteration of stomatal aperture.

GLKs Affect Stomatal Aperture. We measured the stomatal aperture (as the ratio of width/length) of each plant grown in normal light conditions or kept in dark conditions for 1 d before the experiments. We found, as expected, that the *35S:GLK1/2-SRDX* plants had much smaller apertures than WT, with average apertures of 55% and 72% (for *35S:GLK1-SRDX* plants) and 72% and 83% (for *35S:GLK2-SRDX* plants) of that of WT under dark and light conditions, respectively (Fig. 2*A*). By contrast, *35S:GLK1/2* plants had much larger apertures than WT, with average apertures of 210% and 186% (for *35S:GLK1* plants) and 183% and 166% (for *35S:GLK2* plants) of that of WT under dark and light conditions, respectively (Fig. 2*A*). Thermography monitoring also revealed that the leaf surface temperature of *35S:GLK1/2-SRDX* plants was 1.5 °C higher than that of WT and that the water loss of detached seedlings was 13% lower than WT (Fig. 2 *C* and *D* and Fig. S3), showing that the *35S:GLK1/2-SRDX* plants have low transpiration rates. The stomata of *35S:GLK1/2-SRDX* plants closed in response to abscisic acid (ABA) treatment, similar to WT, when the leaf epidermis was treated with 1 or 20 µM ABA for 2.5 h under light
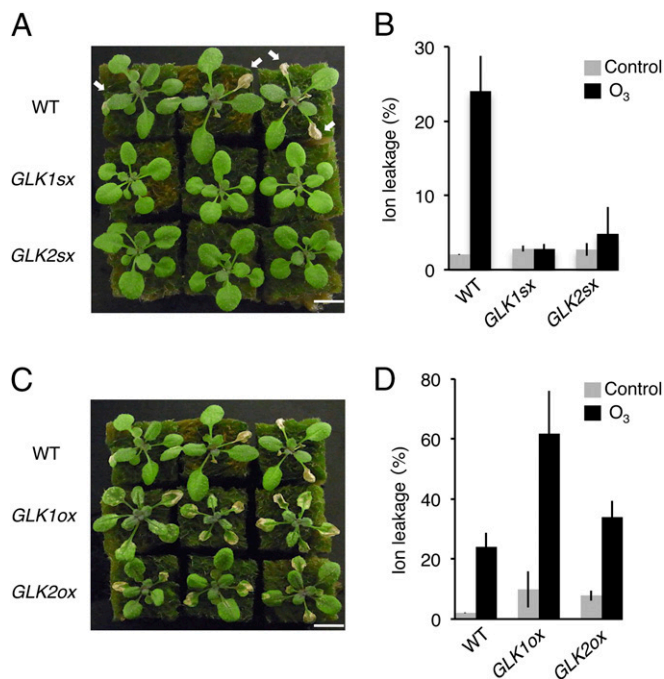

Fig. 1. Sensitivity to ozone of *GLK1/2* transgenic *Arabidopsis*. (*A*) Rosette plants of WT and *35S:GLK1/2-SRDX* (*GLK1sx* and *GLK2sx*) 1 d after exposure to 0.3 ppm O₃ for 7 h. Arrows indicate the damaged leaves. (*B*) Ion leakage of WT and *GLK1/2sx* plants. The gray and black bars represent plants exposed to fresh air or O₃, respectively. The average of three biological replicates (three plants per replicate) is shown. Error bars represent SD. (*C*) Rosette plants of WT and *35S:GLK1/2* (*GLK1ox* and *GLK2ox*) 1 d after exposure to 0.3 ppm O₃ for 7 h. (*D*) Ion leakage of WT and *GLK1/2ox* plants. The gray and black bars represent plants exposed to fresh air or O₃, respectively. The average of three biological replicates (three plants per replicate) is shown. Error bars represent SD.

(Fig. 2*B*), indicating that the alteration of stomatal apertures in *35S:GLK1/2-SRDX* plants does not appear to be attributable to a change in sensitivity to ABA.

GLKs Affect the Expression of Genes Related to Stomatal Movement. We detected the expression of *GLK1* and *GLK2* in guard cells and mesophyll cells (Fig. S4), and our observations were consistent with the previously reported microarray data (29). To examine the possible mechanisms of the closed-stomata phenotype of *35S:GLK1/2-SRDX* plants, we performed microarray experiments to find genes regulated by GLK1. Among transcripts of numerous genes changed in abundance in *35S:GLK1-SRDX* seedlings, we identified a set of genes related to stomatal movement (listed in Table S1). In *35S:GLK1-SRDX* plants, we found significant down-regulation ($P < 0.06$) of *KAT1*, *KAT2*, and *AKT1*, which encode Shaker-type K^+_{in} channels (11, 12). In addition, *BLUE LIGHT SIGNALING1* (*BLUS1*) (30), which encodes a kinase phosphorylated by phototropins and regulates blue light-induced stomatal opening through activation of H⁺-ATPase, and *FLOWERING LOCUS T* (*FT*) (31), which regulates stomatal opening, were also down-regulated ($P < 0.07$).

We analyzed the expression of *KAT1*, *KAT2*, *AKT1*, *BLUS1*, and *FT* by quantitative (q)RT-PCR using RNA isolated from stomata-rich epidermal cells and confirmed the reduction of expression of these genes ($P < 0.05$), except for *AKT1* in *35S:GLK1-SRDX* plants (Fig. 3*A* and Table S2). Expression of *FT* was remarkably suppressed in *35S:GLK1-SRDX* plants ($P < 0.01$). These expression profiles suggest that the severe closed-stomata phenotype of *35S:GLK1/2-SRDX* plants may be caused by a combination of different effects; among these effects are the reduction of blue light signaling and K^+_{in} channel activity in guard cells.
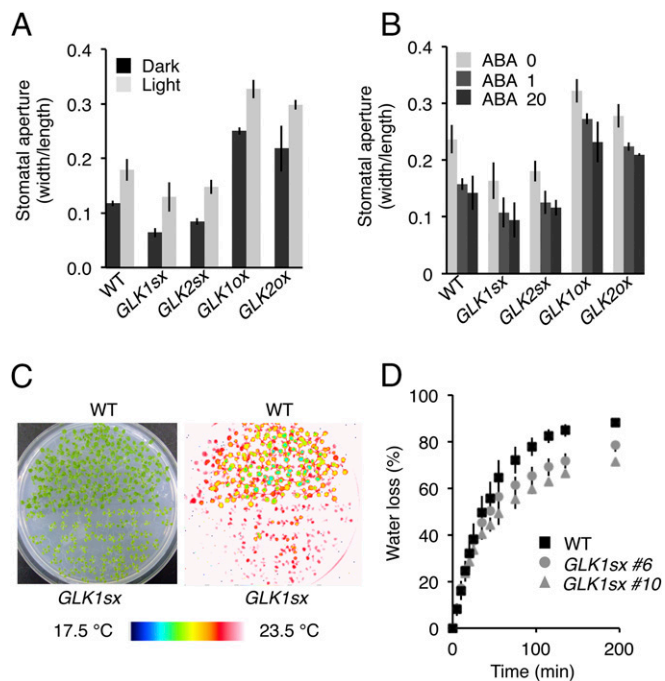
Fig. 2. GLK1/2 control stomatal opening. (*A*) Stomatal aperture of epidermal fragments from the plants under dark conditions, in which plants were incubated 1 d before measurements (dark), and light conditions (light). (*B*) Stomatal aperture of epidermal fragments incubated with 0, 1, and 20 μM ABA under white light for 2.5 h. The average of three independent experiments is shown. Approximately 150 stomata in total (*n* = 3 independent experiments, 50 stomata per experiment) were analyzed in each line. Error bars represent SD. (*C*) Thermal images of WT (*Upper*) and *GLK1sx* (*Lower*) plants grown on MS medium, showing the higher temperature of *GLK1sx* plants. (*D*) Water loss in WT and *GLK1sx* plants for the evaluation of transpiration rate. The average of three biological replicates (five to seven plants per replicate) is shown. Error bars represent SD.

Because *blus1* and *ft* knockout mutants showed a closed-stomata phenotype attributable to the reduction of the H^+-ATPase activity (30, 31), we analyzed the expression of the H^+-ATPase genes and the activity of H^+-ATPases in guard cells using an immunohistochemical method (32) by monitoring the blue light-induced phosphorylation of the penultimate Thr of these H^+-ATPases. Results of qRT-PCR using RNA isolated from stomata-rich epidermal cells showed that the expression of three H^+-ATPase genes, *Arabidopsis* H^+-*ATPase 1* (*AHA1*), *AHA2*, and *AHA5*, which are mainly expressed in guard cells (33), was slightly but significantly reduced in *35S:GLK1-SRDX* plants (*P* < 0.05; Fig. S5*A*). By contrast, the level of blue light-induced phosphorylation and the protein levels of guard cell H^+-ATPases did not significantly differ between *35S:GLK1-SRDX* and WT plants (Fig. S5 *B and C*), implying that the activities of H^+-ATPases may not be directly involved in the closed-stomata phenotype of *35S:GLK1/2-SRDX* plants.

GLKs Regulate K^+_{in}-Related Genes. The expression of major K^+_{in} channel genes was reduced in *35S:GLK1-SRDX* plants (Fig. 3*A*); therefore, we analyzed the activity of K^+_{in} channels using patch-clamp experiments with protoplasts isolated from guard cells. The average of the steady-state whole-cell current at −180 mV was −221.9 ± 22.1 pA in WT but was −105.8 ± 18.9 pA (*P* = 0.0071) in *35S:GLK1-SRDX* plants (Fig. 3 *B* and *C*), indicating that the activity of K^+_{in} channels of *35S:GLK1-SRDX* plants was 52.3% lower than the activity in WT. Our results indicate that GLK1-SRDX down-regulates the gene expression and activity of K^+_{in} channels.

The *A. thaliana KAT1* locus contains three GLK-binding sites in the 3-kb 5′ region upstream of the translation initiation site. Those are two CACGTG, which is also recognized as the G-box

(25), and CCAATC. Transient expression analyses in *Arabidopsis* leaves revealed that the luciferase reporter gene (*LUC*) driven by the 5′ upstream region of *KAT1* (*KAT1pro:LUC*) was up-regulated when coexpressed with *35S:GLK1/2* (Fig. 3*D*; *P* < 0.05). Also, coexpression with *35S:GLK1/2-SRDX* down-regulated the activity of the *35S-KAT1pro:LUC* reporter gene, in which the enhancer region of the CaMV 35S promoter was fused to the upstream region of *KAT1pro:LUC* (Fig. 3*D*; *P* < 0.05). These results suggest that *KAT1* may be a direct target of GLK1/2.

To analyze whether ABA affects GLK1 activity, we treated protoplasts with 2 μM ABA after cotransformation with the effector plasmid and reporter gene and found that ABA did not affect the activation by *35S:GLK1/2* or the repression by *35S:GLK1/2-SRDX* of the *KAT1pro:LUC* or *35S-KAT1pro:LUC* reporter genes (Fig. 3*E*; *P* > 0.05). These results suggest that GLKs regulate *KAT1* expression independently from ABA signaling.

Guard Cell-Specific Expression of GLK1-SRDX Induces Ozone Tolerance and a Closed-Stomata Phenotype. The *glk1 glk2* double mutants exhibit a pale green phenotype due to inhibition of chloroplast development (23). The *35S:GLK1/2-SRDX* plants were paler green than WT but much darker and larger than the *glk1 glk2* mutants (Fig. S6). To limit the activity of GLK1-SRDX specifically to guard cells, we expressed GLK1-SRDX under the control of the *GC1* (*At1g22690*) promoter (*GC1pro:GLK1-SRDX*), which shows strong activity in guard cells (34). *GC1pro:GLK1-SRDX* plants exhibited a closed-stomata phenotype and tolerance to ozone, similar to the *35S:GLK1/2-SRDX* plants (Fig. 4). However, unlike the *35S:GLK1-SRDX* plants or *glk1 glk2* mutants, the *GC1pro:GLK1-SRDX* plants exhibited a green color similar to WT, showing that *GC1pro:GLK1-SRDX* did not affect chloroplast development in mesophyll cells (Fig. 4*A*). In addition, these results indicate that the closed-stomata phenotype of *35S:GLK1-SRDX* plants was not attributable to a defect in the mesophyll chloroplasts.

Discussion

In this study, we showed that transgenic *Arabidopsis* plants that expressed *35S:GLK1/2-SRDX* exhibited a closed-stomata phenotype and tolerance to ozone exposure, whereas *35S:GLK1/2* plants exhibited an open-stomata phenotype and hypersensitivity to ozone. We demonstrated that the activity of K^+_{in} channels was down-regulated in *35S:GLK1-SRDX* plants, and *KAT1* appears to be a direct target of GLKs (Fig. 3), suggesting that GLKs act as positive regulators of K^+_{in} channel genes and stomatal movement. Similar to the GLKs, the AKSs transcription factors positively regulate stomatal opening and activate *KAT1* expression (21). The *aks1 aks2* double mutants have reduced K^+_{in} channel activity and lower rates of light- or fusicoccin (Fc)-dependent stomata opening; AKSs also function in an ABA-dependent manner and are regulated by phosphorylation (21). In contrast to AKSs, ABA does not appear to affect the transcriptional activity of GLK1 (Fig. 3). Although GLKs and AKSs may act redundantly in the regulation of genes for K^+_{in} channels, each of the transcription factors appears to be regulated by a different signaling pathway. This putative functional redundancy might explain why *aks1 aks2* mutants exhibit a weak closed-stomata phenotype (21) and why *glk1 glk2* double mutants do not exhibit a clear closed-stomata phenotype or ozone tolerance (Fig. S7). On the other hand, *35S:GLK1/2-SRDX* plants exhibit a severe closed-stomata phenotype. This observation is probably because the chimeric repressor dominantly suppresses the target genes even in the presence of endogenous or functionally redundant transcription factors (27).

The *kat1* single mutant does not affect or only slightly inhibits Fc-dependent stomatal opening (21, 35). By contrast, the dominant negative form of KAT1 or KAT2 impaired the activity of K^+_{in} channels in guard cells and clearly inhibited light-dependent stomata opening (36, 37), indicating that the activity of K^+_{in} channels in guard cells is essential and might be much higher than
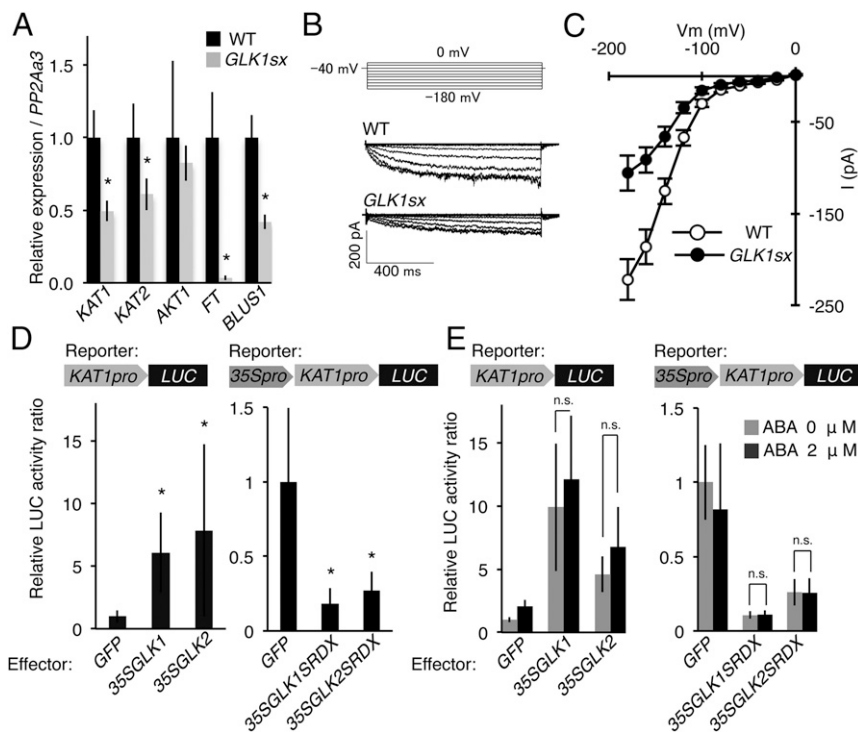
Fig. 3. GLK1/2 regulate K$^+$ channel genes. (*A*) Relative expression of *KAT1*, *KAT2*, *AKT1*, *FT*, and *BLUS1* determined by qRT-PCR, using RNA extracted from guard cell-enriched epidermis. The average of four biological replicates is shown. Error bars represent SD. *$P < 0.05$. (*B*) The whole-cell inward K$^+$ currents in response to membrane potentials by voltage protocol (*Upper*), stepped from a holding potential of −40 mV to pulse potentials from 0 to −180 mV in a 20-mV decrement in guard cell protoplasts of WT and *GLK1sx* plants. (*C*) Steady-state current–voltage relationship in guard cells of WT ($n = 18$ experiments) and *GLK1sx* ($n = 8$ experiments) plants. Error bars represent SE. (*D*) Relative luciferase activities after cobombardment of *Arabidopsis* leaves with *35S:GLK1/2* effectors and the *KAT1pro:LUC* reporter construct and *35S:GLK1/2-SRDX* effectors and the *35S-KAT1pro:LUC* reporter construct, respectively. The luciferase activity is shown as the relative ratio to the value obtained by the combination of *35S:GFP* effector (control) and each reporter construct. The average of six replicates is shown. Error bars represent SD. *$P < 0.05$. (*E*) Relative luciferase activities after cobombardment of *Arabidopsis* protoplasts with *35S:GLK1/2* effectors and the *KAT1pro:LUC* reporter construct and *35S:GLK1/2-SRDX* effectors and the *35S-KAT1pro:LUC* reporter construct, respectively. The black and gray bars indicate samples treated with or without 2 μM ABA, respectively. All luciferase activities are shown as the relative ratio to the value obtained by the combination of *35S:GFP* effector (control) and each reporter construct. The average of five replicates is shown. The relative luciferase activity of individual transient assay is shown in Table S4. Error bars represent SD. n.s., not significant ($P > 0.05$).

the threshold necessary for stomata opening (35–38). Although the severe closed-stomata phenotype of *35S:GLK1/2-SRDX* plants might result from combinatorial effects of multiple genes, we suggest that their closed-stomata phenotype in light conditions was attributable, at least in part, to a decrease in the activity of K$^+_{in}$ channels and a reduction of the expression of K$^+_{in}$ channel genes in guard cells as shown in *aks1 aks2* mutants (21). We found that the expression of numerous genes was affected in *35S:GLK1-SRDX* seedlings. However, we did not find obvious differences in the expression of ABA-responsive genes in our microarray data, probably because ABA sensitivity is not altered in *35S:GLK1-SRDX* plants (Figs. 2*B* and 3*E*). Further analysis of the genes affected by GLK1-SRDX may provide new insights into the mechanisms of stomatal movements regulated by GLK1/2.

The mechanisms of the transcriptional regulation of stomatal aperture are not fully understood. We propose that GLK1/2 positively regulate stomata opening, likely by activating the expression of K$^+_{in}$ channel genes. Light induces the expression of *GLK1* and *GLK2* (23) and ABA decreases the expression of *GLK2* in guard cells (39), similar to the behavior of *KAT1* and consistent with the movements of stomata. Because an increase in K$^+_{in}$ channel activity does not produce additional stomatal opening (21, 36, 37, 40), the open-stomata phenotype of *35S:GLK1/2* plants indicates that GLK1/2 may regulate the expression of genes necessary for stomatal opening other than those for K$^+_{in}$ channels (Fig. 3*A*), which may induce the more-severe closed-stomata phenotype of *35S:GLK1/2-SRDX* plants. Unknown factor(s) that act downstream of GLKs may regulate stomatal movement, because the activity of

H$^+$-ATPases does not seem to be directly involved in the closed-stomata phenotype of *35S:GLK1/2-SRDX* plants (Fig. S5). Guard cell chloroplasts are essential for blue light-dependent stomatal opening (41), implying a relationship between the activity of GLKs and the regulation of stomatal movements. Further study of the functions of GLK1/2 in stomatal movement will be necessary to provide new insights into the relationship between chloroplast development and transpiration, which affect photosynthetic efficiency.

Stomata regulate transpiration rate and absorption of gasses. Modification of stomatal movements could improve both the efficiency of photosynthesis and the tolerance to air pollutants. Transcription factors can be useful tools for the manipulation of plant traits because transcription factors regulate multiple genes and some act as master regulators of phenotype. Regulation of the expression of GLK1-SRDX specifically in guard cells could prove useful to create crops that are tolerant to air pollutants.

Materials and Methods

Construction of Plasmids. The protein-coding regions and the 5′ upstream promoter regions of genes were amplified from a cDNA library or from genomic DNA of *A. thaliana* with the appropriate primer sets (Table S3). The GLK1-SRDX and GLK2-SRDX transgenes were constructed as described previously (42). To prepare the GC1pro:GLK1-SRDX construct, 1,745 bp of the
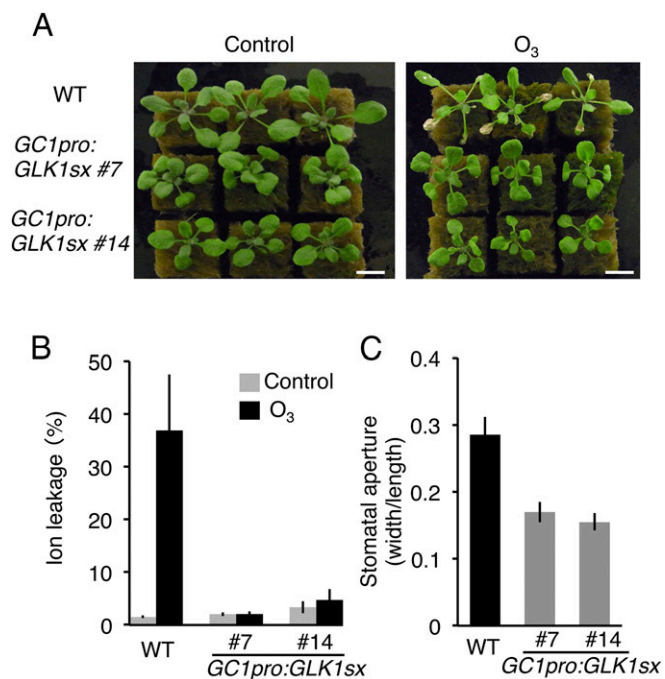
Fig. 4. Phenotype of *GC1pro:GLK1-SRDX* plants. (*A*) WT and two independent lines of *GC1pro:GLK1-SRDX* (*GC1pro:GLK1sx*) 1 d after exposure to fresh air (*Left*) or 0.3 ppm O$_3$ (*Right*) for 7 h. (*B*) Ion leakage of WT and *GC1pro:GLK1sx* plants exposed to fresh air and 0.3 ppm O$_3$ for 7 h. The gray and black bars represent plants exposed to fresh air or O$_3$, respectively. The average of three biological replicates (three plants per replicate) is shown. Error bars represent SD. (*C*) Stomatal aperture of WT and *GC1pro:GLK1sx* plants. The average of three independent experiments is shown. Approximately 150 stomata in total (*n* = 3 independent experiments, 50 stomata per experiment) were analyzed in each line. Error bars represent SD.

GC1 promoter region and the GLK1 protein coding region were cloned into the pDONRG_P4P1R and pDONR207 vectors (42), respectively, using the Gateway BP reaction (Life Technologies), and then were assembled in the R4pGWB5_SRDX multisite gateway vector (43) by the Gateway LR reaction. To prepare the *35S:GLK1*, *35S:GLK2*, *35S:GLK1-SRDX*, and *35S:GLK2-SRDX* effector plasmids for transient effector-reporter experiments, protein-coding regions of *GLK1* and *GLK2* were cloned into the SmaI site of p35SG or p35SSRDXG (42). To prepare the *KAT1pro:LUC* and *35S:KAT1pro:LUC* reporter plasmids, 2,900 bp of 5′ upstream region of the translation initiation site of *KAT1* was cloned into pDONRG_P4P1R (43) using the Gateway BP reaction (Life Technologies) and transferred by the Gateway LR reaction into R4L1pDEST190LUC and 35S_R4L1pDEST190LUC, which has a R4L1 recombination cassette upstream of the TATA box of the p190LUC vector (44) without or with the CaMV 35S enhancer region further upstream, respectively.

O$_3$ and SO$_2$ Treatments and Measurement of Ion Leakage. Two-week-old seedlings grown on solid MS medium (containing 0.8% agar, 0.5% sucrose, and 0.5 g/L Mes, pH 5.7 by KOH) in 150-mm Petri dishes or rock wool were exposed to 0.3 ppm ozone for 7 h or 1.0 ppm sulfur dioxide for 11 h in a growth chamber at 25 °C and 70% relative humidity under continuous light of 350 μmol m^{-2} s^{-1} (photosynthetic photon flux density). Ion leakage was measured with detached second leaves from three individual plants exposed to O$_3$ or SO$_2$ or fresh air as a control, as described previously (6, 45).

Inhibition of Root Elongation by Methyl Viologen. Transgenic seeds were sown on solid MS medium [containing 1.2% (wt/vol) agar, 0.5% sucrose, and 0.5 g/L Mes, pH 5.7 by KOH] containing various concentrations of methyl viologen hydrate (paraquat; Nakalai tesque). The root length of 14-d-old seedlings was measured.

Analysis of Stomatal Responses. The transpiration rate of GLK transgenic plants grown on solid MS medium (containing 0.8% agar, 0.5% sucrose, and 0.5 g/L Mes, pH 5.7 by KOH) in 150-mm Petri dishes was evaluated by thermoimaging using a thermal video system (TVS-8500; Nippon Avionics). Analysis of water loss rate was performed using 2-wk-old seedlings detached

from MS medium by measuring the fresh weight at the indicated periods of time in a plant growth room at 23 °C under 40% relative humidity. For analyses of stomatal aperture, second rosette leaves of 2-wk-old seedlings grown on solid MS medium (containing 0.8% agar, 0.5% sucrose, and 0.5 g/L Mes, pH 5.7 by KOH) in 150-mm dish were used. The leaf epidermis samples, isolated by blender, were incubated in buffer (5 mM Mes/bis-Tris propane, 50 mM KCl, and 0.1 mM CaCl$_2$, pH 6.5) (46–48) with/without ABA for 2.5 h at 23 °C under white light conditions. All measurements were conducted between 3 and 5 h after turning on the light in the growth chamber.

Isolation of RNA and Analysis of RNA Expression. Total RNA was isolated from 2-wk-old seedlings grown on MS medium [containing 0.8% agar, 3% sucrose, 1 mL/L Gamborg's vitamin solution (Sigma) and 0.5 g/L Mes, pH 5.7 by KOH], using an RNeasy Plant Mini kit (Qiagen). Epidermis-enriched cells were isolated from blender-treated leaf samples. For qRT-PCR analyses, 0.5 μg of total RNA was subjected to first-strand cDNA synthesis using the PrimeScript RT Master Mix (Takara Bio). qRT-PCR was performed by the SYBR green method using the GoTaq qPCR Master Mix (Promega) and ABI 7500 real-time PCR system (Applied Biosystems), with the appropriate primers (Table S3). Relative amounts of transcripts were calculated by an absolute quantification method using the *PP2AA3* gene as an internal control.

Immunohistochemical Staining of the Guard Cell H$^+$-ATPase. Detection of the phosphorylated H$^+$-ATPase and total H$^+$-ATPase in guard cells was performed according to the immunohistochemical method (32), with some modifications. Epidermal fragments from rosette leaves were fixed with 4% (wt/vol) paraformaldehyde for 2 h at room temperature and then digested with 2% (wt/vol) Driselase (Sigma) and 0.5% (wt/vol) Macerozyme R-10 (Yakult) for 45 min at 37 °C. The tissues were permeabilized with 3% (wt/vol) Triton X-100 for 30 min at room temperature. After the blocking with 3% BSA (Gibco), the samples were treated with anti-phosphorylated penultimate Thr of the H$^+$-ATPase (anti-pThr) or anti-catalytic domain of the H$^+$-ATPase at a dilution of 1:500 as a primary antibody. The stained signals were visualized using Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen) as a secondary antibody. The signal intensities were quantified and expressed according to the previous method (32).

Patch-Clamp Analysis. Guard cell protoplasts were prepared from 4- to 6-wk-old *Arabidopsis* plants, and the patch-clamp experiments were carried out as described previously (21). The pipette solution contained 30 mM KCl, 70 mM K-Glu, 2 mM MgCl$_2$, 6.7 mM EGTA, 3.35 mM CaCl$_2$, 5 mM ATP, and 10 mM Hepes·Tris (pH 7.1). The bath solution contained 30 mM KCl, 40 mM CaCl$_2$, 2 mM MgCl$_2$, and 10 mM Mes·Tris (pH 5.5). Osmolality was adjusted to 500 mmol/kg (pipette solution) and 485 mmol/kg (bath solution) with D-sorbitol. The voltage protocol was stepped from a holding potential of −40 mV to pulse potentials from 0 to −180 mV in 20-mV decrements. Leak currents were not subtracted.

Transient Effector–Reporter Analysis. For transient expression analysis using rosette leaves, 0.8 μg of reporter plasmid, 0.6 μg of effector plasmid, and 0.4 μg of reference plasmids (Renilla *LUC* gene) were transiently introduced into rosette leaves of 3- to 4-wk-old plants grown on soil with a photoperiod of 12 h/12 h light/dark by particle bombardment. For transient expression analysis using protoplasts, 2.0 μg of reporter plasmid, 3.0 μg of effector plasmid, and 0.4 μg of reference plasmids (Renilla *LUC* gene) were transiently introduced into protoplasts isolated from 4-wk-old plants by the PEG method (49). Relative luciferase activity was quantified and normalized as described previously (50).

Microarray Analysis. The microarray experiments were performed using the Agilent *Arabidopsis* 3 (44,000) microarray (Agilent Technologies) according to the manufacturer's instructions. Total RNA was isolated from 2-wk-old seedlings grown on MS medium (containing 0.8% agar, 0.5% sucrose, and 0.5 g/L Mes, pH 5.7 by KOH) using an RNeasy Plant Mini kit (Qiagen) and used for microarray experiments. Four biological replicates were tested with a one-color method. Spot signal values were calculated with Feature Extraction version 9.1 software (Agilent). The quality control (QC) value was defined as 1 when a spot passed the "FeatNonUnifOL" filter and as 2 when the spot further passed the "FeatPopnOL" filter. The detection value was defined as 1 when a spot passed the "IsPosAndSignif" filter and as 2 when the spot further passed the "IsWellAboveBG" filter. All signal values were divided by the median value among spots with a QC of 2 to enable comparison with other microarray data. Spot-to-gene conversion was accomplished based on a table provided by The *Arabidopsis* Information Resource (TAIR) (ftp://ftp.arabidopsis.org/home/tair/ Microarrays/Agilent/agilent_array_elements-2010-12-20.txt). The average values were used for the genes corresponding to two or more probes. All data were deposited in the National Center for Biotechnology Information Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo) under accession no. GSE42545.

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