Autophagy controls reactive oxygen species homeostasis in guard cells that is essential for stomatal opening

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Reactive oxygen species (ROS) function as key signaling molecules to inhibit stomatal opening and promote stomatal closure in response to diverse environmental stresses. However, how guard cells maintain basal intracellular ROS levels is not yet known. This study aimed to determine the role of autophagy in the maintenance of basal ROS levels in guard cells. We isolated the *Arabidopsis autophagy-related 2* (*atg2*) mutant, which is impaired in stomatal opening in response to light and low CO_2 concentrations. Disruption of other autophagy genes, including *ATG5*, *ATG7*, *ATG10*, and *ATG12*, also caused similar stomatal defects. The *atg* mutants constitutively accumulated high levels of ROS in guard cells, and antioxidants such as ascorbate and glutathione rescued ROS accumulation and stomatal opening. Furthermore, the *atg* mutations increased the number and aggregation of peroxisomes in guard cells, and these peroxisomes exhibited reduced activity of the ROS scavenger catalase and elevated hydrogen peroxide (H_2O_2) as visualized using the peroxisome-targeted H_2O_2 sensor HyPer. Moreover, such ROS accumulation decreased by the application of 2-hydroxy-3-butynoate, an inhibitor of peroxisomal H_2O_2-producing glycolate oxidase. Our results showed that autophagy controls guard cell ROS homeostasis by eliminating oxidized peroxisomes, thereby allowing stomatal opening.

Arabidopsis | autophagy | peroxisome | ROS | stomata

Guard cells surround adjustable stomatal pores on the leaf epidermis and control gas exchange between plants and the atmosphere, allowing CO_2 influx for photosynthetic carbon fixation and nutrient uptake by roots via the transpirational stream. Because plants are sessile, guard cells sense and integrate multiple endogenous and environmental signals such as light, water status, hormones, and CO_2 concentrations, thereby optimizing stomatal aperture to promote plant growth and survival under environmental conditions (1–5). Reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), act as important second messengers in abscisic acid (ABA) signaling and cause a reduction in stomatal aperture (6, 7). In order to be effective as a second messenger and avoid toxicity, the basal intracellular ROS concentrations, which might increase rapidly in response to stress, need to be maintained at low levels.

Numerous studies have shown that various environmental conditions and phytohormones also trigger ROS production in guard cells (6). Elevated ROS cause an increase in guard cell intracellular Ca^{2+} concentration by activating voltage-dependent Ca^{2+}-permeable channels, which leads to the activation of slow-sustained (S-type) anion channels in the plasma membrane (8, 9). Anion efflux from guard cells results in membrane depolarization that leads to K^+ efflux through outward-rectifying K^+ channels, which in turn causes water efflux and stomatal closure. A genetic study in *Arabidopsis* identified a plasma membrane leucine-rich repeat receptor-like pseudokinase GUARD CELL HYDROGEN PEROXIDE-RESISTANT1 that is essential for H_2O_2-mediated activation of Ca^{2+} channels and S-type anion channels (9, 10).

ROS have also been implicated in the inhibition of blue light-dependent stomatal opening, an effect essential for promoting stomatal closure under stress conditions during the day (11). Blue light perception by phototropins, light-activated receptor kinases (12), initiates signaling cascades by phosphorylating a Ser/Thr protein kinase BLUE LIGHT SIGNALING 1 (BLUS1) (13). The activated BLUS1 ultimately activates the plasma membrane H^+-ATPase via phosphorylation (14). Consequently, H^+ extrusion from guard cells hyperpolarizes the plasma membrane that facilitates K^+ uptake through inward-rectifying K^+ channels, eventually leading to water uptake and stomatal opening. H_2O_2 has been shown to inhibit blue light-dependent H^+ pumping by suppressing the phosphorylation of H^+-ATPase, which prevents membrane hyperpolarization and sustains the depolarized state (11). The H_2O_2-induced Ca^{2+} also inhibits H^+-ATPase (15). Moreover, H_2O_2

Significance

Reactive oxygen species (ROS) are key signaling molecules that play an important role in the regulation of stomatal movements in response to stress conditions. However, how basal cellular ROS levels are regulated in stomatal guard cells is not yet known. Our results revealed that autophagy maintains ROS homeostasis by eliminating oxidized peroxisomes, which allows the optimization of stomatal opening for photosynthetic CO_2 fixation and plant growth. This study provides insights on regulatory mechanisms of ROS homeostasis in guard cells and the physiological significance of plant peroxisome-specific autophagy, that is, pexophagy.

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inhibits inward-rectifying K^+ channels (16). Therefore, ROS appear to play multifunctional roles in stomatal regulations.

Cellular ROS levels are regulated by the balance between the rate of ROS production and degradation. Two plasma membrane NADPH oxidases, AtrbohD and AtrbohF, have been implicated as a major source of ROS production in *Arabidopsis* guard cells (6, 7). A recent study on H_2O_2 influx into guard cells suggested the involvement of plasma membrane aquaporin PIP2; 1 in H_2O_2 transport (17). Furthermore, ROS are generated via normal energy and metabolic processes in chloroplasts and peroxisomes (18, 19). The increased ROS in guard cells are removed by antioxidant enzymes such as ascorbate peroxidase (APX1) and catalase (CAT3) (20, 21). Although some mechanistic details of ROS production and signaling by various physiological signals have been elucidated, little is known about how basal ROS homeostasis is maintained in guard cells.

In this study, we isolated and characterized the *Arabidopsis autophagy-related* (*atg*) mutants that are impaired in light and low CO_2-induced stomatal opening. We showed that autophagy is essential for maintaining basal ROS levels by eliminating oxidized peroxisomes in guard cells, which allows stomatal opening.

Results

Isolation of an *Arabidopsis atg2-5* Mutant Defective in Stomatal Opening Responses. Leaf temperature provides a sensitive assay to monitor stomatal opening via transpirational water loss. We developed a system that detects blue light-dependent stomatal opening by thermal imaging (13). By screening *Arabidopsis* T-DNA insertion and ethyl methanesulfonate-mutagenized lines, we isolated a recessive mutant showing defects in leaf temperature decrease in response to blue light (Fig. 1*A*). We named this mutant *atg2-5*, since it is allelic to the *atg2* mutants described later. The stomatal conductance in intact leaves of wild type increased in response to red light, and superimposition of weak blue light elicited additional stomatal opening (Fig. 1*B*). In contrast, both red light- and blue light-dependent stomatal opening were reduced in the *atg2-5* mutant (Fig. 1*B*). Measurements of stomatal aperture in epidermis revealed that *atg2-5* showed reduced stomatal opening in response to blue light and fusicoccin (Fc), an activator of the H^+-ATPase (Fig. 1*C*). Furthermore, the *atg2-5* mutation impaired low CO_2-induced stomatal opening (*SI Appendix*, Fig. S1).

To determine whether *atg2-5* impairs blue light signaling that leads to the activation of H^+-ATPase, we measured H^+ pumping from guard cell protoplasts. In addition to stomatal opening, H^+ pumping by blue light was attenuated in the *atg2-5* mutant compared with that in wild-type control (Fig. 1 *D* and *E*). Similarly, blue light-dependent phosphorylation of H^+-ATPase was reduced in the *atg2-5* mutant (*SI Appendix*, Fig. S2 *A* and *B*). We found that the amount of H^+-ATPase was slightly decreased in *atg2-5* (*SI Appendix*, Fig. S2 *A* and *C*). However, such a decrease could not completely account for the reduced stomatal opening since the disruption of *Arabidopsis AHA1*, a major H^+-ATPase isoform expressed in guard cells, remarkably reduces the total amount of H^+-ATPase, but exhibits a lesser degree of inhibition in stomatal opening and H^+ pumping (22). Taken together, these results suggest that *atg2-5* mutation blocks multiple stomatal opening systems.

Identification of the *ATG2* Gene. To identify the gene responsible for the *atg2-5* mutant, we initially attempted to determine the T-DNA insertion site, since *atg2-5* was isolated from the pool of T-DNA lines. However, such flanking sequence was not amplified by thermal asymmetric interlaced PCR (TAIL-PCR). Therefore, we used map-based cloning and whole-genome sequencing to identify the causal mutation in the *atg2-5* mutant. The *ATG2* locus was mapped to a 231 kbp region on the upper arm of chromosome 3 (Fig. 2*A*), and this region contains 70 annotated genes according to The *Arabidopsis* Information Resource. To narrow down candidate genes, we applied MutMap (23) to F2 progeny that showed

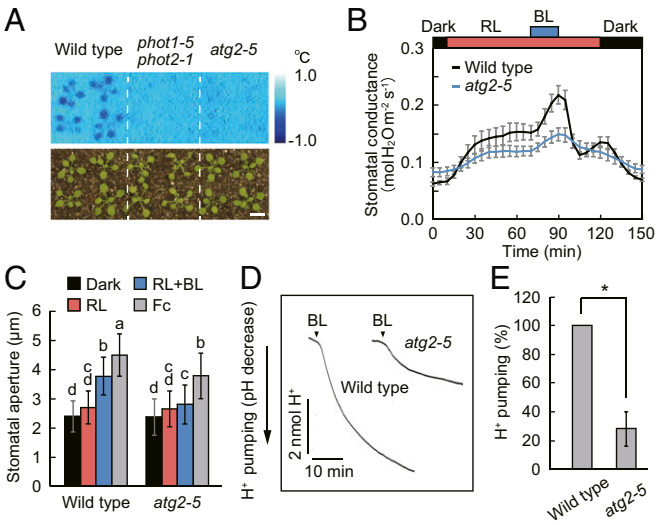

Fig. 1. Impairment of light-dependent stomatal responses in the *atg2-5* mutant. (*A*) Thermal image of a blue light-dependent leaf temperature decrease. Dark-adapted plants were illuminated with red light (RL: 80 μmol m^{-2} s^{-1}) for 50 min, and then blue light (BL: 5 μmol m^{-2} s^{-1}) was superimposed on RL. Subtractive images were obtained by subtracting an initial thermal image (before BL illumination) from an image taken 15 min after BL illumination. (Scale bar: 1 cm.) (*B*) Light-dependent changes in stomatal conductance in intact leaves. Leaves of dark-adapted plants were illuminated with RL (300 μmol m^{-2} s^{-1}) for 1 h, and then BL (10 μmol m^{-2} s^{-1}) was superimposed as indicated. Data represent means ± SEM (n = 5). (*C*) Light- and fusicoccin-dependent stomatal opening in the epidermis. Epidermal strips were incubated in 5 mM MES-bistrispropane (pH 6.5), 50 mM KCl, and 0.1 mM $CaCl_2$ in the dark or under RL (50 μmol m^{-2} s^{-1}) with or without BL (10 μmol m^{-2} s^{-1}) for 2 h. Fusicoccin (Fc) at 1 μM was added to the epidermis and incubated in the dark for 2 h. Data represent means ± SD (n = 75, pooled from triplicate experiments). Different letters indicate significant difference (ANOVA with Tukey's test, $P < 0.01$). (*D*) Blue light-dependent H^+ pumping. Guard cell protoplasts were illuminated with RL (50 μmol m^{-2} s^{-1}) for 2 h, and then BL (10 μmol m^{-2} s^{-1}) was superimposed on RL as indicated. (*E*) Quantification of the magnitude of H^+ pumping. Data represent means ± SEM (n = 4). Asterisk indicates significant difference from the wild type ($P < 0.01$; Student's t test).

the mutant phenotype. The highest SNP-index peak was observed on chromosome 3, consistent with the results of map-based cloning. Accordingly, sequence analysis of this region revealed a 1 bp insertion of G in the fifth exon of At3g19190, which encodes *AUTOPHAGY-RELATED2* (*ATG2*), and this insertion resulted in a frameshift and premature stop codon (Fig. 2*B*). We examined stomatal opening in loss-of-function allele of *peup1-1,* which harbor a single point mutation within *ATG2* (Fig. 2*B*) (24). The *peup1-1* mutant was defective in light-dependent stomatal opening (Fig. 2*C* and *SI Appendix*, Fig. S3 *A* and *B*). The F1 plants from the cross between *atg2-5* and wild type showed the same stomatal opening as in wild type, whereas those from the cross between *atg2-5* and *peup1-1* showed traits of both parents (Fig. 2*C* and *SI Appendix*, Fig. S3 *A* and *B*). The *atg2-5* stomatal phenotypes were complemented by expressing *GFP-ATG2* from its own promoter (Fig. 2*D* and *SI Appendix*, Fig. S3 *C* and *D*). We concluded that the mutation in *ATG2* is the basis for the *atg2-5* phenotype.

The Autophagy-Defective Mutants Exhibit Impairments in Stomatal Opening. Autophagy is a eukaryotic process that degrades cytoplasmic constituents and organelles and is strictly controlled by a set of highly conserved autophagy-related proteins, including ATG2. In yeast, ATG2 forms a complex with the phosphatidylinositol 3-phosphate (PI3P)-binding protein ATG18 at the preautophagosomal structure (PAS) to promote autophagosome formation (25, 26). In *Arabidopsis*, ATG2 has also been shown to

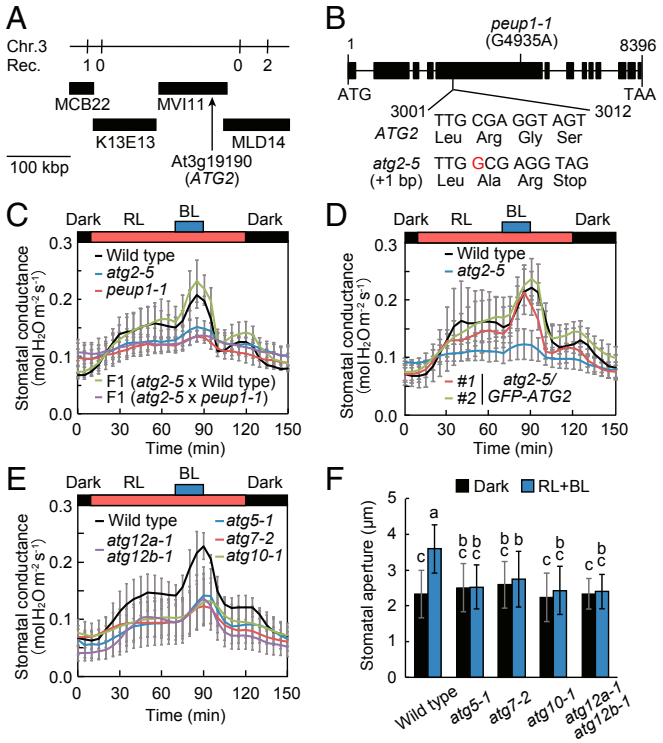

Fig. 2. Identification of *ATG2* gene and impairment of stomatal opening in other autophagy-related mutants. (*A*) Map-based cloning of the *ATG2* gene. *ATG2* was mapped to the 231 kbp region between BAC clones MCB22 and MLD14. Numbers indicate the recombination from a total of 154 chromosomes. (*B*) Genomic structure and mutation sites of *ATG2* gene. Black rectangles indicate the protein-coding region. (*C*) Light-dependent stomatal opening in F1 progenies obtained from crosses between the *atg2-5* and *peup1-1* mutants. (*D*) Rescue of light-dependent stomatal opening by transformation of *atg2-5* mutant with *GFP-ATG2*. (*E* and *F*) Impairment of light-dependent stomatal opening in intact leaves (*E*) and the epidermis (*F*) in other autophagy-related mutants. For *C*–*E*, stomatal conductance was measured as described in Fig. 1*B*. Data represent means ± SEM ($n = 3$). For *F*, epidermal strips were incubated as described in Fig. 1*C*. Data represent means ± SD ($n = 75$, pooled from triplicate experiments). Different letters indicate significant difference (ANOVA with Tukey's test, $P < 0.01$).

be essential for autophagosome formation (27). To determine whether the defective stomatal phenotype of *atg2* mutants is caused by the disruption of general autophagy or the ATG2 specific function, we examined light-dependent stomatal opening in *Arabidopsis* mutants lacking other autophagy-essential genes such as *ATG5*, *ATG7*, *ATG10*, and *ATG12*. ATG12 is a small ubiquitin-like protein that is specifically conjugated to ATG5 through the sequential actions of ubiquitin E1- and E2-like proteins ATG7 and ATG10, respectively. The ATG12–ATG5 conjugate further interacts with ATG16, and the resulting complex facilitates phagophore expansion and maturation (25, 26). Similar to that in *atg2* mutants, the stomatal conductance of *atg5-1*, *atg7-2*, *atg10-1*, and *atg12a-1 atg12b-1* was less responsive to both red light and blue light (Fig. 2*E*). Furthermore, the stomatal opening in epidermis was decreased in these mutants (Fig. 2*F*). Thus, the defect of autophagic process appeared to be linked to the inhibition of stomatal opening.

Autophagy-Defective Mutants Accumulate High Levels of ROS in Guard Cells, and Antioxidants Restore Stomatal Opening. *Arabidopsis* autophagy-defective mutants show high levels of ROS in leaves (28). Considering that ROS play a central role in stomatal regulations (6, 7), we suspected that increased intracellular ROS might be responsible for the defective stomatal phenotype in *atg* mutants. To explore this possibility, we determined ROS levels in guard cells by using H_2DCFDA, an oxidation-sensitive fluorescence probe. In wild type, intracellular ROS levels increased slightly after exposure to light and largely in response to 10 μM ABA (Fig. 3 *A* and *B*). In contrast, the *atg2-5* mutant exhibited elevated ROS levels irrespective of ABA treatment (Fig. 3 *A* and *B*). Furthermore, similar elevated ROS levels were observed in other *atg* mutants (*SI Appendix*, Fig. S4 *A* and *B*).

To determine whether the observed constitutive ROS accumulation in *atg* mutants correlates with the inhibition of stomatal opening, we investigated the effect of antioxidants on light-dependent stomatal opening. Ascorbic acid (ASC) acts as an

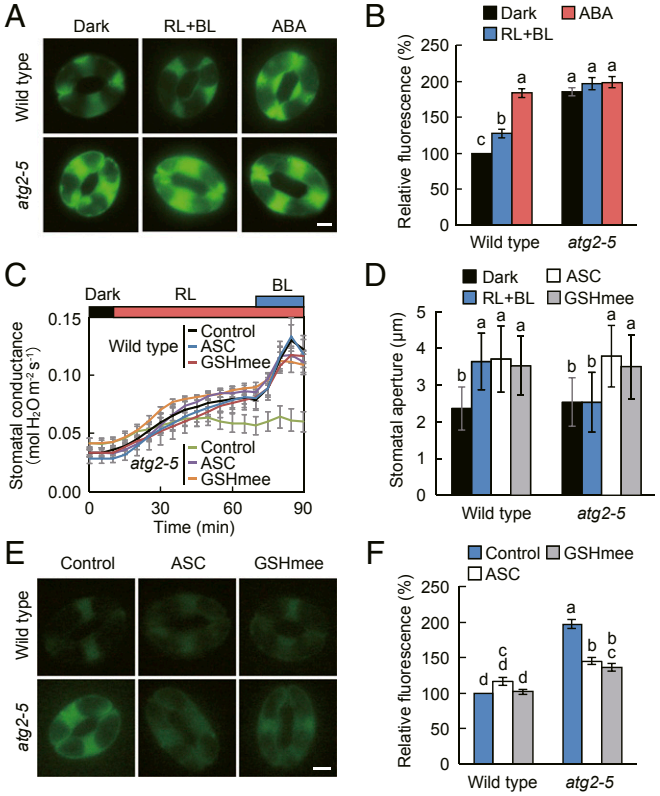

Fig. 3. ROS accumulation in *atg2* guard cells and restoration of stomatal opening and ROS levels by antioxidants. (*A* and *B*) ROS accumulation in *atg2* mutant guard cells. Images of ROS accumulation in guard cells indicated by the fluorescence dye H_2DCFDA (*A*) and quantification of the relative ROS levels analyzed using ImageJ software (*B*). Epidermal strips were incubated in the dark or under red (RL: 50 μmol m^{-2} s^{-1}) and blue light (BL: 10 μmol m^{-2} s^{-1}) for 1.5 h, and then loaded with H_2DCFDA for 30 min. After excess dye was removed by washing, abscisic acid (ABA) at 10 μM was added, and reactions were allowed to proceed under RL and BL for an additional 30 min. (Scale bar: 5 μm.) Data represent means ± SEM ($n = 90$, pooled from triplicate experiments). (*C* and *D*) Restoration of light-dependent stomatal opening in intact leaves (*C*) and epidermis (*D*) of *atg2-5* mutant by ascorbic acid (ASC) and glutathione methyl ester (GSHmee). For *C*, leaves of dark-adapted plants were illuminated with RL and BL as described in Fig. 1*B*. During dark adaptation, 10 mM ASC or 10 μM GSHmee was applied to the soil, and plants were incubated for at least 9 h before the measurements. Data represent means ± SEM ($n = 6$). For *D*, epidermal strips were incubated as described in Fig. 1*C* in the presence of 10 mM ASC or 10 μM GSHmee. Data represent means ± SD ($n = 75$, pooled from triplicate experiments). (*E* and *F*) Scavenging of ROS by ASC and GSHmee in *atg2-5* mutant guard cells. Epidermal strips were prepared from dark-adapted plants as described in Fig. 3*C*, and the ROS levels were determined using H_2DCFDA. (Scale bar: 5 μm.) Data represent means ± SEM ($n = 90$, pooled from triplicate experiments). For (*B*, *D*, and *F*) lowercase letters a, b, c, and d indicate significant difference (ANOVA with Tukey's test, $P < 0.01$).

electron donor for the reduction of H_2O_2 into H_2O, and reduced glutathione (GSH) participates in the regeneration of ASC (19). Application of ASC restored stomatal opening in the *atg2-5* mutant, but had no effects on stomatal opening in the wild type (Fig. 3 *C* and *D*). A similar result was observed with the addition of glutathione monoethyl ester (GSHmee), a membrane-permeable form of GSH (Fig. 3 *C* and *D*) (29). Furthermore, these antioxidants recovered stomatal opening in other *atg* mutants (*SI Appendix*, Fig. S4*C*). Along with stomatal opening, we confirmed that the antioxidants scavenged intracellular ROS in *atg2-5* guard cells (Fig. 3 *E* and *F* and *SI Appendix*, Fig. S4 *D* and *E*). Conversely, the double mutant of ASC- and GSH-deficient *vtc1-1* and *cad2-1*, respectively, displayed ROS accumulation and reduced stomatal opening particularly under high light condition (*SI Appendix*, Fig. S5). Taken together, these data suggest that autophagy has a major function in regulating basal ROS levels and stomatal opening.

Autophagy-Defective Mutants Harbor Aggregated Peroxisomes with High Levels of H_2O_2 in Guard Cells. If autophagy plays a role in maintaining intracellular ROS in guard cells, it might control the degradation of oxidized or damaged organelles. In plant cells, peroxisomes and chloroplasts are the major sites of ROS production (18, 19, 30). Therefore, we rationalized that autophagy could impact ROS homeostasis by eliminating dysfunctional peroxisomes or chloroplasts, known as pexophagy and chlorophagy, respectively (31). To address this, we crossed *atg* mutants with transgenic plants expressing GFP fused with the peroxisome targeting signal 1 (GFP-PTS1), a peroxisomal marker (32), and analyzed peroxisome number in guard cells. A significant increase in peroxisome number was observed in *atg2-5* as well as other *atg* mutants compared with that in wild type (Fig. 4 *A* and *B* and *SI Appendix*, Fig. S6 *A* and *B*). In contrast, such an increase was not noted in chloroplasts, as visualized by chlorophyll fluorescence images (*SI Appendix*, Fig. S6 *C* and *D*). In accordance with these results, the *atg2-5* guard cells exhibited excess accumulation of peroxisomal proteins such as CAT, APX, and glycolate oxidase (GOX) but not chloroplastic ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (RBCL) and mitochondrial chaperonin 10 (Cpn10) (Fig. 4 *C* and *D*).

Notably, the *atg* mutants exhibited aggregation of peroxisomes in guard cells (Fig. 4 *A* and *B* and *SI Appendix*, Fig. S6 *A* and *B*). The aggregated peroxisomes accumulate enzymatically inactive forms of CAT, a main antioxidative enzyme in peroxisomes (24, 33). Disruptions of *CAT3*, the predominant isoform expressed in *Arabidopsis* guard cells (21), and *NO CATALASE ACTIVITY1*

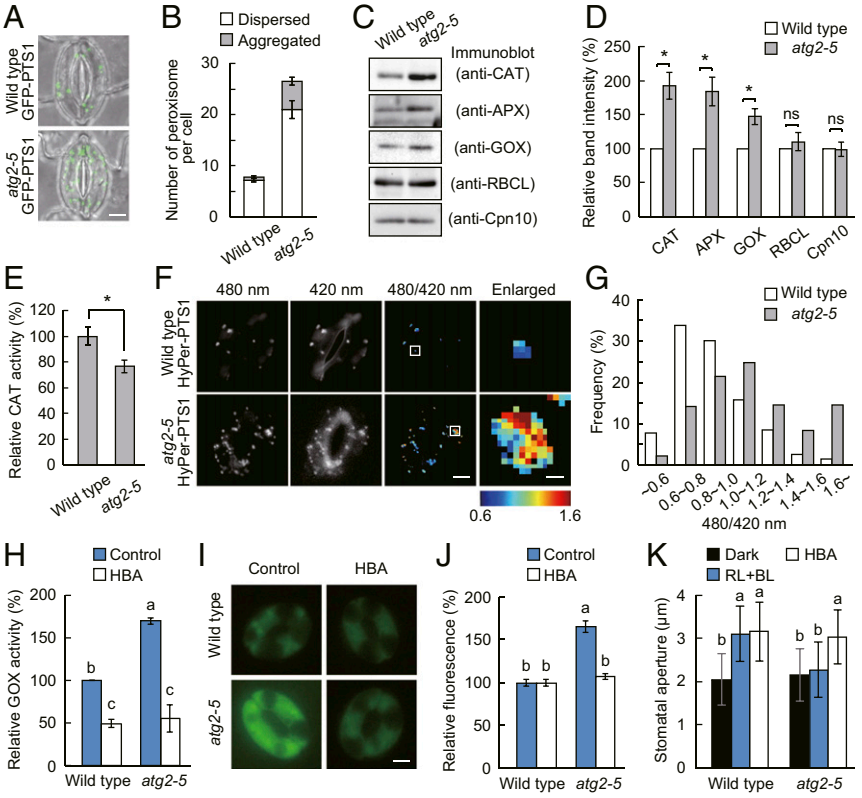

Fig. 4. Autophagy deficiency causes an increase of peroxisome number and H_2O_2 accumulation in guard cells. (*A*) Images of GFP-PTS1 fluorescence and differential interference contrast (DIC) in guard cells. (Scale bar: 5 μm.) (*B*) Quantification of peroxisome number per guard cell. Data represent means ± SEM (n = 16). (*C*) Levels of peroxisomal (CAT, APX, and GOX), chloroplastic (RBCL), and mitochondrial proteins (Cpn10) in the *atg2-5* mutant guard cells. (*D*) Quantification of the protein levels using ImageJ software. Data represent means ± SEM (n = 4). (*E*) Relative CAT activity in the crude extracts from guard cell protoplasts. The values of the total CAT activity were divided by the band intensity values of corresponding CAT. Data represent means ± SEM (n = 4). (*F*) Images of HyPer-PTS1 fluorescence excited with 420 nm and 480 nm light, and the corresponding fluorescence ratio (480/420) in guard cells. (Scale bar: 5 μm.) The boxed areas in the 480/420 nm images are enlarged in the *Right*. (Scale bar: 0.5 μm.) (*G*) Distribution of the fluorescence ratio (480/420 nm) in peroxisomes of wild type (n = 272) and *atg2-5* (n = 275). (*H*) Relative GOX activity in the crude extracts from epidermal strips. Epidermal strips were preincubated in the dark for at least 9 h in the presence or absence of 10 μM 2-hydroxy-3-butynoate (HBA), and then illuminated with red light (RL: 50 μmol m^{-2} s^{-1}) and blue light (BL: 10 μmol m^{-2} s^{-1}) for 2 h. Data represent means ± SEM (n = 3). (*I* and *J*) Restorations of ROS accumulation in *atg2-5* guard cells by HBA. Epidermal strips were preincubated as described in *H*, and the ROS levels were determined using H_2DCFDA. (Scale bar: 5 μm.) Data represent means ± SEM (n = 90, pooled from triplicate experiments). (*K*) Restoration of stomatal opening in *atg2-5* mutant by HBA. Data represent means ± SD (n = 75, pooled from triplicate experiments). For *D* and *E*, asterisks indicate significant difference from the wild type ($P < 0.01$; Student's t test). For *H*, *J*, and *K*, different letters indicate significant difference (ANOVA with Tukey's test, $P < 0.01$).

(*NCA1*), a molecular chaperone required for CAT activity (34, 35), resulted in ROS accumulation and inhibition of stomatal opening (*SI Appendix*, Fig. S7). Thus, such peroxisome aggregates could involve ROS production. Our attempts to isolate insoluble fraction from guard cell protoplasts and measure its CAT activity were unsuccessful. Alternatively, we calculated the relative CAT activity by dividing the total CAT activity measured in the crude extracts from guard cell protoplasts by the band intensity of CAT protein in immunoblot analysis (Fig. 4 *C* and *D*). The relative CAT activity decreased in *atg2-5* mutant guard cells in comparison to wild type (Fig. 4*E*). Consequently, we determined the basal H_2O_2 levels in peroxisomes by using genetically encoded H_2O_2 sensor HyPer fused with PTS1 (HyPer-PTS1) (36). After oxidation with H_2O_2, the excitation peak of HyPer shifts from 420 to 500 nm. Therefore, the ratiometric fluorescent signal provides a sensitive measurement of cellular H_2O_2. The HyPer-PTS1 signal was apparent in guard cells of both wild-type and *atg2-5* mutant (Fig. 4*F*). Unlike in wild type, the 480/420 nm ratio was higher in *atg2-5* mutant, indicating that H_2O_2 is accumulated in the peroxisomes of *atg2-5* mutant guard cells (Fig. 4 *F* and *G*). These findings indicate that autophagy might control ROS levels by selectively eliminating oxidized peroxisomes in guard cells.

To gain further insight into the metabolic source of ROS in *atg* mutants, we investigated the involvement of GOX, which catalyzes the oxidation of glycolate to glyoxylate and concomitantly produces H_2O_2. The *Arabidopsis* genome contains 3 genes encoding GOX, and a public database indicated that *GOX1* and *GOX2* are preferentially expressed in the guard cells (*Arabidopsis* eFP Browser; ref. 37). However, simultaneous knock-down of these 2 genes causes severe growth defects (38). We, thus, utilized 2-hydroxy-3-butynoate (HBA), an irreversible inhibitor of GOX (39). We confirmed that HBA impaired GOX activity for recombinant GOX1 and GOX2 proteins (*SI Appendix*, Fig. S8). In concordance with GOX protein levels (Fig. 4 *C* and *D*), the *atg2-5* mutant showed higher GOX activity than that of the wild type, and application of HBA reduced GOX activity in both wild-type and *atg2-5* mutant (Fig. 4*H*). Consistently, HBA decreased ROS levels and recovered stomatal opening in the *atg2-5* mutant (Fig. 4 *I–K*). Together, these results suggest that peroxisomal GOX activity is responsible for the primary source of ROS accumulation in *atg* mutant guard cells.

Discussion

In the present study, we showed that autophagy controls ROS homeostasis in guard cells, which is crucial for stomatal opening. Using thermal imaging, we isolated the *Arabidopsis* autophagy-defective mutant and found that deletion of autophagy-essential genes results in the impairment of stomatal opening in response to various signals, including light, Fc, and low CO_2 (Fig. 1 *A–C* and *SI Appendix*, Fig. S1). We also found that intracellular ROS were constitutively increased in guard cells of these autophagy-defective mutants (Fig. 3 *A* and *B* and *SI Appendix*, Fig. S4). These findings are consistent with the enhanced ROS accumulation in the leaves of *Arabidopsis atg* mutants (28). Furthermore, exogenous application of antioxidants such as ascorbic acid and glutathione decreased the levels of ROS and recovered stomatal opening in the *atg* mutants (Fig. 3 *C–F* and *SI Appendix*, Fig. S4). From these results, we concluded that higher ROS accumulation is responsible for stomatal phenotypes in the *atg* mutants. Such ROS accumulation might cause the activation and inactivation of the stomatal closing and opening systems, respectively (8–11, 16). Supporting this, blue light-mediated activation of H^+-ATPase was inhibited in the *atg2-5* mutant (Fig. 1 *D* and *E*).

Peroxisomes are eukaryotic organelles that have diverse metabolic functions. In plant peroxisomes, ROS, particularly H_2O_2, are produced during metabolic processes, including photorespiration, fatty acid β-oxidation, and other oxidative reactions (30). We hypothesized that autophagy maintains the quality of peroxisomes by eliminating dysfunctional and oxidized peroxisomes, thereby preventing excess ROS accumulation in guard cells. In support of this hypothesis, the *atg* mutants showed an increased number and aggregation of peroxisomes in guard cells (Fig. 4 *A* and *B* and *SI Appendix*, Fig. S6 *A* and *B*). This aggregate contains enzymatically inactive CAT, implying a decrease in ROS detoxification in these peroxisomes (24, 33). Indeed, the *atg2-5* mutant peroxisomes exhibited high levels of H_2O_2 with reduced CAT activity (Fig. 4 *D–F*). The *atg* mutants also display accumulation of oxidative peroxisomes in leaves but not in roots (24, 33). These findings, combined with those of previous studies, indicate that pexophagy plays crucial roles in ROS homeostasis in aerial plant tissues, including guard cells.

Our data suggest that peroxisomal GOX activity is the predominant metabolic source of ROS accumulation in the *atg* mutant guard cells (Fig. 4 *H–K*). However, it is currently unknown why excessive ROS was accumulated in *atg* guard cells even under dark and low light condition, in which photorespiration is not active (Fig. 3 *A* and *B*). We note that a GOX substrate glycolate is provided by conversion not only from the photorespiratory 2-phosphoglycolate (2-PG) metabolism with 2-PG phosphatase (PGLP) but also from glyoxylate with cytosolic and plastidial glyoxylate reductase (GLYR) (40). Glycolate is also produced by the oxidation of the 1,2-dihydroxyethyl-thiamin-diphosphate intermediate of transketolase (41). Conversely, ROS accumulation of *cat3-1* and *nca1-1* mutants under low light condition was limited compared to that of *atg* mutants (*SI Appendix*, Fig. S7). One possibility is that the highly oxidized peroxisomes are removed immediately as long as autophagy is functional. Further genetic analysis of *atg* mutants will help to dissect the nature of the ROS homeostasis in guard cells.

A recent investigation indicated that phototropins mediate the breakdown of triacylglycerols in guard cells, which leads to the production of ATP through peroxisomal β-oxidation and thus impacts light-dependent stomatal opening (42). We cannot exclude the possibility that intracellular ATP levels are diminished in *atg* mutant guard cells. However, *Arabidopsis atg* mutants retain functional β-oxidation activity (24, 43). Furthermore, mutation of the *Arabidopsis* KAT2/PED1/PKT3, a major isoform of 3-ketoacyl-CoA thiolase that catalyzes the last step of the β-oxidation, exhibited light-dependent stomatal opening comparable to that in wild type (*SI Appendix*, Fig. S9; ref. 44). Thus, β-oxidation is unlikely to be a major cause of the stomatal defects in *atg* mutants.

In addition to peroxisomes, photosynthetic electron transport chain in chloroplasts represents the major site of ROS generation in plant cells (18, 19). Recent studies have provided evidence for the selective elimination of chloroplasts via autophagy. In the leaves of wild-type *Arabidopsis*, the number of chloroplasts decreased following exposure to dark or UV-B, but such decrease was not found in *atg* mutants (45, 46). In contrast, under nonstress conditions, the number of chloroplasts and maximum quantum yield of photosystem II (*Fv*/*Fm*) were not significantly different between wild type and *atg* mutants (24, 46). In line with these findings, our data revealed no significant difference in chloroplast number in guard cells (*SI Appendix*, Fig. S6 *C* and *D*). We found 2 additional chloroplast degradation pathways that are not dependent on autophagy, which include the formation of senescence-associated vacuoles and CV-containing vesicles (47). Furthermore, recent studies have identified selective autophagy of the endoplasmic reticulum and mitochondria in plant cells (31). Further investigation will be required for elucidating the role of autophagy-mediated elimination of these organelles in ROS homeostasis in plant cells.

Plasma membrane NADPH oxidases mediate ROS production in guard cells in response to ABA (48). In addition, cell wall peroxidase is implicated in salicylic acid-induced ROS generation and stomatal closure (49). However, these pathways

are unlikely to account for ROS accumulation in *atg* mutants because diphenyleneiodonium chloride and salicylhydroxamic acid, inhibitors of NADPH oxidase and peroxidase, respectively (16, 49), had little effect on ROS levels in the *atg2-5* mutant (*SI Appendix*, Fig. S10).

In conclusion, this study identified autophagy-mediated ROS homeostasis mechanisms in guard cells. In general, autophagy has been recognized to play a role in nutrient recycling under starvation conditions. However, in sharp contrast, our findings showed that autophagy selectively degrades oxidized peroxisomes in guard cells under nutrient and nonstress conditions. Although selective elimination of organelles via autophagy is thought to maintain the quality control of organelles, the physiological significance of pexophagy in plants has remained unclear. Thus, we propose that plant pexophagy sustains stomatal opening and photosynthetic CO_2 fixation by regulating basal ROS in guard cells. Maintenance of ROS homeostasis by pexophagy might also allow rapid ROS increase and stomatal closure in response to diverse environmental changes; this acts as an adaptive mechanism under natural conditions. Further studies are warranted to elucidate the selective mechanisms underlying plant pexophagy.

Materials and Methods

Plant materials and growth conditions, measurements of stomatal opening, isolation of guard cell protoplasts, measurement of H^+ pumping, immunoblot analysis, identification of *ATG2* gene, construction of transgenic plants, detection of ROS, detection of H_2O_2 in peroxisomes, confocal microscopy, measurement of CAT activity, preparation of recombinant proteins, and measurement of GOX activity are described in *SI Appendix, SI Methods*.

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