



# 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<sub>2</sub> 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<sub>2</sub>O<sub>2</sub>) as visualized using the peroxisome-targeted H<sub>2</sub>O<sub>2</sub> sensor HyPer. Moreover, such ROS accumulation decreased by the application of 2-hydroxy-3-butyrate, an inhibitor of peroxisomal H<sub>2</sub>O<sub>2</sub>-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<sub>2</sub> 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<sub>2</sub> concentrations, thereby optimizing stomatal aperture to promote plant growth and survival under environmental conditions (1–5). Reactive oxygen species (ROS), including hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), 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<sup>2+</sup> concentration by activating voltage-dependent Ca<sup>2+</sup>-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<sup>+</sup> efflux through outward-rectifying K<sup>+</sup> 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<sub>2</sub>O<sub>2</sub>-mediated activation of Ca<sup>2+</sup> 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<sup>+</sup>-ATPase via phosphorylation (14). Consequently, H<sup>+</sup> extrusion from guard cells hyperpolarizes the plasma membrane that facilitates K<sup>+</sup> uptake through inward-rectifying K<sup>+</sup> channels, eventually leading to water uptake and stomatal opening. H<sub>2</sub>O<sub>2</sub> has been shown to inhibit blue light-dependent H<sup>+</sup> pumping by suppressing the phosphorylation of H<sup>+</sup>-ATPase, which prevents membrane hyperpolarization and sustains the depolarized state (11). The H<sub>2</sub>O<sub>2</sub>-induced Ca<sup>2+</sup> also inhibits H<sup>+</sup>-ATPase (15). Moreover, H<sub>2</sub>O<sub>2</sub>

## 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<sub>2</sub> 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.**

Author contributions: S.Y. and A.T. designed research; S.Y., S.M., K.O., K.H., and A.T. performed research; K.M.T. and Y.K. contributed new reagents/analytic tools; S.Y., S.M., K.O., K.M.T., M.N., K.-i.S., and A.T. analyzed data; and A.T. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1910886116/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1910886116/-DCSupplemental).

Published online September 4, 2019.

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.

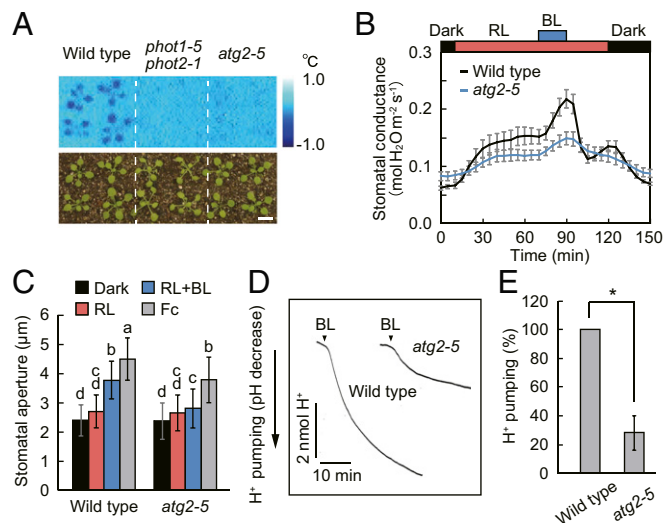
In this study, we isolated and characterized the *Arabidopsis* autophagy-related (*atg*) mutants that are impaired in light and low  $CO_2$ -induced stomatal opening. We showed that autophagy is essential for maintaining basal ROS levels by eliminating oxidized peroxisomes in guard cells, which allows 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. 1A). 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. 1B). In contrast, both red light- and blue light-dependent stomatal opening were reduced in the *atg2-5* mutant (Fig. 1B). 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. 1C). 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. 1D and E). Similarly, blue light-dependent phosphorylation of  $H^+$ -ATPase was reduced in the *atg2-5* mutant (SI Appendix, Fig. S2A and B). We found that the amount of  $H^+$ -ATPase was slightly decreased in *atg2-5* (SI Appendix, Fig. S2A 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.

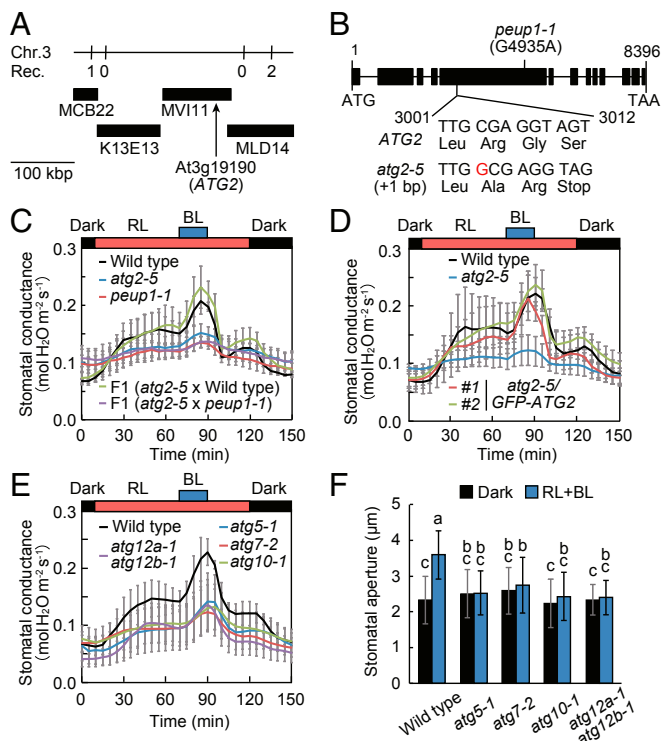
**Identification of the *ATG2* Gene.** To identify the gene responsible for the *atg2-5* mutant, we initially attempted to determine the T-DNA insertion site, since *atg2-5* was isolated from the pool of T-DNA lines. However, such flanking sequence was not amplified by thermal asymmetric interlaced PCR (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. 2A), 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:  $80 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) for 50 min, and then blue light (BL:  $5 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) 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 ( $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) for 1 h, and then BL ( $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) was superimposed as indicated. Data represent means  $\pm$  SEM ( $n = 5$ ). (C) Light- and fusicoccin-dependent stomatal opening in the epidermis. Epidermal strips were incubated in 5 mM MES-bis-trispropane (pH 6.5), 50 mM KCl, and 0.1 mM  $CaCl_2$  in the dark or under RL ( $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) with or without BL ( $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) for 2 h. Fusicoccin (Fc) at  $1 \mu\text{M}$  was added to the epidermis and incubated in the dark for 2 h. Data represent means  $\pm$  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 ( $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) for 2 h, and then BL ( $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) was superimposed on RL as indicated. (E) Quantification of the magnitude of  $H^+$  pumping. Data represent means  $\pm$  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. 2B). We examined stomatal opening in loss-of-function allele of *peup1-1*, which harbor a single point mutation within *ATG2* (Fig. 2B) (24). The *peup1-1* mutant was defective in light-dependent stomatal opening (Fig. 2C and SI Appendix, Fig. S3A 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. 2C and SI Appendix, Fig. S3A and B). The *atg2-5* stomatal phenotypes were complemented by expressing *GFP-ATG2* from its own promoter (Fig. 2D and SI Appendix, Fig. S3C 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



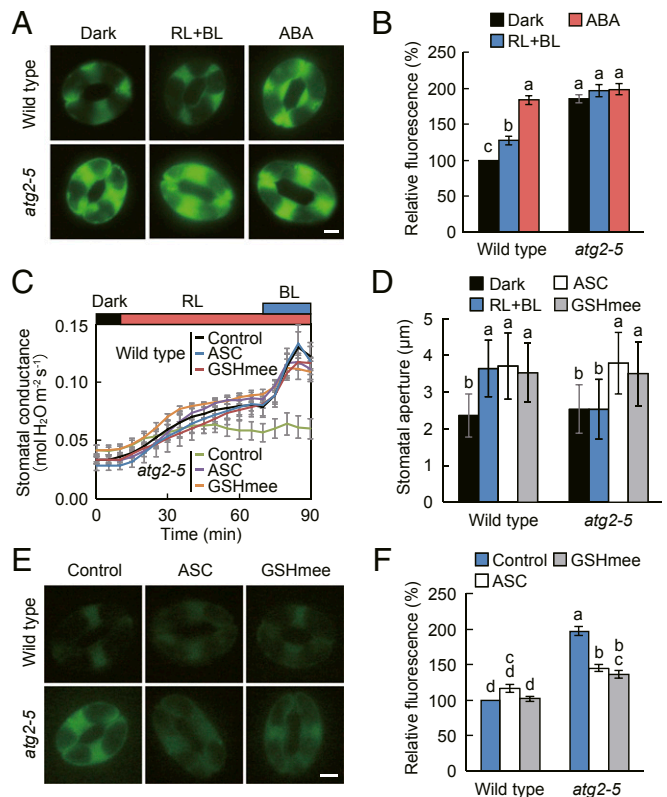
**Fig. 2.** Identification of *ATG2* gene and impairment of stomatal opening in other autophagy-related mutants. (A) Map-based cloning of the *ATG2* gene. *ATG2* was mapped to the 231 kbp region between BAC clones MCB22 and MLD14. Numbers indicate the recombination from a total of 154 chromosomes. (B) 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. 1B. Data represent means  $\pm$  SEM ( $n = 3$ ). For F, epidermal strips were incubated as described in Fig. 1C. Data represent means  $\pm$  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. 2E). Furthermore, the stomatal opening in epidermis was decreased in these mutants (Fig. 2F). 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 *atg2* 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  $\mu M$  ABA (Fig. 3A and B). In contrast, the *atg2-5* mutant exhibited elevated ROS levels irrespective of ABA treatment (Fig. 3A and B). Furthermore, similar elevated ROS levels were observed in other *atg* mutants (*SI Appendix*, Fig. S4A and B).

To determine whether the observed constitutive ROS accumulation in *atg2* 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  $\mu mol m^{-2} s^{-1}$ ) and blue light (BL; 10  $\mu mol m^{-2} s^{-1}$ ) 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  $\mu M$  was added, and reactions were allowed to proceed under RL and BL for an additional 30 min. (Scale bar: 5  $\mu m$ .) Data represent means  $\pm$  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. 1B. During dark adaptation, 10 mM ASC or 10  $\mu M$  GSHmee was applied to the soil, and plants were incubated for at least 9 h before the measurements. Data represent means  $\pm$  SEM ( $n = 6$ ). For D, epidermal strips were incubated as described in Fig. 1C in the presence of 10 mM ASC or 10  $\mu M$  GSHmee. Data represent means  $\pm$  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. 3C, and the ROS levels were determined using  $H_2DCFDA$ . (Scale bar: 5  $\mu m$ .) Data represent means  $\pm$  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,  $P < 0.01$ ).



(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. 4E). Consequently, we determined the basal H<sub>2</sub>O<sub>2</sub> levels in peroxisomes by using genetically encoded H<sub>2</sub>O<sub>2</sub> sensor HyPer fused with PTS1 (HyPer-PTS1) (36). After oxidation with H<sub>2</sub>O<sub>2</sub>, the excitation peak of HyPer shifts from 420 to 500 nm. Therefore, the ratiometric fluorescent signal provides a sensitive measurement of cellular H<sub>2</sub>O<sub>2</sub>. The HyPer-PTS1 signal was apparent in guard cells of both wild-type and *atg2-5* mutant (Fig. 4F). Unlike in wild type, the 480/420 nm ratio was higher in *atg2-5* mutant, indicating that H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub>. 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-butyrate (HBA), an irreversible inhibitor of GOX (39). We confirmed that HBA inhibited 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. 4H). 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<sub>2</sub> (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<sup>+</sup>-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<sub>2</sub>O<sub>2</sub>, are produced during metabolic processes, including photorespiration, fatty acid  $\beta$ -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<sub>2</sub>O<sub>2</sub> 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  $\beta$ -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  $\beta$ -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  $\beta$ -oxidation, exhibited light-dependent stomatal opening comparable to that in wild type (*SI Appendix*, Fig. S9; ref. 44). Thus,  $\beta$ -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<sub>2</sub> 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.

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## Materials and Methods

Plant materials and growth conditions, measurements of stomatal opening, isolation of guard cell protoplasts, measurement of H<sup>+</sup> pumping, immunoblot analysis, identification of *ATG2* gene, construction of transgenic plants, detection of ROS, detection of H<sub>2</sub>O<sub>2</sub> in peroxisomes, confocal microscopy, measurement of CAT activity, preparation of recombinant proteins, and measurement of GOX activity are described in *SI Appendix, SI Methods*.

**ACKNOWLEDGMENTS.** We thank Jyunichi Mano, Masaru Shibata, Tsuneaki Takami, Koichi Sugimoto, Shino Goto-Yamada, and Noriyuki Suetsugu for valuable discussion; Michitaro Shibata for technical information; Michito Tsuyama for equipment support; Ann Cuyper and Takahiro Ishikawa for providing *vtc1-1*, *cad2-1*, and *vtc1-1 cad2-1* mutants; the Salk Institute Genomic Analysis Laboratory for providing the sequence-indexed *Arabidopsis* T-DNA insertion mutants; the Arabidopsis Biological Resource Center and NASC for providing seeds; the Model Plant Research Facility, National Institute for Basic Biology BioResource Center, and Spectroscopy and Bioimaging Facility, NIBB Core Research Facilities, for technical support; and the Iwate Biotechnology Research Center (IBRC) for providing MutMap pipeline. This work was supported by Japan Society for the Promotion of Science KAKENHI (Grant numbers: 18H02468, 26711019, 15K14552 [to A.T.]; 19K16171 [to S.Y.]; 17K07457 [to S.M.]); the Japan Foundation for Applied Enzymology (to A.T.); the Cooperative Research Grant of the Plant Transgenic Design Initiative (PTraD) at Gene Research Center, University of Tsukuba (to A.T.); the Cooperative Research Project Program of the Medical Institute of Bioregulation, Kyushu University (to A.T.); and NIBB Collaborative Research Program Number 17-518 (to K.O.).