Autophagy Contributes to Nighttime Energy Availability for Growth in Arabidopsis[1][W][OA]

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Autophagy is an intracellular process leading to the vacuolar degradation of cytoplasmic components. Autophagic degradation of chloroplasts is particularly activated in leaves under conditions of low sugar availability. Here, we investigated the importance of autophagy in the energy availability and growth of Arabidopsis (*Arabidopsis thaliana*). *autophagy-deficient* (*atg*) mutants showed reduced growth under short-day conditions. This growth inhibition was largely relieved under continuous light or under short-day conditions combined with feeding of exogenous sucrose, suggesting that autophagy is involved in energy production at night for growth. Arabidopsis accumulates starch during the day and degrades it for respiration at night. Nighttime energy availability is perturbed in starchless mutants, in which a lack of starch accumulation causes a transient sugar deficit at night. We generated starchless and *atg* double mutants and grew them under different photoperiods. The double mutants showed more severe phenotypes than did *atg* or starchless single mutants: reduced growth and early cell death in leaves were observed when plants were grown under 10-h photoperiods. Transcript analysis of dark-inducible genes revealed that the sugar starvation symptoms observed in starchless mutants became more severe in starchless *atg* double mutants. The contents of free amino acids (AAs) increased, and transcript levels of several genes involved in AA catabolism were elevated in starchless mutant leaves. The increases in branched-chain AA and aromatic AA contents were partially compromised in starchless *atg* double mutants. We conclude that autophagy can contribute to energy availability at night by providing a supply of alternative energy sources such as AAs.

Plants get energy for growth and survival via photosynthetic carbon assimilation during the day. Energy availability needs to be maintained to allow continuous plant growth throughout the day/night cycle (Smith and Stitt, 2007). In Arabidopsis (*Arabidopsis thaliana*), a portion of the photoassimilate is accumulated as starch and degraded during respiration at night. When diel energy availability is perturbed, a transient sugar deficit can occur, leading to temporal interruption of plant growth (Gibon et al., 2004; Graf et al., 2010). Thus, plants have to adapt to fluctuations of sugar availability due to changes in day/night periods or growth environments. Energy availability can be strongly perturbed by several suboptimal conditions, such as shading, flooding, drought, and extreme temperatures, that interfere with carbon assimilation and/or respiration (Baena-González et al., 2007; Baena-González and Sheen, 2008). Such low-energy stress is mimicked in starchless mutants, in which the lack of starch accumulation causes a sugar deficit at night (Gibon et al., 2004; Bläsing et al., 2005). Since plants cannot simply move out of suboptimal environments, they must metabolically produce alternative respiratory substrates as an energy source for survival and growth under these energy-limited conditions (Araújo et al., 2011).

Protein is an important respiratory substrate in heterotrophic mammalian systems. Because plant respiration is primarily dependent on sugar oxidation, the importance of protein and amino acids (AAs) in plant respiration remains unclear (Plaxton and Podesta, 2006). However, enzymatic pathways for the catabolism of alternative respiratory substrates seem to be induced in vegetative organs of plants when sugar availability strongly declines (Buchanan-Wollaston et al., 2005). It has been reported that an electron-transfer flavoprotein (ETF)/ETF:ubiquinone oxidoreductase (ETFQO) complex functions to transfer electrons to the ubiquinone pools in order to support respiration during continuous darkness (Ishizaki et al., 2005,

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2006). Isovaleryl-CoA dehydrogenase (IVDH) and 2-hydroxyglutarate dehydrogenase (D2HGDH) have been identified as enzymes for AA breakdown and electron donors to the ETF/ETFQO complex (Araújo et al., 2010). Glu dehydrogenase (GDH) has also been suggested to play a key role in AA catabolism (Miyashita and Good, 2008). These reports demonstrate that AAs can be catabolized and become a respiratory substrate in plant systems.

Autophagy is a ubiquitous recycling system in eukaryotic cells by which proteins and organelles are transported for degradation in the vacuoles of yeast and plants or the lysosomes of animals (for review, see Nakatogawa et al., 2009; Li and Vierstra, 2012; Liu and Bassham, 2012; Yoshimoto, 2012). During autophagy, a portion of the cell's cytoplasmic contents is sequestered by a double-membrane vesicle called an autophagosome and delivered to the vacuole/lysosome. The outer membrane of the autophagosome then fuses with the vacuolar/lysosomal membrane, and the inner membrane structure (called the autophagic body) is degraded by resident hydrolases. Autophagy-related genes (*ATGs*) are well conserved across yeast, plants, and animals (Meijer et al., 2007). Numerous analyses using RNA interference knockdown and transfer DNA-insertional knockout mutants of *ATGs* in Arabidopsis have revealed that the core machinery for autophagosomal membrane elongation is conserved in plants (Yoshimoto et al., 2004; Thompson et al., 2005; Xiong et al., 2005; Phillips et al., 2008; Chung et al., 2010; Suttangkakul et al., 2011). Autophagy is considered to play an important role in nutrient recycling under starvation conditions similar to its role in yeast and animals (Li and Vierstra, 2012; Liu and Bassham, 2012; Yoshimoto, 2012). Recently, Guiboileau et al. (2012) demonstrated the importance of autophagy in nitrogen remobilization of Arabidopsis.

We have demonstrated that chloroplastic proteins are degraded by autophagy via Rubisco-containing bodies (RCBs), a type of autophagic body containing chloroplast stroma (Chiba et al., 2003; Ishida et al., 2008). The chloroplast is an organelle specific to photoautotrophs and has a central role not only in photosynthesis but also in the assimilation of several mineral nutrients. Chloroplasts are the major source of material for autophagic recycling in plants, because the majority of plant nutrients are distributed to chloroplasts, such that chloroplastic proteins account for 75% to 80% of total leaf nitrogen in C3 plants (Makino et al., 2003). The RCB/autophagy system contributes to Rubisco degradation during leaf senescence (Ono et al., 2013), and RCB production is particularly activated in leaves under conditions of low sugar availability, such as in individually darkened leaves, leaves at the end of the night in a diurnal cycle, or leaves of starchless mutants (Wada et al., 2009; Izumi et al., 2010), suggesting the involvement of the RCB/autophagy system in energy production. Although autophagy is an important catabolic pathway regulating energy homeostasis in mammalian systems (Singh and Cuervo, 2011), a substantial role for autophagy in energy availability has not been reported in plant systems.

In this study, we investigated the importance of autophagy in diel energy availability and growth of Arabidopsis. *autophagy-deficient* (*atg*) mutants showed more severe phenotypes when sugar availability during the night was reduced due to shorter photoperiods and/or starchless mutations. Metabolic and transcript analyses indicated that autophagy provides a supply of energy sources such as AAs as an alternative to sugars. Our data demonstrate that autophagic recycling can contribute to plant energy availability.

RESULTS

Growth Retardation of Autophagy-Deficient Mutants Occurs under Short-Day Conditions But Not under Continuous Light

Photoperiod is a major factor controlling plant energy availability under nonstressed conditions: sugar availability from photosynthetic carbon assimilation is reduced under short days (SD; Gibon et al., 2004, 2009; Smith and Stitt, 2007). Although *atg* mutants of Arabidopsis can complete their life cycle irrespective of photoperiod conditions (Doelling et al., 2002; Hanaoka et al., 2002), their growth rates are clearly lower than those of wild-type plants under SD conditions (Guiboileau et al., 2012). First, we examined the vegetative growth rates of several *atg* mutants under SD (10 h of light/14 h of dark) and continuous light (CL) conditions (Fig. 1). All *atg* mutants grown in soil culture under SD displayed growth retardation: shoot fresh weight in *atg* mutants was 36% to 48% of the wild-type level (Fig. 1B). This reduced growth was not observed under CL. Furthermore, we grew wild-type and *atg* mutants under SD conditions with elevated CO_2 concentrations (1,000–1,200 μL L^{-1}), which increase the CO_2 assimilation rate during the day and sugar availability (Cheng et al., 1998). Shoot fresh weight of *atg* mutants was 60% to 65% of the wild-type level; therefore, the SD-dependent reduced growth of the *atg* mutants was partially but not fully compensated for by elevated CO_2 concentration. These results suggest that SD-dependent growth retardation of *atg* mutants is especially related to nighttime carbon utilization.

We grew wild-type and *atg* mutant plants on mineral-rich medium with or without Suc to create sugar-excess conditions throughout the day/night cycle of SD (Fig. 1). Reduced growth of *atg* mutants was observed in Suc-free medium, similar to that observed in soil; however, *atg* growth was not significantly different from the wild type in Suc-rich medium (Fig. 1B). Shoot fresh weight for the wild type and *atg* mutants under all conditions corresponded to the observed plant size (Fig. 1). These findings suggest that autophagy contributes to plant growth under SD conditions via carbon metabolism at night.

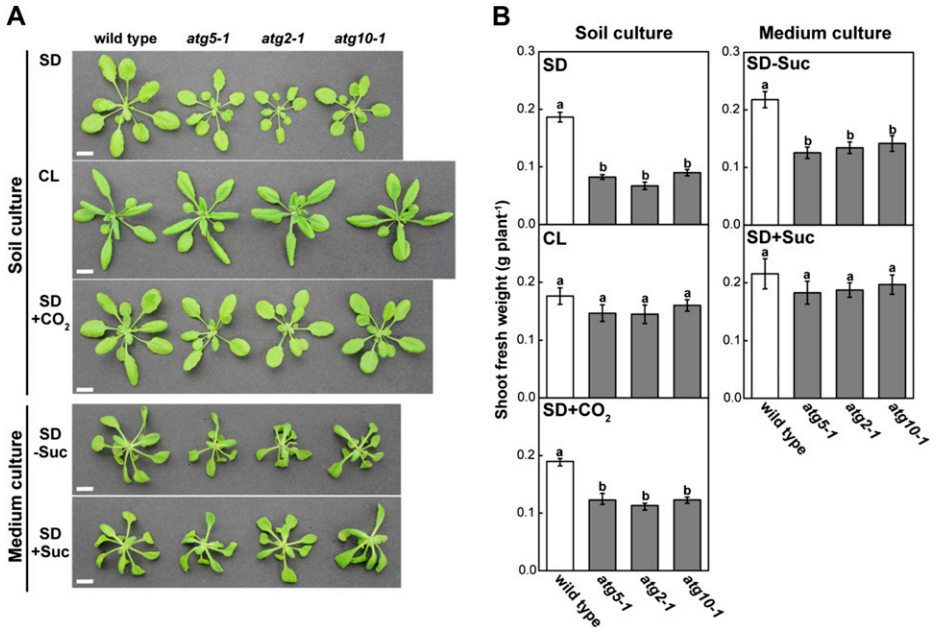

Figure 1. SD-dependent growth retardation of *atg* single mutant plants. Wild-type, *atg5-1*, *atg2-1*, and *atg10-1* plants were grown in soil culture for 30 d under SD (SD), for 23 d under CL (CL), or for 24 d with CO_2 supply under SD (SD+CO_2). The same lines were also grown in mineral-rich medium culture under SD for 28 d without Suc (SD−Suc) or for 23 d with 1% (w/v) Suc (SD+Suc). Images (A) and shoot fresh weight (B) were obtained when the shoot fresh weight of the wild type was approximately 0.2 g under the indicated conditions. Bars = 10 mm. The data represent means ± SE (n = 5–8). Statistical analysis was performed using Tukey's test; values with the same letter were not significantly different among individual genotypes ($P \leq 0.05$).

Autophagy Is Required for Adaptation and Growth during Fluctuations in Energy Availability Due to Starchless Carbohydrate Metabolism

Sugar availability at night is severely perturbed in starchless mutants due to the lack of starch accumulation and partially perturbed in starch-excess mutants due to a reduced capacity for starch degradation, resulting in reduced energy availability at night (Caspar et al., 1985, 1991; Lin et al., 1988). To characterize further the role of autophagy in carbon metabolism, we generated double mutants containing a starchless mutation, *phosphoglucomutase* (*pgm*; Caspar et al., 1985) or *ADP-glucose pyrophosphorylase1* (*adg1*; Lin et al., 1988), with an *atg5* mutation (*pgm atg5* and *adg1 atg5*). The *starch-excess1* (*sex1*; Caspar et al., 1991) and *atg5* double mutant was also generated (*sex1 atg5*). The wild-type and mutant plants were grown under different photoperiod conditions (SD, long days [LD; 14 h of light/10 h of dark], or CL) in soil culture. Under SD, *pgm atg5* and *adg1 atg5* exhibited early cell death in leaves before the transition to the reproductive stage (Fig. 2A). This phenotype was not observed under LD or CL conditions or in *sex1 atg5* even under SD (Fig. 2A). Although a similar cell death phenomenon of early senescence is observed in *atg* single mutants (Yoshimoto et al., 2009), almost all the leaves of *pgm atg5* and *adg1 atg5* died visibly earlier than those of *atg5*, despite slower growth (Fig. 2A). Early cell death in leaves under SD was also observed in double mutants of *adg1* with another *atg* mutation, *atg2* (*adg1 atg2*; Supplemental Fig. S1).

The starchless *atg* double mutant plants were much smaller than wild-type plants and the respective single mutants under SD but were not different under CL conditions (Fig. 2A). To compare plant growth, we measured shoot fresh weight of the mutant lines (Fig. 2B). Shoot fresh weight in *pgm*, *adg1*, and *sex1* was lower than that in the wild type under SD and LD. Thus, the growth retardation observed in starchless and starch-excess single mutants under shortened photoperiods was confirmed, and the *atg5* mutation led to further reductions of shoot fresh weight in all backgrounds under SD and LD conditions. Under CL, there were no differences in shoot fresh weight among the different genotypes. The shoot fresh weight of the respective single and double mutants corresponded to the observed plant size (Fig. 2A). The growth retardation observed under shortened photoperiods was similar between *atg* mutants and starchless mutants, and the phenotypes were additive in the starchless *atg* double mutants under SD (Fig. 2). These findings also suggest that autophagy is involved in carbon utilization at night, particularly when sugar availability is perturbed.

We measured leaf starch and sugar contents during the 14-h night under SD in the wild type, *atg5*, starchless mutants, and starchless *atg* double mutants to check the nighttime sugar availability (Fig. 3). In the wild type and *atg5*, starch accumulated at the end of the day gradually decreased during the night and was almost entirely consumed at the end of the night. Suc levels were maintained throughout the night in both lines. These diurnal changes represent normal carbohydrate metabolism in Arabidopsis (Smith and Stitt, 2007). In *pgm* and *adg1*, a lack of accumulation of starch led to an obvious accumulation of soluble sugars at the end of the day; however, these sugars rapidly decreased at the beginning of the night, and sugars were almost exhausted before the middle of the night (Fig. 3). In *pgm atg5* and *adg1 atg5*, carbohydrate metabolism

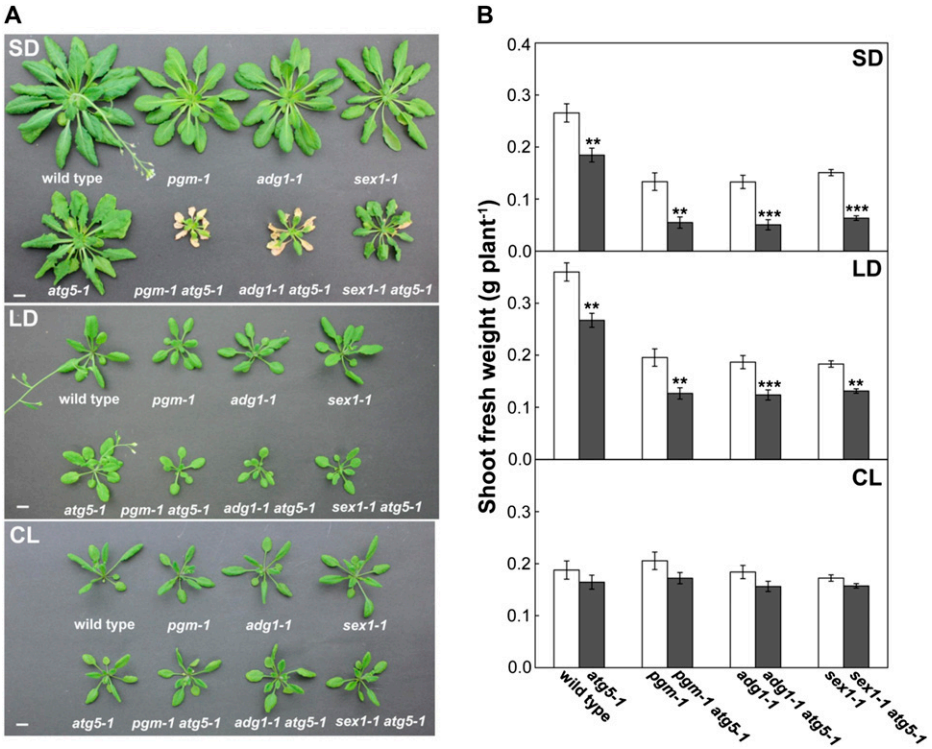

Figure 2. Starchless and autophagy-deficient double mutants display an early-cell-death phenotype in leaves and reduced growth under SD. A, The wild type, single mutant lines (*atg5-1*, *pgm-1*, *adg1-1*, *sex1-1*), and double mutant lines (*pgm-1 atg5-1*, *adg1-1 atg5-1*, *sex1-1 atg5-1*) were grown in nutrient-rich soil for 60 d under SD, for 30 d under LD, or for 23 d under CL. Bars = 10 mm. B, The same lines were grown for 40 d under SD, for 30 d under LD, or for 23 d under CL, and plant shoot fresh weight was measured. The data represent means ± SE (n = 4–5). Statistical analysis was performed by Student's t test; asterisks indicate significant differences between the lines with and without the *atg5* mutation at the 5% (*), 1% (**), and 0.1% (***) levels, respectively.

during the night was the same as that observed in starchless single mutants, with the exception that Suc contents tended to be higher and Glc and Fru contents were lower than in starchless single mutants at the end of the day (Fig. 3). Thus, the occurrence of sugar exhaustion during the night was obvious in starchless *atg* double mutants.

Autophagy Deficiency Makes Starvation Symptoms More Severe under a Transient Sugar Deficit

Next, to assess the extent of the starvation symptoms during the night, we measured transcript levels of *DIN6* (Fujiki et al., 2001b), *ATL8* (Graf et al., 2010), and *DIN1* (Oh et al., 1996), as dark-inducible genes (*DIN*s), in leaves at the end of the night of SD (Fig. 4). *DIN* transcripts are acutely induced upon the initiation of sugar starvation, and the *DIN*s analyzed here have been established as appropriate reporters of sugar starvation symptoms (Baena-González et al., 2007; Graf et al., 2010). Transcript levels of *ATL8* and *DIN1* were significantly elevated in *atg5* compared with the wild type. However, transcript levels of analyzed *DIN*s were much higher in *pgm* and *adg1* than in the wild type and *atg5*, indicating that sugar starvation was indeed occurring in leaves of starchless mutants at the end of the night. In addition, transcript levels of *DIN6* and *ATL8* were further elevated in *pgm atg5* and *adg1 atg5*, implying that the extent of the starvation symptoms was more severe in starchless *atg* double mutants than in starchless single mutants.

To provide additional support for this conclusion, we analyzed transcript levels of *DIN*s during extended night in the *atg* single mutant (Supplemental Fig. S2). Wild-type and *atg5* plants were grown for 30 d under SD and then subjected to a 6-h extended night beyond the normal 14-h night. The extended night treatment has been established as an experimental system for temporal sugar starvation symptoms in Arabidopsis, because it causes starch exhaustion and acute responses to sugar deficits (Smith and Stitt, 2007; Graf et al., 2010). While *DIN6*, *ATL8*, and *DIN1* transcript levels were strongly elevated in wild-type leaves during the extended night, this elevation occurred earlier in *atg5* (Supplemental Fig. S2). Two hours after the start of the extended night, *DIN6*, *ATL8*, and *DIN1* transcript levels in *atg5* leaves were 2.1-, 2.2-, and 1.5-fold greater than in wild-type leaves, respectively. These results support the notion that autophagy deficiency causes more severe starvation symptoms during a sugar deficit.

Increases in AA Contents of Starchless Mutants Were Partially Compromised by Deficiency in Autophagy

We hypothesized that autophagic protein degradation supplies free AAs as an alternative respiratory substrate. To examine the effects of starchless and/or *atg* mutations on the free AA pool, we performed capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) analysis and measured AA contents in the leaves of wild-type, *atg5*, *pgm*, and *pgm*

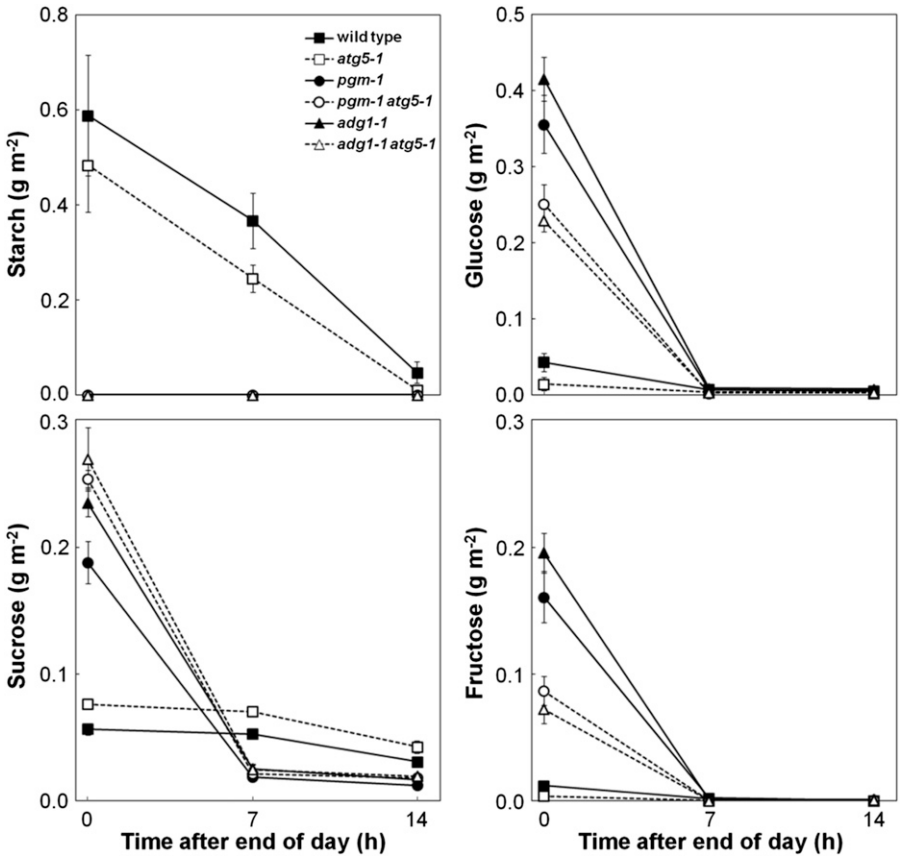

Figure 3. Sugar exhaustion in starchless mutant leaves at night. Starch, Suc, Glc, and Fru contents were measured in the leaves of the wild type, single mutant lines (*atg5-1*, *pgm-1*, *adg1-1*), and double mutant lines (*pgm-1 atg5-1*, *adg1-1 atg5-1*) at 0, 7, and 14 h after the end of the day during the 14-h night 40 d after sowing. The data represent means ± SE ($n = 4$).

atg5 plants (Fig. 5). The contents of the individual AAs largely increased in *pgm* compared with the wild type, with the exception of Asp, Gln, and Pro. Therefore, the total AA content in *pgm* leaves increased to 2.3-fold of the wild-type amount. The increases in branched-chain amino acids (BCAAs; Leu, Ile, and Val), aromatic AAs (Phe, Trp, and Tyr) except Phe, and Thr, His, and Met observed in *pgm* were partially compromised in *pgm atg5*. Because the content of Asp, a quantitatively major AA, was higher in *pgm atg5* than in *pgm*, the total AA content did not differ between the two genotypes. In *atg5* single mutants, individual AA contents in leaves did not decrease compared with the wild type.

Free AAs can be catabolized, and the resulting carbon skeletons are possibly supplied to the tricarboxylic acid (TCA) cycle for respiration (Araújo et al., 2011). Among TCA cycle-related organic acids, the contents of fumarate, succinate, 2-oxoglutarate, pyruvate, and isocitrate increased in *pgm* and *pgm atg5* leaves (Supplemental Fig. S3), suggesting that AA catabolism was activated in starchless leaves.

Recently, the importance of BCAAs, aromatic AAs, and Lys for respiration during sugar starvation has been demonstrated through the identifications of IVDH, D2HGDH, ETF, and ETFQO as enzymes and proteins related to the catabolism of these AAs (Ishizaki et al., 2005, 2006; Araújo et al., 2010). In addition, GDH has been suggested to play an important role in AA catabolism by supplying 2-oxoglutarate for deamination reactions (Miyashita and Good, 2008). Thus, we measured transcript levels of *ETFα*, *ETFβ*, *ETFQO*, *IVDH*, *D2HGDH*, *GDH1*, and *GDH2* in leaves of the wild type, *atg5*, starchless mutants, and starchless *atg5* double mutants at the end of the night (Fig. 6). The transcript levels of *IVDH*, *ETFQO*, and *GDH2* were much higher in starchless lines than in the wild type and *atg5*. The transcript pattern of *ETFQO* was similar to that of *DIN6* and *ATL8* (Fig. 4).

To examine the changes of autophagic activity in starchless mutants, we measured transcript levels of *ATG8* gene family members (*ATG8a* to *ATG8i*) as *ATGs* (Supplemental Fig. S4). The ATG8 protein is a component of the autophagosome membrane and is turned over with autophagic bodies via vacuolar degradation (Yoshimoto et al., 2004; Suttangkakul et al., 2011). *ATG8e*, *ATG8f*, and *ATG8h* mRNA levels in starchless lines were approximately twice those in the wild type and *atg5* (Supplemental Fig. S4). We also examined RCB production in the mutant leaves under SD (Supplemental Fig. S5), using concanamycin A treatment to inhibit the vacuolar degradation of RCBs (Izumi et al., 2010). Leaves were excised at the end of the day and incubated with concanamycin A in darkness for 14 h, the same period as the night under SD. Small vesicles exhibiting GFP fluorescence without chlorophyll fluorescence, which represent RCBs,

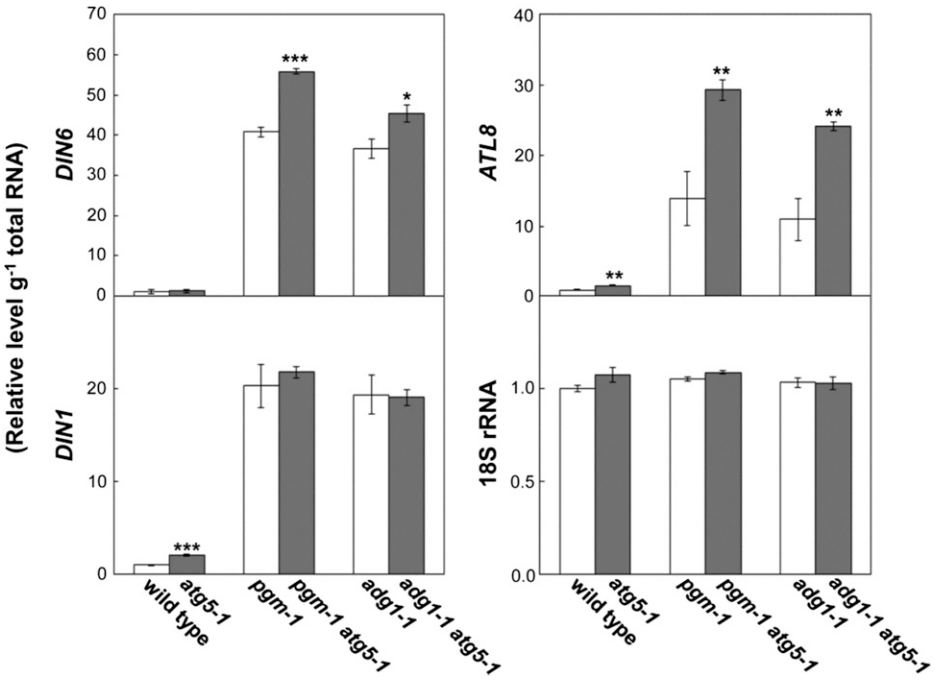

Figure 4. Transcript levels of *DINs* as a marker of sugar starvation symptoms during the night. Total RNA was isolated from leaves of the wild type, single mutant lines (*atg5-1*, *pgm-1*, *adg1-1*), and double mutant lines (*pgm-1 atg5-1*, *adg1-1 atg5-1*) at the end of the night 40 d after sowing and subjected to qRT-PCR analysis to measure transcript levels of *DIN6*, *DIN1*, and *ATL8*. 18S rRNA was used as an internal control. mRNA levels are shown relative to the wild-type level, which is represented as 1. The data represent means ± SE (n = 4). Statistical analysis was performed by Student's t test; asterisks indicate significant differences between lines with or without the *atg5* mutation at the 5% (*), 1% (**), and 0.1% (***) levels, respectively.

accumulated in the central vacuole of wild-type, *pgm*, and *adg1* leaves (Supplemental Fig. S5A). The number of accumulated RCBs in *pgm* and *adg1* was 3.2- and 2.5-fold higher than in the wild type, respectively (Supplemental Fig. S5B). We also confirmed that RCBs were absent in *atg5*, *pgm atg5*, and *adg1 atg5* leaves (Supplemental Fig. S5). These results support the notion that autophagy and AA catabolism are activated for the supply of alternative respiratory substrates during a sugar deficit in starchless mutant leaves.

Other Effects of Autophagy Deficiency in the Catabolism of Possible Energy Sources

CE-TOFMS analysis also revealed large metabolic changes other than in AAs due to autophagy deficiency (Supplemental Data S1). We focused on the changes in intermediates of the catabolism of possible energy sources (Table I). The contents of several intermediates in AA catabolism significantly decreased in *pgm atg5* compared with *pgm* (Table I). These included 4-methyl-2-oxovaleric acid and 3-methyl-2-oxovaleric acid, which are initial intermediates of Leu and Ile catabolic processes (Binder et al., 2007). Saccharopine and 2-aminoadipic acid, intermediates of Lys catabolism (Galili et al., 2001), were also decreased in *pgm atg5* relative to *pgm*, as was Lys itself (Fig. 5). These findings support our conclusion that autophagy provides a supply of AAs as alternative energy sources.

Decreases in allantoin and urate, which are intermediates of purine catabolism (Zrenner et al., 2006), were also observed (Table I). Nucleotides are also possible energy sources (Zrenner et al., 2006) and could be targets of autophagic degradation, so that ribosomes containing ribosomal RNA (rRNA) would be degraded by an autophagic process similar to yeast ribophagy (Hillwig et al., 2011). Although fatty acids and lipids can be alternative respiratory substrates during sugar starvation (Kunz et al., 2009), the contents of choline, glycerophosphocholine, and phosphorylcholine, which are phospholipid-related metabolites, increased with *atg5* mutation (Table I). These metabolites are derived from phospholipase-mediated degradation of phosphatidylcholine, a major phospholipid in plants (Bargmann and Munnik, 2006; Inoue and Moriyasu, 2006). Phospholipid degradation may thus be compensatorily accelerated by deficiency in autophagy.

DISCUSSION

Recent work has greatly developed our understanding of the role of autophagy in several biological processes of plants (Liu et al., 2005, 2012; Fujiki et al., 2007; Xiong et al., 2007; Ishida et al., 2008; Hofius et al., 2009; Wada et al., 2009; Yoshimoto et al., 2009; Kwon et al., 2010; Derrien et al., 2012; Guiboileau et al., 2012; Ono et al., 2013). Although the importance of autophagy in carbon utilization has been suggested based on the observed activation of autophagy during sugar starvation and the sensitivity of *atg* mutants to continuous darkness (Thompson et al., 2005; Phillips et al., 2008; Chung et al., 2010; Izumi et al., 2010; Suttangkakul et al., 2011), a substantial role for autophagy in the energy availability of plant systems has not been established experimentally. In this study, we found that the growth retardation of *atg* mutants under SD is

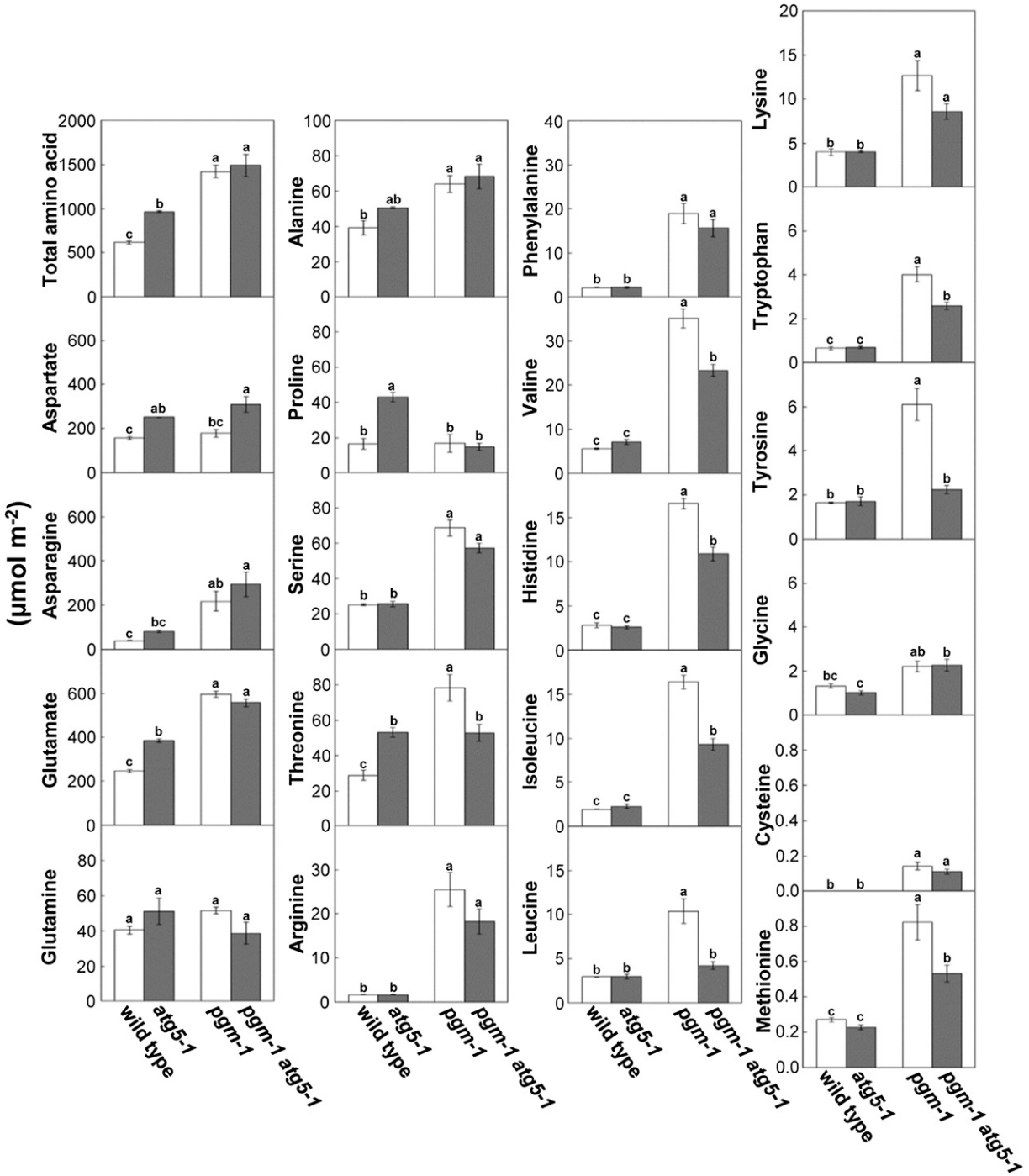

Figure 5. Increases in leaf AA contents in starchless mutants were partially compromised in starchless *atg* double mutants. AA contents were measured in leaves of the wild type, *atg5-1*, *pgm-1*, and *pgm-1 atg5-1* at the end of the night under SD by CE-TOFMS analysis. The data represent means ± SE ($n = 3$). Statistical analysis was performed by Tukey's test; values with the same letter were not significantly different among the individual genotypes ($P \leq 0.05$).

closely related to nighttime energy availability. Thus, we generated starchless *atg* double mutants because sugar availability at night is reduced in starchless mutants. The double mutants showed further severe starvation symptoms and phenotypes: reduced growth and early cell death in leaves were observed under SD. CE-TOFMS analysis indicated that autophagy provides a supply of energy sources such as AAs instead of sugars. Our data thus support a model in which autophagy contributes to energy availability at night

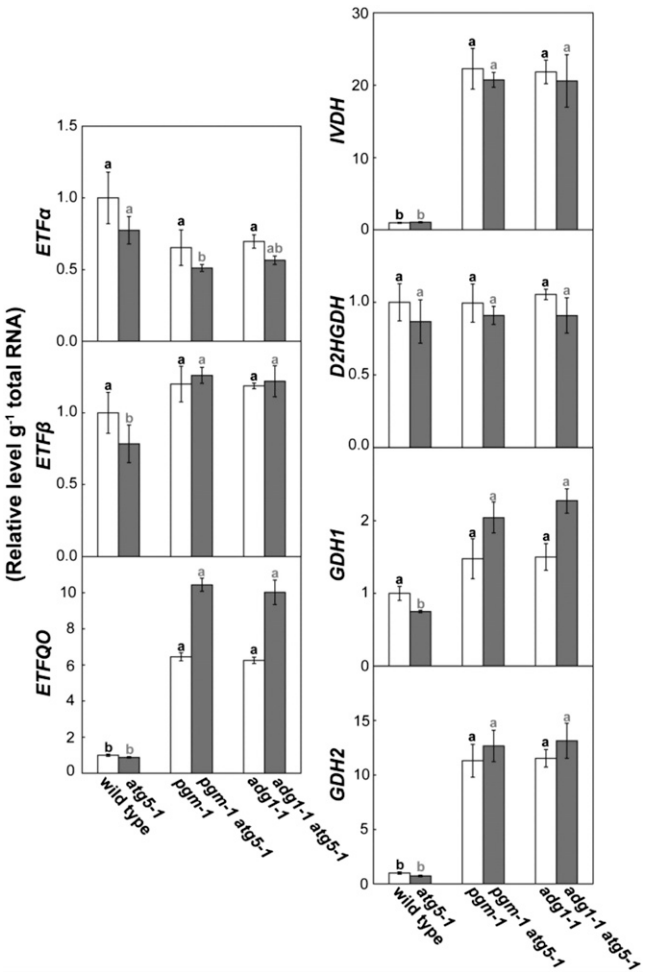

Figure 6. Transcripts of several genes involved in AA catabolism were elevated in starchless leaves during the night. Total RNA was isolated from leaves of the wild type, single mutant lines (*atg5-1*, *pgm-1*, *adg1-1*), and double mutant lines (*pgm-1 atg5-1*, *adg1-1 atg5-1*) at the end of the night 40 d after sowing and subjected to qRT-PCR analysis to measure transcript levels of *ETFα*, *ETFβ*, *ETFQO*, *D2HGDH*, *IVDH*, *GDH1*, and *GDH2*. mRNA levels are shown relative to the wild-type level, which is represented as 1. The data represent means ± SE ($n = 4$). Statistical analysis was performed by Tukey's test; columns with the same letter were not significantly different among the wild type, *pgm-1*, and *adg1-1* (black letters; $P \leq 0.05$) or among *atg5-1*, *pgm-1 atg5-1*, and *adg1-1 atg5-1* (gray letters; $P \leq 0.05$).

for growth by supplying energy sources under conditions of limited sugar availability.

The Importance of Autophagy during Fluctuations in Energy Availability

We established the importance of autophagy under unstable energy availability using starchless mutant plants. Fluctuations or disruptions in energy availability can be caused during several biotic or abiotic stresses (Baena-González et al., 2007; Baena-González and Sheen, 2008). In starchless mutants, photosynthesis is not limited, but a transient sugar deficit of several hours occurs at night (Fig. 3). Starchless mutation, therefore, seems to mimic energy limitation caused by environmental stresses more closely than artificial sugar starvation in which plants are grown in darkness for several days.

Starchless *atg* double mutants exhibited a more severe phenotype under shortened photoperiods (Fig. 2). The early-cell-death phenotype of *atg* single mutants has been shown to be dependent on the excessive accumulation of salicylic acid (SA; Yoshimoto et al., 2009). SA was only detected in *pgm atg5* leaves by CE-TOFMS (Supplemental Data S1), suggesting that SA accumulation was directly responsible for the cell death phenomenon in starchless *atg* double mutants. However, the trigger for the excessive accumulation of SA remains unknown. Arabidopsis plants seem to decrease respiration activity in order to maintain an energy supply and survive when they are exposed to extended darkness (Keech et al., 2007). The proper balance of several catabolic pathways for controlled energy supply is likely to be disrupted by autophagy deficiency, such as an acceleration of phospholipid degradation (Table I). This imbalanced energy supply may stimulate cell damage and SA accumulation and ultimately may cause early cell death. Increased oxidative damage in *pgm atg5* and *atg5* was implied by the increases in the oxidized form of glutathione compared with the wild type and *pgm* (Supplemental Data S1; Meyer and Hell, 2005).

When energy availability is perturbed due to interference with photosynthesis, photosynthetic proteins in chloroplasts cannot fully function and are likely an optimal energy source via autophagic degradation. Partial degradation via RCBs without dismantling of the whole chloroplast would appear to be an efficient mechanism for maintaining some basal functions of the chloroplast until growth conditions improve. Whereas autophagy has generally been considered to be a bulk degradation system for nutrient recycling, several lines of evidence for selectivity in plant autophagy have recently been provided (Floyd et al., 2012; Li and Vierstra, 2012). In terms of organelle selectivity, autophagic degradation of the endoplasmic reticulum during endoplasmic reticulum stress was recently reported (Liu et al., 2012). In addition, chloroplastic proteins may be selectively degraded via the RCB/autophagy system for proper carbon recycling, although the mechanism underpinning such selectivity is not known.

The Link between Autophagic Protein Degradation and AA Catabolism

Recently, *etfβ*, *etfqo*, and *ivdh*, which are mutants of genes involved in BCAA or aromatic AA catabolism, were identified and found to show increased accumulation of BCAA and aromatic AAs compared with the wild type during extended darkness of several

Table I. *Selected metabolites involved in the catabolism of possible energy sources that were significantly increased or decreased in leaves of the Arabidopsis pgm atg5 mutant compared with pgm*

Metabolite Description	Fold Change (*pgm-1 atg5-1*/*pgm-1*)	Involved Catabolic Pathway
Glycerophosphocholine	2.65	Phospholipid degradation
Phosphorylcholine	1.67	Phospholipid degradation
Choline	1.40	Phospholipid degradation
4-Methyl-2-oxovaleric acid 3-Methyl-2-oxovaleric acid	0.69	Leu and Ile catabolism
2-Aminoadipic acid	0.55	Lys catabolism
Saccharopine	0.50	Lys catabolism
Allantoin	0.33	Purine catabolism
Urate	0.19	Purine catabolism

days (Ishizaki et al., 2005, 2006; Araújo et al., 2010). Here, increases of these AA contents in starchless mutants were partially compromised in starchless *atg* double mutants (Fig. 5). These findings imply that autophagy contributes to AA catabolism via free AA supply. BCAAs and aromatic AAs are quantitatively minor in AA pools; however, the contents of these AAs and transcript levels of several genes for their catabolism drastically increase during sugar starvation (Fujiki et al., 2001a, 2002; Buchanan-Wollaston et al., 2005; Araújo et al., 2010). Furthermore, *etfβ*, *etfqo*, and *ivdh* mutants show reduced tolerance to sugar starvation: these mutants die earlier than wild-type plants during extended darkness (Ishizaki et al., 2005, 2006; Araújo et al., 2010). Although GDH catalyzes the conversion between Glu and 2-oxoglutarate, the *gdh1 gdh2* double mutants also display high accumulation of BCAA and early cell death during extended darkness, which is suggested to be a result of decreased 2-oxoglutarate production for the initial step of BCAA catabolism (Miyashita and Good, 2008). These phenomena clearly indicate a key role of BCAAs and aromatic AAs in the metabolic adaptation to sugar starvation conditions. Autophagy deficiency appears to affect BCAA and aromatic AA contents in starchless *atg* double mutants, because these AAs were actively catabolized in starchless mutants (Fig. 5).

Although high sensitivity to extended darkness of several days is also observed in several *atg* mutants (Thompson et al., 2005; Phillips et al., 2008; Chung et al., 2010), metabolic changes have not been investigated during such conditions. Further metabolic analysis using mutants of autophagy and AA catabolism are needed to confirm the metabolic link between autophagic protein degradation and AA catabolism.

Contribution of Autophagy to Energy Availability at Night for Normal Growth

The growth retardation with shortened photoperiods in *atg* single mutants is similar to that observed in starchless and starch-excess mutants in which nighttime sugar availability is reduced (Figs. 1 and 2; Caspar et al., 1985, 1991; Lin et al., 1988). This suggests that autophagy supports energy availability at night for normal growth. However, a major regulator of diel energy availability in response to photoperiods is the rate of starch synthesis and starch degradation (Smith and Stitt, 2007; Graf et al., 2010). In fact, the extent of growth retardation with shortened photoperiod was more severe in starchless mutants than in *atg* mutants (Fig. 2). In our CE-TOFMS analysis, clear evidence that autophagy provides AAs as energy sources at night during SD was not observed in *atg5* single mutants (Fig. 4). It is conceivable that autophagic protein degradation constitutively supports carbon utilization and plant growth each night under SD conditions at lower levels than during a sugar deficit.

Plant productivity was recently shown to be closely associated not only with carbon assimilation capacity during the day but carbon utilization at night (Sulpice et al., 2009; Graf et al., 2010). When plant growth is environmentally restricted by carbon utilization, such as under SD conditions, it may be more efficient to have a portion of proteins degraded and consumed as an energy source. In fact, a metabolic shift that is dependent on light intensity for growth conditions was shown to result in increases in BCAA, aromatic AA, and Lys contents under low light compared with normal and high light in Arabidopsis leaves (Jänkänpää et al., 2012). In addition, some reports have suggested the involvement of protein degradation in diel carbon metabolism. GDH activity increases with photoperiod shortening (Gibon et al., 2009). Global gene expression analysis of diurnal changes showed that *ATG8e* transcripts elevate toward the end of the night, along with some genes involved in ubiquitin-dependent protein degradation (Bläsing et al., 2005). The carbon concentration in seeds is lower in *atg5* than in the wild type (Guiboileau et al., 2012), suggesting the possible involvement of autophagy in whole-plant carbon utilization. The link between protein degradation and carbon metabolism during photoautotrophic growth has been uncharacterized in plant systems. Our findings suggest a metabolic strategy in which autophagy can contribute to carbon utilization and plant growth. However, protein is a less efficient respiratory substrate than carbohydrate in plants (Araújo et al., 2011). The role of autophagy in energy availability should be further characterized not only during

severe stress conditions but also during nonstressed conditions that moderately alter sugar availability.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia was used for all experiments. Transgenic plants expressing stroma-targeted GFP (CT-GFP) and *pgm-1*, *adg1-1*, and *sex1-1* background lines with CT-GFP were described previously (Izumi et al., 2010). The autophagy-deficient mutants *atg5-1* (SAIL_129_B07), *atg2-1* (SALK_076727), and *atg10-1* (SALK_084434) were described previously (Thompson et al., 2005; Phillips et al., 2008; Yoshimoto et al., 2009). Double mutant lines were generated by sexual crossing. Plants were grown in soil (Metro-Mix 350; SunGro Horticulture) or on mineral-rich medium in chambers at 23°C to 24°C with fluorescent lamps (100 μmol quanta m^{-2} s^{-1}) under different photoperiods (10 h for SD, 14 h for LD, and 24 h for CL). A 1:1,000 dilution of Hyponex (Hyponex Japan) was added to the soil once per week as a source of mineral nutrients. Mineral-rich medium was produced using a previously described hydroponic solution (Izumi et al., 2012) with or without 1% (w/v) Suc. Growth analysis was performed after different numbers of days of growth depending on photoperiod or culture conditions. For the *atg* single mutants (Fig. 1), shoot fresh weight and images were obtained at the point when the shoot fresh weight of wild-type plants was approximately 0.2 g in the various conditions. In medium culture, individual plants were germinated and grown in Suc-rich medium for 1 week and transplanted to the same Suc-rich medium or Suc-free medium. For the double mutant lines (Fig. 2), images and shoot fresh weight were obtained approximately 5 d after bolting of wild-type plants under CL and LD conditions. Under SD, images of plants were obtained 60 d after sowing and shoot fresh weight was obtained 40 d after sowing because the start of bolting was later under SD compared with the other conditions. Plants grown for 40 d were used in the other experiments using starchless *atg* double mutants under SD.

Measurement of Carbohydrates

Third to sixth rosette leaves were harvested at 0, 7, and 14 h after the end of the day during the 14-h night of SD. Starch, Suc, Glc, and Fru contents were measured using an F-kit for α-Glc/Suc/Fru (Roche Diagnostics) and an F-kit for starch (Roche Diagnostics) as described previously (Izumi et al., 2010).

Quantitative Reverse Transcription-PCR Analysis

Total RNA was isolated from third to sixth rosette leaves using the RNeasy kit (Qiagen). Isolated RNA was treated with DNase (DNA-free; Ambion), and complementary DNA was synthesized using random hexamer and oligo(dT) primers with the PrimeScript reverse transcription reagent kit (Takara). Quantitative reverse transcription (qRT)-PCR analysis was performed as described previously (Izumi et al., 2012) using an aliquot of the synthesized complementary DNA derived from 1.8 ng of total RNA. The sequences of the primers used are shown in Supplemental Table S1. Several primers were used previously (Baena-González et al., 2007; Miyashita and Good, 2008; Carbonell-Bejerano et al., 2010; Graf et al., 2010; Kwon et al., 2010). Amplicon sequences of original primers were confirmed by sequencing after TA cloning. The level of 18S rRNA was measured as an internal standard (Fig. 4; Supplemental Fig. S2), and rRNA levels did not differ between wild-type and mutant plants, indicating the validity of the method employed.

Measurement of Ionic Metabolites by CE-TOFMS

Third to sixth rosette leaves were homogenized with methanol (internal control). After additions of water and chloroform, the upper aqueous layer was filtered through a Millipore 5-kD cutoff filter to remove proteins. The filtrate was lyophilized, dissolved in water, and analyzed by CE-TOFMS using the Agilent CE Capillary Electrophoresis System (Agilent Technologies). Cationic metabolites were analyzed with a fused silica capillary (50 μm i.d. $\times$ 80 cm total length), with Cation Buffer Solution (Human Metabolome Technologies) as the electrolyte (Soga and Heiger, 2000). Anionic metabolites were analyzed with a fused silica capillary (50 μm i.d. $\times$ 80 cm total length), with Anion Buffer Solution (Human Metabolome Technologies) as the electrolyte (Soga et al., 2007). Raw data obtained by CE-TOFMS were processed with MasterHands (Sugimoto et al., 2010). Signal peaks corresponding to isotopomers of 108 compounds including intermediates of the glycolytic system, intermediates of the TCA cycle, and AAs were extracted. Their migration times were normalized using those of the internal standards, and the resultant relative area values were further normalized by sample leaf area.

Analysis of RCB Production Activity

RCB production was analyzed using the previously described method with a slight modification (Supplemental Fig. S5; Izumi et al., 2010). Fourth rosette leaves were excised at the end of the day from three to four independent CT-GFP plants. The leaves were incubated in 10 mM MES-NaOH (pH 5.5) with 1 μM concanamycin A for 14 h, the same period as the usual night period of SD, at 23°C in darkness. After incubation, each leaf was divided into four parts, and two square regions (188 μm $\times$ 188 μm each) in each section were monitored by laser scanning confocal microscopy and images were obtained. The number of accumulated RCBs in the images was counted. Each image was considered an independent data point and subjected to statistical analysis. Laser scanning confocal microscopy was performed with a Nikon C1si system equipped with a CFI Plan Apo VC 60$\times$ water-immersion objective (numerical aperture = 1.20; Nikon) as described previously in detail (Ishida et al., 2008).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Early-cell-death phenotype in leaves of *atg2* and *adg1* double mutants under SD.

Supplemental Figure S2. Early induction of *DIN* transcripts by autophagy deficiency during an extended night.

Supplemental Figure S3. Contents of organic acids of the TCA cycle were increased in starchless mutant leaves.

Supplemental Figure S4. Transcript levels of several members of the *ATG8* gene family were elevated in starchless mutant leaves.

Supplemental Figure S5. Higher RCB production activity in starchless mutant leaves.

Supplemental Table S1. List of primer sequences for qRT-PCR analysis.

Supplemental Data S1. Changes in metabolite contents caused by *atg5* mutation according to CE-TOFMS analysis.

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