Autophagy Negatively Regulates Cell Death by Controlling NPR1-Dependent Salicylic Acid Signaling during Senescence and the Innate Immune Response in Arabidopsis

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Autophagy is an evolutionarily conserved intracellular process for vacuolar degradation of cytoplasmic components. In higher plants, autophagy defects result in early senescence and excessive immunity-related programmed cell death (PCD) irrespective of nutrient conditions; however, the mechanisms by which cells die in the absence of autophagy have been unclear. Here, we demonstrate a conserved requirement for salicylic acid (SA) signaling for these phenomena in autophagy-independent mutants (atg mutants). The atg mutant phenotypes of accelerated PCD in senescence and immunity are SA signaling dependent but do not require intact jasmonic acid or ethylene signaling pathways. Application of an SA agonist induces the phenotypes in the defective mutants (unclear. Here, we demonstrate a conserved requirement for salicylic acid (SA) signaling for these phenomena in autophagy-independent Arabidopsis plants, suggesting that the cell death phenotypes in the atg mutants are dependent on the SA signal transducer NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1. We also show that autophagy is induced by the SA agonist. These findings imply that plant autophagy operates a novel negative feedback loop modulating SA signaling to negatively regulate senescence and immunity-related PCD.

INTRODUCTION

Autophagy is an intracellular degradation process that delivers cytoplasmic constituents to the vacuole/lysosome (Klionsky and Ohsumi, 1999; Klionsky, 2005, 2007). During the autophagic event, bulk cytoplasmic constituents and organelles are engulfed into a double membrane vesicle called an autophagosome. The outer membrane of the autophagosome then fuses to the vacuolar membrane, delivering an inner membrane structure called an autophagic body, into the vacuolar lumen for degradation by resident hydrolases. The autophagy-mediated degradation process has been well elucidated at the molecular level in yeast (Saccharomyces cerevisiae). Genetic analyses in yeast identified 18 autophagy-related (ATG) genes that are essential for autophagosome formation (Tsukada and Ohsumi, 1993; Thumm et al., 1994; Barth et al., 2001). Most of the ATG genes are well conserved across plant and animal kingdoms, suggesting that the molecular basis of the core autophagy machinery is essentially the same in higher eukaryotes, although four out of 18 ATG genes have not been identified in plants yet (Meijer et al., 2007). Indeed, Arabidopsis thaliana ATG-deficient mutants are defective in autophagy, confirming that ATG proteins are essential for plant autophagy (Bassham et al., 2006).

Arabidopsis atg mutants, such as atg7-1 (Doelling et al., 2002), atg9-1 (Hanaoka et al., 2002), atg4a4b-1 (Yoshimoto et al., 2004), atg5-1 (Thompson et al., 2005), atg10-1 (Phillips et al., 2008), ATG6-RNA interference (RNAi; Patel and Dinesh-Kumar, 2008), and ATG18a-RNAi (Xiong et al., 2005) are all defective in autophagy but are able to complete normal embryogenesis, germination, cotyledon development, shoot and root elongation, flowering, and seed production under normal nutrient-rich conditions. These plants are, however, hypersensitive to nutrient-limiting conditions, and transcripts of some of the ATG genes are induced during starvation (Rose et al., 2006; van der Graaff et al., 2006; Chung et al., 2009). Recently, we found that chloroplast-derived ribulose-1,5-bisphosphate carboxylase/oxygenase–containing bodies and whole chloroplasts are delivered to the vacuole and degraded by autophagy under nutrient-limiting conditions (Ishida et al., 2008; Wada et al., 2009). Based on these data, plant autophagy has been proposed to play an important role mainly in nutrient recycling.

There are several lines of evidence that plant autophagy may also have other functions. For instance, ATG proteins are expressed even under nutrient-rich conditions (Doelling et al., 2002; Hanaoka et al., 2002; Yoshimoto et al., 2004; Thompson et al., 2005; Phillips et al., 2008; Chung et al., 2009). In nutrient-rich conditions, several atg mutants exhibit an early senescence phenotype (Doelling et al., 2002). In addition, autophagic defects
can cause excessive immunity-related programmed cell death (PCD), irrespective of nutrient conditions (Liu et al., 2005; Patel and Dinesh-Kumar, 2008). Silencing of ATG6 in Nicotiana benthamiana resulted in reduced autophagy and spread of chlorotic cell death around the infection site after inoculation with Tobacco mosaic virus (TMV; Liu et al., 2005). This phenotype was not due to virus movement because transient expression of the elicitor protein (TMV-p50) alone was sufficient to induce the spread of chlorotic cell death in leaves of ATG6-silenced tobacco plants. A similar phenotype was also observed in ATG6-RNAi plants when infected with Pseudomonas syringae pv tomato bacteria DC3000 expressing avrRpm1 (Pst-avrRpm1) (Patel and Dinesh-Kumar, 2008); however, mechanisms underlying this phenomenon have not been elucidated, and, thus, physiological roles of plant autophagy under nutrient-rich conditions have still remained elusive.

Here, we investigated the mechanism that induces early senescence and excessive immunity-related PCD in the autophagy-defective plants under nutrient-rich conditions. By biochemical analysis, we found that salicylic acid (SA) accumulated to high levels in the atg2 and atg5 mutants. The early senescence in atg5 was suppressed by expressing NahG, which encodes an SA hydroxylase, to deplete endogenous SA, or by reducing SA was suppressed by expressing NahG atg2 analysis, we found that salicylic acid (SA) accumulated to high levels in 4-week-old plants grown on nutrient-rich soil under short-day conditions. The visible senescence phenotype was observed under long-day conditions, although the onset of visible senescence was later than under long-day conditions. The visible senescence phenotype was observed at around weeks 6 and 8 in atg2 and atg5, respectively. At these stages, wild-type plants were still green and healthy. In both long- and short-day conditions, atg2 exhibited a more severe phenotype than atg5 for unknown reasons. In addition to the senescence phenotype, the atg2 and atg5 mutants showed growth retardation (Figures 1A and 3B). These results suggest that autophagy has an additional function apart from the role in recycling of proteins to serve as a source of amino acids in plants.

**RESULTS**

**Autophagy-Defective Mutants Exhibit an Early Senescence Phenotype under Nutrient-Rich Conditions**

To investigate the function of autophagy in nutrient-rich conditions, we conducted phytohormone analyses in the wild type and atg5. Four-week-old plants grown on nutrient-rich soil under short-day conditions were used to minimize a potentially confounding influence of aging. Endogenous levels of SA, jasmonic acid (JA), jasmonic acid isoleucine (JA-Ile), gibberellin (GA), and isopentenyl adenine (iP), auxin (indole-3-acetic acid [IAA]), abscisic acid (ABA), cytokinin, and cytokinin derivatives (trans-zeatin [Tz], dihydrozeatin [DHZ], isopentenyladenine [IP], trans-zeatin riboside [iZR], and isopentenyl adenosine [iPR]) were measured using liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) (Table 1). We found that endogenous levels of SA were approximately threefold higher in atg5 than in the wild type. The increase of SA levels was much more apparent in older (7-week-old) plants rather than younger (4-week-old) ones (see Supplemental Figure 1 online). Moreover, levels of JA and JA-Ile were increased approximately twofold in atg5 mutants (Table 1). By contrast, IAA, ABA, and iP levels were only slightly increased in atg5 when compared with the wild type. No significant difference was observed for the other phytohormones in wild-type and atg5 plants.

To investigate the early senescence phenotype in the atg mutants at the transcriptional level, expression analyses were performed, especially focusing on senescence- and defense-related genes (Figure 2). We found that a senescence marker gene SAG12 was not at detectable levels in the 3-week-old wild-type and atg2 and atg5 mutants, whereas SAG12 transcripts accumulated to much higher levels in 4-week-old atg2 and to a lesser extent in atg5 than in the wild type. The timing of these alterations in transcript levels correlates with the onset of visible senescence phenotype in the long- or short-day conditions. Transcript levels of the SA-responsive defense markers, PR1 and PR2, were abundant in 3-week-old atg2 and atg5, whereas transcript levels of these genes in the wild type remained low at this stage, consistent with the observation that SA hyperaccumulated in the atg2 and atg5 mutants. Transcript accumulation of PDF1.2, a JA-responsive marker, was also higher in the atg2 and atg5 mutants at this stage, whereas the transcript levels of VSP2, another JA-responsive marker, and PAL, another defense-related marker, were similar in the wild type and the atg2 and atg5 mutants. Taken together, these data suggest that SA signaling is activated in the atg2 and atg5 mutants before the onset of a visible senescence phenotype.
The Senescence Phenotype in *atg2* and *atg5* Mutants Is SA Dependent but JA and Ethylene Independent

To investigate if the early senescence phenotype in the *atg* mutants is SA dependent, we generated *atg2* and *atg5* mutants expressing the heterologous *NahG* transgene. Plants overexpressing *NahG* (*NahG* plants) completely suppressed the early senescence phenotype in both *atg2* and *atg5* (Figure 3A; see Supplemental Figure 2A online). To further confirm the SA dependence of the senescence phenotype, the *atg2* and *atg5* mutants were crossed to two other SA-related mutants, *sid2* and *npr1*. *SID2* encodes an isochorismate synthase, a rate-limiting enzyme for SA biosynthesis (Wildermuth et al., 2001), whereas *NPR1* encodes an ankyrin repeat protein that acts downstream of SA and regulates *PR1* expression through a direct interaction with a TGA-type bZIP transcription factor (Dong, 2004). Similar to *NahG* plants, both *sid2* and *npr1* suppressed the early senescence phenotype in *atg2* and *atg5* (Figure 3B; see Supplemental Figure 2B online), although to a lesser extent in *sid2*, possibly due to the existence of other SA biosynthesis pathways.

Since JA and ethylene have been shown to modulate senescence pathways (Oh et al., 1997; He et al., 2002) and because *PDF1.2*, a JA marker, was induced in the *atg* mutants, we examined if the JA and ethylene pathways are also involved in the senescence phenotype in the *atg* mutants. For this purpose, we created *atg5 coi1*, *atg5 jar1*, and *atg5 ein2* double mutants. COI1 is an F-box protein that has a pivotal role in JA signaling, whereas JAR1 catalyzes the conjugation of JA to amino acids. Biological functions of EIN2 are still unclear, but it is required for general ethylene signaling. In contrast with *atg5 sid2*, *atg5 npr1*, and *NahG atg5* plants, these double mutants retained the early senescence phenotype in *atg5* even though JA and ethylene signaling were impaired (Figure 3B). Similar results were also obtained for the *atg2* -based double mutants, further supporting our hypothesis that JA and ethylene signaling are not required for

![Figure 1. Early Senescence Phenotype of Autophagy-Defective Mutants under Nutrient-Rich Conditions.](image)
the senescence phenotype in the atg2 and atg5 mutants (see Supplemental Figure 2B online).

**Pathogen-Induced Spread of Chlorotic Cell Death in atg5 Requires SID2 and NPR1**

Since autophagy deficiency leads to the spread of immunity-related PCD (Liu et al., 2005; Patel and Dinesh-Kumar, 2008), we investigated if this is also due to activation of the SA pathway. Infiltration of the avirulent bacterium *P. syringae* pv *tomato* bacteria DC3000 expressing *avrRpm1* (*Pst-avrRpm1*) into atg5 leaves led to the spread of chlorotic cell death, whereas in the wild type, cell death was limited within the infected site (Figure 3C; see Supplemental Figure 3 online), consistent with previous reports. This phenotype was clearly seen in the older leaves of 7- to 8-week-old plants grown in the short-day condition; however, we noticed that in the younger leaves or at an early stage (4 to 5 weeks), no clear difference was detected between wild-type and atg5 plants (data not shown). Thus, the phenotype is leaf age and plant age dependent. The growth of *Pst-avrRpm1* bacteria was not significantly different between wild-type and atg5 plants, and the bacteria were not found in tissues outside the infection site (see Supplemental Figure 4 online). We also found that infiltration of *Pst-avrRpm1* did not lead to the spread of chlorotic cell death in the atg5 sid2 and atg5 npr1 double mutants (Figure 3C; see Supplemental Figure 3 online). Therefore, we concluded that the pathogen-induced spread of chlorotic cell death in autophagy-deficient plants requires the activation of the SA signaling pathway.

**Dark-Induced Senescence and Growth Inhibition of Primary Roots under Nitrogen-Depleted Conditions Are Not Suppressed by Inactivation of SA Signaling**

Previous reports have shown that autophagy-defective mutants exhibited a dark-induced senescence phenotype and reduced growth rate of primary roots under nutrient-starved conditions (Doelling et al., 2002; Hanaoka et al., 2002; Surpin et al., 2003; Yoshimoto et al., 2004; Thompson et al., 2005; Xiong et al., 2005; Patel and Dinesh-Kumar, 2008; Phillips et al., 2008). Consistent with these reports, the atg2 and atg5 mutants induced senescence earlier than the wild type when 1-week-old seedlings were kept in the dark for 1 week. (Figure 4A, top and middle panels). In addition, detached leaves from 2-week-old atg5 kept in the dark for several days exhibited senescence, whereas those of the wild type still remained green (Figure 4D). In contrast with the early senescence phenotype, these phenotypes were not suppressed by overexpression of the NahG gene (Figures 4A and 4D, bottom panels). Furthermore, when seedlings were grown on nitrogen-depleted medium for 14 d, the growth rate of primary roots in atg5 was approximately half that of wild-type primary roots (Figures 4B and 4C). This phenotype was also not suppressed by overexpression of the NahG gene (Figures 4B and 4C). These results indicate that there are at least two different types of senescence phenotypes in the autophagy-defective mutants; one is an SA-dependent, developmentally controlled, early senescence phenotype and the other is an SA-independent, starvation- and dark-inducible phenotype.

**BTH Sensitivity in the Autophagy-Defective Mutants Is NPR1 Dependent**

To further characterize the SA-dependent senescence pathway in the atg mutants, the SA agonist BTH, which is not

### Table 1. Comprehensive Analysis of Phytohormones in Wild-Type and atg5 Mutant Plants

<table>
<thead>
<tr>
<th>Hormones (ng/gFW)</th>
<th>Wild Type</th>
<th>atg5</th>
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<tbody>
<tr>
<td>SA</td>
<td>53.3 ± 3.85</td>
<td>159.6 ± 32.2</td>
</tr>
<tr>
<td>JA</td>
<td>68.2 ± 28.1</td>
<td>141.9 ± 40.6</td>
</tr>
<tr>
<td>JA-Ile</td>
<td>5.50 ± 2.32</td>
<td>10.3 ± 4.13</td>
</tr>
<tr>
<td>GA1</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>GA4</td>
<td>0.11 ± 0.05</td>
<td>0.17 ± 0.09</td>
</tr>
<tr>
<td>IAA</td>
<td>5.00 ± 0.04</td>
<td>6.25 ± 0.25</td>
</tr>
<tr>
<td>ABA</td>
<td>5.28 ± 0.22</td>
<td>7.00 ± 0.21</td>
</tr>
<tr>
<td>tZ</td>
<td>0.43 ± 0.05</td>
<td>0.30 ± 0.01</td>
</tr>
<tr>
<td>DHZ</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>iP</td>
<td>0.05 ± 0.002</td>
<td>0.08 ± 0.001</td>
</tr>
<tr>
<td>tZR</td>
<td>1.44 ± 0.27</td>
<td>1.12 ± 0.15</td>
</tr>
<tr>
<td>iP1R</td>
<td>0.75 ± 0.11</td>
<td>0.94 ± 0.13</td>
</tr>
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Data represent the mean ± SD of three experiments. nd, not detected; FW, fresh weight.

**Figure 2.** Expression Patterns of Senescence- or Pathogen-Related Genes in Wild-Type and Autophagy-Defective Mutant Plants.

Total RNAs from leaves of wild-type, atg2, and atg5 plants grown on rockwool supplied with a rich nutrient solution for 3 to 4 weeks under long-day conditions were isolated and subjected to cycle-optimized RT-PCR using gene-specific primers and 18S rRNA as an internal control. SYBR-green was used for staining the gels. Gel pictures were rearranged for presentation purposes. Results were reproduced in three independent experiments.
Figure 3. Early Senescence and Excessive Immunity-Related PCD Phenotypes of Autophagy-Defective Mutants Suppressed by Inactivation of the SA Signaling Pathway.

(A) The NahG gene was introduced into atg5 by crossing. Photographs of 6-week-old plants of the indicated genotypes grown on vermiculite supplied with a rich nutrient solution under long-day conditions.

(B) The phenotype of the atg5 double mutants with sid2, npr1, coi1, jar1, and ein1. Photographs of 5-week-old plants grown on rockwool supplied with a rich nutrient solution under long-day conditions.

(C) The fifth to eighth leaves of each plant grown under short-day conditions for 8 weeks were infected with Pst-avrRpm1 (2 × 10^7 colony-forming units/mL) or 10 mM MgCl₂ (mock). Photographs were taken 9 d after infection. Results were reproduced in at least three independent experiments using four or more plants in each experiment.
catalyzed by NahG, was applied to NahG atg5 plants and to NahG and wild-type plants as controls. As shown in Figure 5A, application of BTH to the NahG atg5 plants restored senescence within a week, whereas wild-type and NahG plants were unaffected by this treatment. These results indicate that a BTH-inducible component(s) in the SA signaling pathway is required for the early senescence phenotype in the atg mutants. Since overexpression of NahG has a side effect due to accumulation of catechol, which is a by-product of SA degradation (Saskia and van Wees, 2003; van Wees and Glazebrook, 2003), atg5 sid2 plants were also treated with BTH to verify the above results. After BTH treatment, atg5 sid2 plants exhibited a senescence phenotype, whereas no effects were observed in sid2 (Figure 5B). Because most of the BTH-inducible genes are controlled by NPR1 (Wang et al., 2006), we investigated if the BTH effect on atg5 is suppressed by npr1. Unlike NahG atg5 and atg5 sid2 plants, atg5 npr1 double mutants did not show the early senescence phenotype after BTH treatments (Figure 5C). Thus, BTH hypersensitivity in the autophagy-defective mutants requires NPR1.
Autophagy-Defective Mutants Accumulate High Levels of Reactive Oxygen Species

Leaf senescence is a form of PCD that is often associated with pronounced accumulation of reactive oxygen species (ROS) (Overmyer et al., 2003). Thus, we monitored levels of hydrogen peroxide ($\text{H}_2\text{O}_2$) in $\text{atg}2$ and $\text{atg}5$, the SA-related mutants, and their double mutant combinations. Sixth or seventh leaves from 8-week-old plants grown under short-day conditions were stained with 3,3'-diaminobenzidine tetrahydrochloride (DAB), a histochemical reagent for $\text{H}_2\text{O}_2$ (Thordal-Christensen et al., 1997). Although these leaves showed no visible phenotypes (Figure 6, bottom panels), $\text{atg}2$ and $\text{atg}5$ leaves accumulated high levels of $\text{H}_2\text{O}_2$, whereas those of wild-type, $\text{NahG}$, $\text{sid}2$, and $\text{npr}1$ showed much weaker staining, except for the vascular tissue (Figure 6, top and middle panels). Interestingly, $\text{NahG atg}5$ and $\text{atg}5$ $\text{sid}2$ plants, in which the early senescence phenotype in the $\text{atg}$ mutant was suppressed, still exhibited enhanced accumulation of $\text{H}_2\text{O}_2$, although $\text{H}_2\text{O}_2$ levels appeared to be lower than those of $\text{atg}2$ or $\text{atg}5$ single mutants. In addition, $\text{npr}1$ $\text{atg}5$ plants also showed enhanced accumulation of $\text{H}_2\text{O}_2$ compared with $\text{npr}1$ plants (Figure 6, top panels). This accumulation of $\text{H}_2\text{O}_2$ in the $\text{npr}1$ $\text{atg}5$ was also lower than those of $\text{atg}$ single mutants. In summary, ROS accumulated in the $\text{atg}2$ and $\text{atg}5$ mutants in a partly SA-independent manner. These results suggest a partial role for autophagy in limiting ROS levels.

Figure 5. Phenotypes of BTH-Treated Wild-Type, $\text{NahG}$, $\text{NahG atg}5$, $\text{sid}2$, $\text{atg}5$ $\text{sid}2$, $\text{npr}1$, and $\text{atg}5$ $\text{npr}1$ Plants.

(A) Mock-treated (left) and BTH-treated (right) wild-type, $\text{NahG}$, and $\text{NahG atg}5$ plants 7 d after treatment. BTH (100 $\mu$M) was sprayed on 6-week-old plants grown under long-day conditions, and after 4 d it was repeated. Photographs were taken 3 d after the second BTH treatment. (B) and (C) BTH-treated $\text{sid}2$, $\text{atg}5$ $\text{sid}2$, $\text{npr}1$, and $\text{atg}5$ $\text{npr}1$ plants 10 d after treatment. BTH (100 $\mu$M) was sprayed on 7-week-old plants grown under short-day conditions, and after 4 d it was repeated. Then, after 6 d, photographs were taken. Results were reproduced in at least three independent experiments using four or more plants in each experiment.
Autophagy Is Induced by SA Agonist Treatment

Since plant autophagy appears to be involved in the negative regulation of SA signaling, we examined the effect of BTH in induction of autophagosome formation in wild-type plants. For this purpose, we used green fluorescent protein (GFP)-tagged ATG8a as a marker for autophagy (Yoshimoto et al., 2004; Contento et al., 2005; Thompson et al., 2005). Roots of 7-d-old seedlings expressing GFP-ATG8a grown on nutrient-rich Murashige and Skoog (MS) solid medium were transferred to MS liquid medium with or without 100 μM BTH for 8 h and then observed by fluorescence microscopy. Numerous green dots or ring structures, likely representing pre-autophagosome or autophagosome structures, were detected in root cells after the BTH treatment, and less of these structures were present in non-treated root cells (Figures 7A and 7B). The results were further confirmed by the following cytological method. When roots of wild-type seedlings were treated with BTH and E-64d, a Cys protease inhibitor that blocks degradation in vacuole, autophagic bodies accumulated more rapidly compared with roots treated with E-64d alone (see Supplemental Figure 5 online). As expected, accumulation of autophagic bodies was not found in BTH-treated atg2 and atg5 mutant roots (see Supplemental Figure 5 online). The induction of autophagy by the SA agonist appears to be NPR1 dependent because npr1 mutant roots did not show rapid accumulation of autophagic bodies after BTH treatment (see Supplemental Figure 5 online). These observations imply that autophagy can be induced by SA and suggest that plant autophagy positively acts to eliminate SA signaling (Figure 7C).

DISCUSSION

The autophagy-defective atg2 and atg5 Arabidopsis mutants exhibited both an early senescence and an excessive immunity-related PCD regardless of nutrient conditions, suggesting that plant autophagy has an important regulatory role in a prosurvival function during such cell death progressions. Here, using biochemical, pharmacological, and genetic approaches, we have clearly demonstrated that excessive SA signaling is a major factor in atg-dependent chlorotic cell death.

Autophagy under Nutrient-Rich Conditions

Since autophagy is induced under starvation conditions, its physiological role had been thought to be nutrient recycling in general. Indeed, intracellular free amino acid levels decrease in autophagy-defective yeast cells (Onodera and Ohsumi, 2005) and mice (Kuma et al., 2004) under nutrient-limiting conditions; however, recent studies in mammals have indicated that autophagy plays much broader roles in many biological processes,
such as development, anti-aging, elimination of microorganisms, cell death, tumor suppression, and antigen presentation (Mizushima, 2005). In this study, we present several lines of evidence that plant autophagy has a role distinct from nutrient recycling. We found that the \textit{atg2} and \textit{atg5} mutants exhibit an early senescence phenotype under both short-day and long-day conditions even when nutrients are sufficient (Figure 1). Furthermore, our biochemical and molecular dissection revealed that the \textit{atg5} mutants accumulate SA to high levels (Table 1) and express a senescence marker gene, \textit{SAG12}, and SA-responsive defense marker genes, \textit{PR1} and \textit{PR2} (Figure 2), before showing any microscopically visible phenotypes. Because there was no significant decrease in free amino acid levels in \textit{atg5} compared with those in the wild type (see Supplemental Table 1 online), starvation is not likely to be the cause of such phenotypes. In addition, even under nutrient-rich conditions, ATG proteins are expressed throughout all developmental stages (Doelling et al., 2002; Hanaoka et al., 2002; Yoshimoto et al., 2004; Thompson et al., 2005; Phillips et al., 2008; Chung et al., 2009). These results strongly support the notion that plant autophagy has additional functions other than just recycling proteins as a source of amino acids.

Figure 7. Negative Regulation of SA Signaling by Plant Autophagy.

\textbf{(A)} Autophagy is induced by BTH treatment. Roots of 7-d-old seedlings stably expressing GFP-ATG8a were excised and transferred to MS liquid medium with (right) or without (left) BTH (100 \text{ \textmu}M) for 8 h and then observed by fluorescence microscopy. Bars = 20 \text{ \mu}m.

\textbf{(B)} Quantification of autophagosome-related structures. Numbers of autophagosome-related structures per root section were counted and the average number was determined for seven seedlings per treatment. Error bars indicate the SD. Results were reproduced in three independent experiments. An asterisk indicates a significant difference (\textit{P} < 0.01; Student’s \textit{t} test).

\textbf{(C)} Hypothetical model for the role of autophagy during aging and immunity-related PCD. From this study, the following hypothetical model is proposed. During senescence and pathogen infection, SA signaling is accelerated by induction of SA biosynthesis, making an amplification loop through EDS1. Autophagy is induced by this SA signaling via NPR1 to operate a negative feedback loop modulating SA signaling that limits senescence and pathogen-induced chlorotic cell death. Based on Hofius et al. (2009) (*).
Autophagy Negatively Regulates Cell Death via the SA Signaling Pathway during Aging and the Innate Immune Response

Our findings demonstrated that autophagy negatively regulates SA signaling, which is required for the early senescence phenotype, as well as for the excessive immunity-related PCD phenotype in the atg mutants. The importance of SA in senescence has been demonstrated by a report that transcript levels of SAG12 and PR1 were reduced in NahG plants, pad4, or npr1, and these SA-related mutant plants showed a delayed senescence phenotype (Morris et al., 2000); however, SA or BTH treatment alone was insufficient to induce senescence or immunity-related PCD in wild-type, sid2, or NahG plants (Figure 5). By contrast, BTH was able to induce the atg-dependent early senescence phenotype that was suppressed within the NahG or sid2 background (Figure 5). Thus, apart from SA, there must be other factors regulating early senescence in the atg2 and atg5 mutants. These factors should function with NPR1 or its downstream component (s) because BTH is not able to override the npr1-dependent suppression of the early senescence in atg5.

A possible candidate for such factors is ROS since we found that autophagy also negatively regulates ROS accumulation. In yeast and mammals, cells lacking autophagy accumulate dysfunctional mitochondria, resulting in an increase in ROS production (Zhang et al., 2007; Tal et al., 2009). In Arabidopsis, however, high levels of H2O2 in the atg2 and atg5 mutants might not be due to dysfunctional mitochondria. Instead, high ROS accumulation in the atg2 and atg5 mutants was reduced to nearly one-half in the NahG and sid2 background plants (Figure 6). Thus, ROS accumulated by both SA-dependent and SA-independent mechanisms in atg2 and atg5 mutant plants. Since increasing ROS levels induce SA biosynthesis (Neuenschwander et al., 1995, Takahashi et al., 1997, Chammongol et al., 1998), SA and ROS are likely to form a positive amplification loop (Shah, 2003; Figure 7). The cellular redox state modulated by SA-ROS amplification can be sensed by NPR1 (Mou et al., 2003). This observation can explain why chlorotic cell death in the atg2 and atg5 mutants is suppressed by npr1, and even exogenous application of BTH is not able to recover the phenotype.

What could be the physiological role(s) of autophagy during cell death in plants? The fact that SA signaling is activated in the atg2 and atg5 mutants led us to hypothesize that autophagy is induced to eliminate SA signaling during aging and immunity-related PCD. Using an autophagosome marker, GFP-ATG8a, a number of autophagosomes were detected in wild-type root cells after 8 h of BTH treatment, but significantly fewer autophagosomes were observed in nontreated cells (Figures 7A and 7B), implying that autophagy is induced by SA. This result is consistent with an earlier report that autophagy is induced by pathogen infections (Liu et al., 2005). In addition, although the molecular mechanism is still unclear, autophagy can be induced by H2O2 in Arabidopsis (Xiong et al., 2007). In mammalian cells, autophagy can also be induced by H2O2 via ATG4 protein activity (Scherz-Shouval et al., 2007). These findings suggest a hypothetical model in which autophagy actively downregulates SA-ROS amplification signaling that controls cell death during aging and immunity-related PCD (Figure 7C).

Autophagy-Defective Mutants Are Similar to mlo2, a Powdery Mildew–Resistant Mutant, with Regard to the Age-Related Early Cell Death Phenotype

While carrying out the phenotypic analyses, we noticed similarities in the phenotypes between atg2 and atg5 and the mlo2 mutants in Arabidopsis. Arabidopsis mlo2 exhibits resistance to a virulent powdery mildew pathogen, Golovinomyces orontii (Consonni et al., 2006). In addition to resistance to powdery mildew, mlo2 also shows a developmentally controlled early senescence phenotype that is accompanied by the accumulation of H2O2. Reminiscent of atg2 and atg5 mutants, the mlo2-mediated early senescence phenotype is suppressed by inactivation of SA signaling but not by inactivation of JA or ethylene signaling. In addition, mlo2 exhibits a developmentally controlled increase in SA levels that precedes the onset of the visible senescence phenotypes. Furthermore, we found that, similar to atg2 and atg5 mutants, BTH treatments restore the mlo2-dependent early senescence phenotype in the NahG or sid2 background (see Supplemental Figure 6 online). Similarities between atg5 and mlo2 were further highlighted by the fact that the early senescence phenotype in both atg5 and mlo2 was accelerated by pen1 and partially suppressed by pen2 (see Supplemental Figure 7 online; Consonni et al., 2006); however, unlike mlo2, atg2 and atg5 mutants did not exhibit significant resistance to powdery mildew (see Supplemental Figure 8 online), indicating that these mutants are similar to mlo2 only with regard to the early senescence phenotype. In fact, powdery mildew resistance in mlo2 was not dependent on SA (Consonni et al., 2006). Similar to its barley (Hordeum vulgare) ortholog, MLO2 is thought to function as a modulator of SNARE protein-dependent and vesicle transport-associated processes at the plasma membrane (Panstruga, 2005). Consequently, loss of MLO2 may cause the accumulation of particular vesicle populations that result in the age-related early cell death phenotype. In this scenario, autophagy may be involved in degradation of such vesicles, and the normal level of autophagy may not be sufficient for removing an increased number of vesicles, thus leading to similar senescence phenotypes.

Does Autophagy Have a Pro-Death Function in Plants?

Morphological studies suggested that autophagic processes execute developmental PCD in plants (Matile and Winkenbach, 1971; Smith et al., 1992; Filonova et al., 2000). However, various atg mutants that completely lack autophagy could achieve normal embryogenesis, seed germination, xylem differentiation, root development, and petal senescence, processes that were previously assumed to involve autophagic cell death (Lam, 2004; Bozhkov et al., 2005; van Doorn and Woltering, 2005, 2008). Recently, Hofius et al. (2009) proposed that autophagy has a pro-death function during Pst-avrRps4–induced cell death in Arabidopsis, as partially reduced cell death rates, measured by an ion leakage assay, were detected in autophagy-defective mutants. The pro-death function appears to be very specific as only minor or no reduction of cell death was observed when Pst-avrRpm1 or Pst-avrRpt2 was used. Interestingly, however, both Pst-avrRps4 and Pst-avrRpm1 clearly induced cell death lesions at the...
infection site, and no spread of chlorotic cell death was observed (Hofius et al., 2009). This is markedly different from what we and others observed (Figure 3C; Liu et al., 2005). This apparent discrepancy can be explained by our observation that the spreading cell death phenotype of the atg2 and atg5 mutants was only seen in older leaves of older plants, not in younger plants or younger leaves. The age dependency is likely due to the developmentally controlled increase of SA levels in the atg2 and atg5 mutants (see Supplemental Figure 1 online). It is possible that in case of Pst-avrRps4, proper SA levels are critical to fully execute cell death. Consistent with this possibility, EDS1, a modulator of SA amplification signal, is important for Pst-avrRps4-dependent SA induction (Feyts et al., 2001) as well as for Pst-avrRps4-dependent autophagy induction (Hofius et al., 2009). These facts suggest that SA can induce autophagy, further supporting our model (Figure 7C).

In mammalian cells, autophagy can also be a part of the cell death mechanism (Levine and Yuan, 2005). The interaction between anti-apoptotic Bcl2 protein and ATG6/Bcl11 governs the threshold for transition from cell survival to cell death (Pattinck et al., 2005). In most of these cases, apoptosis-associated proteins, such as caspases, Bax, Bak, and Bcl2, were related to these autophagic cell death phenotypes; however, orthologs of these proteins have not been identified at the primary sequence level in plant genomes. Thus, unlike in mammals, plant autophagy may not have such a pro-death function. However, we cannot exclude the possibility that excessive autophagy promotes programmed cell death under some specific condition in plants, since plants might have functional homologs of apoptosis-related proteins. Further analyses using NahG atg, atg sid2, or atg np1 plants, in which the early senescence phenotype is suppressed, and further identification of functional homologs of apoptosis-related proteins would help to unravel the potential pro-death function in plant autophagy.

METHODS

Plant Material and Growth Conditions

A. thaliana ecotype Columbia was used in this study. The seeds of T-DNA knockout mutants of ATG2 and ATG5 (atg2-1 [SALK_076727] and atg5-1 [SAIL_129B07], respectively) were obtained from the Nottingham Arabidopsis Resource Center. Homozygous plants were isolated by PCR as described by Inoue et al. (2006). For long-day conditions, plants were grown on rockwool or vermiculite at 22°C with 16-h-light/8-h-dark cycles using hydroponic media for nutrients. For short-day conditions, plants were grown on soil at 22°C with 8-h-light/16-h-dark cycles. For plate-grown plants, seeds were surface sterilized, chilled at 4°C for 4 d, and then sown and grown on MS or nitrogen-depleted MS medium (MS-N). The MS-N medium was prepared by replacing the KNO3 with KCl.

Quantification of Phytohormones

Phytohormones were quantified using a 6410 Triple Quad LCMS (Agilent) that includes an Agilent 1200 series rapid resolution liquid chromatography system fitted with a ZORBAX Eclipse XDB-C18 column (1.8 μm, 2.1 × 50 mm), d5-GA3, d5-GA4, d5-iP, d5-iPR, d5-tZR, and d5-tZ obtained from OICheml, d5-ABA obtained from Icon isolopes, d5-IAA and d5-SA obtained from Sigma-Aldrich, d5-JA obtained from Tokyo Kasei, and synthesized 13C6-JA-Ile (Jikumaru et al., 2004) were used as internal standards. Aerial parts of Arabidopsis grown for 4 weeks were frozen with liquid nitrogen, ground with 10-mm ceramic beads, and extracted with 5 volumes of 80% (v/v) methanol containing 1% (v/v) acetic acid and internal standards for 1 h. Extracts were centrifuged at 4°C, 14,000g, 10 min, and the supernatant was collected. This procedure was repeated once, and methanol was removed in a SpeedVac (Thermo Fisher). Acidic water extracts were loaded onto an Oasis HLB extraction cartridge (30 mg, 1 mL; Waters) and washed with 1 mL of water containing 1% acetic acid to separate high polar impurities. Phytohormones were eluted with 2 mL of 80% methanol containing 1% acetic acid, and the methanol in this eluent was removed in a SpeedVac. Acidic water extracts were loaded onto an Oasis MCX extraction cartridge (30 mg, 1 mL). After washing with 1 mL of water containing 1% acetic acid, acidic and neutral compounds were eluted with 2 mL of methanol (AN fraction). After washing with water containing 5% (v/v) ammonia, basic compounds were eluted with 2 mL of 60% (v/v) methanol containing 5% (v/v) ammonia. After drying these basic fractions, 20 μL of water containing 1% acetic acid was added and the basic hormones (I2 and I3) in these fractions were analyzed by LC-MS/MS. Subfractions (10%) of the AN fractions were collected and prepared as basic fractions to analyze for SA. The remaining 90% of the AN fractions were concentrated to acidic water in the SpeedVac to remove methanol and loaded onto Oasis WAX extraction cartridges (30 mg, 1 mL). After washing with 1 mL of water containing 1% acetic acid, neutral compounds were eluted with 2 mL of methanol, and acidic compounds were eluted with 2 mL of 80% methanol containing 1% acetic acid. After concentrating these acidic fractions to dryness, 20 μL of water containing 1% acetic acid was added and acidic hormones (I2A, I2B, I3, A1, GA1, and GA2) in these fractions were analyzed. LC conditions and parameters for LC-ESI-MS/MS analysis are described in Supplemental Tables 2 and 3 online.

Expression Analyses

To examine the expression of senescence- and defense-related genes, such as SAG12, PR1, PR2, PDF1.2, VSP2, and PAL1, total RNA was isolated from leaves using the RNeasy plant mini kit (Qiagen). The isolated total RNA was treated with DNase (Takara) prior to the synthesis of first-strand cDNA by the SuperScript III first-strand synthesis system with oligo(dT)20 primer (Invitrogen). cDNA derived from 0.2 μg of total RNA was used as a template for each PCR reaction. Gene-specific primers used are as follows: for SAG12, 5′-CTTTTGCAGAACAGCTGGT-3′ and 5′-ATGGCAAGACCACATAGTCC-3′; for PR1, 5′-CTGTCCTGAGTCTGTGACC-3′ and 5′-ATGGAGATGCTTCAGACCTCATCCACC-3′; for PDF1.2a, 5′-ATGCGCTGATGTCCAACCCCTATC-3′ and 5′-ATGGGACGTTACAGTACACTTGTTGCGG-3′; for PR2, 5′-ATGGCTAAGTTTGCTTCCATCATCACCCTTATC-3′ and 5′-ATGGGACGTTACAGTACACTTGTTGCGG-3′; for VSP2, 5′-CTTTACTCTCCCTTCTGTTCTTGCCCTACCT-3′ and 5′-AGTGATTGTGGAGCTTAAACCCCTCCC-3′; for PAL1, 5′-ATGGAGATTAACGGGGCACAACAGAACGCGG-3′ and 5′-CAAGACGGTCAAGATTTGGGAGCGACGAGATTAG-3′ and QuantumRNA 18S internal standards for 18S rRNA (Ambion). PCR was terminated after 24 cycles for SAG12, PR1, PR2, PDF1.2a, VSP2, PAL1, and 20 cycles for 18S rRNA to ensure that the RT-PCR was in the logarithmic amplification range. The products were electrophoresed on a gel containing SYBR Green I (Takara) and detected using a CCD camera with SYBR Green fluorescence filter.

Genetic Analyses

Homozygous NahG in ecotype Col-0 (B15; Delaney et al., 1994), obtained from Syngenta Biotechnology, was crossed to atg2 and atg5, respectively. The F3 seeds were sown on MS medium, and then atg2 and atg5

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mutants homozygously expressing the NahG gene were identified by PCR using gene-specific primers. Gene-specific primers used are as follows: for verifying atg2 mutant, 5'-GCTTGAGCCGCTTGTGCACT-3' designated as LB1, and 5'-GACTGACTATTTTGGTCA-3' and 5'-CATCAGGATTTGGCCTAAC-3'; for atg5 mutant, 5'-TAGCATCTGATTTCTCATACAACTGACTGACA-3' and 5'-TTGGTCATGCAAGGATA-AGCG-3'; for NahG, 5'-ATGAAAAAACATATAACTTGGCTGCGC-3' and 5'-CTGCTCTAGGGCGCGACAGCA-3'.

Arabidopsis mutant alleles sid2-2 (Dewdney et al., 2000), npr1-1 (Cao et al., 1997), jar1-1 (Staswick et al., 2002), coi1-1 (Xie et al., 1998), and ein2-1 (Alonso et al., 1999) were used for mutant crosses with either atg2 or atg5. atg sid2 was identified by PCR using the following gene-specific primers: 5'-TTCTTCAATGAGGAGGAGGAG-3' and 5'-CAGCCAAGGCGTACCAGC-3' and 5'-AGCAGAAAATGTTTGAGTCAGCA-3'. Only the 879-bp fragment was amplified from homozygous Col-0, two fragments (879 and 581 bp) were amplified from heterozygous plants, and only the 581-bp fragment was amplified from homozygous sid2-2. atg npr1 was created by cleaved-amplified polymorphic sequence (CAPS) selection of npr1-1 homozygous F3 families by use of the Nia III polymorphism of npr1-1. atg jar1 was selected with CAPS using Hpy188 III polymorphism. For isolation of atg coi1, first atg/atg coi1/coi1 was selected by CAPS using XcmI polymorphism as described before (Xie et al., 1998). Then, double mutants segregating from the atg/atg coi1/coi1 were used for phenotypic analyses. atg ein2 was confirmed by selection of kanamycin-resistant F2 seedlings insensitive to 500 μM 1-aminoacyclopropane-1-carboxylic acid. After the selection, sequencing of ein2 in atg ein2 confirmed that the mutant was homozygous for the ein2-1 allele.

Phenotypic Analyses

Phenotypic analyses were performed as described previously (Hanaoka et al., 2002; Yoshimoto et al., 2004), except for examination of pathogen infection. For observation of the pathogen-induced cell death phenotype, seeds were sown on soil and grown under short-day conditions for 8 weeks, and then Pst-avrRpm1 (2 × 10^7 colony-forming units/mL) or 10 mM MgCl2 (mock) was hand-infiltrated using a needleless syringe. After the infection, plants were transferred to continuous light conditions for 9 d and then photographed after M for 8 h and then observed with a Zeiss laser scanning microscope (LSM 510, version 3.2) and Adobe Photoshop Element 5.0 (Adobe Systems).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: ATG2 (At3g19180), ATG5 (At5g17290), SAG12 (At5g45880), PR1 (At2g14610), PR2 (At3g57260), PDF1.2a (At5g44420), VSP2 (At5g24770), PAL1 (At2g37040), SIS2 (At1g74710), NPR1 (At1g64260), JAR1 (At2g43370), COI1 (At2g39940), EIN2 (At5g32380), and MLO2 (At1g11310).

Supplemental Data

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

Supplemental Figures 1. The atg5 Mutant Shows a Developmentally Controlled Increase in SA Levels.

Supplemental Figure 2. Early Senescence Phenotype of Autophagy-Defective Mutants Was Suppressed by Inactivation of the SA Signaling Pathway.

Supplemental Figure 3. Pathogen-Induced Chlorotic Cell Death Suppressed by SA Signaling Pathway.

Supplemental Figure 4. Bacterial Growth Analysis of Pst-avrRpm1 in Wild-Type and atg5 Plants.

Supplemental Figure 5. Autophagy Is Induced by BTH Treatment.

Supplemental figure 6. Phenotypes of BTH-Treated mlo2-5, mlo2-11, NahG mlo2-5, NahG mlo2-11, and mlo2-11 sid2 Plants.

Supplemental Figure 7. Early Senescence Phenotype of Autophagy-Defective Mutants Was Accelerated by pen1 and Partially Suppressed by pen2.

Supplemental Figure 8. The atg Mutants Do Not Show Obvious Resistance against a Powdery Mildew, G. orontii. Unlike mlo2.

Supplemental Table 1. Relative Levels of Free Amino Acids on Wild-Type and atg5 Mutant Plants.

Supplemental Table 2. LC Conditions.

Supplemental Table 3. Parameters for LC-ESI-MS/MS Analysis (Agilent 1200-6410).

Supplemental Methods.

Supplemental References.

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Role of Autophagy in Cell Death
Autophagy Negatively Regulates Cell Death by Controlling NPR1-Dependent Salicylic Acid Signaling during Senescence and the Innate Immune Response in *Arabidopsis*

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