Pollination induces autophagy in petunia petals via ethylene

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Abstract

Autophagy is one of the main mechanisms of degradation and remobilization of macromolecules, and it appears to play an important role in petal senescence. However, little is known about the regulatory mechanisms of autophagy in petal senescence. Autophagic processes were observed by electron microscopy and monodansylcadaverine staining of senescing petals of petunia (Petunia hybrida); autophagy-related gene 8 (ATG8) homologues were isolated from petunia and the regulation of expression was analysed. Nutrient remobilization was also examined during pollination-induced petal senescence. Active autophagic processes were observed in the mesophyll cells of senescing petunia petals. Pollination induced the expression of PhATG8 homologues and was accompanied by an increase in ethylene production. Ethylene inhibitor treatment in pollinated flowers delayed the induction of PhATG8 homologues, and ethylene treatment rapidly upregulated PhATG8 homologues in petunia petals. Dry weight and nitrogen content were decreased in the petals and increased in the ovaries after pollination in detached flowers. These results indicated that pollination induces autophagy and that ethylene is a key regulator of autophagy in petal senescence of petunia. The data also demonstrated the translocation of nutrients from the petals to the ovaries during pollination-induced petal senescence.

Key words: autophagy, ethylene, flower, nutrient remobilization, petunia, pollination, senescence.

Introduction

Petals in flowering plants senesce and are eventually shed after pollination or at a certain period after flower opening, regardless of pollination. As petals serve to attract potential pollinators, once pollination is accomplished or a flower is no longer receptive to pollination, petals become tissues that are no longer needed and are costly to maintain (Stead, 1992). Pollination dramatically accelerates petal senescence in many plant species, including petunia (Petunia hybrida), and is accompanied by a burst of ethylene production (Borochov and Woodson, 1989). This endogenous ethylene production has been shown to mediate pollination-accelerated petal (corolla) senescence in petunia (Whitehead et al., 1984; Singh et al., 1992; Jones, 2008). In plant species showing ethylene-dependent petal senescence, exogenous ethylene treatment accelerates the increase in ethylene production and petal senescence, and a chemical or genetic inhibition of ethylene biosynthesis or perception delays petal senescence (Woltering and van Doorn, 1988; Borochov and Woodson, 1989; Shibuya and Clark, 2006).

During petal senescence, cellular constituents are degraded, and nutrients are believed to be recycled for reallocation to developing tissues. In support of this recycling function, the nitrogen, phosphorus, and potassium content of petunia petals is significantly reduced during pollination-induced senescence (Chapin and Jones, 2007). Many of the senescence upregulated genes that have been identified from petals encode catabolic enzymes that are probably involved in the breakdown and relocation of cellular constituents (van...
Doorn and Woltering, 2008). In petunia, it has been shown that protease and nuclease (PhNUC1) activities increase during petal senescence and that ethylene modulates the timing of the upregulation of cysteine protease genes and nuclease activity (Jones et al., 2005; Langston et al., 2005). Chapin and Jones (2009) also reported that the expression of phosphate transporter (PhPT1) is upregulated during petal senescence of petunia under the control of ethylene.

Autophagy is a conserved system that degrades intercellular components and, thus, seems to play an essential role in the degradation and remobilization of macromolecules during petal senescence. In the senescing petals of common morning glory (Ipomoea purpurea), Matile and Winkenbach (1971) observed numerous vesicles and cytoplasmic components in the vacuoles by electron microscopy that are indicative of autophagic processes. We showed previously that the percentage of cells containing autophagic structures increases in senescing petals of Japanese morning glory (Ipomoea nil), which indicates that autophagic activity increases during petal senescence (Shibuya et al., 2009).

Autophagy consists of the two major fluxes of macro- and microautophagy in plants (Thompson and Vierstra, 2005; Bassham et al., 2006). In macroautophagy, proteins of the cytosol are encapsulated in double-membrane vesicles called autophagosomes and then transported to the vacuoles. The outer membranes of autophagosomes fuse with the tonoplast, and the internal vesicle is released as autophagic bodies for degradation. Microautophagy, by contrast, involves sequestration of the cytoplasm by invagination of the tonoplast. Several homologues of budding yeast (Saccharomyces cerevisiae) autophagy-related genes (ATG) have been isolated in Arabidopsis thaliana (Doelling et al., 2002; Hanaoka et al., 2002). One of the ATG proteins, ATG8, is an ubiquitin-like peptide tag that is necessary for autophagosome formation (Ohsumi, 2001). Previous studies have shown that ATATG8 mRNA levels increase in senescing leaves of Arabidopsis (Doelling et al., 2002; Yoshimoto et al., 2004; Thompson et al., 2005). We reported previously that the transcript levels of ATG8 homologues increase during petal senescence in Japanese morning glory (Shibuya et al., 2009, 2011). However, the regulatory mechanisms of autophagy in petal senescence remain largely unknown.

In this study, we showed that autophagy is induced by pollination and that ethylene regulates its induction during petal senescence in petunia. We also discuss the role of autophagy in the recycling of nutrients in petal senescence.

Materials and methods

Plant material and treatments

Petunia hybrida cv. Mitchell Diploid were grown in commercial potting soil (Kureha soil; Kureha Chemical, Tokyo, Japan) in 12 cm pots with fertilization with 1 g l⁻¹ of Hyponex 15-30-15 (Hyponex Japan, Osaka, Japan) twice a week. The plants were grown in a glass greenhouse (15 °C minimum and 25 °C maximum) permitting sunlight irradiation in Tsukuba (E 140° 05’, N 36° 02’) from March to May or from September to November. All flowers used in experiments were emasculated just before anthesis to prevent self-pollination. At anthesis, flowers were pollinated and kept on the plant or detached and placed in vials containing distilled water or treatment solution and then pollinated. Detached flowers were kept in an incubator at 23 °C, 70% relative humidity in a 12h/12h (light/dark) photoperiod at 10 μmol m⁻² s⁻¹ with white-fluorescent lamps unless indicated otherwise.

For ethylene treatment of unpollinated flowers, detached flowers were sealed in a chamber with 2 μl l⁻¹ of ethylene for 4, 16 or 24 h. For 1-methylecyclopropene (1-MCP) plus ethylene treatment of unpollinated flowers, detached flowers were sealed in a chamber with 2 μl l⁻¹ of 1-MCP (EthylBloc™ Sachet; Floralife, Walterboro, SC, USA) for 24 h, followed by 1 h in air to allow the accumulated 1-MCP to defuse from the tissues. The 1-MCP-treated flowers were then placed in a chamber with 2 μl l⁻¹ of ethylene for 16 h, followed by 24 h in air. For the control, flowers were kept in air for the same period (65h). During the ethylene and 1-MCP treatments, chambers were held under continuous light at 10 μmol m⁻² s⁻¹. For 1-MCP treatment of pollinated flowers, detached flowers were pollinated and then sealed in a chamber with 2 μl l⁻¹ of 1-MCP for 10 d. Chambers were opened every 24 h to sample petals and replenish 1-MCP.

For concanamycin A (Wako, Osaka, Japan) treatment, detached flowers were placed in 5 μM concanamycin A solution in vials and then pollinated. Concanamycin A is a vacuolar H⁺-ATPase inhibitor that inhibits vacuolar hydrolase activity by increasing the internal vacuolar pH (Drose et al., 1993), thereby preventing vacuolar protein degradation, which prevents autophagic flux (Yoshimoto et al., 2004; Bassham et al., 2006; Klionsky et al., 2012; Yoshimoto, 2012). Concanamycin A was prepared as a 100 μM stock solution in absolute dimethyl sulphoxide. For the control, flowers were placed in distilled water containing dimethyl sulphoxide at the same concentration as for the treatment group. Both groups were incubated for 3 d.

Electron microscopy

To visualize autophagic processes in petunia petals, petal limbs were observed with transmission electron microscopy. Detached flowers were treated with concanamycin A prior to observation in order to enhance the visualization of autophagic bodies by inhibiting the degradation of vacuolar proteins (Yoshimoto et al., 2004; Bassham et al., 2006; Yoshimoto, 2012). Flowers detached immediately prior to anthesis were placed in vials of distilled water, pollinated or not, and then held for 24 h. Pollinated and unpollinated flowers were transferred to vials containing 5 μM concanamycin A and held in the incubator for 24 h.

Electron microscopy analysis was performed on sections prepared from at least two different pollinated or unpollinated flowers. Petal limbs of the flowers were fixed with 4% paraformaldehyde and 2% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) at 4 °C overnight. After fixation, samples were rinsed with 0.05 M cacodylate buffer, followed by post-fixation with 2% osmium tetroxide in 0.05 M cacodylate buffer at 4 °C for 3 h. Dehydrated specimens were embedded in epoxy resin (Quetol-651; Nisshin EM, Tokyo, Japan). Ultra-thin sections (70 nm) were made with a diamond knife using an ultramicrotome (Ultracut UCT; Leica, Wetzlar, Germany) and sections were placed on copper grids. Sections were stained with 2% uranyl acetate at room temperature for 15 min and rinsed with distilled water, followed by secondary staining with lead stain solution (Sigma-Aldrich, St Louis, MO, USA) at room temperature for 3 min. The sections were observed under a transmission electron microscope (JEM-1200EX; JEOL, Tokyo, Japan) at an acceleration voltage of 80 kV. Digital images were taken with a CCD camera (Veleta; Olympus, Tokyo, Japan).

Monodansylecadaverine (MDC) staining

Autophagic structures were visualized by fluorescent MDC staining (Contento et al., 2005). Petals were sampled from at least five different flowers for each treatment. Petal limbs were separated into epidermis and mesophyll cells with forceps and stained with 0.05 mM MDC (Sigma-Aldrich) in PBS supplemented with 0.4 M mannitol.
for 10 min at room temperature, and then washed twice with PBS containing 0.4 M mannitol. MDC-stained tissues were visualized by fluorescence microscopy (AX70; Olympus) with a narrow band-pass filter for 4’,6-diamino-2-phenylindole (U-MNUA2; Olympus). Bright-field and fluorescence images were taken with a digital camera (DP30BW; Olympus).

Ethylene measurement

Petals were placed individually in 15-ml glass test tubes, sealed and incubated at 23 °C for 1 h. Headspace gas samples (1 ml) were taken and injected into a gas chromatograph (GC-7A, Shimadzu, Kyoto, Japan) equipped with an alumina column and a flame ionization detector.

Identification of ATG8 homologues in petunia

To identify the ATG8 homologues in petunia, a BLAST search was performed on the petunia expressed sequence tag (EST) database at the Sol Genomics Network (http://solgenomics.net/) using sequences of Arabidopsis ATG8 genes (AtATG8a, At4g21980; AtATG8b, At4g04620; AtATG8c, At1g62040; AtATG8d, At2g05630; AtATG8e, At2g45170; AtATG8f, At4g16520; AtATG8g, At3g60640; AtATG8h, At3g06420; and AtATG8i, At3g15580). cDNA clones of petunia ATG8 homologues were obtained by RT-PCR of the identified ESTs and fully sequenced. Sequence alignment of ATG8 homologues and phylogenetic analysis were performed with petunia ATG8 (PhATG8), AtATG8, and budding yeast ATG8 (ScATG8; GenBank accession o. AY692870) using the ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and Phylogeny.fr (http://www.phylogeny.fr/; Dereeper et al., 2008) programs.

Quantitative real-time RT-PCR

Total RNA was isolated from petal limbs using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and treated with cloned DNase I (Takara Bio, Otsu, Japan). Synthesis of cDNA was carried out with random hexamer primers using the SuperScript III first-strand synthesis system for RT-PCR (Invitrogen). PCR was performed using SYBR Premix Ex Taq II (Takara Bio) with a Thermal Cycler Dice Real Time System (TP600, Takara Bio). Thermocycling conditions were 95 °C for 10 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Primers for real-time RT-PCR were designed to target the 3'-untranslated region of target genes with the Primer3 program (http://frodo.wi.mit.edu/; Table S1, at JXB online). Melter curves were generated to check amplification specificity, and relative target gene expression was normalized to PhACTIN expression for each cDNA sample, as described by Chapin and Jones (2009). Mean values from three separate experiments were graphed.

Nutrient analysis

Petals, ovaries, and receptacle with sepals were collected from 30 flowers from each treatment. Tissues were dried at 80 °C for 2 d and dry weights were taken. For nutrient analysis of each tissue, dried samples from ten flowers were combined and ground with a mortar and pestle. Total nitrogen content analysis was conducted on three samples from ten flowers were combined and ground with a mortar and pestle. Total nitrogen content analysis was conducted on three separate experiments were graphed.

Results

Autophagy in senescing petals

Petunia flowers that were emasculated and left to age on the plant exhibited petal wilting at 9–10 d after anthesis, while flowers pollinated at anthesis showed petal wilting at 2–3 d after pollination (Fig. 1A). Petunia petals consisted of adaxial and abaxial epidermis with one layer of cells and mesophyll cells, which form net-like layers with large intercellular spaces between epidermal layers. Vascular bundles dotted the petal mesophyll.

To examine whether autophagy occurs in senescing petals, petal limbs of pollinated and unpollinated petunia flowers were observed with transmission electron microscopy following treatment with concanamycin A. If autophagy occurs in plant cells, this treatment is known to lead the accumulation of autophagic bodies in the vacuole by inhibiting the degradation of vacuolar proteins and thus enhances the visualization of autophagic flux (Yoshimoto et al., 2004; Bassham et al., 2006; Yoshimoto, 2012). This chemical has been used to visualize autophagic processes in Arabidopsis (Yoshimoto et al., 2004; Thompson et al., 2005). In the mesophyll cells of petals located between vascular bundles from pollinated flowers at 2 dap, which showed petal wilting, spherical structures or granules were occasionally observed in the vacuoles, and some spherical bodies contained cytoplasmic structures (Fig. 1B). The vacuoles of some cells appeared to have lost membrane integrity in these flowers. In contrast, the vacuoles in mesophyll cells of petals from unpollinated flowers at 2 d after anthesis contained few of these structures (Fig. 1B). At the edges of the vascular bundles, cells containing vacuoles of various sizes were observed, and the vacuoles contained many vesicles and granules in pollinated flowers at 2 dap (Supplementary Fig. S1 at JXB online).

To analyse further the autophagic processes in senescing petals of petunia, petal limbs were stained with the fluorescent dye MDC. Many MDC-stained structures were observed in mesophyll cells of petals from pollinated flowers at 2 and 3 dap, but few were observed at 0 and 1 dap (Fig. 1C, Supplementary Fig. S2 at JXB online). These structures were also observed in epidermal cells of senescing flowers but to a lesser extent. MDC-stained structures were observed in the mesophyll cells of petals in unpollinated flowers showing petal wilting at 10 d (data not shown). These findings indicated that autophagy occurs in the senescing petals of petunia.

ATG8 gene family in petunia

A BLAST search of the petunia EST database identified four AtATG8 homologues: PhATG8a (GenBank accession no. AB721297), PhATG8b (AB721298), PhATG8c (AB721299), and PhATG8d (AB721300). Phylogenetic analysis of the full-length ATG8 proteins revealed that PhATG8a–c clustered together (Supplementary Fig. S3 at JXB online). PhATG8d was grouped with AtATG8b and AtATG8i in another clade. All of the PhATG8 members in both clades contained Gly in the carboxyl-terminal region, which is conserved among ATG8 proteins (Supplementary Fig. S4 at JXB online).

Induction of PhATG8 homologues by pollination

Unpollinated flowers exhibited petal wilting at 9–10 d after anthesis, accompanied by an increase in ethylene production, and petals abscised by 11 d. mRNA levels of PhATG8
Fig. 1. Microscopy analysis of senescing petals of petunia flowers after pollination. (A) Flowers pollinated at anthesis are shown at 0, 1, 2, and 3 d after pollination (dap). Bars, 1 cm. (B) Electron micrographs of mesophyll cells in the petals of petunia flowers. Detached flowers were pollinated or not at anthesis and treated with concanamycin A. Mesophyll cells located in the middle between vascular
homologues started to increase from 8 d, paralleling the increased ethylene production (Fig. 2A, B). In pollinated flowers, petal wilting was observed from 2 dap and the petals were completely wilted by 3 dap (Fig. 1A). Ethylene production and mRNA levels of \( \text{PhATG8} \) homologues increased rapidly at 2 dap and then decreased (Fig. 2C, D). Thus, the increase in mRNA levels of \( \text{PhATG8} \) homologues corresponded with increased ethylene production, suggesting that pollination accelerates the induction of \( \text{PhATG8} \) homologues.

**Induction of \( \text{PhATG8} \) homologues by exogenous ethylene**

As the expression levels of \( \text{PhATG8} \) homologues paralleled ethylene production, we analysed whether ethylene induces the expression of these homologues. Exposure of detached flowers to ethylene (2 µl l\(^{-1}\)) for 16 h accelerated petal wilting. While these flowers did not exhibit petal wilting when removed from ethylene at 16 h, the flowers showed increased ethylene production and petal wilting within the next 24 h. In contrast, wilting was not accelerated with ethylene treatment for 4 h and these flowers showed petal wilting at 6–7 d after treatment, as for the flowers held in air. Increased mRNA levels of \( \text{PhATG8} \) homologues in petals were detected in flowers treated with ethylene for 4 h, and the levels increased further in flowers treated for 16 and 24 h (Fig. 3). These results indicated that \( \text{PhATG8} \) homologues are upregulated by ethylene.

Induction of \( \text{PhATG8} \) homologues by ethylene was examined further using treatment with 1-MCP, an inhibitor of ethylene perception. Treatment with ethylene for 16 h induced ethylene production and expression of \( \text{PhATG8} \) homologues over the next 24 h (Fig. 4A, B). However, treatment with 1-MCP prior to exposure to ethylene prevented the induction of ethylene production and expression of \( \text{PhATG8} \) homologues (Fig. 4A, B). These results support the hypothesis that expression of \( \text{PhATG8} \) homologues is regulated by ethylene.

**Induction of \( \text{PhATG8} \) homologues and MDC-stained structures by pollination via ethylene**

To examine whether upregulation of \( \text{PhATG8} \) homologues by pollination depends on ethylene, we analysed the expression patterns of \( \text{PhATG8} \) homologues in detached flowers that were pollinated and held in air or in the presence of 1-MCP. Flowers held in air started to show petal wilting at 2 dap, while flowers held under continuous 1-MCP showed petal wilting at around 10 dap. Ethylene production increased rapidly at 2 dap in the flowers held in air, while ethylene

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**Fig. 2.** Ethylene production and expression of \( \text{PhATG8} \) homologues in petals during natural and pollination-induced senescence. Ethylene production (A) and expression of \( \text{PhATG8} \) homologues (B) was determined in the petals of naturally senescing (unpollinated) flowers. Ethylene production (C) and expression of \( \text{PhATG8} \) homologues (D) was determined in the petals of pollinated flowers. Each bar represents the mean±standard error (SE) from three different samples.

Bundles in the petals of unpollinated flowers (left) and pollinated flowers (right) at 2 d after anthesis are shown. The spherical body indicated by an arrow is shown in an inset at higher magnification. Bars, 5 µm (main pictures); 500 nm (inset). (C) MDC staining in mesophyll cells of petunia petals. Petal limbs collected from flowers at 0, 1, 2, and 3 dap were stained. A micrograph of the petal limb at 2 dap taken at higher magnification is shown as an inset. Bars, 500 µm (main pictures); 50 µm (inset).
production was low until 4 dap and then increased after 6 dap in the flowers held in continuous 1-MCP (Fig. 5A). mRNA levels of PhATG8 homologues paralleled the patterns in ethylene production with a rapid increase at 2 dap for flowers held in air and no clear increase in mRNA levels for flowers held in 1-MCP for PhATG8a, -b, and -d until 6 dap (Fig. 5B). The mRNA level of PhATG8c increased from 1 dap in the flowers held in 1-MCP, showing a pattern that was similar to that for flowers kept in air but to a lesser extent. These results...
indicated that the induction of \( \text{PhATG}_8 \) homologues by pollination is regulated via ethylene. mRNA levels of \( \text{PhATG}_8 \) homologues in flowers exposed to 1-MCP increased at later stages of senescence, starting around 8 dap (Fig. 5B).

For mesophyll cells of petals from pollinated flowers held in air at 3 dap, which showed petal wilting, many MDC-stained structures were observed (Fig. 6A; Supplementary Fig. S5 at JXB online), but few were observed in the mesophyll cells from pollinated flowers held in 1-MCP at 3 dap (Fig. 6B; Supplementary Fig. S5). These results indicated that the induction of MDC-stained structures by pollination is regulated via ethylene. MDC-stained structures were observed in mesophyll cells of petals from pollinated flowers held in 1-MCP at 10 dap, which started to show petal wilting (Supplementary Fig. S6 at JXB online).

Nutrient reallocation during pollination-induced petal senescence

The decrease in nutrients in the petals during senescence is believed to be due to the reallocation to the developing ovaries. However, it was not clear whether the nutrients in the petals were actually translocated to the ovaries because the increased nutrients in the ovaries could have come from other parts of the plant if flowers attached to the plant were used. To circumvent this problem, we used detached flowers, which were placed in distilled water. In this system of petunia flowers that were detached from the plants at anthesis, emasculated, placed in distilled water, and then pollinated or left unpollinated, the petals showed similar patterns of senescence as flowers that remained attached to the plant: pollinated flowers exhibited petal wilting, while the petals of unpollinated flowers were still turgid at 3 d. The dry weight of the petals was significantly lower in pollinated flowers than in unpollinated flowers (Fig. 7). The dry weights of the ovaries and receptacles with sepals were significantly higher in pollinated flowers than in unpollinated flowers. Nitrogen content was significantly lower in the petals of pollinated flowers and it was significantly higher in the ovaries of pollinated flowers (Fig. 7). These data support the idea that nutrients are remobilized from the petals to the ovaries during pollination-induced senescence of petals.

To examine the involvement of autophagy in the remobilization of nutrients during petal senescence, pollinated flowers were treated with concanamycin A, which inhibits autophagic flux by blocking the degradation of vacuolar proteins. This chemical has been used to inhibit the degradation of vacuolar proteins in seedlings of \( \text{Arabidopsis} \) treated through their roots (Xiong et al., 2007). In pollinated flowers held incubated with concanamycin A, the dry weight of the ovary was significantly lower than that in untreated flowers (Fig. 8), indicating that concanamycin A treatment inhibits...
an increase in dry weight of the ovary in pollinated flowers. The changes in nitrogen content showed patterns similar to the changes in dry weight, but the differences were not significant in this set of experiments (Fig. 8).

Discussion

Pollination dramatically accelerates petal senescence in petunia. Through this mechanism, costly tissues that are no longer needed can be removed and nutrients from cellular components can be remobilized to developing tissues following degradation by autophagic mechanisms. In this study, we examined the regulatory mechanisms of autophagy in petal senescence.

The occurrence of autophagy in senescing petals was confirmed by electron microscopy and MDC staining. Many MDC-stained structures were observed in the mesophyll cells of senescing petals, while few were observed in those of presenescent petals, suggesting that autophagy flux is more active in senescing petals. However, as MDC has been found to stain other acidic components in addition to autophagosomes in mammalian cells (Klionsky et al., 2012), detailed analysis on the induced MDC-stained structures will be required. Autophagy-like processes were also observed in cells at the edges of vascular bundles by electron microscopy, as previously reported in senescing petals of common morning glory (Matile and Winkenbach, 1971) and carnation (Smith et al., 1992).

Pollinated petunia flowers showed faster petal senescence, and pollination accelerated the increase in ethylene production and mRNA levels of PhATG8 homologues. mRNA levels for PhATG8 homologues also increased during natural senescence in unpollinated flowers, indicating that pollination is not an absolute requirement for the induction of PhATG8 homologues. Expression patterns of PhATG8 homologues following pollination paralleled the changes in ethylene production, and exogenous ethylene treatment induced the expression of PhATG8 homologues, suggesting that ethylene regulates the induction of PhATG8 homologues. As the expression of PhATG8 homologues was induced by the 4h ethylene treatment, which was not sufficient to accelerate petal senescence, the induction appeared to be regulated more directly by ethylene than by secondary responses to ethylene as a result of accelerated senescence.

In pollinated flowers held in continuous 1-MCP, an ethylene perception inhibitor, mRNA induction of the PhATG8 homologues, except for PhATG8c, was clearly delayed compared with flowers held in air. These results suggested that the induction of PhATG8 homologues by pollination is mediated by ethylene. It should be noted here that the mRNA levels of all PhATG8 homologues eventually increased after pollination for flowers kept in 1-MCP. As ethylene perception is practically blocked in the continuous 1-MCP-treated flowers, the increase in mRNA levels for these genes in later stages of senescence is likely to be regulated independently of the ethylene signal. Age-related factors, in addition to ethylene, may also regulate autophagy in petal senescence. This pattern is similar to the regulation of nuclease and cysteine protease activities during petal senescence: increases in nuclease activity and mRNA levels of cysteine protease genes were delayed but not blocked in transgenic petunias (35S::etr1-1) with reduced ethylene sensitivity (Jones et al., 2005; Langston et al., 2005). However, ethylene production increased in the continuous 1-MCP-treated flowers in later stages. A similar increase in endogenous ethylene production has been reported in 35S::etr1-1 transgenic petunias (Langston et al., 2005). If inhibition of ethylene perception is incomplete in later stages of petal senescence in these plants due to age-related changes, the endogenous ethylene may lead to the induction of PhATG8 homologues. The induction of MDC-stained structures was also delayed by 1-MCP treatment in the mesophyll cells of petals from pollinated flowers, further suggesting that the induction of autophagy by pollination is mediated by ethylene.

The expression pattern of PhATG8c in 1-MCP-treated flowers differed from that of other homologues: the mRNA level of PhATG8c increased from 1 dap, even in the presence of 1-MCP. The Arabidopsis genome has been shown to encode nine ATG8 isoforms (AtATG8a–i; Doelling et al., 2002; Hanaoka et al., 2002) and the corresponding genes show tissue-specific expression, suggesting that the AtATG8 isoforms serve multiple non-redundant functions (Slavikova et al., 2005; Thompson et al., 2005). The expression patterns of PhATG8 homologues may also reflect differing regulatory mechanisms and functions in petal senescence.

Nutrients in the petals have been shown to decrease during pollination-induced senescence in petunia (Chapin and Jones, 2007). Using detached flowers placed in distilled water, thus blocking the supply of nutrients from other
plant parts, we suggested that decreases in nutrients in the petals are due to translocation to the ovaries in pollination-induced senescing flowers. Recently, autophagy was shown to play an important role in nitrogen remobilization to the seeds from other parts of the plant during senescence in Arabidopsis (Guiboileau et al., 2012). Treatment with concanamycin A, which inhibits autophagic flux, suppressed the increase in ovary dry weight for pollinated flowers. However, the nitrogen content in the ovaries of concanamycin A-treated flowers was not significantly lower than in control flowers. The lower dry weight of ovaries in concanamycin A-treated flowers might reflect the inhibited translocation of total nutrients, including carbon, phosphorus, and potassium in addition to nitrogen, but the dry weight of the petals in concanamycin A-treated flowers was not significantly higher than that of the control flowers. These findings appear to support the role of autophagy in the remobilization of nutrients from the petals to the ovaries, although the effect of concanamycin A in the inhibition of autophagy in the petals of detached flowers treated through cut stems remains to be confirmed. Thus, a model system that offers more effective inhibition of autophagy during petal senescence may be needed to provide more definitive results. It may be possible to produce autophagy-defective transgenic petunias, but there will probably be difficulty in doing so due to the pleiotropic effects of genes on senescence.

In autophagy-defective Arabidopsis mutants, leaf senescence symptoms are accelerated (Doelling et al., 2002; Hanaoka et al., 2002; Thompson et al., 2005; Xiong et al., 2005; Bassham et al., 2006), suggesting that autophagy delays leaf senescence. Autophagy is required to keep cells healthy by removing damaged proteins and contributes to cell survival by recycling nutrients, such that suppression of autophagy may cause precocious cell death. Recently, the over-accumulation of salicylic acid or high concentrations of reactive oxygen species have been proposed to be involved in early cell death phenotypes in leaves of autophagy-defective Arabidopsis mutants (Yoshimoto et al., 2009). On the other hand, petals are to be discarded after pollination and induced autophagy would cause massive degradation of cellular components for the recycling of nutrients along with cell death. It will be of particular interest to determine whether autophagy is a direct cause of cell death in petal senescence.

In this study, we showed that autophagy is induced in senescing petals of petunia and that pollination accelerates the induction of autophagy. The results indicated that ethylene is a key regulator of autophagy in petal senescence. The data on nutrient recycling reported here suggest support for the role of autophagy in the remobilization of nutrients during petal senescence, but further analyses are required.

**Supplementary data**

Supplementary data are available at JXB online.

**Table S1.** Sequences of primers used in real-time RT-PCR.

**Fig. S1.** An electron micrograph of a cell at the vascular bundle in a petal from a pollinated flower at 2 dap.

**Fig. S2.** MDC staining in mesophyll cells of petunia petals during the progression of senescence.

**Fig. S3.** Phylogenetic analysis of ATG8 homologues.

**Fig. S4.** Amino acid sequence alignment of ATG8 homologues.

**Fig. S5.** MDC staining in mesophyll cells of petunia petals in 1-MCP-treated flowers at 3 dap.

**Fig. S6.** MDC staining in mesophyll cells of petunia petals in 1-MCP-treated flowers at 10 dap.

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