InPSR26, a Putative Membrane Protein, Regulates Programmed Cell Death during Petal Senescence in Japanese Morning Glory

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The onset and progression of petal senescence, which is a type of programmed cell death (PCD), are highly regulated. Genes showing changes in expression during petal senescence in Japanese morning glory (Ipomoea nil) were isolated and examined to elucidate their function in PCD. We show here that a putative membrane protein, InPSR26, regulates progression of PCD during petal senescence in Japanese morning glory. InPSR26 is dominantly expressed in petal limbs and its transcript level increases prior to visible senescence symptoms. Transgenic plants with reduced InPSR26 expression (PSR26r lines) showed accelerated petal wilting, with PCD symptoms including cell collapse, ion and anthocyanin leakage, and DNA degradation accelerated in petals compared to wild-type plants. Transcript levels of autophagy- and PCD-related genes (InATG4, InATG8, InVPE, and InBI-1) were reduced in the petals of PSR26r plants. Autophagy visualized by monodansylcadaverine staining confirmed that autophagy is induced in senescing petal cells of wild-type plants and that the percentage of cells containing monodansylcadaverine-stained structures, most likely autophagosomes, was significantly lower in the petals of PSR26r plants, indicating reduced autophagic activity in the PSR26r plants. These results suggest that InPSR26 acts to delay the progression of PCD during petal senescence, possibly through regulation of the autophagic process. Our data also suggest that autophagy delays PCD in petal senescence.

Senescence is a highly regulated developmental process that is tightly controlled by multiple genes. Petal senescence is usually classified as a developmental programmed cell death (PCD; Pennell and Lamb, 1997; Rogers, 2006; van Doorn and Woltering, 2008). van Doorn and Woltering (2005) categorized plant PCD into three morphological types: apoptotic, autophagic, and nonlysosomal PCD. In plants, true apoptosis does not seem to occur, whereas autophagic and nonlysosomal PCD are commonly observed (van Doorn and Woltering, 2005).

Morning glory (including Ipomoea nil, Ipomoea purpurea, and Ipomoea tricolor), with its ephemeral flowers that open in the morning and show petal senescence symptoms within the same day has been used in several studies on petal senescence (Matile and Winkenbach, 1971; Kende and Baumgartner, 1974; Baumgartner et al., 1975; Hanson and Kende, 1975). Using electron microscopy, Matile and Winkenbach (1971) observed numerous vesicles and cytoplasmic components in the vacuoles of senescing I. purpurea petals that indicate autophagic processes. We previously analyzed PCD symptoms in senescing Japanese morning glory (Ipomoea nil) petals and showed that chromatin condensation occurred at flower opening and that DNA degradation increased as petals senesce (Yamada et al., 2006).

As autophagy is one of the main mechanisms responsible for the degradation and remobilization of macromolecules, it seems to play an essential role in petal senescence. In plants, transport to the vacuole is described by two major autophagic pathways of micro- and macroautophagy (Thompson and Vierstra, 2005; Bassham et al., 2006). Microautophagy involves sequestration of the cytoplasm by invagination of the tonoplast. Macroautophagy, by contrast, involves entrapment of proteins of the cytosol by double-membrane vesicles called autophagosomes. The outer membrane of autophagosome fuses with the tonoplast to release the internal vesicle as an autophagic body (Thompson and Vierstra, 2005). Several homologs of yeast (Saccharomyces cerevisiae) autophagy genes, including genes involved in autophagosome formation, have been isolated in Arabidopsis (Arabidopsis thaliana). Loss-of-function mutants or transgenic plants have been shown to have accelerated leaf senescence under both normal and nutrient-deficient conditions.
suggesting that autophagy plays a role in delaying PCD during leaf senescence. On the other hand, there has been little research on whether and, if so, how autophagy is involved in PCD during petal senescence.

Previous studies to identify genes that regulate PCD during petal senescence, using differential screening and microarray analysis have identified up- or down-regulation of numerous genes in several plant species, including *Hemerocallis hybrid* (Panavas et al., 1999), *Iris hollandica* (van Doorn et al., 2003), and *Alstroemeria* (Breeze et al., 2004). However, no genes specific for cell death have yet been identified (van Doorn and Woltering, 2008). The lack of effective transformation methods makes it difficult to determine the function of isolated genes in these plant species.

We have previously isolated genes showing changes in expression during petal senescence (petal senescence-related genes [PSRs]) in Japanese morning glory (Yamada et al., 2007). In this study, we produced transgenic plants with reduced expression of PSRs to determine function. We show here that one of the PSRs, *InPSR26*, which encodes a putative membrane protein, regulates the progression of PCD in petal senescence. We also discuss the role of autophagy in petal senescence.

**RESULTS**

**Characterization of *InPSR26***

*InPSR26* (*In26*, accession no. AB267825) was isolated and identified as a gene that is up-regulated during petal senescence in Japanese morning glory (cv Violet; Yamada et al., 2007). The deduced *InPSR26* peptide consists of 152 amino acids and shows 50% amino acid sequence identity over the length of the entire peptide with putative membrane proteins of Arabidopsis (At4g15610 and At3g06390), which have unknown function. The deduced *InPSR26* protein was predicted to include a conserved domain spanning amino acids 1 to 134 that may have a catalytic activity, and transmembrane helices based on a conserved domain database search (Marchler-Bauer et al., 2007). Homologs of *InPSR26* were found in several plant species, including rice (*Oryza sativa*), grape (*Vitis Vinifera*), and *Panax ginseng* (Supplemental Fig. S1).

**Expression of *InPSR26* in Wild-Type Plants**

Wilting and inward rolling of the petals was observed to start at about 10 h after flower opening (t10) in wild-type Japanese morning glory (Fig. 1A). While little mRNA for *InPSR26* was detected in the petal limbs of buds at 12 h prior to opening (t-12), the level increased after flower opening, reached a peak at t8, and then decreased (Fig. 2A). *InPSR26* mRNA levels at t4 were lower in carpel, style, petal tube, sepal, and vegetative tissue compared to that in the petal limb (Fig. 2B). No significant changes in *InPSR26* mRNA level were observed during leaf senescence (data not shown; Yamada et al., 2007).

**InPSR26 Transgenic Plants**

To analyze the function of *InPSR26*, we produced transgenic Japanese morning glory plants with reduced *InPSR26* expression. A total of six independent primary transgenic lines were obtained and four lines, PSR26r-A, -B, -K, and -V, produced T1 seeds. T1 plants of the PSR26r-A, -K, and -V lines showed accelerated petal wilting compared to wild-type plants (Supplemental Fig. S2). No morphological differences in floral organs and tissue structures of the petals, as well as...
vegetative tissue, in these transgenic lines were observed (Supplemental Fig. S3). Leaf senescence patterns were indistinguishable between wild type and these transgenic plants. As the primary and T1 transformants of the PSR26r-B line exhibited severe delay in growth and had a few small flowers, these were not examined further.

InPSR26 expression was lower in the petal limbs of the PSR26r-A5 and PSR26r-V2 plants compared to wild-type plants throughout the experimental period (Fig. 2A) and was 5.6% and 6.0% of that in wild type at t8, respectively. In PSR26r-K2 plants, InPSR26 mRNA was 38% of that in wild type at t8, but no decrease was observed at other time points (Fig. 2A).

Petal Senescence in PSR26r Transgenic Plants

In wild-type plants, petal wilting started between t10 and t12, accompanied by inward rolling of the petals. In PSR26r-A5, -K2, and -V2 plants, wilting at the distal petal edge between the ribs was observed between t4 and t8 with variation among flowers, and wilting expanded to the proximal regions (Fig. 1, A and B; Supplemental Fig. S2). Inward rolling for PSR26r-A5, -K2, and -V2 plants started at almost the same time as for wild-type flowers, at between t10 and t12.

Water potential is a common index of petal wilting and is known to decrease as petals senesce (Doi et al., 2000). The water potential of petal limbs of wild-type plants showed no significant changes to t8 and then decreased by t12 (Table I), concurrent with wilting of the petals. Water potential of petal limbs was significantly lower at t4 and t8 in PSR26r-A5 plants, at t8 in PSR26r-K2 plants, and at t4 in PSR26r-V2 plants than in wild-type plants, which is consistent with visible petal wilting. These results indicate that the time to petal wilting is accelerated in these PSR26r transgenic plants.

PCD in the Petals of PSR26r Transgenic Plants

We selected two transgenic lines, PSR26r-A5 and PSR26r-K2, which showed strong and mild acceleration of visible petal wilting, respectively, for use in subsequent experiments. Epidermal cells of the petal limbs maintained conical shape at t8 in wild-type plants but collapsed to irregular shapes at t8 at the edge of the petal limbs of PSR26r-A5 plants, which showed accelerated petal wilting (Fig. 1, C and D). In PSR26r-K2 plants, a similar collapse of the epidermal cells was observed at t8. Collapse of epidermis cells was not observed until about t14 in wild-type plants. These results indicate that wilting of the petals in PSR26r transgenic plants is accompanied by cellular collapse of epidermal cells and that the collapse is accelerated in flowers of transgenic lines.

Ion and anthocyanin leakage from petal limbs at t8, when a significant decrease in water potential was detected in PSR26r plants, was higher in the PSR26r plants than in wild-type plants (Fig. 3, A and B). There were no significant differences in the total amount of anthocyanin in petal limbs between wild-type and the transgenic plants (data not shown). These results indicate increased leakiness of the plasma membrane and tonoplast at t8 in the transgenic plants.

DNA extracted from the petal limbs of wild-type plants showed slight degradation at t8 and increased degradation at t12, with faint DNA laddering (Fig. 4). In PSR26r-A5 and PSR26r-K2 plants, DNA showed slight degradation at t4 and increased degradation at later time points (Fig. 4). Similar DNA degradation results were obtained in three separate experiments. These data indicate that the time to DNA degradation is accelerated in the PSR26r transgenic plants, suggesting that progression of PCD is accelerated in the petals of PSR26r transgenic plants.

Expression of Autophagy- and PCD-Related Genes in PSR26r Transgenic Plants

The function of InPSR26 in petal senescence was analyzed through expression of Japanese morning glory.
glory homologs of autophagy- and PCD-related genes in PSR26r transgenic plants. These homologs were identified by a search of the Japanese morning glory EST database (T. Yamada and K. Ichimura, unpublished data). Homologs of InATG4 (BJ578707), InATG8 (BJ562998), and InPI3K (BJ567637), which are involved in autophagy, showed the highest homology with deduced amino acid sequence of Arabidopsis genes Autophagy 4a (NP_850412), Autophagy 8h (NP_566518), and phosphatidylinositol 3-kinase (PI3K; BAD94035), respectively. InVPE (BJ572512) encodes a vacuolar processing enzyme and the deduced amino acid sequence shows the highest homology with γ-VPE (NP_195020) in Arabidopsis. InBI-1 (BJ576044) encodes a Bax inhibitor that shows the highest homology with ATBI-1 (NP_199523) in Arabidopsis. InPSR15 (AB267820) is a PSR encoding a Cys protease precursor, which shows homology with PRT22 (AAD05851) in Sandersonia (Yamada et al., 2007).

All of the autophagy- and PCD-related genes identified here were up-regulated during petal senescence in the petal limbs of wild-type plants (Fig. 5). mRNA levels of InATG4, InATG8, InPI3K, and InBI-1 reached maximum levels at t8, about 2 h prior to petal wilting and then decreased. mRNA levels of InVPE and InPSR15 increased during petal senescence and remained a high expression level through the experimental period. In PSR26r-A5 and PSR26r-K2 plants, InATG4, InATG8, InVPE, and InBI-1 mRNA levels at t8 were much lower than in wild-type plants (Fig. 5). On the other hand, clear differences in mRNA levels between wild-type and transgenic plants for these genes were not observed at other time points. Levels of InPI3K and InPSR15 mRNA were slightly lower at t8 in transgenic plants than in wild-type plants, but clear changes in mRNA levels were not observed throughout the experimental period.

**Table 1.** Water potential (MPa) of the petal limbs in wild-type and PSR26r-A5, -K2, and -V2 plants

Water potential was measured in buds at t-12 and opened flowers every 4 h (from t0 to t12). Mean ± sd (n ≥ 3); different letters indicate significant differences at each time point at P < 0.05 (Tukey-Kramer test).

| Line     | Time |   |   |   |   |   |   |   |   |   |   |
|----------|------|---|---|---|---|---|---|---|---|---|---|
|          | -12  | 0 | 4 | 8 | 12|   |   |   |   |   |   |
| Wild type| -1.25 ± 0.30a | -1.39 ± 0.19a | -1.19 ± 0.27a | -1.25 ± 0.10a | -2.05 ± 0.13 |   |   |   |   |   |   |
| A5       | -1.28 ± 0.08a | -1.95 ± 0.20a | -2.32 ± 0.59b | -2.03 ± 0.59b | n.d.      |   |   |   |   |   |   |
| K2       | -1.18 ± 0.11a | -1.64 ± 0.10a | -1.78 ± 0.46a | -2.02 ± 0.50b | n.d.      |   |   |   |   |   |   |
| V2       | -1.59 ± 0.11a | -1.54 ± 0.32a | -1.86 ± 0.22b | -1.76 ± 0.08a | n.d.      |   |   |   |   |   |   |

The morning glory contained anthocyanins in the vacuole, indicating their origin in epidermal cells (Fig. 6, A, B, D, and E), while other protoplasts did not contain anthocyanins, indicating that they are likely from mesophyll cells (Fig. 6C). We first confirmed that no clearly fluorescent structures were present in the absence of MDC staining; vacuoles containing anthocyanins slightly fluoresced (Fig. 6E). MDC-stained structures were observed in protoplasts from both epidermal and mesophyll cells of wild-type petal limbs at t8 (Fig. 6, A–C). Most of the structures were observed in the cytoplasm, but some appeared to be present within the vacuole (Fig. 6, A and B). In protoplasts prepared from the petal limbs of buds at t-16, MDC-stained structures were rarely observed (Fig. 6D).

To characterize MDC-stained structures in more detail, we treated flowers with concanamycin A and PSR encoding a Cys protease precursor, which shows homology with PRT22 (AAD05851) in Sandersonia (Yamada et al., 2007).

**Figure 3.** Ion and anthocyanin leakage from petal limbs of wild-type (WT) and PSR26r-A5 and -K2 plants. Ion (A) and anthocyanin (B) leakage from petal limbs at t8 was measured. Each bar represents the mean ± sd from six different samples. Asterisks indicate significant differences at P < 0.01 (***) and P < 0.05 (*) compared to wild type (Tukey-Kramer test).
(conA), which inhibits degradation of autophagic bodies in the vacuole (Yoshimoto et al., 2004). Here, we used Japanese morning glory line Q0262, which has white flowers, to clearly visualize the structures inside the vacuole. Treatment of conA resulted in acceleration of petal senescence (data not shown). MDC-stained structures showing movement were observed in the vacuoles of protoplasts prepared from both control and conA-treated flowers at t8. However, the vacuoles of protoplasts prepared from conA-treated flowers contained markedly more MDC-stained structures (Fig. 6F).

The percentage of protoplasts containing MDC-stained structures was determined for protoplasts from epidermal cells of the petal limbs at several time points during senescence in wild-type plants (Fig. 7A). The percentage was 8.9% in buds at t-16 and increased to 49% in opened flowers at t0. The percentage increased during petal senescence. These findings indicate that autophagy is induced during flower opening and petal senescence.

MDC-stained structures were also observed in protoplasts prepared from the petal limbs of PSR26r-A5 and PSR26r-K2 plants. However, the percentages of protoplasts containing MDC-stained structures were significantly lower at t8 in PSR26r plants than in wild-type plants (Fig. 7B). There were no significant differences in the percentages at t4 between wild-type and PSR26r plants (data not shown). These results suggest that autophagic processes are suppressed in PSR26r transgenic plants at t8.

DISCUSSION

Japanese morning glory has ephemeral flowers with flower longevity under the control of PCD. Among the isolated genes showing changes in expression during senescence in Japanese morning glory petals (Yamada et al., 2007), InPSR26 was identified as a gene that is up-regulated during petal senescence, thus making it a candidate gene for regulating PCD. InPSR26 shows high identity to genes encoding putative membrane proteins of Arabidopsis with unknown function. InPSR26 is dominantly expressed in petal limbs, and its transcript level increases after flower opening and reaches a maximum a few hours prior to visible petal senescence, indicating that InPSR26 is involved in petal senescence.

Flowers of PSR26r transgenic plants, which have reduced InPSR26 expression, showed accelerated visible petal senescence. In the PSR26r plants, the collapse of epidermal cells of the petals occurring in concert with petal wilting is observed to occur earlier than in wild-type plants. Further, ion and anthocyanin leakage was higher in petal limbs of PSR26r transgenic plants than in those of wild-type plants at t8, suggest-
ing that there are more dead cells in the petals of transgenic plants. Ion and anthocyanin leakage from the petals of \textit{Hemerocallis} was also documented to increase during petal senescence (Bieleski and Reid, 1992; Panavas and Rubinstein, 1998). Hastened PCD in the petals of PSR26r transgenic plants is also reflected in the acceleration of DNA degradation. Compared to the PSR26r-A5 line, the PSR26r-K2 line showed less acceleration in the time to visible senescence, less increase in ion and anthocyanin leakage, and less acceleration in the time to DNA degradation. The weaker expression of senescence phenotypes in the PSR26r-K2 line seems to be a result of a less reduction in $\text{InPSR26}$ expression, likely due to the positional effect of the transgene. On the whole, a reduction of $\text{InPSR26}$ expression resulted in an acceleration of PCD in the petals of the transgenic plants, suggesting that $\text{InPSR26}$ plays a role in the delay of PCD in petal senescence.

Although autophagy has been shown to occur during petal senescence (Matile and Winkenbach, 1971), the mechanism of autophagy in PCD during petal senescence remains unclear. We analyzed the expression of Japanese morning glory gene homologs known to be involved in autophagy and PCD: $\text{ATG4}$ and $\text{ATG8}$ are autophagy-related proteins involved in early steps of autophagosome formation (Yoshimoto et al., 2004), and $\text{PI3K}$ is a class III phosphatidylinositol-3 kinase, which likely regulates autophagy (Codogno and Meijer, 2005). mRNA levels of $\text{InATG4}$, $\text{InATG8}$, and $\text{InPI3K}$ increased prior to visible senescence in wild-type petals, suggesting that their up-regulation equates to an increase in autophagic activity during petal senescence. We visualized autophagy using MDC staining, which has recently been shown to specifically stain autophagosomes in Arabidopsis (Contento et al., 2005). In senescing petals of morning glory, the presence of autophagosomes has been demonstrated by electron microscopic analyses (Matile and Winkenbach, 1971). MDC-stained structures were observed mostly in the cytoplasm but occasionally were observed within the vacuoles of protoplasts prepared from petals of wild-type plants at t8. Treatment with conA, which is known to inhibit degradation of autophagic bodies in the vacuole (Yoshimoto et al., 2004), appeared to increase accumulation of MDC-stained structures in the vacuole. These observations are similar to those in suspension cells and roots of Arabidopsis (Yoshimoto et al., 2004; Contento et al., 2005) and are consistent with the function of autophagy in the transfer of cytoplasmic materials into the vacuole for degradation, implying that the MDC-stained structures are most likely autophagosomes. However, the specificity of MDC staining for autophagosomes in this cell type should be confirmed, for example, by expressing GFP-ATG8 fusion proteins. The percentage of protoplasts containing MDC-stained structures was greater in the petal limbs of opened flowers compared to buds and it increased as the petals senesce. These results suggest that autophagic activity is up-regulated during flower opening and petal senescence.

A reduction of $\text{InPSR26}$ expression appears to affect expression of autophagy-related genes. In the petal limbs of both the PSR26r-A5 and PSR26r-K2 plants, the usual rapid up-regulation of $\text{InATG8}$ at $t_8$ was not observed. $\text{InATG4}$ mRNA level at $t_8$ was also reduced in transgenic plants compared to wild-type plants. These data suggest that $\text{InPSR26}$ is involved in induction of these genes. In PSR26r-A5 plants, however,
In the petals of PSR26r transgenic plants showing suppressed autophagy, PCD symptoms were accelerated. This could mean that autophagy is a mechanism that delays PCD during petal senescence. Treatment with an autophagy inhibitor, 3-methyladenine, was shown to hasten PCD in Japanese morning glory petals (T. Yamada and K. Ichimura, unpublished data). Arabidopsis loss-of-function mutants on autophagy-related genes (ATG4, ATG5, ATG7, ATG9, and ATG18) showed acceleration of leaf senescence under both normal and nutrient-deficient conditions (Doelling et al., 2002; Hanoaka et al., 2002; Thompson et al., 2005; Xiong et al., 2005; Bassham et al., 2006), suggesting that autophagy aids in cell survival and delays PCD in leaf senescence. These reports appear to corroborate our findings that suppression of autophagy results in acceleration of petal senescence. However, it seems paradoxical that there is a mechanism to delay cell death in the petals that will be discarded. One interpretation is that autophagy is essential to the recovery and translocation of nutrients from dying tissue to growing tissues of plants, such as developing seeds, in that it delays precocious death for this to be accomplished. Indeed, PSR26r transgenic plants produced fewer seeds per flower compared to wild-type plants (data not shown).

VPE is a Cys protease that activates various proteins in the vacuole and is thought to be a key executioner of PCD in some types of plant cells (Hatsugai et al., 2006). In PSR26r-A5 and PSR26r-K2 plants, InVPE expression was reduced at t8 compared to wild-type plants, similar to expression of InATG4 and InATG8. As VPE activates several vacuolar proteases, VPE might be involved in autophagic degradation processes and its expression might be regulated in concert with autophagy-related genes. Expression of InBI-1 was also reduced in PSR26r transgenic plants at t8. Bax Inhibitor-1 (BI-1) is an antagonist of Bax, which is a proapoptotic protein of the Bcl-2 family in animal cells. No members of the Bcl-2 family have been identified in plants, but plants contain a homolog of BI-1, and BI-1 has been shown to function as an anti-PCD protein in plants (Kawai-Yamada et al., 2001; Watanabe and Lam, 2006). Although involvement of BI-1 in petal senescence remains obscure, a decrease in InBI-1 mRNA level might be associated with the acceleration of cell death in PSR26r plants. BI-1 may prevent precocious cell death until recycling of nutrients via autophagy is accomplished in the petals. These autophagy-related genes seem to regulate progression of PCD by orchestrated expression. InPSR26 may regulate the induction of these genes during petal senescence.

We showed that InPSR26 is a putative membrane protein that is involved in the progression of PCD during petal senescence. Our results highlight a link between autophagy and PCD in petal senescence, although the biochemical function of InPSR26 remains largely unknown. Analyses on the function of this protein will provide new insights on PCD and autophagy in petal senescence.
MATERIALS AND METHODS

Plant Materials

Seedlings of Japanese morning glory (Ipomoea nil 'Violet') were planted in soil in 12-cm pots and grown in a growth chamber at 24°C in a 12/12-h photoperiod at 100 μmol m−2 s−1 with white-fluorescent lamps. Plants were fertilized with 1 g L−1 Hypoex 15-30-15 (Hypoex) once a week. In these experiments, we designated the onset of the light period as time 0 (t0), as described in Yamada et al. (2007). Flowers were almost fully open at t0. For conA treatment, Japanese morning glory line Q0262, which has white flowers, was used.

Production of Transgenic Plants

Transgenic plants with reduced expression of InPSR26 were produced using RNAi strategy. Briefly, a 486-bp segment of InPSR26 cDNA spanning nucleotides 17 to 502 was cloned into pDONR210 (Invitrogen) and then the segment was cloned into the binary transformation vector pHYGWIWG(II) containing the hygromicine resistance (Hyg) gene (Karimi et al., 2002) in antisense and sense orientations using Gateway BP and LR clonase enzyme mix (Invitrogen). The resulting binary vector, pH7-PSR26, contains the target segment downstream of the cauliflower mosaic virus 35S promoter in the antisense and sense orientations, and upstream of the 35S terminator. Tissue-cultured cells derived from an immature embryo of Japanese morning glory cv Violet were transformed with pH7-PSR26 using Agrobacterium AGL0 according to the methods of Shimizu et al. (2003) and Ono et al. (2000) with modifications. Regenerated shoots were selected from independent calluses. The presence of the transgene was confirmed through PCR by amplifying a segment of the Hyg gene and through Southern-blot analysis by detecting the transgene present in the Hyg gene containing the hygromicine resistance (Hyg) gene (Supplemental Fig. S4). All subsequent experiments were performed on T1 and T2 progenies of transgenic plants.

Quantitative Real-Time Reverse Transcription-PCR

Total RNA was isolated using Trizol reagents (Invitrogen) and treated with cloned DNase I (Takara Bio). Synthesis of cDNA was carried out with random hexamer primers using the SuperScript III first-strand synthesis system for reverse transcription (RT)-PCR (Invitrogen). Primers for real-time RT-PCR were designed to target the 3′-untranslated region of target genes with the Primer3 program (Supplemental Table S1). PCR reactions were performed using QuantiTech SYBR Green PCR kit (Qiagen) on the LightCycler quick system (model 350S; Roche Diagnostics). Thermal cycling conditions were 95°C for 15 min followed by 50 cycles of 94°C for 15 s, 60°C for 20 s, and 72°C for 15 s. Data were normalized by calculating the transcript level ratios of target genes and Actin 4 within the same sample, as described in Yamada et al. (2007).

Water Potential, Ion Leakage, and Anthocyanin Measurements

Petal water potential was measured psychrometrically with a Wescor HR-33 microvolt meter (Wescor) and C-52 sample chambers (Wescor) as described in Doi et al. (2000). Disks (9-mm diameter) were sampled from the fresh petal and equilibrated in a chamber for 1 h prior to measurement. Ion leakage from petals was measured on cut petal limbs that were immersed in 50 mL distilled water in a plastic vial and covered with nylon mesh to prevent floating up. Distilled water was immediately renewed and the vials were shaken slowly for 1 h at room temperature. Following incubation, conductivity (value A) of the bathing solution was measured with a conductivity meter (W-W22EP; TOA DKK). The bathing solution was then returned to the plastic vial containing the petal limb and incubated at 95°C for 30 min. After cooling to room temperature conductivity (value B) of the bathing solution was measured again. Ion leakage was expressed as a percentage of value A relative to value B. Anthocyanin leakage was measured on a petal limb and was immersed in 20 mL distilled water and incubated for 30 min at room temperature. Anthocyanin concentration in the solution was measured at the absorption maximum of 530 nm with a UV/VIS spectrophotometer (V-530, JASCO). For the measurement of the total amount of anthocyanin in petals, cut petal limbs were immersed in 15 mL methanol containing 0.3 N HCl and incubated at 4°C for 20 h. Anthocyanin concentration in the solution was measured at its absorption maximum, 510 nm.

DNA Degradation Analysis

Total DNA was extracted according to Yamada and Marubashi (2003). Extracted DNA was immediately electrophoresed on 3% agarose gel and stained with ethidium bromide. The gel pattern was photographed with an electronic UV transilluminator system (FAS-III mini+ DS-30; Toyobo) as described in Yamada et al. (2006).

MDC Staining of Protoplasts

Protoplasts were prepared by digesting petal strips with 1.5% (w/v) cellulase and 0.2% (w/v) macerozyme (Yakult Pharmaceutical) for 2 h at 25°C and staining through 80-μm nylon mesh. MDC staining of protoplasts and visualization of MDC-stained autophagosomes were performed according to the methods of Contento et al. (2005). Briefly, protoplasts were stained with a 0.05-mM MDC (Sigma) in phosphate-buffered saline supplemented with 0.4 mM mannitol for 10 min, then washed twice with phosphate-buffered saline containing 0.4 mM mannitol. MDC-stained protoplasts were visualized using fluorescence microscopy (Olympus AX70) with a filter for 4′,6-diamidino-2-phenylindole. Bright-field (light) and fluorescence images were taken with a digital color camera (DP71; Olympus). The number of protoplasts containing autophagosomes was determined as shown in Xiong et al. (2005). Protoplasts were prepared from three different samples for each time point, and at least 300 protoplasts were counted for each preparation.

ConA Treatment

Buds of Japanese morning glory line Q0262 were excised at t-12 and placed in solutions of 5-μM conA (Wako) in 1.5-mL centrifuge tubes. ConA was prepared as a 100 μM stock solution in absolute dimethyl sulfoxide. For the control, buds were placed in sterile distilled water containing an amount of dimethyl sulfoxide equal to that used in the treatment. Cut buds were kept under the same conditions as for growing plants, as described above.

Microscopic Analysis

Petal surfaces shown in Figure 1, C and D, were observed using digital microscopy (VH-8000C; Keyence).

Supplemental Data

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

Supplemental Figure S1. Alignment of predicted amino acid sequences of InPSR26 homologs.

Supplemental Figure S2. Time course of visible senescence in wild-type and PSR26r-A5, -K2, and -V2 plants.

Supplemental Figure S3. Agar sections of petals of wild-type and PSR26r-A5 plants.

Supplemental Figure S4. Detection of the transgene in PSR26r plants.

Supplemental Table S1. Primers used for real-time RT-PCR analyses.

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