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Gene expression in opening and senescing petals of morning glory (*Ipomoea nil*) flowers

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Abstract We isolated several senescence-associated genes (SAGs) from the petals of morning glory (*Ipomoea nil*) flowers, with the aim of furthering our understanding of programmed cell death. Samples were taken from the closed bud stage to advanced visible senescence. Actinomycin D, an inhibitor of transcription, if given prior to 4 h after opening, suppressed the onset of visible senescence, which occurred at about 9 h after flower opening. The isolated genes all showed upregulation. Two cell-wall related genes were upregulated early, one encoding an extensin and one a caffeoyl-CoA-3-O-methyltransferase, involved in lignin production. A pectinacetylesterase was upregulated after flower opening and might be involved in cell-wall degradation. Some identified genes showed high homology with published SAGs possibly involved in remobilisation processes: an alcohol dehydrogenase and three cysteine proteases. One transcript encoded a leucine-rich repeat receptor protein kinase, putatively involved in signal transduction. Another transcript encoded a 14-3-3 protein, also a protein kinase. Two genes have apparently not been associated previously with senescence: the first encoded a putative SEC14, which is required for Golgi vesicle transport, the second was a putative ataxin-2, which has been related to RNA metabolism. Induction of the latter has been shown to result in cell death in yeast, due to defects in actin filament formation. The possible roles of these genes in programmed cell death are discussed.

Keywords Actinomycin D · DNA degradation · Nuclear fragmentation · Petal senescence · Programmed cell death · Senescence-associated genes

Abbreviations ActD: Actinomycin D · ADH: Alcohol dehydrogenase · DAPI: 4,6-Diamino-2-phenylindole · HR: Hypersensitive response · LTP: Lipid transfer protein · PCD: Programmed cell death · RACE: Rapid amplification of cDNA ends · SAG: Senescence-associated gene · SCA2: Spinocerebellar ataxia type 2

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Introduction

Senescence is highly regulated and known to be under the control of numerous genes. Senescence can be initiated by an environmental signal, such as shortening of the day length in leaves of deciduous trees in temperate zones. It can also be induced by a plant signal that is external to the cell, for example signals that indicate lack of minerals or water. The time to visible petal senescence is specific for each species and seems regulated by genes in the petal cells. In some species the time to visible petal senescence is regulated by endogenous ethylene, whereas in other species it is ethylene-independent.

At the ultrastructural level, petal senescence is characterized by the disappearance, initially, of endoplasmic reticulum and attached ribosomes, and by the disappearance of plastids and Golgi bodies. The mitochondria and the nucleus, in contrast remain until a late stage. Autophagic processes are indicated by the degradation of various organelles in the vacuole, rupture of the tonoplast, and subsequent autolysis of the whole cell (Matile and Winkenbach 1971; Smith et al. 1992).

Genes that exhibit enhanced expression during senescence have been called senescence-associated (Nam 1997) or senescence-related (Jones 2004) genes. Here, we will use the term senescence-associated genes (SAGs). Many SAGs have been identified in the senescing petals of flowers such as *Hemerocallis* (Panavas et al. 1999), *Narcissus* (Hunter et al. 2002), *Sandersonia* (Eason et al. 2002), and *Petunia* (Jones 2004). Profiling of gene expressions in the petals during flower opening and senescence have also been performed using microarray analysis in *Iris* (van Doorn et al. 2003) and *Alstroemeria* (Breeze et al. 2004).

We used the ephemeral *Ipomoea nil* flowers. This species and the closely related *I. tricolor* have been used in several previous studies on petal senescence (Matile and Winkenbach 1971; Kende and Baumgartner 1974; Baumgartner et al. 1975; Yamada et al. 2006). We previously determined the time line of various visible symptoms of programmed cell death (PCD; here taken to be synonymous to senescence) and processes such as DNA degradation, chromatin condensation, and nuclear fragmentation, during petal PCD in *I. nil* (Yamada et al. 2006). The purpose of the present paper was to relate the expression of identified SAGs to the visible senescence symptoms and to some of the known changes at the cellular level. The experiments are an attempt to furthering our understanding of PCD and the role of gene expression in this process.

Materials and methods

Plant materials

Seedlings of *I. nil* (L.) Roth *cv.* Violet (seeds from Marutane, Kyoto, Japan) were grown in a controlled chamber at $24 \pm 1°C$, about 70% relative humidity (RH) and 13 h per day cool-white fluorescent light (100 μmol m^{-2} s^{-1}). The light period was from 8:00 a.m. to 9:00 p.m. The flowers were almost fully open when the lights turned on. Some experiments used excised flowers. Data on gene expression and gene identification were obtained by sampling petals from intact plants, at various intervals after the closed bud stage.

Treatment with RNA synthesis inhibitor

Excised flowers were transferred to sterile distilled water as a control or an aqueous solution of 20 μM actinomycin D (ActD). Cut flowers were kept at $24 \pm 1°C$ and about 70% RH, under continuous illumination (100 μmol m^{-2} s^{-1}). Treatments were continuous but started at 2 h intervals from the onset of flower opening.

Petal inward rolling

The abaxial surface of the petals was photographed horizontally. The diameter aligned with the midrib of the fused petals was measured by SimpleDigitizer version 3.01 software for image analysis (free software downloaded from http://www.vector.co.jp/). The mean was calculated from the diameters of five positions on each corolla. The inrolling index was calculated as the percentage decrease from the maximum diameter.

Preparation of total RNA

Total RNA was isolated from each plant material using ISO-GEN (Nippongene, Toyama, Japan). Subsequently, genomic DNA included in the RNA sample was digested with RNase-Free DNase set (Qiagen, Hilden, Germany), and the RNA was purified using RNeasy mini kit (Qiagen). All reagent kits and instruments were used according to the manufacturer's instructions. Quality and quantity of RNA were checked using spectrophotometer DU640 (Beckman Coulter, Fullerton, CA) and formaldehyde gel electrophoresis.

cDNA subtraction

Poly(A)$^+$mRNA was isolated from total RNA sample, which was prepared from the petals of five flowers, using Oligotex-MAG mRNA purification kit (Takara Bio, Shiga, Japan). cDNA synthesis and subtraction were performed with PCR-select cDNA subtraction kit (Clontech, Palo Alto, CA). Twenty-seven cycles of primary PCR and 12 cycles of secondary PCR were performed using Advantage 2 PCR polymerase kit (Clontech). The efficiency of the subtraction was evaluated using control regents provided in the kit.

Differential screening

Differential screening of subtracted cDNA pools was performed using PCR-select differential screening kit (Clontech). Briefly, secondary PCR products from each forward subtraction were subcloned into the pGEM-T-easy vector using T/A-based cloning system (Promega, Madison, WI). Clones were picked from the subtracted cDNA library and their cDNA inserts were amplified respectively.

The PCR products were blotted on Hybond-N$^+$ nylon membrane (Amersham, Piscataway, NJ) and four identical cDNA arrays were replicated. Hybridization was carried out with these arrays and alkaline phosphatase-labeled probes, which were prepared from four cDNA pools generated by subtractive hybridization, and performed overnight at 72°C using AlkPhos direct labeling and detection system (Amersham). Hybridization signals were quantitatively analyzed by fluorescence scanning system, STORM 860 (Amersham). The ratios of signal intensity for the combinations of subtracted probes (forward/reverse) or unsubtracted probes (tester/driver) were calculated from the values of two separate experiments in the hybridization. cDNA clones whose averaged signal ratios for subtracted probes were > 4 and unsubtracted probes were > 2, were selected to assign to be differentially expressed. Selected clones were sequenced by Shimadzu Biotech, Kyoto, Japan, and their homologies were annotated by BLAST program (Llop Tous et al. 2000).

RACE-PCR

To isolate full-length cDNAs, rapid amplification of cDNA ends (RACE) was performed using SMART RACE cDNA amplification kit (Clontech). Gene specific primers for 3′- and 5′-RACE were picked from a DNA sequence of each clone using Primer3 (http://frodo.wi.mit.edu/primer3/primer3_code.html). The RACE products were subcloned into the pGEM-T-easy vector and sequenced using the BigDye terminator cycle sequencing ready reaction kit (PE Biosystems, Foster City, CA) on the ABI PRISM 3100 genetic analyzer (PE Biosystems). Sequence analyzing software DNASIS version 3.7 (Hitachi software engineering, Yokohama, Japan) was used to trim the vector sequence from raw sequence data, and to assemble contigs. Full-length cDNAs were PCR amplified using the template cDNAs for 5′-RACE and primers picked from the contig sequences. The PCR products were directly sequenced according to the above method. Putative functions or homologies of the isolated genes were examined with BLASTX and the conserved domain database using Reverse Position Specific BLAST (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

Real-time PCR

Total RNA samples were prepared as above mentioned. Synthesis of cDNA was carried out with primers of random hexamer using the SuperScript first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA). Primers for real-time RT-PCR were picked from the sequence of target genes using Primer3 for product size ranges between 100 and 300 bp, primer size of 20 bp, and primer Tm of 60°C

(Table S1). The primer pair for the constitutively expressed control gene, *Actin 4* (AB054978, Yamaguchi et al. 2001) was chosen to span an intron. The specificity of primer sets and the absence of genomic DNA in the synthesized cDNA samples were confirmed by agarose gel electrophoresis of the PCR products. PCR reactions were performed in glass capillaries with the LightCycler Quick System model 350S (Roche Diagnostics, Basel, Swiss), using QuantiTect SYBR Green PCR kit (Qiagen). Each reaction was performed upon 2 μL aliquot of 100 μL cDNA solution derived from 5 μg of total RNA. Thermal cycling conditions were 95°C for 15 min followed by 55 cycles of 15 s at 94°C for denature, 20 s at 60°C for annealing, and 15 s at 72°C for extension. The absolute transcript level was determined with fit point method using dilution series of target sequence as external standards. To standardize the data, the ratio between the absolute transcript levels of target gene and *Actin 4* within the same sample was calculated for each sample.

Three separate experiments were carried out, showing similar results. Data are the means of absolute transcript level or their ratios, from these three replicate experiments.

Results

Flower opening and visible senescence symptoms: relationship with PCD parameters

I. nil plants have ephemeral flowers, which open in the morning and show petal senescence symptoms the same day. In the present experiments we designated the onset of the light period (8:00 a.m.) as time 0 ($t = 0$ h). At $t = -6$ h the flower was a closed bud. Opening took place mainly between $t = -3$ and 0 h, but was complete only by $t = 3$ to 5 h (between 11:00 a.m. and 1:00 p.m.). Flower opening was accompanied by an increase in petal surface. Inward rolling of the petals, the first visible symptom of senescence, started at about $t = 9$ h. Inward rolling proceeded until most of the corolla had shriveled together (later than $t = 24$ h). This was followed by petal desiccation.

A number of parameters relating to programmed cell death occur prior to, concomitant with, or later than the onset of petal inward rolling. These parameters include: an increase in the activity of a variety of degradative enzymes, a decrease in lipid, protein, and nucleic acid levels, a larger number of DNA masses with decreased 4,6-diamino-2-phenylindole (DAPI) staining, showing less DNA per DNA mass (nucleus), an increase in the number of DNA masses, rupture of the tonoplast, and degradation of the cell walls (Winkenbach 1970; Yamada et al. 2006)
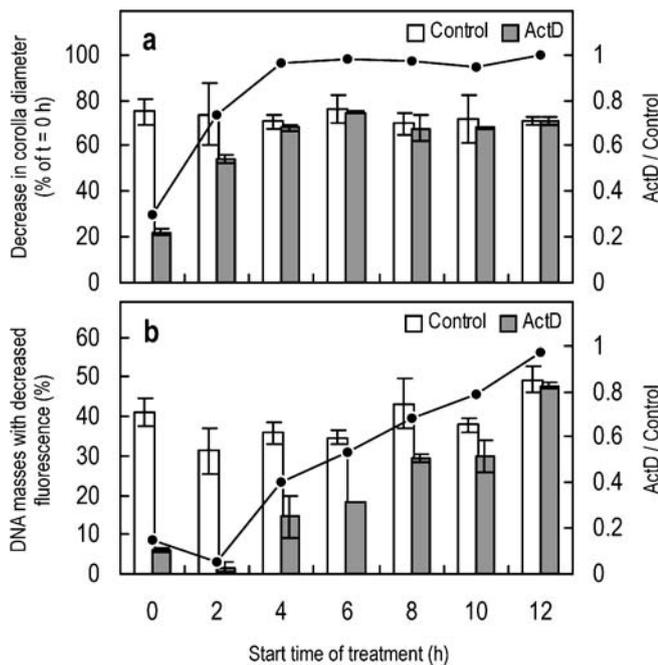
Fig. 1 Inrolling and DNA fluorescence per DNA mass in the petals of flowers treated with actinomycin D (ActD), a RNA synthesis inhibitor, at 2-h intervals. Flowers were excised at $t = 0, 2, 4, 6, 8, 10,$ and 12 h and then transferred to sterile distilled water (control) or an aqueous solution of 20 μM ActD. **a** Changes in the effects of ActD on inrolling as a function of the onset of the treatment. Decrease in corolla diameter (% of $t = 0$ h) was detected at $t = 18$ h. **b** Changes in the effects of ActD on DNA fluorescence per DNA mass, as a function of the onset of the treatment. DNA masses with decreased fluorescence were detected using flow cytometry at $t = 30$ h. **a** and **b** The histogram values are means ± SD of five separate experiments using three flowers each. The lines connect the ratios of the values after ActD treatment and the control values

Effect of actinomycin D

We tested the effect of ActD on petal opening and petal inrolling. ActD was supplied continuously. The treatments started at various times from $t = -3$ h, $t = 0$ h, and subsequently after 2 h intervals until $t = 12$ h. The degree of petal inward rolling was measured at $t = 18$ h. ActD treatment, if started at $t = -3$ h, completely suppressed flower opening (results not shown). Flowers were almost fully open (and went on to open) if ActD treatment started at $t = 0$ h. Petal inward rolling was much inhibited if ActD treatment started at $t = 0$ h. Treatments that started at $t = 2$ h

had much less effect on inward rolling. If the treatments started at $t = 4$ h or later they no longer inhibited petal inward rolling (Fig. 1a).

We also studied the effect of ActD on the number of DNA masses with decreased amount of DNA. A decrease in the amount of DNA per DNA mass was previously found to be due to (a) DNA degradation, and (b) a large increase in the number of DNA masses, concomitant with a decrease in the diameter of these masses. The latter was most likely due to nuclear fragmentation. An increase in the number of DNA masses with decreased fluorescence started at about $t = 12$ and was still found between $t = 24$ and 30 h (Yamada et al. 2006). In the present experiments, the DAPI fluorescence of 5,000 DNA masses was determined at $t = 30$ h. At this time the total amount of DNA in the untreated control has become degraded to about half, and the number of DNA masses has considerably increased (Yamada et al. 2006). ActD inhibited the measured DAPI staining parameter, if the onset of treatment was $t = 0$ or 2 h. If ActD treatment started later, the effect gradually decreased. No effect was found if the treatment started at $t = 12$ h (Fig. 1b).

Isolation of genes showing a change in expression

To isolate genes possibly related to petal senescence, we performed four sequential experiments using cDNA subtraction and differential screening (Table 1). The purpose of the first experiment was to obtain genes decreasing in expression in the petals from $t = 0$ to 4 h. A total of 192 cDNA clones were picked from a subtracted cDNA pool between $t = 0$ h (tester) and $t = 4$ h (driver), and 10 clones remained after differential screening. In the second experiment, 17 clones were selected on the basis of an increase in expression between $t = 0$ and 4 h. We used 288 clones picked from a subtracted cDNA pool between $t = 4$ h (tester) and $t = 0$ h (driver). In the third and fourth experiment, we followed the same procedure to obtain genes with a change in expression from $t = 2$ to 12 h. A total 48 clones were selected from the four tests.

Sequencing and homology analysis

After sequencing of the cDNA fragments, their deduced amino acid sequences were compared with databases

Table 1 Summary of cDNA subtraction and differential screening for selection of candidate genes related to senescence in the petals

	Collection times of RNA sample (h)		No. of cDNA clones picked from subtracted cDNA pools	No. of cDNA clones selected by differential screening
Exp	Tester (A)	Driver (B)		
I	0	4	192	10
II	4	0	288	17
III	2	12	48	5
IV	12	2	144	16

Table 2 Partial sequences of senescence-associated genes (SAGs) selected by cDNA subtraction and differential screening in four experiments (I–IV). The clones designated In03 etc. in the last column are further described in Table 3

| Exp | Gene ID[a] | Clone ID[a] | EST ID[b] | Ratios of signal intensity | | Putative function/homology from EST sequence |
				Subtracted	Unsubtracted	
I	01	su0604a04	AB189184	10.5 ± 0.3	6.3 ± 0.8	Dihydroflavonol 4-reductase
	02	su0605a05	AB189185	4.6 ± 0.1	2.7 ± 0.3	UDP-glycose: flavonoid glucosyltransferase
	03	su0613b01	AB189186	4.6 ± 0.1	2.4 ± 0.1	In03
	04	su0616b04	AB189187	12.1 ± 1.1	2.1 ± 0.2	In04
	05	su0617b05	AB189188	22.5 ± 0.6	2.4 ± 0.3	CONSTANS-like protein
	06	su0622b10	AB189189	24.2 ± 0.6	10.9 ± 1.7	In06
	07	su0627c03	AB189190	8.0 ± 0.6	3.5 ± 0.6	In07
	08	su0634c10	AB189191	11.7 ± 1.4	4.7 ± 0.7	Anthocyanidin synthase
	09	su0647d11	AB189192	14.9 ± 0.1	9.4 ± 0.4	In04
	10	su0656e08	AB189193	25.9 ± 0.6	3.3 ± 1.1	In10
II	11	su1008a08	AB189194	4.9 ± 1.0	2.6 ± 0.4	Ubiquitin-protein ligase
	12	su1010a10	AB189195	12.6 ± 4.0	3.1 ± 0.6	In12
	13	su1014b02	AB189196	12.5 ± 0.8	2.2 ± 0.2	In12
	14	su1027c03	AB189197	4.3 ± 0.3	2.1 ± 0.2	In12
	15	su1041d05	AB189198	5.5 ± 1.0	3.0 ± 0.2	In15
	16	su1044d08	–	6.5 ± 1.4	2.5 ± 0.5	Not sequenced
	17	su1045d09	AB189199	5.6 ± 0.4	2.2 ± 0.2	In17
	18	su1047d11	AB189200	4.2 ± 0.2	2.7 ± 0.1	Auxin-repressed protein
	19	su1049e01	AB189201	8.6 ± 0.8	5.4 ± 0.5	Cold circadian rhythm RNA binding protein
	20	su1054e06	AB189202	7.9 ± 0.2	5.1 ± 0.3	In12
	21	su1056e08	AB189203	13.7 ± 3.1	2.4 ± 0.2	In21
	22	su1068f08	AB189204	6.0 ± 1.4	2.7 ± 0.7	Novel
	23	su1071f11	AB189205	5.4 ± 0.2	2.7 ± 0.3	In23
	24	su1072f12	AB189206	4.9 ± 0.1	7.3 ± 0.3	In15
	25	su1073g01	AB189207	17.5 ± 0.1	3.9 ± 0.7	In25
	26	su1086h02	AB189208	8.3 ± 0.9	2.1 ± 0.1	In27
	27	su1092h08	AB189209	8.8 ± 0.6	4.6 ± 0.7	In15
III	28	su0807a06	AB189210	25.1 ± 1.4	6.8 ± 0.1	In06
	29	su0810a10	AB189211	7.8 ± 0.8	2.7 ± 0.3	In29
	30	su0818b06	AB189212	6.9 ± 0.8	3.1 ± 0.2	In30
	31	su0823b11	AB189213	8.8 ± 0.8	2.3 ± 0.2	In29
	32	su0826c02	AB189214	4.6 ± 0.1	2.6 ± 0.1	In32
IV	33	su1852e04	AB189215	10.1 ± 0.7	2.7 ± 0.9	In33
	34	su1854e06	AB189216	7.0 ± 0.1	2.5 ± 0.5	In33
	35	su1855e07	AB189217	40.1 ± 2.9	2.5 ± 0.3	In35
	36	su1863f03	AB189218	30.7 ± 0.4	4.0 ± 0.6	In36
	37	su1872f12	AB189219	9.8 ± 2.1	3.1 ± 1.1	In33
	38	su1874g02	AB189220	10.5 ± 0.6	2.2 ± 0.2	In33
	39	su1895h11	AB189221	15.6 ± 1.3	3.2 ± 0.7	In33
	40	su1810a10	AB189222	4.5 ± 0.8	2.6 ± 0.5	Novel
	41	su1818b06	AB189223	12.1 ± 1.9	2.6 ± 0.4	In33
	42	su1836c12	AB189224	19.7 ± 0.9	2.4 ± 0.7	In42
	43	su1843d07	AB189225	31.3 ± 6.9	2.6 ± 0.9	In35
	44	su1855e07	AB189226	4.7 ± 1.7	2.8 ± 0.6	Expressed protein, unknown function
	45	su1866f06	AB189227	7.7 ± 0.1	2.7 ± 1.0	In45
	46	su1872f12	AB189228	43.3 ± 3.8	3.8 ± 1.0	In35
	47	su1878g06	AB189229	41.3 ± 1.2	3.2 ± 0.1	In35
	48	su1889h05	AB189230	19.7 ± 0.1	2.2 ± 0.4	Expressed protein, unknown function

[a] Gene and clone IDs are arbitrary.

[b] EST IDs are the accession numbers in the DDBJ/EMBL/GenBank databases.

Table 3 Summary of identification for full length senescence-associated genes (SAGs) based on BLAST search

Gene ID	Accession	Putative protein function/homology	Identity[a]	Plant organs (References)[b]
In03	AB267814	cytochrome P450	46%	–
In04	AB267815	caffeoyl-CoA O-methyltransferase	55% <	A (1), P (2, 3)
In06	AB267816	hypothetical protein	30%	–
In07	AB267817	extensin like protein	51%	P (4)
In10	AB267818	GPI-anchored protein	72%	O (5)
In12	AB267819	putative ripening protein	63%	A (6)
In15	AB267820	cysteine proteinase precursor	70% <	F (7), L (8), A (9)
In17	AB267821	alcohol dehydrogenase	57%	–
In21	AB267822	cysteine proteinase precursor	59% <	F (10), L (11), A (9)
In23	AB267823	pectinacetylesterase family protein	58%	–
In25	AB267824	ataxin-2 related protein	37%	–
In26	AB267825	integral membrane family protein	41%	–
In29	AB267826	leucine-rich repeat transmembrane protein kinase	62%	–
In30	AB267827	lipid transfer protein 3 precursor	47%	O (12)
In32	AB267828	SEC14 cytosolic factor / phosphoglyceride transfer family protein	64%	–
In33	AB267829	cysteine proteinase precursor	91%	L (13)
In35	AB267830	expressed protein	52%	–
In36	AB267831	putative senescence-associated protein	96%	–
In42	AB267832	14-3-3 protein	91% <	P (14)
In45	AB267833	nodulin-like protein	55%	–

[a] Identity was obtained from BLASTX analysis for deduced amino acid sequence of each In.

[b] Type of related phenomena addressed in the references; F, flower senescence; L, leaf senescence; P, pathogen-defense responses; A, abiotic-stress responses; O, others.

Note. Reference numbers confined to this table; 1, Ibdah et al. (2003); 2, Schmitt et al. (1991); 3, Busam et al. (1997); 4, Takemoto et al. (2003); 5, Hashimoto and Yamamoto (1998); 6, Hara et al. (2002); 7, Eason et al. (2002); 8, Xu and Chye (1999); 9, Koizumi et al. (1993); 10, Coupe et al. (2003); 11, Hayden and Christopher (2004); 12, Liu et al. (2000); 13, Chen et al. (2002); 14, Lapointe et al. (2001).

(Table 2). Out of the 48 genes that we isolated, one could not be sequenced and 31 showed homology with identified genes. Most of the cDNA fragments contained only a partial sequence. Full-length cDNAs were isolated using RACE-PCR. Full-length cDNA was obtained from 20 genes. The amino acid sequences deduced from these 20 full-length cDNAs were compared again with the database. The results are shown in Table 3. We only used the full-length clones for expression analysis.

Expression profile of SAG in the petals and other plant parts

Absolute transcript levels of SAGs were measured using real-time RT-PCR. The change in transcript levels of SAGs differed widely. For example, the difference in level exceeded 10^7 copies per ng RNA in clone In30 but did not exceed 10^4 copies in In25 and In29.

Relative transcript levels of SAGs in the petals (corolla), from closed buds to advanced visible senescence, were analysed by using *Actin 4* as an internal standard. During the period of study, the mRNA levels of *Actin 4* was constant, both in petals and in leaves (Fig. 2). As shown in Fig. 3, most genes studied showed upregulation followed by down-regulation, some early during the sampling period and others later. Maximum expression of these genes occurred around $t = 0$ h (In03, In04, In06, In07, In10, In17, In29, In32), $t = 4$–6 h (In12, In21, In23, In30), or later (In15, In35). Detectable expression was relatively short in ln07 and was found throughout the experimental period in ln03 and ln17, with other genes intermediate. Four genes showed upregulation towards the end of the experimental period and no subsequent down-regulation. Two of these showed a brief period of expression shortly before the end of the experimental period (In33 and In45), while the other two exhibited expression throughout the experimental period (In42) or almost throughout this period (In36).

Gene expression during petal senescence was compared with that in parts of young seedlings (stem, root, and nonsenescent cotyledon) and in leaves at a few stages of development (young, fully expanded, fully yellow). All genes studied showed very low expression in the young seedlings. In33, In35, and In42 increased in abundance in senescent leaves. The transcript levels of the other genes were extremely low or undetectable in leaves at the times of sampling.
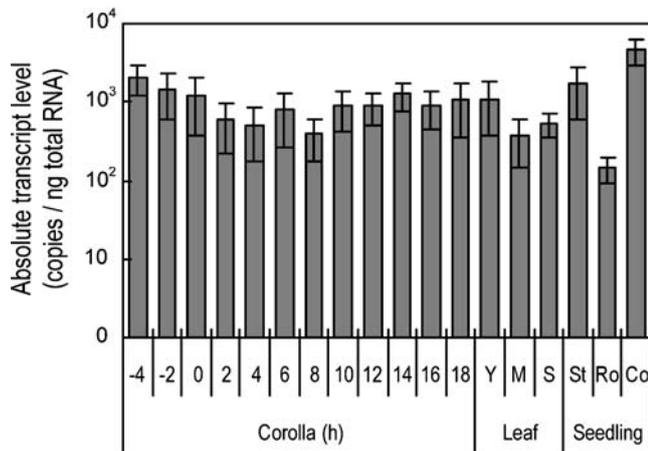
Fig. 2 Absolute transcript levels of *Actin 4* in the petals and other organs measured using real-time RT-PCR. Petals were collected every 2 h from $t = -4$ to 18 h, and each sample was mixed from three individual flowers. Leaves were collected from the seedlings 30 days after germination: Y, young leaf before full expansion; M, mature leaf after full expansion; S, senescent leaf after full yellowing. Young organs were corrected from the seedlings 14 days after germination: St, stem; Ro, root; Co, cotyledon. Data are shown as the means ± SE from eight separate experiments in three different samples

Appearance of senescence parameters compared with gene expression

The temporal relation between various processes during petal senescence and expression of SAGs is shown in Fig. 4. A color change started from $t = 3$ h and was clear by $t = 5$ h. Inward rolling started about $t = 9$ h and went on until at least $t = 22$ h. Using agarose gels, DNA degradation was observed from $t = 9$ to 30 h. Apparent nuclear fragmentation probably started at about $t = 0$ h but was clearly shown between $t = 12$ and 18 h. It persisted at least until $t = 24$ h (Yamada et al. 2006). Expression of SAGs was related to visible senescence symptoms and some PCD parameters. We used the period of genes expression during which the relative transcript level exceeded 10% of total, from $t = -4$ to 18 h. The 10% limit was arbitrarily chosen. Data are shown in Fig. 4. The data indicate that most of the genes studied started expression prior to the onset of any of the parameters of senescence/PCD. Some other genes, in contrast, were expressed when the senescence and PCD processes measured were already underway.

Discussion

The flowers opened from about 5:00 a.m. and were almost open at 8:00 a.m. If ActD treatment started at 5:00 a.m. the flowers did not open. This shows that the gene expression required for opening occurs very closely in time with the actual opening process.

Petal inward rolling is the main visible senescence symptom in *I. nil*. It is reportedly due to loss of turgor in the midrib of the fused petals. This loss of turgor, in turn, is due to senescence (Winkenbach 1970). ActD, if applied at $t = 0$ h strongly inhibited the inward rolling of the petals, but if given at $t = 4$ h it no longer had an effect (Fig. 1). Inward rolling started at about $t = 9$ h. It is therefore tempting to speculate that the window of gene expression leading to this senescence symptom (ending at about $t = 2$ to 4 h) preceded the onset of the symptom by about 5–7 h.

Interestingly, the effect of ActD on the DNA fluorescence per DNA mass differed from the ActD effect on petal wilting/inward rolling. If ActD was applied from $t = 0$ h, it almost fully inhibited both inward rolling and the DNA fluorescence per DNA mass. This indicates that the transcription processes that lead to these senescence effects was active at $t = 0$ h. However, if ActD was applied at $t = 4$ h, it came too late to affect inward rolling, but still strongly inhibited the DNA fluorescence parameter. This suggests that the transcription leading to petal inward rolling—the earlier senescence symptom—apparently ceased earlier than that resulting in the later senescence symptom/parameter.

Two enzymes related to cell wall synthesis and cell wall reassembly were expressed very early during the investigated time span. In07 was similar to tobacco NtEIG-C29, and encoded a putative extensin-like protein (Takemoto et al. 2003). Extensins are plant structural cell-wall proteins, implicated in growth and in stress responses (Merkouropoulos and Shirsat 2003). In04 encoded caffeoyl-CoA-3-O-methyltransferase, an enzyme that catalyses the methylation of caffeoyl-CoA into feruoyl-CoA, a precursor of lignin. It is involved in growth and in disease resistance response (Pakusch et al. 1989). Flower opening is based on movements and therefore on elongation growth. During the period of flower opening the petals also grew in width and length. The expression, during opening, of some genes related to cell-wall growth or cell-wall degradation might therefore relate to flower opening, but it cannot be excluded that they were related to cell-wall degradation prior to cell death. It is known that cell death in the mesophyll can start very early, even before flower opening (Wagstaff et al. 2003), so early expression might also be part of cell-wall remobilization rather than growth.

In23 encoded a putative pectinacetylesterase. It showed some similarity with a gene expressed in petals of daffodil flowers (Hunter et al. 2002). This gene was expressed much later than the two other cell-wall related enzymes. The expression of this gene also occurred after the end of flower opening. This gene is therefore probably related to cell-wall degradation as part of remobilization. Winkenbach (1970)
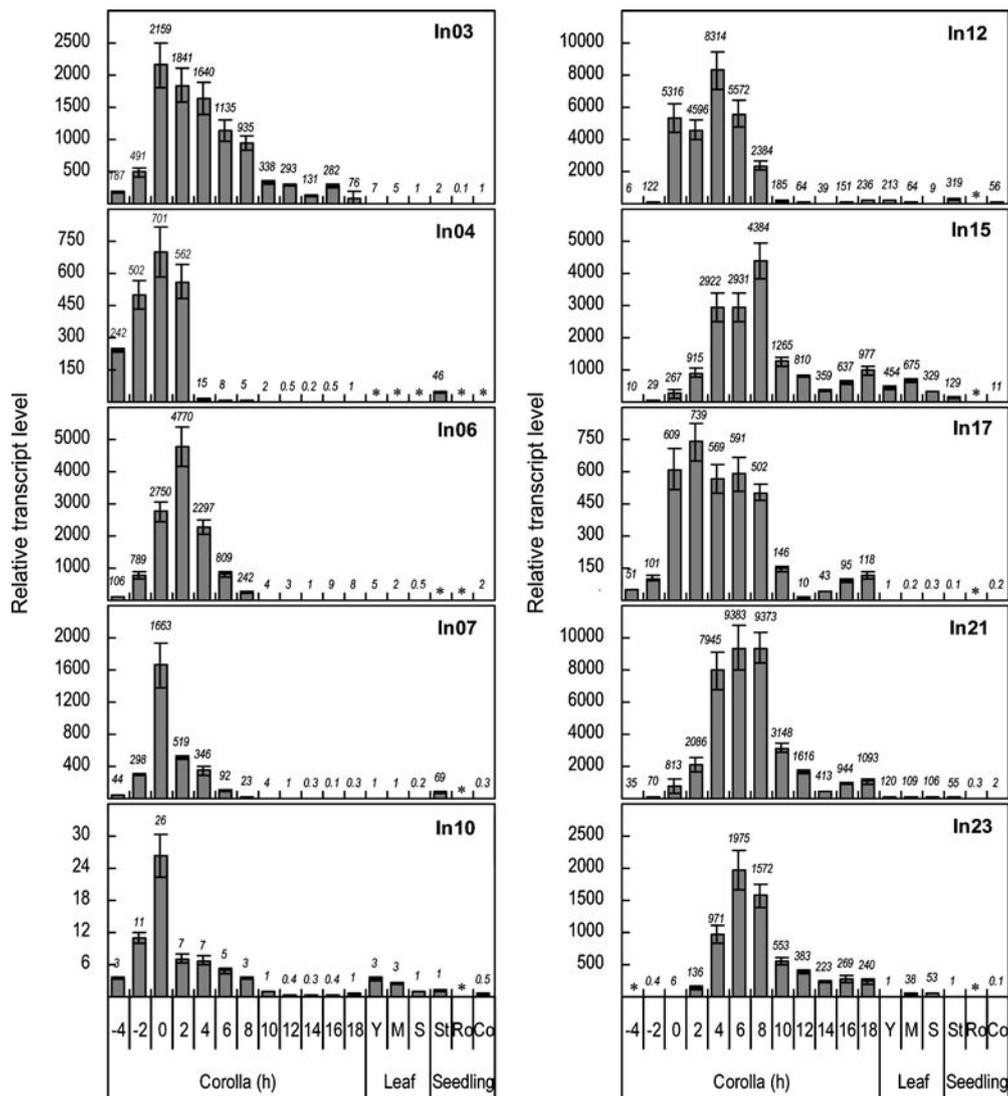
Fig. 3 Change of the relative transcript levels for senescence-associated genes (SAGs) in the petals and other organs analysed using *Actin 4* as an internal standard. Petals were collected every 2 h from *t* = −4 to 18 h, and each sample was mixed from three individual flowers. Leaves were collected from the seedlings 30 days after germination: Y, young leaf before full expansion; M, mature leaf after full expansion; S, senescent leaf after full yellowing. Young organs were corrected from the seedlings 14 d after germination: St, stem; Ro, root; Co, cotyledon. Data are shown as the means ± SE from three different samples. Asterisks indicate the level were lower than detectable limit of real-time RT-PCR

showed extensive cell-wall breakdown during petal senescence in *I. nil*.

A number of SAGs showed high homology with known SAGs in other species. In15, a putative cysteine protease, showed similarity with PRT22 related to tepal senescence of *Sandersonia* (Eason et al. 2002). In21, a putative cysteine protease with a granulin domain, was similar to several genes related to senescence, i.e., BoCP2 of broccoli (Coupe et al. 2003), ALSCYP1 of *Alstroemeria* petals (Wagstaff et al. 2002), SEN102 of daylily petals (Guerrero et al. 1998), and DCCP1 of carnation petals (Jones et al. 1995). The sequence of the putative cysteine protease In33 was similar to that of genes related to leaf senescence, i.e., SPG31 of

sweet potato (Chen et al. 2002), SAG12 of *Arabidopsis* (Noh and Amasino 1999a), and BnSAG12 of *Brassica* (Noh and Amasino 1999b). As inhibitors of cysteine proteases delayed the senescence of *Sandersonia* petals flowers (Eason et al. 2002) and postharvest floret senescence was delayed in transgenic broccoli plants bearing a down-regulated cysteine protease gene (Eason et al. 2005), some cysteine proteases are apparently required for production of the visible senescence symptoms.

Cytochrome P450 genes were expressed in senescing petals of daylily (Panavas et al. 1999) and *Alstroemeria* (Breeze et al. 2004). A cytochrome P450 was also expressed in the *Brassica* leaves at the onset of senescence (Buchanan-
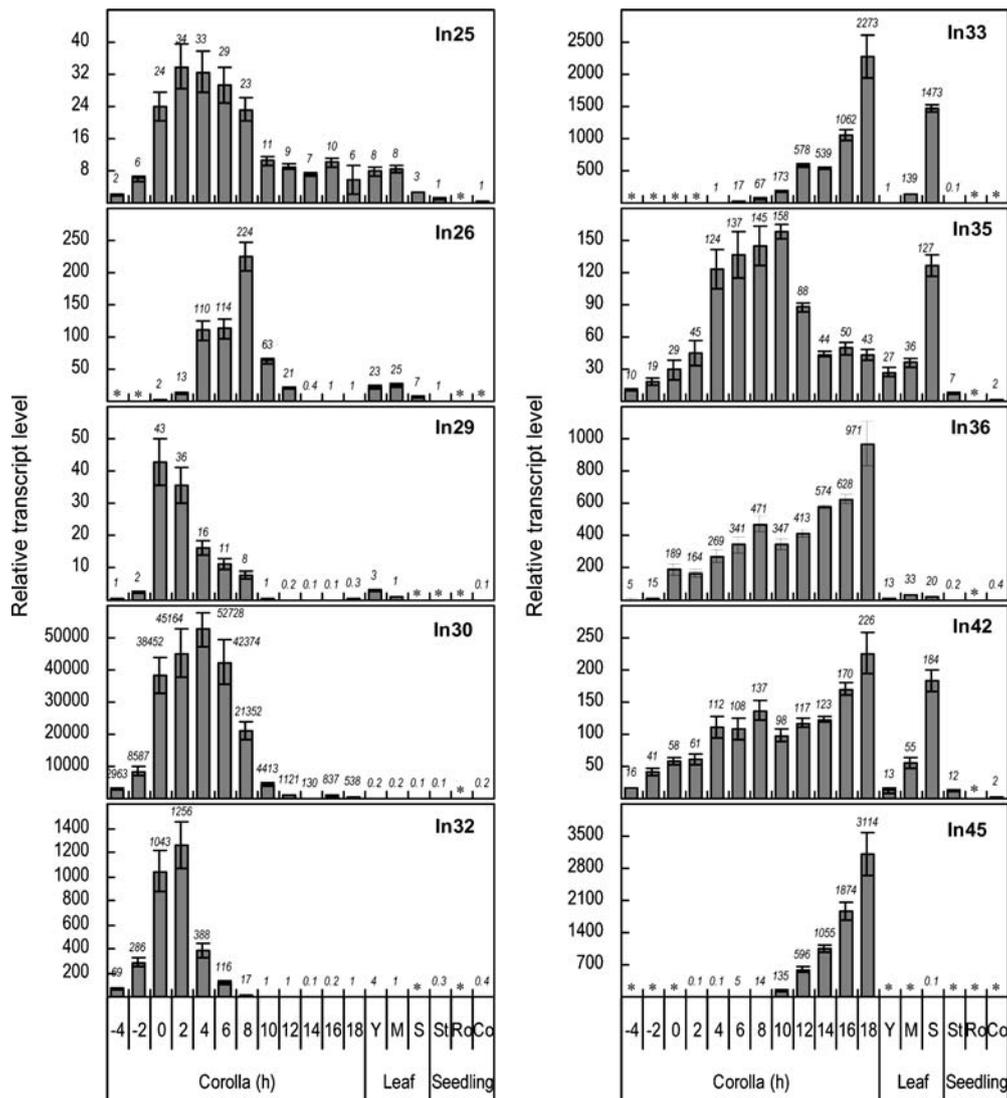
Fig. 3 Continued

Wollaston 1997). Cytochrome P450 containing proteins are involved in numerous biochemical processes. An *Arabidopsis* cytochrome P450 was expressed prior to cell death in the hypersensitive response (HR) and was associated with cell death during leaf senescence (Godiard et al. 1998). The function of cytochrome P450 in senescence is as yet unknown.

Both an alcohol dehydrogenase (ADH), with a different sequence than our In17, and a so-called lipid transfer protein (LTP), which showed low similarity to In30, have been identified as SAGs in *Arabidopsis* leaves (Gepstein et al. 2003). The role of ADH during senescence is as yet unclear. ADH is upregulated in response to hypoxia, but is also known to become upregulated as a result of aerobic fermentation in yeast. ADH was also shown to be involved in conversion of C6-volatiles such as n-hexanal to the corresponding alcohols, during fruit flavor production in tomato and has been impli-

cated in aromatic compounds in *Petunia* flowers (Garabagi et al. 2005). LTPs are now known not to be involved in intracellular lipid trafficking—the role they were initially proposed to have—but rather in plant resistance to biotic and abiotic stresses (Blein et al. 2002). Their function is as yet unclear.

One SAG showed sequence similarity with known stress-responsive genes in other species. In12 was similar to a gene encoding a putative abscisic acid/water deficit-induced protein of *Calystegia soldanella*. It is a homolog of tomato ASR (abscisic acid, stress, and ripening) gene family. The expression of the members of this family is induced by a number of abiotic stresses (Hara et al. 2002). In15 and In21 (putative cysteine proteases) were similar to PD19 and RD21 of *Arabidopsis*. The expression of these two genes was induced by drought stress (Koizumi et al. 1993), but it is not clear if this occurred independently of water-stress induced senescence.
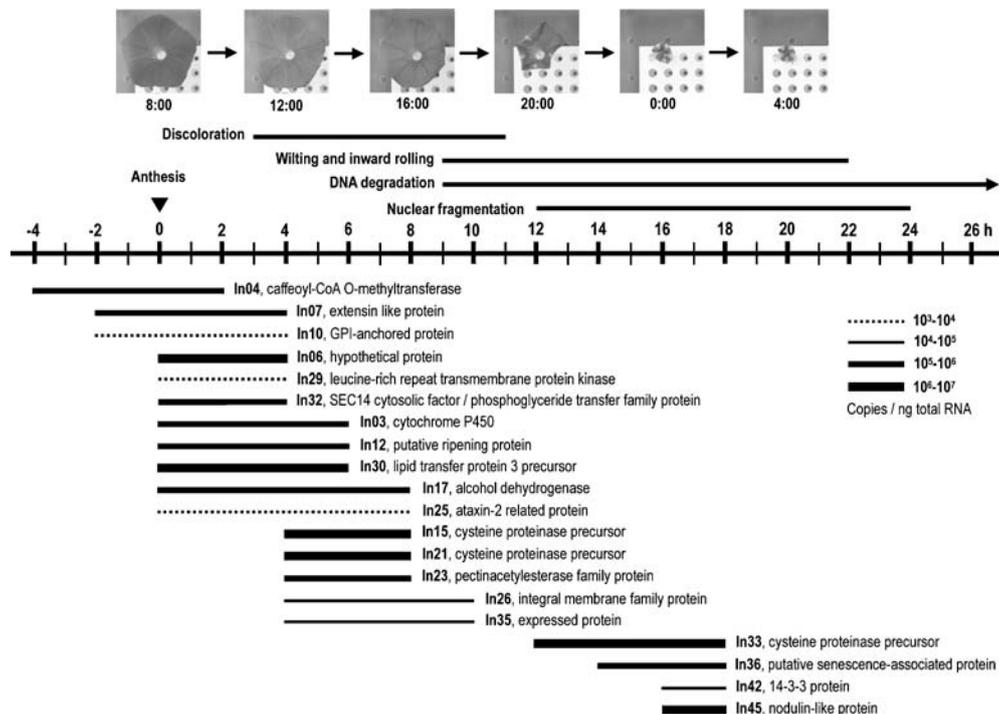
Fig. 4 Summary of the expression of SAGs and time courses of senescence processes in the petals. The expression period of each gene is indicated as range of the times when the relative transcript levels are more than 10% of total levels of all times. Width of the horizontal lines indicates the average absolute transcript level of each gene during the expression periods. The time course of the mentioned processes (of senescence/programmed cell death) have been reported previously (Yamada et al. 2006)

In29 encoded a putative leucine-rich repeat transmembrane receptor protein kinase. Since other protein kinases are involved in signal transduction, it is tempting to speculate that the present protein has a similar function. Another leucine-rich repeat transmembrane protein kinase (called SIRK) has been reported to become upregulated, rather specifically, during leaf senescence in *Arabidopsis*. The transcriptional activation of SIRK depended on the transcription factor WRKY6. In *Arabidopsis* the strongest WRKY6 expression was observed during leaf senescence, but expression was also found in some other tissues including floral organ abscission zones. SIRK had nine W boxes (TGACC/T) typical for binding of WRKY transcription factors (Robatzek and Somssich 2002). The currently isolated putative protein kinase (In29) contains three W boxes, so seems also regulated by a WRKY transcription factor.

In42 showed high similarity with genes encoding a 14-3-3, protein kinases that are ubiquitous in eukaryotic cells. 14-3-3 proteins control processes such as progression through the cell cycle, initiation and maintenance of DNA damage checkpoints, and prevention of apoptosis (Wilker and Yaffe 2004). In higher plants, 14-3-3 has been related to pathogen defense responses (Lapointe et al. 2001), and other stress responses (Roberts 2003). In contrast to the leucine repeat rich receptor protein kinase, 14-3-3 proteins reported from plants do not seem to have a W box. The 14-3-3 now isolated from *Ipomoea* also does not contain one.

In25 had a homology domain of ataxin-2, a protein of unknown function in animal cells, involved in causing spinocerebellar ataxia type 2 (SCA2), a late-onset neurodegenerative disorder (Huynh et al. 2003). Ataxin-2 has an ortholog in *Arabidopsis*. Its action has been related to RNA metabolism (Bravo et al. 2005). Ataxin-2 reportedly has an important role in regulating the susceptibility of neuroblastoma cells to apoptotic stimuli (Wiedemeyer et al. 2003). If ataxin-2 is overexpressed in yeast it causes premature cell death due to defects in actin filament formation (Satterfield et al. 2002). Actin depolymerisation has been shown to be an adequate cause of cell death in pollen tubes (Thomas et al. 2006), suggesting that the relation between cell death and lack of actin structure is also present in angiosperms. It is not clear if the presently observed induction of ataxin-2 in *Ipomoea* petals is also an essential part of the process leading to cell death.

In32 had a homology domain of SEC14, a major phosphatidylcholine/phosphatidylinositol transfer protein in yeast. SEC14 is required for normal Golgi vesicle transport. Secretion of some enzymes to the cell wall depends on SEC14, probably because this secretion depends on vesicle transport (Henneberry et al. 2001). Ablation of *SEC14* function not only prevented Golgi-mediated protein transport, but also resulted in cell death (Cleves et al. 1991).

Except for a few genes, such as cysteine proteases and cell-wall-related proteins, we do at present not know the

possible function of the isolated genes in the process leading to visible senescence.

A putative cysteine protease (In33), a protein of unknown biochemical function (In35) and the putative 14-3-3 protein were expressed both in senescing petals and in senescing leaves. They might therefore also be involved in leaf senescence. Results from studies on gene expression during leaf senescence (Buchanan-Wollaston 2003) and petal senescence (van Doorn et al. 2003; Breeze et al. 2004) indicate that the two are not fundamentally different.

If one is to compare the period of gene expression with the other parameters during senescence, a number of pitfalls should be kept in mind. First, the expression of genes that are necessary to produce the parameter might considerably precede the onset of the parameter, as previously concluded in the case of genes required to produce petal wilting and inward rolling. Secondly, the petals are not necessarily homogeneous with regard to the onset of the various senescence processes in different cell types. In *Iris*, for example, the mesophyll cells senesced two days prior to the epidermis cells, whereby visible petal wilting was a result of senescence in the epidermis cells and unrelated to the death of the mesophyll cells (van Doorn et al. 2003). This means that gene expression in cells that die early can considerably precede the gene expression in the cells that produce a visible senescence symptom. Examples of tissue-specific gene expression include developing roots (Birnbaum et al. 2003) and programmed cell death in the seed pericarp and endosperm (Sreenivasulu et al. 2006).

Despite these caveats it might be possible to exclude some gene expression, at least tentatively, from the production of a visible symptom. If a process such as wilting is clearly underway, but a gene still has to start its increase in expression, it is likely that this gene is not involved in producing the process. The present results therefore indicate that the genes In33 (encoding a putative cysteine protease), In36 (encoding a putative senescence-associated protein), In42 (encoding a 14-3-3 protein), and In45 (encoding a putative nodulin-like protein) are not involved in petal discoloration and probably also not in the early stages of petal wilting, DNA degradation, and nuclear fragmentation.

In conclusion, the data indicate that the end of gene expression leading to flower opening occurred very closely in time with the opening process itself. This follows from the effect of ActD, which fully inhibited opening if applied less than an hour prior to the onset of floral opening. This was different from the relation between gene expression and the onset of visible senescence. Here, the end of gene expression required to produce visible senescence preceded the onset of the symptom by about 5 h. In addition, several of the isolated SAGs have been isolated previously, in association with petal or leaf senescence. The present data corroborate the relation-

ship between the expression of these genes and the processes leading to senescence/PCD. The detailed function of several of these genes is still unknown. Two genes have not been previously noted in relation to senescence. One of these, encoding an ataxin-2 is particularly interesting. If overexpressed, it caused premature cell death in yeast. Another gene, encoding a putative receptor protein kinase also seems interesting since the upregulation of a similar gene was rather specific for leaf senescence. The results, as those of earlier studies on gene expression in relation to senescence/PCD, confirm the ideas of highly regulated gene expression and the presence of a concerted mechanism of cellular degradation.

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