Effect of cytokinins on delaying petunia flower senescence: a transcriptome study approach

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Abstract

Flower senescence is a fascinating natural process that represents the final developmental stage in the life of a flower. Plant hormones play an important role in regulating the timing of flower senescence. Ethylene is a trigger and usually accelerates the senescence rate, while cytokinins are known to delay it. The aim of this work was to study the effect of 6-benzylaminopurine (BA) on petal senescence by transcript profile comparison after 3 or 6 h using a cross-species method by hybridizing petunia samples to a 4 × 44 K Agilent tomato array. The relative content of ethylene, abscisic acid, anthocyanins, total carotenoids and total phenols that determine the physiological behaviours of the petal tissue were measured. BA treatment prolonged the flower life and increased the concentrations of phenols and anthocyanins, while total carotenoids

did not increase and were lower than the control. The ethylene biosynthetic and perception gene expressions were studied immediately after treatment until 24 h and all genes were repressed, while ethylene production was strongly induced after 4 days. The microarray analyses highlighted that BA strongly affected gene regulation after 3 h, but only 14 % of genes remained differentially expressed after 6 h. The most affected pathways and genes were those related to stress, such as heat shock proteins, abscisic acid (ABA) catabolism and its signalling pathway, lipid metabolism and antioxidant defence systems. A gene annotation enrichment analysis using DAVID showed that the most important gene clusters were involved in energy generation and conservation processes. In addition to the ethylene pathway, cytokinins seem to be strongly involved the regulation of the ABA response in flower tissues.

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Keywords

Flower senescence
6-Benzyladenine
BA
Cytokinins
Petunia × *hybrid*
ABA

Electronic supplementary material

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Introduction

Flower senescence is one of the most fascinating natural processes. It is temporally programmed and characterised by sequential events that begin with molecular changes and involve the physiology and biochemistry of a part of or the entire organism. The regulation of senescence has been extensively studied, but many aspects have not been elucidated. This process is influenced by a number of endogenous phytohormones; in fact, exogenously applied ethylene, abscisic acid (ABA) and cytokinins have been shown to accelerate or delay senescence. Plant hormone biosynthesis

and accumulation change during a flower's life, and tissue sensitivities also change with development. Transgenic petunias that constitutively express the ethylene receptor gene *etr1* (P35S::etr1-1) have delayed corolla senescence and a flower longevity that is twice that of non-transformed Mitchell flowers (Jones et al. 2009). In addition, earlier experiments showed analogous results using inhibitors of ethylene biosynthesis or its activity (Singh et al. 1992; Mensuali-Sodi et al. 2005).

The initiation of the senescence process is known to involve alterations in the levels of other plant hormones, the cytokinins. Increasing cytokinin levels in plants is a strategy for delaying flower senescence. Previous studies have shown that either exogenous application (Taverner et al. 1999) or increased exogenous production of cytokinins in transgenic lines overexpressing a senescence-associated gene (SAG12)-specific promoter for driving the expression of the isopentenyl transferase gene (SAG12-ipt) delays senescence (Gan and Amasino 1995; Xu et al. 2010). Transgenic lines of Petunia transformed with SAG12-ipt exhibit delayed senescence, and in some plants, total cytokinins were 25-fold higher than in wild type (Chang et al. 2003).

Exogenous applications of BA for 24 h or 5–6 days using concentrations ranging from 2 to 200 μM almost completely prevented senescence (Taverner et al. 1999). Examining the metabolism of cytokinins in petunias demonstrated that a reduction of the isoprenoid side chain of zeatin riboside reduced the senescence inhibitory effect. The target genes of these cytokinins during flower senescence are unknown. Therefore, the aim of this work was to study the role of BA in petunia flower senescence by transcriptome profile analysis. Because we were interested in primary responses in gene activation, the sampling was performed after 3 and 6 h. These time points were selected on the basis of previous experiments and literature confirmation on genome-wide expression in *Arabidopsis* treated with BA (Brenner et al. 2005). As the sequences of mRNAs in petunia are not publically available, a microarray cross-species approach was adopted. The differentially expressed genes obtained were validated using qRT-PCR for genes involved in ethylene biosynthesis perception and signal transduction and for a set of genes selected among the list of differentially expressed genes (DEGs).

Materials and methods

Plant material

Petunia (*Petunia* × *hybrida* L.) flowers cv. Dreams Appleblossom were grown in the greenhouse under natural conditions (April–August) with 28/18 °C day/night temperatures. The development of the flowers from the bud stage was monitored daily, and the flowers were harvested the first day of flower opening no later than 11:00.

Flower life and chemical treatments

Flowers were treated by placing their pedicels in a solution containing distilled water (control) or 200 μM BA (Sigma, Italy). The effect of the treatments was evaluated in a postharvest room under the following conditions: photon flux density of 15 μmol m^{-2} s^{-1}, 12-h photoperiod, 60 % RH and 20 °C temperature. Flower life was determined by daily observation and was considered finished at the first symptom of senescence (wilting).

Abscisic acid and ethylene determinations

Petals [100 mg fresh weight (FW)] were collected, weighed, frozen in liquid nitrogen, and then stored at −80 °C until analysis. ABA was measured after an extraction in distilled water (water:tissue ratio = 10:1 v/w) overnight at 4 °C. Then, ABA was determined by an indirect enzyme-linked immunosorbent assay based on the use of the DBPA1 monoclonal antibody raised against S(+)-ABA (Vernieri et al. 1991) as described previously (Trivellini et al. 2011).

Ethylene production was measured by enclosing flower organs in airtight containers (250 ml). Two-millilitre gas samples were taken from the headspace of the containers after 1 h incubation at room temperature. The ethylene concentration in the sample was measured by a gas chromatograph (HP5890, Hewlett-Packard, Menlo Park, CA) using a flame ionisation detector, a stainless steel column (150 × 0.4 cm ø packed with Hysep T) with column and detector temperatures of 70 and 350 °C, respectively, and nitrogen carrier gas at a flow rate of 30 ml min^{-1}.

Anthocyanin, carotenoid and phenol determinations

Total carotenoids for petunia petals were determined by extraction using 99.9 % methanol as a solvent. Petal samples were kept in a dark, cold room

at 4 °C for 24 h. Quantitative measurement of carotenoids were performed immediately after extraction. Absorbance readings were performed at 470 nm. Total carotenoids were calculated using Lichtenthaler's formula (1987).

Anthocyanins from petals of the frozen tissue (50 mg) were extracted into methanolic HCl (1 %). Samples were incubated overnight at 4 °C in darkness. The concentration of anthocyanins was expressed in cyanidin-3-glucoside equivalents determined spectrophotometrically at 535 nm using an extinction coefficient of 29,600 (ε).

Phenolic compounds were extracted from 30 to 50 mg of petals using 5 ml of 1.2 M HCl in 99 % methanol. Absorbance measurements were taken after overnight incubation at 4 °C. Total phenolic compounds were estimated by measuring absorbance at 320 nm using an UV–Vis spectrophotometer (Ke and Saltveit 1989). The amounts of total phenol were expressed as gallic acid equivalents.

Microarrays analysis: cDNA synthesis, labelling and hybridisation

Total RNA was amplified using the Amino Allyl MessageAmp II aRNA Kit (Ambion) to obtain amino allyl antisense RNA (aaRNA), following the method developed by Eberwine and coworkers. Briefly, mRNA was reverse transcribed into single-stranded cDNA; after the second strand synthesis (in the second round of amplification), cDNA was in vitro transcribed in aaRNA including amino allyl modified nucleotides (aaUTP). Both dsDNA and aaRNA underwent a purification step using columns provided with the kit.

Labelling was performed using NHS ester Cy3 or Cy5 dyes (Amersham Biosciences) that are able to react with the modified RNA. The mRNA quality was checked using RNA 6000 nano chip assays (Agilent Technologies). At least 5 μg of mRNA for each sample was labelled and purified with columns. Equal amounts (0.825 μg) of labelled sample and reference specimens were combined, fragmented and hybridised to oligonucleotide glass arrays representing the *S. lycopersicum* transcriptome. All steps were performed using the In Situ Hybridisation Kit Plus (Agilent Technologies) and followed the 60-mer oligo microarray processing protocol (Agilent Technologies). Then, the slides were washed

with the Agilent wash procedure and scanned with a dual-laser Agilent G2505B microarray scanner. A tomato gene expression microarray of *S. lycopersicum* from the Agilent catalogue (http://earray.chem.agilent.com) was used. The microarray design format was 4 × 44 K. Each slide contained four microarrays. The total number of oligos was 45,220 with 1,417 Agilent controls. The length of the probes was 60 bp, and the probes were randomly located on the microarray slide. Five replicates for each probe were used. The labelled RNA was hybridised to the sequences on the chip as described in Janssen et al. (2008). In brief, for each of the two biological replicates, RNA from each treatment was labelled with Cy3 and hybridised in the presence of Cy5-labelled petunia onto the tomato genome set. Both labelling experiments were then repeated so that each RNA population was hybridised four times, twice with Cy5 and twice with Cy3. The data were analysed by fitting linear models to both the log2 Cy5/Cy3 ratios (ratio analysis) and log2 Cy3 and log2 Cy5 intensities (separate channel analysis) using the limma software package (Smyth and Speed 2003).

Total RNA isolation and gene expression analyses

Total RNA was isolated using Trizol reagent (Invitrogen, Italy) following the manufacturer's instructions. Total RNA (1 µg) was treated with DNaseI (Sigma, Italy) and used as a template for cDNA synthesis. The efficiency of DNaseI was confirmed by ~~gel~~ electrophoresis gel. cDNA synthesis was performed using Superscript III (Invitrogen, Italy) with a mix of random primers (10 µM) and an 18 S rRNA reverse primer.

Genes involved in the ethylene pathway that were studied were *PhACS* (Z18953), *PhACO* (M90294), *PhERS1* (DQ154118), *PhETR2* (DQ154119), *PhETR3* (AF145975), *PhEIN2* (AY353249) and *PhEIL1* (AY353248). The expression of these genes was determined by qRT-PCR (ABI7300, Applied Biosystems, Italy) using specific primers. Moreover, to confirm the microarrays results, eight genes were randomly selected among the DEGs. All primers are listed in Suppl. Table 7 (S7).

SYBR green chemistry was used for gene expression analyses. Dissociation curves were performed to check the absence of primer dimers and other amplification by-products.

The amplification programme was set to the following: 1 cycle at 50 °C for

2 min, then 95 °C for 2 min; 40 cycles of 95 °C for 30 s, 55 °C for 1 min and 72 °C for 30 s (signal acquisition stage); 72 °C for 10 min and a dissociation curve. *18S rRNA* and *PhACT* were used as internal controls (S7).

Gene annotation enrichment analysis

The Database for Annotation, Visualisation and Integrated Discovery (DAVID 6.7; Huang et al. 2009; http://david.abcc.ncifcrf.gov/home.jsp) was used to aid biological interpretation of the gene sets by identifying enriched biological themes using over 40 annotation categories, including gene ontology terms, protein–protein interactions and protein functional domains. The enrichment score of a cluster was obtained using the functional annotation clustering (FAC) and Gene Functional Classification tools that clusters functionally related annotations into groups and ranks them in importance with an enrichment score. An enrichment score of 1.3 for a cluster is equivalent to a non-log scale of 0.05; therefore, scores ≥1.3 are considered enriched. To provide an overall idea of gene distributions among terms, a functional annotation chart was used. The number of genes involved in a given term is divided. Medium stringency was applied for the analyses.

Statistical analysis

The physiological experiments were repeated at least two times, and each experiment represents the mean from three biological samples ($n = 3$). The qRT-PCR reactions were performed on two biological and three technical replicates.

Data were subjected to one-way ANOVA analyses, and significant differences among means were determined using a Bonferroni post-test ($P < 0.05$).

All array data were normalised using LOWESS normalisation. Data were excluded for genes with poor spot quality or genes that did not have a mean intensity greater than 10 for one of the two channels (green and red) in at least 70 % of the experiments. The log2 ratio of the mean red intensity over the mean green intensity was calculated for each gene followed by LOWESS normalisation.

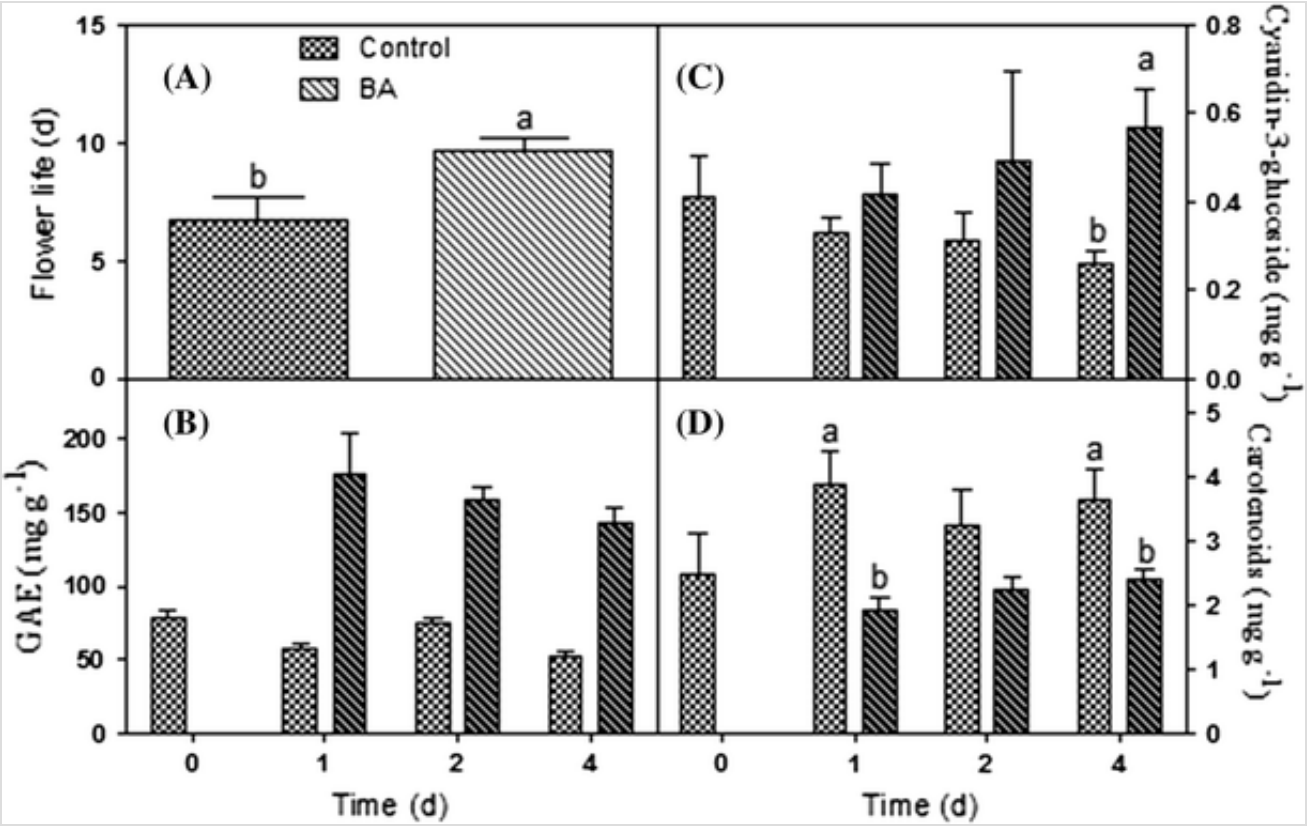
Results

Flower life, anthocyanins, total phenols and carotenoids

The flower lifespan of untreated petunia was on average 6.7 days. Treatments with BA delayed flower senescence to 9–10 days (Fig. 1A ~~a~~). The differences in the endogenous content of the main antioxidant compounds were evaluated over a period of 4 days. The total phenols in BA-treated flowers increased by threefold after 1 day of vase life and slightly declined during the experimental period, while in control flowers, total phenols did not change (Fig. 1B ~~b~~). The levels of anthocyanins increased in treated flowers, while levels declined in control flowers (Fig. 1C ~~c~~). After 4 days of vase life, the anthocyanins were 2.2-fold higher than the controls. Total carotenoids in BA-treated flowers, however, did not increase among the different time points, and values ranged from 1.9 to 2.4 mg g^{-1} (Fig. 1D ~~d~~). However, a general reduction in total carotenoid content was observed between the treated petals and control petals.

Fig. 1

Flower life (A) ~~(a)~~, total phenols expressed as gallic acid equivalents (B) ~~(b)~~, anthocyanins expressed as cyanidin-3-glucoside (C) ~~(c)~~ and total carotenoids (D) ~~(d)~~ in petals of fully open petunia flowers treated with a solution containing distilled water (control) or 200 μM BA. Values are the means with SE (n = 5). Data were subjected to one-way ANOVA analysis. Different letters indicate significant differences at $P < 0.05$

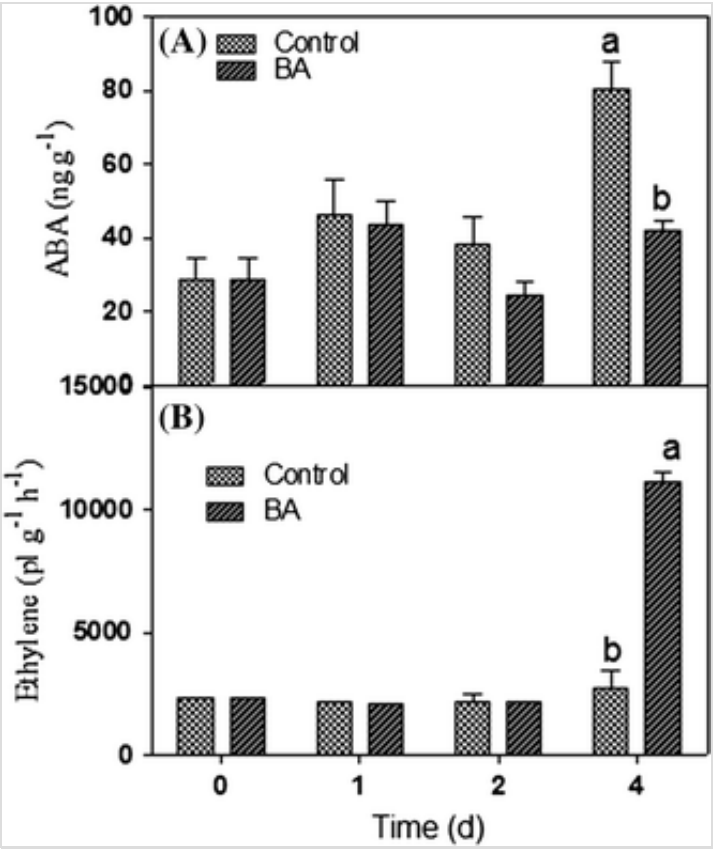


Measurement of endogenous plant hormones: ABA and ethylene

Both ethylene and ABA influence flower senescence, especially in those flowers that are sensitive (Reid and Chen 2007). The levels of these plant hormones strongly affect the speed of flower senescence. Therefore, both hormones were monitored after BA treatment. Endogenous ABA content in control flowers increased with flower aging. The BA treatment prevented the increase in ABA at 4 days that was observed in the control flower. The endogenous content of ABA in BA-treated flowers was 42 ng g^{-1} FW, while in the control, this content was double and reached 80 ng g^{-1} FW (Fig. 2A ~~a~~).

Fig. 2

Endogenous ABA concentration (~~a~~)(A) and ethylene production (B) in petals of fully open petunia flowers treated with a solution containing distilled water (control) or 200 µM BA. Values are the means with SE (n = 4). Data were subjected to one-way ANOVA analysis. Different letters indicate significant differences at $P < 0.05$



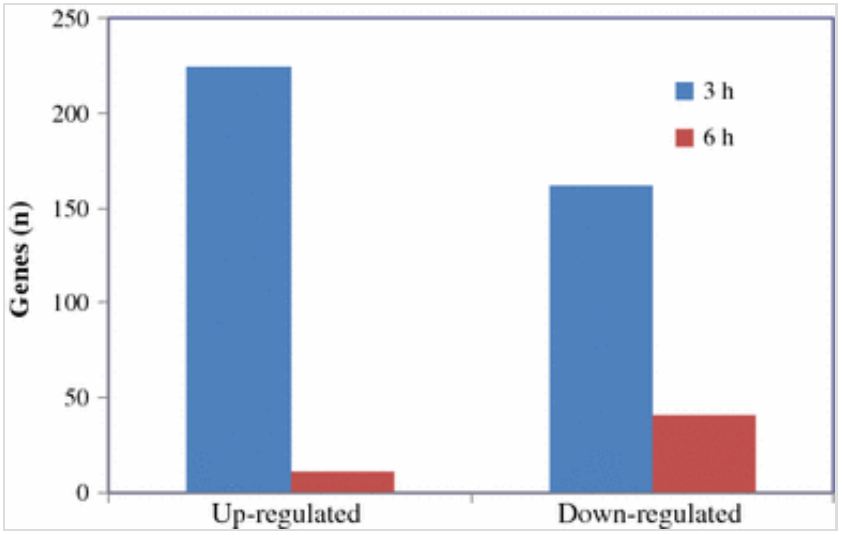
Ethylene biosynthesis slightly increased in control flowers, although the change was not significant, while it was strongly stimulated by treatment with BA (Fig. 2B ~~b~~). After 4 days of vase life, the ethylene production in BA-treated flowers was four fold higher than control flowers.

The length of exogenous application of BA differentially affected the response of petunia petals.

The number of DEGs between control and BA-treated petals after 3 h was 386, based on two fold changes and $P < 0.01$. Among them, 162 DEGs were up-regulated and 319 were down-regulated (Suppl. Table S1 and S2). After 6 h, only 52 DEGs were identified, and 41 were up-regulated and 11 were down-regulated (Fig. 3).

Fig. 3

Genes *up*-or *down-regulated* by 200 μM BA after 3 or 6 h of treatment



The most repressed genes at 3 h encoded two arabinogalactan proteins with eight to twelvefold changes. At 6 h, the most repressed genes were hydrolase and endo-beta-1,4-D-glucanase with sixfold changes and cytokinin oxidase/dehydrogenase 2 with a 5.85-fold change. The highest up-regulated gene at 3 h was HSP 70 with 27–48-fold changes. After 6 h, the highest expressed gene was temperature-induced lipocalin, binding/transporter, putative.

Venn diagrams were used to illustrate the relationship between the numbers of genes whose transcripts showed a two fold or greater change in abundance among the experimental timing and the number of genes showing common induction and/or repression in the two treatments.

The Venn diagram showed that only 16 genes were significantly expressed in treated petals at both the 3- and 6-h sampling times (Table 1). Considering only the down-regulated genes, a comparison between 3 and 6 h showed that no DEGs were commonly expressed. A comparison of up-regulated genes at 3 h and down-regulated genes at 6 h showed that 12 genes were in common (Table 2).

Table 1

The 16 genes that were significantly differentially expressed at both the 3- and 6-h sampling times in petals treated with BA

Accession no.	Gene annotation	Fold change	
		3 h	6 h
AC215453	Chloroplast genome	2.38	−5.44
AC239738	NADH dehydrogenase subunit 3	3.89	−2.75

AF123259	Heat shock protein 90	8.32	2.16
AK323459	LYM2 (LYSM DOMAIN GPI-ANCHORED PROTEIN 2 PRECURSOR), putative	−2.76	2.36
AK324636	Putative UDP-glucoronosyl/UDP-glucosyl transferase family protein	5.79	−2.86
AK325451	α-Tubulin	−2.18	2.37
AK329594	Ran-binding protein 1b (RanBP1b)	3.87	−2.77
AM087200	Chloroplast genome	2.38	−4.19
AY034149	Alternative oxidase 1b	2.44	−2.34
AY971876	Ascorbate oxidase	2.75	−3.31
BM412459	Unknown	3.76	−2.42
BT012711	α-Tubulin	−2.11	2.68
BT012714	Pectate lyase family protein, putative	2.61	−3.04
BT013487	Cytidine triphosphate synthase (CTP synthase)	−3.08	2.01
BT013505	Myo-inositol-1-phosphate synthase (INS-1P)	4.32	−2.15
TA39797_4081	F1-ATPase alpha subunit (atpA)	4.48	−2.11

Table 2

The 12 genes that were significantly repressed after 3 h and up-regulated after 6 h in BA-treated petals

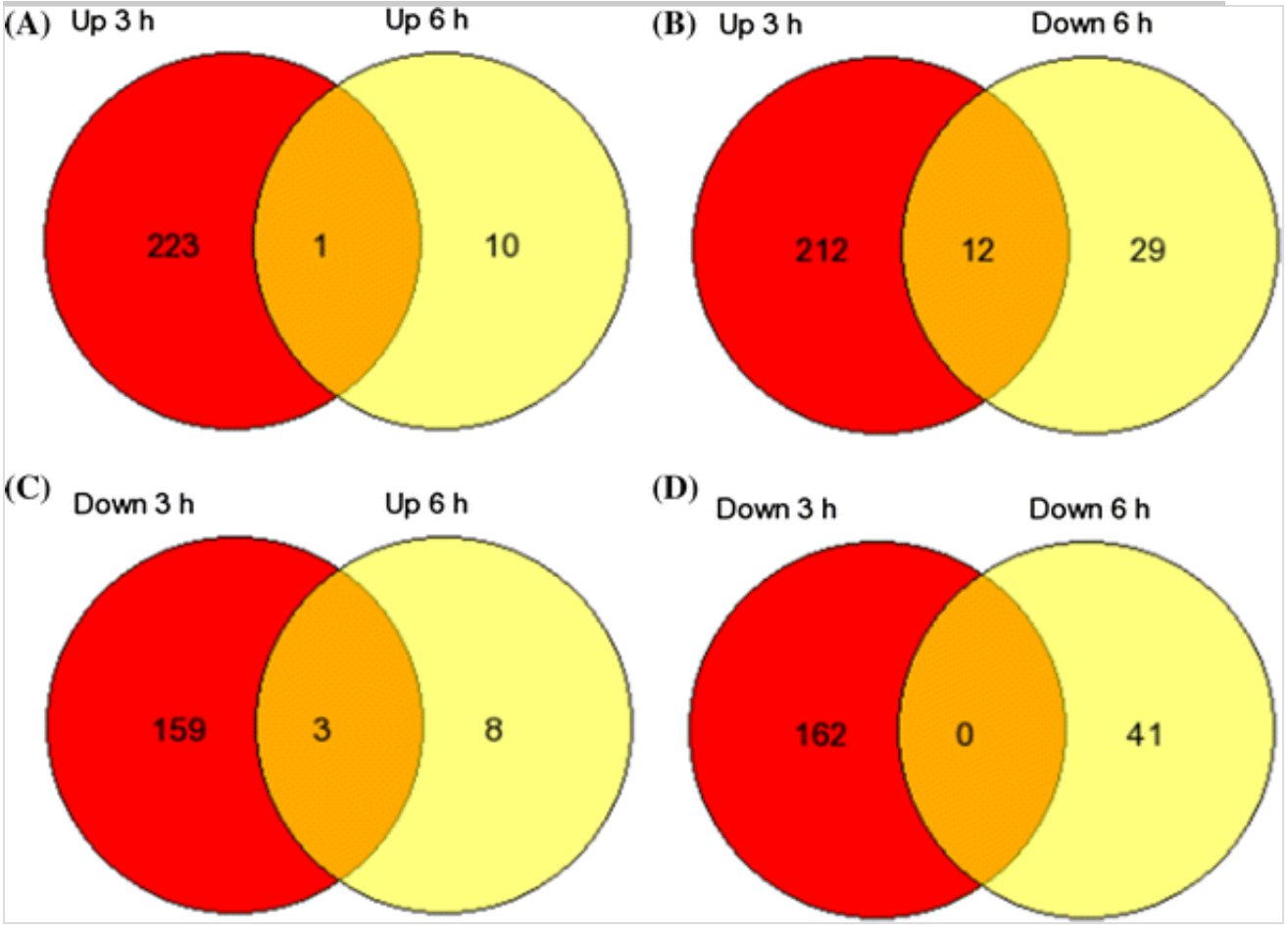
Accession no.	**Gene annotation**	**Fold change**	
		3 h	**6 h**
AC215453	Chloroplast genome	2.30	−5.44
AC239738	NADH dehydrogenase subunit 3	3.89	−2.75
AK324636	Putative UDP-glucoronosyl/UDP-glucosyl transferase family protein	5.79	−2.86
AK329594	Ran-binding protein 1b (RanBP1b)	3.87	−2.77
AM087200	Chloroplast genome	2.38	−4.19
AY034149	Alternative oxidase 1b	2.44	−2.34
AY971876	Ascorbate oxidase	2.75	−3.31

BM412459	Unknown	3.76	−2.42
BT012714	Pectate lyase family protein, putative	2.61	−3.04
BT013487	Cytidine triphosphate synthase (CTP synthase)	3.08	−2.01
BT013505	Myo-inositol-1-phosphate synthase (INS-1P)	4.32	−2.15
TA39797_4081	F1-ATPase alpha subunit (atpA)	4.48	−2.11

A comparison of up-regulated genes at both 3 and 6 h showed that only one gene was constantly expressed. The Venn diagram with up-regulated genes at 3 and 6 h showed that only one gene was repressed at both sampling times, which was HSP 90 (heat shock proteins, BT013102) (Fig. 4A ~~a~~).

Fig. 4

Venn diagrams illustrating the number of genes that were up-regulated, down-regulated and shared between 3- and 6-h BA treatments



Only three genes were down-regulated at 3 h and up-regulated after 6 h

(AK323459, AK325451, BT012711) (Fig. 4C ~~c~~). These genes encode unknown sequences.

Twelve genes were up-regulated after 3 h but down-regulated after 6 h (AC215453, AC239738, AK324636, AK329594, AM087200, AY034149, AY971876, BM412459, BT012714, BT013487, BT013505, and TA39797_4081) (Fig. 4B ~~b~~).

Genes related to ABA biosynthesis metabolism that were affected by the BA treatment after 3 h were ABA INSENSITIVE 2 (ABI2), WRKY16, the ABA-response element binding factors (AREBs) and ABA 8′-hydroxylase. All genes were up-regulated by 2.15 and 2.5-fold. After 6 h, only one gene related to ABA was differentially regulated, the putative ABA-responsive protein-related gene. This gene was down-regulated by 2.07-fold. No genes down-regulated at both 3 and 6 h (Fig. 4D ~~d~~).

Validation of microarray data by real-time RT-PCR

To confirm the microarray results, eight genes were randomly selected among the up- and down-regulated DEGs and were analysed by real-time RT-PCR analysis at 3 and 6 h after BA treatment. Genes were identified using BLAST tools to search against ESTs and nucleotide sequences available in GenBank. Primers for these genes are listed in Suppl. Table 7, and the results are reported in Suppl. Table 8. A quarter of the total genes selected for validation (two genes) showed the opposite behaviour compared to the microarray results, and these genes are UDP-glucuronosyl/UDP-glucosyltransferase (*PhUGG*) and endo-beta-1,4-D-glucanase (*PhGLUC*) (Ross et al. 2001; Buchanan et al. 2012). These results can be due to the presence of different genes belonging to the same family. The *UGGs* are a superfamily with over 100 genes encoding *UGGs*, while 22–29 GLUC genes have been identified in different plant species. The remaining 75 % of the selected genes showed a similar expression pattern between the array and the real time PCR.

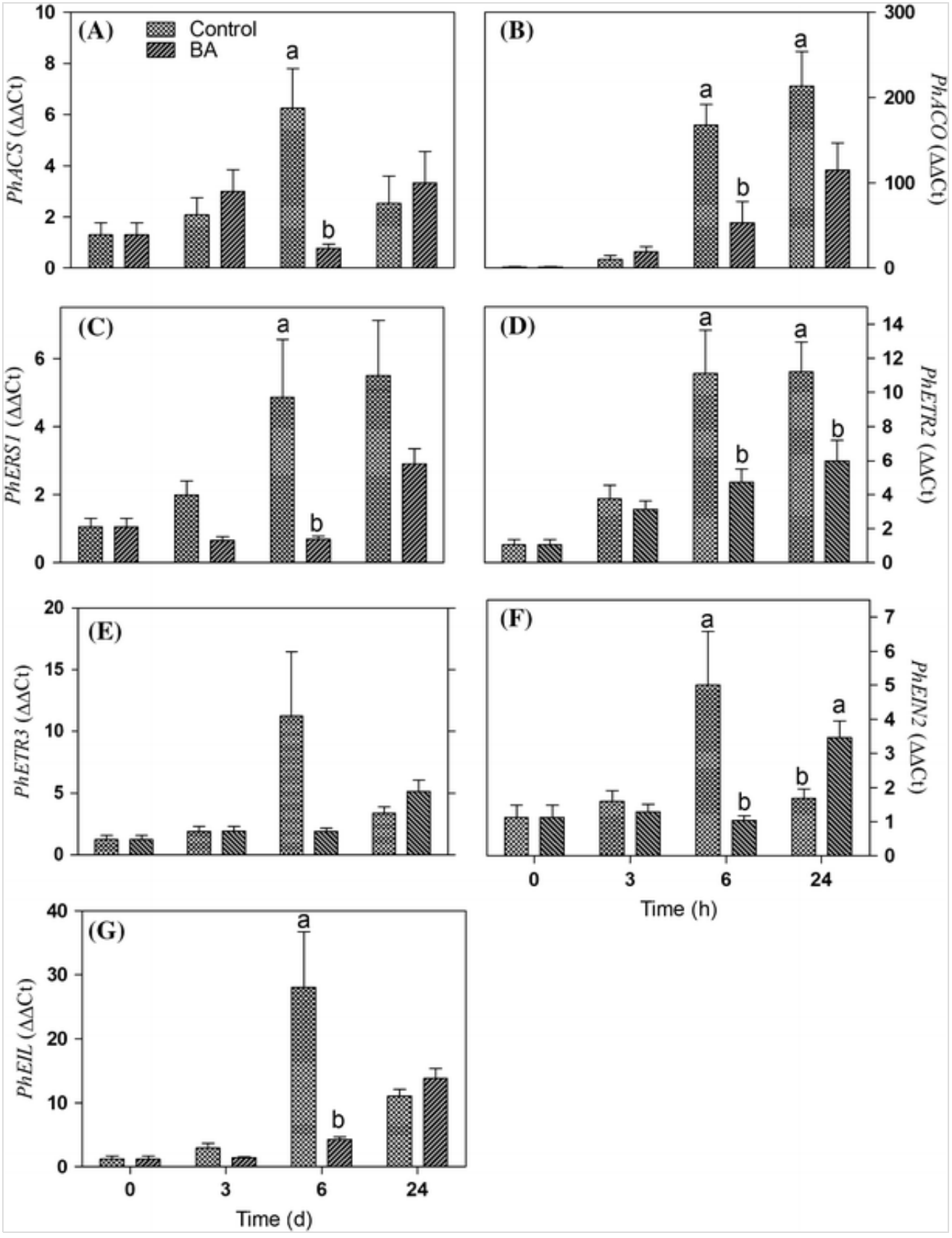
Transcript accumulation of ethylene pathway genes

The effect of BA treatment was also studied by monitoring the transcript accumulation of the most important genes involved in ethylene biosynthesis and perception. Petunia is an ethylene-sensitive species, and this plant hormone plays an important role in flower senescence. Because

BA extends flower life, the ethylene biosynthetic and receptor genes were studied to understand how cytokinins affect its signalling pathway. Petunia ACC synthase (*PhACS*) was down-regulated by BA treatment after 6 h and was six to seven fold lower (Fig. 5A ~~a~~). After 24 h, no differences were observed between the treated and control flowers. The last enzyme involved in ethylene biosynthesis, ACC oxidase (*PhACO*), was down-regulated in BA-treated flowers compared with control flowers after 6 and 24 h, but BA treatment did not prevent the increase in its expression compared with the initial values at time 0. After 6 h, *PhACO* expression in BA-treated flowers was sevenfold lower than control flowers, and expression was fourfold lower after 24 h (Fig. 5B ~~b~~).

Fig. 5

Gene expression analyses of the *PhACS* (A~~a~~), *PhACO* (B~~b~~), *PhERS1* (C~~c~~), *PhETR2* (D~~d~~), *PhETR3* (E~~e~~), *PhEIN2* (F~~f~~) and *PhEIL* (G~~g~~) genes as measured using qRT-PCR at different time points (0–24 h) in control (distilled water) or 200 μM BA-treated samples. Data are the means with standard deviations of ΔΔCt between genes, and *PhACT* was used as the internal control. The reactions were repeated four times using two biological samples



The expression of ethylene receptor sensor 1 (*PhERS1*) was repressed by BA, while it increased in control flowers during the experiment. In BA-treated flowers, *PhERS1* increased after 24 h but was lower than control flowers (Fig. 5C~~e~~).

The expression of ethylene receptors 2 (*PhETR2*) and 3 (*PhETR3*), was

differently affected by BA treatment. *PhETR2* was slightly repressed during vase life and continued to increase after 24 h, but transcripts were always lower than control flowers (Fig. 5D ~~d~~), with significant differences observed after 6 and 24 h. *PhETR3* was strongly induced in control flowers after 6 h, while BA inhibited its expression, and a slight increase was found after 24 h with higher transcript expression in the treated flowers (Fig. 5E ~~e~~).

The ethylene insensitive 2 (*PhEIN2*) gene showed the same trend as *PhEIL*. Control petals showed a strong induction, demonstrating sevenfold higher expression than treated flowers. After BA treatment, *PhEIN2* increased after 24 h with twice the transcript accumulation compared with control flowers (Fig. 5 f).

The ethylene insensitive-like (*PhEIL*) transcription factor was up-regulated after 6 h of vase life in the control petals. In BA treatment, *PhEIL* increased after 24 h (Fig. 5G ~~g~~).

Identification of enriched biological themes in the cytokinin-induced and -repressed genes

As a step toward understanding the biological significance of the molecular changes underlying the effect of cytokinins during the early senescence reprogramming events, we used the gene functional classification (GFC), FAC and functional annotation chart (FACh) tools from the DAVID programme to examine whether there was a significant enrichment in particular biological processes in the set of 386 3-h B-regulated genes and the set of 52 6-h BA-regulated genes listed in Suppl. Tables 1 and 2 (S1 and S2). Among the DEGs after 3 h, a GFC analysis identified five groups that showed significant enrichment with enrichment scores (ES) ranging from 12.89 to 3.03 (S4). The first group included DEGs involved in the energy flux of photosystems, oxido-reductase reactions and energy transfer though membranes. The second group contained DEGs encoding ribosomal proteins (ES 9.44). The third group of DEGs included DNA-directed RNA polymerase subunits beta and alpha (ES 6.9). The fourth group of DEGs involved proteins with catalytic activity (ES 5.31), and the last group involved chloroplast ATP synthase subunits.

The FAC analysis annotated term members in 13 groups, and among these

groups, five showed significant ES >1.3 (S3). The most enriched cluster, with an ES of 26.14, contained terms associated with plastids/chloroplasts. The second cluster, with an ES of 6.48, contained terms associated with electron transport and photosynthetic processes, including chloroplasts, light reactions, and reaction centres. The third cluster, with an ES of 4.17, showed genes involved in protein biosynthesis, ribosomal structure and translation. The fourth and fifth clusters, with ESs of 2.69 and 2.02, respectively, grouped genes involved in oxido-reductase processes with NADH or NADPH cofactors, which are essential for the electron transport chain, photosynthesis and light reactions.

The FACh analyses performed with medium stringency reported that 61–62 % of genes were related to plastid and chloroplast terms. In addition, 37 % of genes were associated with membrane and thylakoids. 10–18 % of genes were involved in photosynthesis or light reactions. Lastly, 15 % of genes were involved in the electron transport chain.

Similar to the 3-h BA-regulated group, the GFC analysis identified four significantly enriched clusters in the set of 52 6-h BA-regulated genes, with ESs ranging from 14.56 to 3.28 (S6). The first group included DEGs involved in the energy flux of photosystems, oxido-reductase reactions and energy transfer though membranes. The second group contained DEGs encoding ribosomal proteins (ES 10.47). The third group included DNA-direct RNA polymerase subunits beta and alpha (ES 7.76), and the last group involved chloroplast ATP synthase subunits.

The FAC analyses annotated term members in 13 groups, and among these, six groups showed a significant ES >1.3 (S5). The most enriched cluster included plastid and chloroplast terms with an ES of 29.45. The second cluster, with an ES of 10.92, contained terms associated with thylakoids, transmembrane and photosynthesis. The third cluster, with an ES of 4.47, included translation terms, while the fourth group, with an ES of 4.37, comprised photosynthesis and photosystem terms. The fifth cluster involved electron transport, photosystem I, oxido-reductase and metal ion binding terms, and the last cluster included terms associated with dehydrogenase activity.

The FACh analysis showed that the SP_PIR_KEYWORDS and GOTERM_CC_FAT categories of thylakoids and chloroplasts include 78–79 % of genes, followed by trans-membrane, metabolites and terms

involved in energy metabolism.

Discussion

Flower senescence is a complex genetic programme in which changes in the contents of hormones, such as ethylene, cytokinins, and ABA, play important roles and can affect tissue sensitivity (Doorn and Woltering 2008; Rogers 2012). In many species, petal senescence is controlled by ethylene, and these species are called ethylene-sensitive, while in others, this process is not affected by ethylene (ethylene-insensitive species), and the endogenous senescence signals could be attributed to ABA or remain to be elucidated (Hunter et al. 2004; Doorn 2001).

The role of phytohormones in flower senescence is generally correlated to their endogenous content, and the flower senescence processes as well as the estimation of flower longevity is monitored after exogenous treatments (Jones et al. 2009).

Petunia is an ethylene-sensitive flower; in fact, an increase in endogenous ethylene levels drives corolla senescence, and treating flowers with exogenous ethylene induces and/or accelerates flower senescence (Jones et al. 2009).

A heterologous microarray study was used to obtain rapid information on the effect of cytokinins during flower senescence in petunia. This method cannot replace species-specific hybridisations, but it may provide a useful and inexpensive alternative for a general overview of the effect of BA on gene expression profiles during senescence.

Cross-species hybridisation can provide satisfactory results, especially as a broad overview of transcriptome profiles, even if species-specific hybridisation greatly improves the results and enriches the quality and amount of information (Bar-Or et al. 2007). However, Petunia belongs to the *Solanaceae* family, which is the same family as *Solanum lycopersicum* L. whose sequences were used in the microarray in this study. Satisfactory results were previously obtained for a potato cross-species hybridisation on tomato microarrays to compare the different expression profiles of potatoes infected by nematodes (Bar-Or et al. 2006).

Exogenous treatments with cytokinins delays flower senescence in several

plant species. The positive effect of cytokinins on delaying flower senescence has been demonstrated in many flowers. In cut lotus flowers (*Nelumbo nucifera*), BA and thidiazuron were both able to prevent petal blackening (Imsabai and Doorn 2013). Analogous results were found in wallflowers treated with BA, and the use of an inhibitor of cytokinin oxidase confirmed the important role of this plant hormone in flower senescence (Price et al. 2008). Cytokinins may also extend flower life by acting indirectly on the enrichment of antioxidant compounds, such as phenols and anthocyanins. No data on the effect of BA on total phenols in flowers during senescence can be found in the literature. Our findings could support the hypothesis that exogenously applied cytokinin possibly stimulated defence mechanisms by enhancing the accumulation of phenols, as has been previously observed in transgenic *ipt* tobacco plants that overproduce cytokinins (Schnablová et al. 2006). Phenolic compounds have been shown to play an important role in the resistance of plants to biotic and abiotic stresses and endogenous perturbations (Siranidou et al. 2002).

BA treatments extend the vase life of many cut flowers, such as *Eustoma*, *Lilium* cut and potted (Han 2003; Asil and Karimi 2010). *Nicotiana mutabilis* has flowers that change colour during their display life from white through pink to red as the flowers age. The chalcone synthase gene is induced prior to anthocyanin accumulation (Mcnisch et al. 2010). The application of ethylene increases anthocyanin biosynthesis, while 1-methylcyclopropene, which acts by blocking ethylene perception, delays petal colouration and, hence, senescence. The higher amount of anthocyanins in BA-treated petunia petals versus untreated petals could be related to the protective role of anthocyanins as scavengers of reactive oxygen species during petal senescence (Rogers 2012). Thus, the rise in anthocyanins could act to slow the flower senescence process in BA-treated petals. The effect of cytokinins on anthocyanin accumulation was studied in *Arabidopsis* plants (Deikman and Hammer 1995). In these species, exogenously applied BA induced the activation of the phenylalanine ammonia lyase 1, chalcone synthase and chalcone isomerase genes. During leaf growth and expansion, BA treatment increased the anthocyanin concentration by 50-fold (Deikman and Hammer 1995).

Notably, BA enhanced ethylene biosynthesis but retarded senescence, as opposed to accelerating senescence. The application of thidiazuron, a

cytokinin-like compound, enhanced ethylene production but simultaneously extended vase life by inhibiting leaf yellowing in cut stock flowers (Ferrante et al. 2012). In transgenic petunias overexpressing the *ipt* gene and thus over accumulating cytokinins, ethylene production was much higher than control plants, but nevertheless, the vase life was extended (Cheng et al. 2003).

In our study, an increase in ethylene was found during petunia senescence, and the lack of a negative effect can be explained considering that the expression of the ethylene receptors was down-regulated by treatment with BA. In an experiment reported by Chang et al. (2003), transgenic lines displayed a lower endogenous ABA level compared to wild type, and this condition was confirmed by our results. BA treatment delayed senescence by lowering the ABA content, which is known to increase during flower development and senescence in both ethylene-sensitive and -insensitive species (Trivellini et al. 2011; Reid and Chen 2007).

The harvested petunia flowers used in this study were exposed to exogenous cytokinin (BA) treatment for 3 and 6 h. Here, we examined the transcriptome after these time points to explore primary responses to cytokinins that are involved in the signalling pathway that orchestrate the extension of flower life. From our experience (unpublished data), a subtractive library after 24 h did not provide any ESTs. In our opinion, depicting this rapid response in transcriptome reprogramming is a key feature that could potentially allow the identification of specific signalling pathways that are involved in the extension of flower life. When the first symptoms of delayed senescence are visible, all transcriptome machinery has been established on downstream targets and not on the initial triggers of the process. Moreover, the transcriptional machinery is readily activated immediately after a stimulus or treatment with BA (Brenner et al. 2005).

The microarray analyses showed DEGs after 3 or 6 h of BA treatment. Most of the changes induced by the treatment were visible immediately after 3 h and declined after 6 h.

Among the most up-regulated genes were the HSPs. The activation of HSP gene expression after cytokinin treatments have been reported in several heat stress studies. Heat stress increases cytokinin degradation. The exogenous application of this hormone increased heat tolerance, delayed leaf senescence and promoted root growth in heat-sensitive species, such

as creeping bentgrass (Xu et al. 2009; Verdonk et al. 2008). Through the up-regulation of HSPs, exogenously applied BA might protect cellular functions or slow the action of proteases that cause the breakdown of proteins (Veerasamy et al. 2007) and thus delay the senescence process.

Several genes induced by BA affected ABA biosynthesis, catabolism and signalling pathways. Among those gene involved in ABA catabolism, ABA 8′-hydroxylase was up-regulated. The ABA 8′-hydroxylase (CYP707A1) transcripts were 2.5-fold higher than control plants. This gene is most likely responsible for ABA degradation to phaseic acid, and this reason may explain the lower content of ABA in treated flowers. Genes involved in ABA transcription regulation were positively affected; in fact, after 3 h, the transcription factor AREB-like protein was up-regulated. AREB-like protein is involved in the regulation of ABA-regulated genes. Functional studies performed in tomato demonstrated that the overexpression of AREB genes induced a lower accumulation of ABA in the tissues (Bastìas et al. 2011). Among the genes involved in ABA signalling was ABI2. ABI2 was up-regulated, and its function is to reduce the plant's responsiveness to ABA (Merlot et al. 2001). Another regulator of the ABA signalling pathway activated by BA was the WRKY16 transcription factor. Studies performed on knockout mutants showed ABA-hypersensitive phenotypes. Several WRKYs negatively regulate ABA responses (Rushton et al. 2012). Together, these results demonstrate that BA delays floral senescence, lowers the ABA concentration in petals and reduces the tissue's sensitivity. These gene expression studies are in agreement with the lower concentration of ABA found in the BA-treated flowers.

Other up-regulated genes correlated with lipid metabolism. In particular, phospholipase A1 (PLA1, 6.32-fold), myo-inositol-1-phosphate synthase (4.32-fold) and lipase class III were highly expressed. PLA1 is an enzyme that hydrolyses phospholipids and produces 2-acyl-lysophospholipids and fatty acids. The role of PLA1 is focused on membrane maintenance and in the production of different lysophospholipid mediators, such as lysophosphatidylserine and lysophosphatidic acid (Richmond and Smith 2011). Myo-inositol-1-phosphate synthase (EC: 5.5.1.4) is responsible for the conversion of D-glucose-6-phosphate to 1-myo-inositol-1-phosphate, which is the first step in the production of all inositol-containing compounds, including phospholipids. The inositol phosphates play an important role in signal transduction against biotic and abiotic stresses. In

our study, these findings also suggest a role in preventing symptoms of senescence.

The transcriptomic analysis showed the up-regulation of genes involved in the defence against reactive oxygen species (ROS), such as catalase, ascorbate peroxidase and ascorbate oxidase. Senescence is associated with ROS formation (Rogers 2012), and BA seems to activate protection systems to stimulate defence mechanisms (Schnablová et al. 2006).

Some of the genes that were most strongly down-regulated by BA treatment in petunia petals were associated with the arabinogalactan proteins. These proteins are cell wall proteoglycans and are widely distributed in the plant kingdom. They are involved in plant development and signalling. In flowers, the arabinogalactans are highly expressed in pistils (Majewska-Sawka and Nothnagel 2000). In particular, in apple flowers, arabinogalactan accumulation was found in concomitance with stigma receptivity, indicating the role of these proteins in flower pollination (Losada and Herrero 2012). Arabidopsis plants that overexpress inflorescence deficient in abscission showed earlier flower organ abscission and an over-accumulation of arabinogalactan protein above the separation zone (Stenvik et al. 2006). These studies indicate that arabinogalactan proteins are involved in the senescence process; therefore, the down-regulation of these genes after BA treatment in petunia flowers may correlate with the slowing of flower senescence.

Petunia is an ethylene-sensitive flower, and pollination accelerates senescence; therefore, endogenous or exogenous ethylene accelerates petal senescence. During petunia flower senescence, the increase in ethylene production is primarily associated with pollination events (Jones et al. 2008). Thus, senescence can be significantly delayed by treating flowers with inhibitors of ethylene action (Goren et al. 2011). In this work, the ethylene biosynthesis and regulatory pathways were studied in detail to elucidate the effect of BA on petal senescence. BA lowered the transcript accumulation of genes involved in the biosynthesis and perception after 6 and 24 h (Fig. 5). These results were confirmed in other cut flowers, such as *Freesia*, where the expression of *FsACS* was lower than control flowers, especially after the fully open stage until senescence (Yuan et al. 2012). Few reports have demonstrated that BA represses the ethylene receptors (Ma and Wang 2003), but to the best of our knowledge, no studies have

reported this role in flower organs.

Microarray analyses generate large amounts of transcriptional information. To better understand the biological significance of the changes, we used the bioinformatics tool DAVID to identify significant enrichment of particular biological themes.

The most enriched annotation clusters (Suppl. Tables S3 and S5) identified by DAVID analysis were associated with chloroplast function and electron transport though the cell membrane.

Although the experiments were focused on petals, energy generation or conservation processes were strongly affected by the cytokinins. This result explains the plant responses to treatment with cytokinins or substances with cytokinin-like activity. Cytokinins can restore chloroplast activity and reduce leaf yellowing in many cut flower species (Ferrante et al. 2009). The increase in chloroplast-associated genes suggests a role for chloroplasts in flower development and likely in the senescence process. Some studies reported the presence of chloroplasts in the corolla of *Arabidopsis*, petunia and carnation flowers (Pyke and Page 1996 AQ2 ; Weiss et al. 1988; Vainstain and Sharon 1993). These studies observed that the chloroplasts present in petals were able to perform photosynthesis, and the thylakoid complex cyt b6/f accumulated to higher levels compared to the leaf tissue.

In conclusion, transcriptome reprogramming occurs immediately after BA treatment (3 h), and after 6 h, only 14 % of genes are still differentially expressed. In addition to the ethylene pathway, the cytokinins seem to be strongly involved in the regulation of ABA biosynthesis and degradation in flower tissues. BA acts on the regulation of signalling pathways and directly on catabolism. Moreover, the microarray data suggest that in addition to ethylene, ABA plays a primary role in the senescence process in petunia flowers.

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Electronic supplementary material

Below is the link to the electronic supplementary material.

Supplementary material 1 (XLSX 85 kb)

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