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Authors: Santiago A. Trupkin, Francisco H. Astigueta, Amilcar H. Baigorria, Martín N. García, Verónica C. Delfosse, Sergio A. González, Mariana Cecilia Pérez de la Torre, Sebastián Moschen, Verónica V. Lía, Paula Fernández, Ruth A. Heinz

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Identification and expression analysis of *NAC* transcription factors potentially involved in leaf and petal senescence in *Petunia hybrid*

Santiago A. Trupkin[a, c, 1], Francisco H. Astigueta[c, d, 1], Amilcar H. Baigorria[d], Martín N. García[b], Verónica C. Delfosse[c, d], Sergio A. González[c], Mariana Cecilia Pérez de la Torre[a], Sebastián Moschen[c], Verónica V. Lía[b, c, e], Paula Fernández[b, c, d, 2, *], and Ruth A. Heinz[b, c, e, 2, *]

[a] Instituto de Floricultura, Centro de Investigación de Recursos Naturales, Instituto Nacional de Tecnología Agropecuaria, Hurlingham, Buenos Aires, Argentina,

[b] Instituto de Agrobiotecnología y Biología Molecular (IABiMo – INTA-CONICET), Centro de Investigaciones en Ciencias Agronómicas y Veterinarias, Instituto Nacional de Tecnología Agropecuaria, Hurlingham, Buenos Aires, Argentina,

[c] Consejo Nacional de Investigaciones Científicas y Técnicas, Ciudad Autónoma de Buenos Aires, Argentina,

[d] Escuela de Ciencia y Tecnología, Universidad Nacional de San Martín, San Martín, Buenos Aires, Argentina,

[e] Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Autónoma de Buenos Aires, Argentina

*** Corresponding authors**

E-mail address: fernandez.pc@inta.gob.ar (P. Fernández)

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E-mail address: heinz.ruth@inta.gob.ar (R. A. Heinz)

[1 and 2] These authors contributed equally to this work.

Figures: 1-6.

Tables: 1-2.

Highlights

- Progression of natural leaf senescence in *P. hybrida* showed similarities with other plant species.
- Leaf senescence-associated *NAC* genes were identified in *P. hybrida*.
- Expression analysis showed *PhNACs* active at different stages of leaf and also petal development and senescence.
- *NAC* gene expression patterns were generally similar between Arabidopsis and *P. hybrida*.
- Phylogenetic analysis helped to select key candidates for senescence regulation in petunia.

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Abstract

Progression of leaf senescence depends on several families of transcription factors. In Arabidopsis, the NAC family plays crucial roles in the modulation of leaf senescence; however, the mechanisms involved in this NAC-mediated regulation have not been extensively explored in agronomic species. *Petunia hybrida* is an ornamental plant that is commonly found worldwide. Decreasing the rate of leaf and petal senescence in *P. hybrida* is essential for maintaining plant quality. In this study, we examined the NAC-mediated networks involved in regulating senescence in this species. From 41 *NAC* genes, the expression of which changed in Arabidopsis during leaf senescence, we identified 29 putative orthologs in *P. hybrida*. Analysis using quantitative real-time-PCR indicated that 24 genes in *P. hybrida* changed their transcript levels during natural leaf senescence. Leaf-expressed genes were subsequently assessed in petals undergoing natural and pollination-induced senescence. Expression data and phylogenetic analysis were used to generate a list of 10 to 15 candidate genes; 7 of these were considered key regulatory candidates in senescence because of their consistent upregulation in the three senescence processes examined. Altogether, we identified common and distinct patterns of gene expression at different stages of leaf and petal development and during progression of senescence. The results obtained in this study will contribute to the understanding of NAC-mediated regulatory networks in petunia.

Keywords: *Petunia hybrida*; NAC transcription factors; leaf senescence; petal senescence; expression profiles; crop breeding

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1. Introduction

Leaf aging is accompanied by a genetically programmed process called senescence. Senescence is a form of programmed cell death in which nutrients from dying leaves are recycled to support the growth of younger tissues [1,2]. Delaying senescence can keep the photosynthetic machinery active for prolonged periods, which can affect crop yields. However, stressed plants normally exhibit premature senescence, which results in diminished yield [3,4].

Multiple variables, including endogenous signals such as leaf age, reproductive development, hormones and several external stress-related signals, can modulate the onset of senescence [2,5]. The process involves dramatic changes in gene expression, hormone balance, metabolism, and structure; it also involves downregulation in the expression of photosynthesis-related genes [such as *senescence down-regulated genes* (*SDGs*)] and upregulation of multi-functional genes required for senescence to proceed [such as *senescence-associated genes* (*SAG*s)] [6–9]. Several families of transcription factors (TFs) often regulate these changes in global gene expression [8–12]. Particularly, many NAC (NAM, ATAF1, 2 and CUC2) family members, approximately one third in Arabidopsis, vary their expression during the process [9,12,13]. The NAC family is one of the largest plant-specific TF families, and members of this family participate in the mediation of leaf senescence and other processes in plants [5,14–16]. NAC proteins contain a highly conserved N-terminal dimerization and DNA-binding domain encompassing five subdomains (A-E) and a highly diversified C-terminal region that allows specific interactions with DNA and other proteins [15,17,18]. For example, in Arabidopsis, AtNAP [19], ORE1 [20], ORS1 [21], ANAC016 [22] and ATAF1 [23] promote leaf senescence, whereas JUB1 [24] and VNI2 [25] delay this process. These TFs exert their effects via molecular interactions with hormones such as abscisic acid (ABA) and ethylene [5]. Several crop species, including monocots and

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dicots, contain NAC proteins that play roles in senescence; however, the molecular mechanisms that drive the progression of senescence are still unclear [26–33].

In flowering plants, petal senescence is influenced by endogenous age-dependent signals; in many species, pollination activates or accelerates the process [34–36]. The development and physiological roles of leaves and petals are different. In petals, senescence is less influenced by environmental factors and involves a lower extent of nutrient remobilization than that in leaves [37–39]. Global analysis of gene expression in the leaves and petals of the ornamental wallflower plant has shown common and distinct patterns in expression and physiology. Several remobilization-related genes, including senescence-associated gene12 (*SAG12*), encoding a cysteine protease, are upregulated in both leaves and petals with age [40]. Common events are observed in Arabidopsis, although unique events are also identified in individual tissues [41]. Expression of the NAC family is particularly increased during leaf and petal development [41], indicating that these TFs may use similar signaling mechanisms in leaves and petals.

In ethylene-sensitive flowers, the first sign of visible senescence correlates with a transient and sudden rise in ethylene production [42]. *P. hybrida* has ethylene-sensitive corollas; both natural (age-dependent) and pollination-induced petal senescence are associated with endogenous peaks of ethylene production [43]. The corollas of transgenic *P. hybrida* plants, which express a mutant ethylene receptor that generates ethylene-insensitive petals, show notable changes in gene expression and downregulation of six *NAC* genes [44]. Similarly, an early transcriptome analysis of corollas in pollinated petunia revealed variations in the transcription of *NAC* genes [36], indicating that these genes may participate in petal senescence. Moreover, the petals of the ornamental plant *Ipomoea nil* show increased expression of putative orthologs of Arabidopsis *ORE1* (*InEPH1*) and *AtNAP* [45,46]. Interestingly, InEPH1 promotes petal

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senescence and currently represents the only NAC factor involved in regulation of petal senescence [45]. Transcript level-based identification of regulatory networks controlling senescence is important in determining potential regulators of senescence. Indeed, Ha-NAC01, which is a putative ortholog of Arabidopsis ORE1, shows a similar transcriptional regulation network in the sunflower [47]. Thus, signaling pathways mediated by NAC factors may be preserved among species, which can be useful in plant breeding.

P. hybrida is one of the most economically important ornamental plants worldwide. Unlike Arabidopsis, *Petunia* belongs to a different phylogenetic group of higher eudicots [48] and is an important comparative genetic model for studying the molecular basis and regulation of leaf and petal senescence. Moreover, the results obtained using *Petunia* can be extrapolated to other related crops within the Solanaceae family [49]. Therefore, in this study, we performed phylogenetic classification and detailed expression analysis of several *NACs* that may regulate senescence in petunia.

2. Materials and methods

2.1. Identification of NAC transcription factors in P. hybrida

The sequences of NAC proteins present in *Arabidopsis thaliana* were retrieved from PlantTFDB 3.0 (Plant Transcription Factor Database) [50]. The resulting dataset was screened to remove redundant and splicing isoforms, and sequences were manually evaluated for presence of the NAC domain using the PFAM database (NAM domain PF02365) and Araport [51]. Forty-one Arabidopsis *NAC* genes, including 36 *SAGs* and 5 *SDGs*, were selected from published studies on the leaf transcriptome [9,12,41] and from online repositories of leaf expression data such as Leaf Senescence

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DataBase [52] and Arabidopsis eFP Browser [53]. A leaf transcriptome dataset of *P. hybrida* [54] (SGN, http://solgenomics.net) was used to create a repository database (Petunia Transcriptome Repository ATGC v1.0) [55]. Putative petunia orthologs in this repository, and in parental genomes of *P. hybrida* deposited at SOL Genomics Network [56], were then searched using tBLASTn. In addition, BLASTp was used to search other species for the presence of putative ortholog/s to the predicted protein sequences in *P. hybrida* (NCBI, https://www.ncbi.nlm.nih.gov/).

2.2. Alignment and phylogenetic analysis of NAC protein sequences

For phylogenetic reconstruction of the Arabidopsis NAC tree, we aligned the N-terminal region containing the NAC domain (subdomains A–E) of 109 protein sequences using BioEdit software [57]. The Jones, Taylor, and Thornton (JTT) model was selected as best-fitting amino-acid substitution model using ProtTest v3.4 software [58]. The best-fit model of protein evolution and parameters were incorporated into PhyML 3.0 software to estimate large phylogenies by maximum likelihood [59]. For phylogenetic reconstruction of the senescence-associated NAC tree, we aligned the NAC-domain—containing N-terminal region in 161 proteins from different species; these proteins included those with reported roles in senescence. The best evolutionary model was JTT; a neighbor joining (NJ) tree was built using MEGA5 software [60], which is suitable for large datasets. Both phylogenetic trees were visualized using Figtree v1.4.2 software (http://tree.bio.ed.ac.uk/software/figtree/).

2.3. Identification of conserved motifs in the C-terminal region of PhNACs

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The identification of conserved motifs and sequence logos in the divergent C-terminal region of NAC proteins was performed via MEME program available online [61], using parameters described previously by You et al. (2015) [62].

2.4. Plant material and growth conditions

For all experiments, the seedlings of *Petunia* x *hybrida* 'F1 Ultra™ White' (obtained from Syngenta Flowers, Inc., USA) were grown in 10-cm diameter pots containing moistened Grow Mix soil (Terrafértil, Argentina). The pots were placed in a growth chamber and maintained at 20°C and long-day photoperiods of warm-white fluorescent light (16-h light/8-h darkness; 240 μmol m^{-2} s^{-1}; TLD 36W/830, Philips, France). The plants were watered as needed using sub-irrigation with a nutrient solution (Hakaphos® Rojo, COMPO) and cycled regularly to avoid position-related effects.

Natural leaf senescence was analyzed using samples of leaf 11 selected from a total of 14 rosette leaves during bolting. Leaves were tagged with a thread when foliar primordia were approximately 70% of their final size; this was designated as the first sampling time point (day -3, denoting 3 days before full leaf expansion). Samples were collected at different time points until yellowing of approximately 40% of the total leaf area; this yellowing was visible on day 33 after full leaf expansion).

Maximum leaf area was observed approximately 8 d after primordia were evident to the naked eye (~0.5 cm length). Leaves were harvested on each sampling day at 4 h into the light period, resulting in six different time points. At each time point, leaf 11 was sampled from 10 randomly selected plants, and was divided into 3 biological replicates with 3-4 leaves per replicate. The leaves were rapidly frozen in liquid nitrogen and stored at -80°C until use. Each experiment was performed twice,

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and similar results were obtained with respect to plant growth, leaf development, and senescence.

Natural petal senescence was assessed by tagging flowers at anthesis (day 0), and leaving them unpollinated to senesce naturally. Conversely, in experiments using induction of pollination, flowers on the first day of anthesis (day 0) were hand-pollinated by brushing pollen onto the stigma. In both experiments, corollas were collected 8 h after the start of the light period at five different time points. The collected corollas were rapidly frozen in liquid nitrogen and stored at -80°C until use. At each time point, we collected 12 flowers, which were divided into 3 biological replicates with 4 corollas per replicate.

2.5. Chlorophyll and protein measurements

Leaf samples were ground in liquid nitrogen. Chlorophyll was extracted using approximately 50 mg of fresh tissue, which was placed into 1mL of *N,N'*-dimethylformamide, vortexed, and incubated in the dark at -20ºC for 3 days. The samples were then centrifuged for 5 min at 12,000 ×*g*, and absorbance for each extract was measured at 647 and 664 nm with Multiskan EX microplate photometer (Thermo Scientific, Inc., Waltham, MA, USA). Chlorophyll levels were calculated according to Moran (1982) [63] and expressed with respect to fresh weight of each sample.

Total proteins were extracted from fresh tissue (~150 mg) using 500 μl extraction buffer (50 mM sodium phosphate pH 7.0, 10 mM EDTA, 0.1% Triton X-100, and 10 mM beta-mercaptoethanol). Soluble proteins were measured at 600 nm via the Bradford method using a protein assay (Bio-Rad Laboratories, Inc., Richmond, CA, USA) and a calibration curve obtained using bovine serum albumin. Levels of the large subunit of Rubisco (RbcL) were assessed by SDS-PAGE. Protein extracts were

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normalized for leaf weight equivalent to 650 μg of fresh tissue, placed into cracking buffer (10% SDS, 50% glycerol, 25% beta-mercaptoethanol, 0.01% bromophenol blue, and 1.5 M Tris-HCl pH 6.8), boiled for 5 min, and centrifuged for 5 min at 12,000 ×*g* before electrophoresis. Polypeptides were separated on a 12% polyacrylamide gel via SDS-polyacrylamide gel electrophoresis [SDS-PAGE] [64], visualized by Coomassie blue staining (Coomassie G-250 Brilliant Blue, Sigma); a molecular-weight size marker was used to indicate the molecular weight of RbcL.

2.6. Gene expression analysis using quantitative real-time PCR

Total RNA was isolated from selected samples of leaves and petals. High quality RNA was obtained from 150 mg of frozen tissue using TRIzol per manufacturer's instructions (Invitrogen, Argentina). Genomic DNA was eliminated using DNase I (Invitrogen, Argentina). RNA concentration was measured using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The purity and integrity of total RNA were determined at the ratio of 260/280 nm, and via agarose gel electrophoresis using ethidium bromide staining. For each sample, 2 μg DNase-treated RNA was reverse-transcribed using a Superscript III first strand synthesis kit (Invitrogen, USA) and random hexamer primers according to the manufacturer's instructions. Specific primer pairs for quantitative real-time PCR (qPCR) were designed using Beacon designer 6.0 software (Premier Biosoft International, Palo Alto, CA, USA) (Supplementary Table S1). All qPCR reactions were performed using 13 μl containing 4.75 μl of water, 0.5 μl of each primer (200 nM), 1 μl cDNA sample, and 6.25 μl FastStart Universal SYBR Green Master (Roche Diagnostics, Mannheim, Germany). The assays included negative controls (no RT added) and non-template controls. Reactions were performed using a 96-well plate StepOne Plus cycler and software (Applied Biosystems, USA). The thermal profile was set at 95°C for 10 min

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and 40 cycles at 95ºC for 15 s; hybridization temperature was set at 60°C for 1 min. Amplicon specificity was verified by melting curve analysis (60 to 95ºC) after 40 PCR cycles, and products were visualized using agarose gels. The assay was performed using two technical replicates and three biological replicates for each condition. Amplification efficiencies and raw Ct values for the expression of each gene at each time point were determined with the slope of a linear regression model using LinRegPCR software [65]. These profiles were estimated with respect to first sampling and reference gene using fgStatistic software [66], which is based on Pfaffl's algorithm [67]. Final expression values were analyzed using one-way ANOVA followed by Tukey's post-test to assess each gene for significant changes in expression between time points (Supplementary Table S2). All data were analyzed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA).

2.7. Gene clustering and heatmap analysis

Clustering was performed using *cmeans* function [68], which is a fuzzy clustering method included in the 'e1071' R package [69] and R Core Team (https://www.R-project.org/). Heatmap was generated with 'heatmaply' R package [70].

3. Results

3.1. Selection of SAG and SDG genes in the NAC family of Arabidopsis

To identify potential regulators of senescence in petunia, we searched for *NAC* genes showing altered expression throughout the progression of leaf senescence (see Materials and methods). Approximately 39% of *NAC* genes showed changes (Table 1); of the 41 selected genes, 36 were upregulated (88%), whereas only 5 were downregulated during progression of senescence. Proteins encoded by 10 of these

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genes are currently known as regulators of leaf senescence (Table 1). Of the 36 upregulated genes, 7 and 21 (78%) genes showed changes in expression at the very early (VE) and early (E) stages, respectively, whereas 4 and 1 genes showed expression changes at mid (M) and late senescence (L), respectively (Table 1). Of the 5 downregulated genes, one showed changes in expression at the early stages (Table 1). Overall, these results indicate that most genes showed changes in expression patterns at the early stages, with important differences observed in approximately 75% of these genes (Table 1).

We also assessed expression changes in genes of petals in closed flowers, immediately before opening (stage 12), and in opened-pollinated flowers (stage 15) (Table 1). Of the 36 genes upregulated in the leaves, 20 (~56%) also increased their expression levels during petal senescence, with 12 of these genes showing consistent changes in expression (Table 1). All 5 genes downregulated in the leaves also showed downregulation during petal development (Table 1). These findings suggest that numerous NACs in Arabidopsis participate in petal senescence. However, to date, none have been associated with these processes, likely because of the small size and short lifespan of petals in Arabidopsis.

Finally, a phylogenetic tree analysis revealed that none of the selected senescence-associated genes showed a particular clustering (Supplementary Fig. S1); indeed, these 41 genes were widely distributed. These results agree with those reported in the study by Shen et al. (2009) [71], indicating that these genes represent six out of eight different NAC subfamilies.

3.2. Identification of putative NAC transcription factors in P. hybrida and its parental species

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To identify putative NAC orthologs in petunia, a public transcriptomic leaf database of *P. hybrida* [54] was incorporated into a web-based application platform to conduct a BLAST search [55]. Using this tool, we assessed the cDNA sequences of 41 selected members of Arabidopsis and obtained 29 *P. hybrida* genes (*PhNACs*) with 21 best hits and 8 lower-order hits (Table 1 and Supplementary Table S3).

The petunia genomic sequence was unavailable when we conducted these searches. Thus, the transcriptomic leaf database was the best representation of *P. hybrida* sequences and enabled us to examine the genes expressed in leaves. The draft genomes of *P. hybrida* parental species (*P. axillaris* and *P. inflata*) became publicly available while we were analyzing our results [56]. Searches in genome databases recovered sequences of parental species with scores and e-values consistently similar to those obtained for *P. hybrida* sequences (Supplementary Table S3). Using BLASTp, we analyzed the predicted proteins of *P. hybrida* and retrieved their equivalents for *P. axillaris* and *P. inflata* (Supplementary Table S3). Thus, using BLAST to search both databases allowed us to identify several putative NAC orthologs in petunia with identity values above 50% for most of the genes. These results show a high level of conservation between Arabidopsis and *P. hybrida* proteins (Table 1 and Supplementary Table S3).

3.3. Alignment and phylogenetic analysis of NAC proteins identified in P. hybrida

To confirm that *P. hybrida* genes encode NAC domain-containing proteins, we used the highly conserved residues in the NAC DNA-binding domain to construct an alignment and phylogenetic tree (Fig. 1). The conserved residues defining the five NAC subdomains (A-E) have been characterized previously [14]. Our analyses indicated that these were, indeed, NAC proteins (Fig. 1). To evaluate the sequence structures of PhNACs, we aligned the 29 sequences with their equivalents in *P. axillaris* and *P.*

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inflata (Supplementary Table S3 and Supplementary Fig. S2). Our results indicate that 16 PhNACs were full-length proteins (Supplementary Fig. S2). Twenty of 29 proteins were highly similar to both parental equivalents (69%) as shown by the score, identity, and query cover. These results show that these PhNACs were highly conserved. PhNAC099, PhNAC085, PhNAC051, PhNAC082, PhNAC052, and PhNAC058 were related to *P. axillaris* equivalents (21%), whereas PhNAC004, PhNAC101, and PhNAC017 were related to *P. inflata* equivalents (10%).

3.4. Characterization of natural leaf senescence

We then examined the relationship of NAC proteins with the process of natural leaf senescence. For this, we first analyzed the progression of senescence by measuring the levels of chlorophyll and soluble protein used as physiological parameters. We also examined the expression profiles of putative orthologs of chlorophyll a/b–binding protein 2 (*CAB2,* a gene involved in Arabidopsis photosynthesis) and the late senescence marker *SAG12*, which were used as molecular parameters [12,72,73].

Chlorophyll levels declined significantly at day 33 (Fig. 2A and 2F; $P < 0.05$). However, soluble protein levels declined earlier, at day 18 (Fig. 2B; $P < 0.001$). Correspondingly, the levels of the large subunit of Rubisco (RbcL) were similar between days -3 and 4, but declined steadily thereafter (Fig. 2E). Breeze et al. (2011) have proposed that early degradation of Rubisco may contribute to the synthesis of proteins required for senescence without appreciably affecting the rate of photosynthesis [12]. *EF1a* is a highly ranked reference gene in various species [74–77]. Therefore, *PhEF1a* was selected as reference gene for gene quantification studies in petunia. The expression of *PhCAB2* declined significantly at day 11 and reached its lowest levels after day 18 (Fig. 2C; $P < 0.0001$), whereas the expression of *PhSAG12*

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increased significantly at day 25 and continued to increase up to day 33 (Fig. 2D; $P < 0.0001$). Thus, the parameters used in this study allowed us to assess the extent of senescence in *P. hybrida*.

3.5. Expression analysis of PhNACs during natural leaf senescence

Gene expression profiles of *PhNACs* were assessed via quantitative real-time PCR (qPCR, Fig. 3) performed during six different stages of leaf development (Fig. 2). Most *PhNAC* genes (24) were expressed at the evaluated time points (Fig. 3), and the expression of 23 of these genes varied significantly throughout leaf senescence (Supplementary Table S2). Expression of the remaining genes (*PhNAC086*, *PhNAC005*, *PhNAC030*, *PhNAC050*, and *PhNAC059*) was undetectable even using different pairs of primers.

We then performed a clustering analysis of these 23 genes using a *cmeans* algorithm [68] to determine differences in expression profiles (Fig. 3). The genes were classified into four clusters and divided into two major groups: upregulated (clusters 1 to 3) and downregulated (cluster 4). Cluster 1 contained only one gene, (*PhNAC024*), the expression of which increased at mid-senescence (day 11) and reached its highest level at day 33, showing approximately a 10-fold difference in expression between day 11 and 33 (Fig. 3A). Cluster 2 contained 13 genes, half of which showed weak upregulation in the early stages. Higher expression levels were observed for all the genes at mid and late stages. The maximum expression difference was approximately 2 to 4 fold and occurred between early and late stages (Fig. 3B). Cluster 3 consisted of four genes upregulated in the early stages, with increased expression levels at mid and late stages (Fig. 3C). Cluster 4 contained five downregulated genes, four of which showed decreased expression in the mid stages (days 11 and 18), whereas the expression levels of *PhNAC025* decreased at day 33 (Fig. 3D).

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Overall, the expression profiles were consistent; expression of most genes changed in the relatively early stages, suggesting their involvement in the initial steps of senescence.

3.6. Expression analysis of PhNACs during natural petal senescence

Recently, Shibuya et al. (2014) [45] reported that a NAC (InEPH1) in morning glory acts as a positive regulator of petal senescence. Therefore, we investigated whether the same 24 leaf-expressed *PhNACs* occurred in the naturally senescing petals of petunia. The cultivar used in our study had shorter stamens than pistils. This characteristic prevented auto-pollination and therefore facilitated the study of natural petal senescence. To estimate the progression of senescence, we measured soluble protein levels and expression of the *PhSAG12* gene. Soluble proteins began to decrease at the early stages (day 3) and declined markedly at the late stages (~70%; Fig. 4A; $P < 0.0001$); the expression of *PhSAG12* was highly upregulated at the late stages (days 9 to 11) (Fig. 4A; $P < 0.0001$), which agrees with results reported previously for *SAG12* in Arabidopsis [41].

We then analysed the expression profiles of 24 *PhNACs* that have shown detectable expression in leaves (Fig. 3). Nineteen genes showed significant differences in expression (Supplementary Table S2) and were, therefore, selected for clustering analysis (Fig. 4). Clusters 1 and 2 contained the upregulated genes (Fig. 4B and C). All the genes in cluster 1 (*PhNAC017*, *PhNAC024*, *PhNAC058*, and *PhNAC106*) showed considerable changes in their expression levels at mid (days 3 and 6) and late (days 9 and 11) stages. The expression of seven genes in cluster 2 (*PhNAC074*, *PhNAC101*, *PhNAC099*, *PhNAC089*, *PhNAC033*, *PhNAC095*, and *PhNAC097*) showed moderate to strong changes in the late stages. Conversely, cluster 3 consisted of four genes

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(*PhNAC004*, *PhNAC051*, *PhNAC052,* and *PhNAC053*) downregulated in the relatively early stages (Fig. 4D).

3.7. Expression analysis of PhNACs during pollination-induced petal senescence

In *P. hybrida*, pollination accelerates corolla senescence and petal wilting, which occur 48-72 hours after pollination (hap) [43,78]. To estimate senescence progression and the role of *PhNACs* in this process, we measured soluble protein levels and expression of *PhSAG12*. The levels of soluble proteins decreased early (day 3) and then plateaued until the late stages, with an overall reduction of approximately 38% (Fig. 5A; $P = 0.0003$); *PhSAG12* was highly expressed in the late stages (48 and 72 hap; $P < 0.0001$) (Fig. 5A). Eighteen *PhNACs* showed significant changes in expression, as shown in Supplementary Table S2. A clustering analysis of these 18 genes resulted in three clusters (Fig. 5): cluster 1 contained 2 early and 2 late consistently upregulated genes (Fig. 5B); cluster 2 contained 11 genes that were up- and downregulated (Fig. 5C), but showed minimal expression changes (~± 1-fold difference); cluster 3 consisted of 3 genes that were downregulated at mid and late stages (Fig. 5D).

Overall, the expression and clustering analysis of *PhNACs* showed common and distinct patterns of gene expression at different stages of leaf and petal development and during progression of senescence.

3.8. Identification of putative senescence regulators via hierarchical clustering of PhNAC expression profiles and phylogenetic analysis

A clustering analysis of expression profiles was used to further assess gene expression and to simultaneously visualize the three types of senescence examined in

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this study (Fig. 6). Overall, this analysis showed a reduction in the number of genes that consistently changed their expression throughout the different types of senescence. Indeed, most of the *PhNACs* (at least 20) showed changes in expression during natural leaf senescence, followed by natural petal senescence (15 genes) and pollination-induced senescence (7 genes) (Fig. 6). Additionally, most of these genes showed changes in expression during the early and intermediate stages of leaf development, while changes in petal processes were predominantly observed during the late stages (Fig. 6).

To generate a list of candidate genes involved in petunia senescence, we constructed a phylogenetic tree to investigate the relationship of senescence-associated PhNACs with NAC members in other species. As shown in Supplementary Figs. S3 and S4, we analyzed the sequences of 41 selected proteins in Arabidopsis and 29 proteins identified in *P. hybrida*, and their putative orthologs in related Solanaceae species (tomato, potato, and *Nicotiana tomentosiformis*). We also included NACs that participate in leaf senescence, and InEPH1, the regulator of petal senescence in *Ipomoea nil*. In addition, we conducted a motif search of the divergent transcriptional activation region of the C-terminal portion (TAR).

The seven genes in cluster 1 were consistently upregulated in the three types of senescence (Fig. 6). Four genes (*PhNAC106*, *PhNAC017*, *PhNAC074*, and *PhNAC101*) were expressed in the early stages, and two (*PhNAC024*, *PhNAC089*) showed relatively early expression during natural leaf senescence (Fig. 6). Six genes in this cluster have putative orthologs that play roles in leaf senescence, indicating that genes in this category may regulate senescence in petunia (Table 2).

Genes in cluster 3 were upregulated during natural leaf and petal senescence. *PhNAC085* was included in cluster 3, although this gene did not show differences in expression during natural petal senescence (Supplementary Table S2). Therefore, we

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designated *PhNAC085* as a leaf-expressed gene. *PhNAC033* and *PhNAC097*, classified into the adjacent phylogenetic groups IV and V (NAC-a subfamily), are putative orthologs of the positive regulator of Arabidopsis ATAF1 (Table 2). *PhNAC099* and *PhNAC95*, classified into groups XIV and XVII, respectively, of the NAC-b subfamily, are not currently reported to have any senescence-associated orthologs (Table 2).

Genes in cluster 2 were predominantly upregulated during natural leaf senescence. *PhNAC085* and *PhNAC051* showed consistent and similar expression profiles with changes in early and mid- senescence. These two genes were classified into group IX (Fig. 6 and Table 2) and, therefore, this result suggests redundancy; these genes also do not have any currently reported orthologs that play roles in senescence. *PhNAC023*, *PhNAC094*, *PhNAC105*, and *PhNAC096* showed upregulation in early stages of leaf development (Fig. 6); three of these genes have putative orthologs that play roles in senescence (Fig. 6 and Table 2). Thus, these three genes likely participate in senescence regulation in petunia. The expression of *PhNAC082* was strongly upregulated during early stages, although currently, this gene has no orthologs associated with senescence (Fig. 6 and Table 2).

In cluster 4, *PhNAC004* (group VI), and *PhNAC052* and *PhNAC053* (group VII), were downregulated in the three types of senescence (Fig. 6 and Table 2). PhNAC004 has two putative orthologs with function in leaf senescence in different species (JUB1 and GmNAC81), whereas PhNAC052 and PhNAC053 currently have no orthologs associated with senescence (Table 2).

4. Discussion

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In this study, we examined the NAC family of transcription factors in *P. hybrida* in order to understand transcriptional regulation governing leaf and petal senescence. Characterization of natural leaf senescence demonstrated that initiation and progression of this process occurred similarly to those observed in other species (Fig. 2) such as dicots and monocots [11,12,20,79–82]. Thus, leaf senescence may be a generally conserved process. In Arabidopsis, numerous *NACs* showed changes in expression at the early stages of leaf development (~70%) (Table 1). Similarly, most upregulated *PhNACs* (83%) (Fig. 3) showed increased expression at relatively early stages of senescence, whereas nearly all downregulated *PhNACs* showed decreased expression at mid-senescence. In most *PhNACs*, changes in expression occurred at similar times and with similar magnitude (Table 1 and Fig. 3). However, some genes (*PhNAC058*, *PhNAC095*, and *PhNAC097*) showed differences in the time of expression changes. In addition, genes such as *PhNAC004*, *PhNAC082*, and *PhNAC108*, showed expression profiles that were the opposite of those shown by the corresponding Arabidopsis orthologs (*JUB1*, *At1g62700/VND5*, and *VNI2*, respectively) (Table 1 and Fig. 3).

Pollination-induced petal senescence is the type of senescence that normally occurs in Arabidopsis. Comparative expression analysis revealed that the proportion of leaf-expressed genes changing their expression during this type of senescence was similar in Arabidopsis and *P. hybrida* (Table 1 and Fig. 5). Of 36 genes upregulated in the leaves of Arabidopsis, 20 increased their expression (56%), whereas 4 were downregulated (11%), during pollination-induced petal senescence (Table 1). In petunia, of 18 upregulated *PhNACs* in leaves, 11 were upregulated (61%), whereas 2 were downregulated (11%), during pollination-induced senescence (Table 1; Fig. 3 and Fig. 5). In Arabidopsis all downregulated genes in the leaves were also downregulated in petals (Table 1); in petunia, of the five downregulated *PhNACs* in leaves, three were also downregulated in petals (*PhNAC052*, *PhNAC053*, and *PhNAC004*) (Table 1; Fig.

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3 and Fig. 5). Similar expression patterns were observed between Arabidopsis and petunia during leaf and petal senescence, although these plants belong to different groups within the higher eudicots [48]. This finding suggests that senescence-associated *NAC* gene expression patterns are conserved between the two species.

Simultaneous visualization of *NAC* expression in the three types of senescence indicates that the PhNACs identified in this study can act as regulatory factors during leaf and petal senescence (Fig. 6); this trend is also observed in other species [11,12,36,83–86]. The expression of genes contained in cluster 1 (Fig. 6) increased strongly during progression of the three types of senescence, making these some of the most interesting genes characterized in this study. *PhNAC017*, *PhNAC024*, and *PhNAC106* possess equivalents in Arabidopsis that were upregulated in senescing leaves, petals, and pods [41]. Moreover, the putative homologs of these genes also tended to be upregulated in the corollas of pollinated petunia flowers [36] and after 16 h of treatment with ethylene [87]. Moreover, putative homologs of PhNAC058 and PhNAC101 also displayed a similar behavior. Pollination signals in the stigma induce a rapid increase (first 7 hours) in ethylene production in the stigma and style of petunia flowers [88,89] that, much later, leads to the production of ethylene in the corollas. The expression of *PhNAC024*, *PhNAC074*, and *PhNAC089* was upregulated only 6 hours after pollination (Fig. 6), a moment noticeable before fertilization and the production of ethylene in the corollas [36]. Interestingly, an alignment of PhNAC024, PhNAC074, and PhNAC089, showed high identity with both parental equivalents (Supplementary Fig. S2), suggesting a conserved function of these members in the initial steps of senescence regulation.

PhNAC024 is a putative ortholog of positive regulators of leaf senescence in various dicot and monocot species (Table 2). The putative orthologs AtNAP, GhNAP, SlNAP2, and OsNAP function via ABA-associated signaling pathways [19,27,28,90].

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Moreover, AtNAP is associated with ethylene signaling, which promotes leaf senescence [91], indicating a likely cross-talk between these two hormonal pathways. PhNAC024, which showed early and strong changes in expression, may regulate leaf and petal senescence in petunia, and is one of the most interesting candidates identified in this study.

Although they belong to different subfamilies (Table 2), PhNAC089 and PhNAC106 represent two phylogenetically close members of cluster 1 (Fig. 6). Both genes show similar expression profiles, and their putative orthologs in Arabidopsis were simultaneously upregulated during leaf and petal development (Table 1) [41]. PhNAC106 in particular showed the highest expression changes in natural and pollination-induced petal senescence (Fig. 6); these expression changes are mirrored by the putative equivalent of PhNAC106 in aging petals of the wallflower [40]. Interestingly, ONAC016 and VNI2, the putative orthologs of PhNAC089 and PhNAC106 (Table 2), function as repressors of leaf senescence in rice and Arabidopsis, respectively. In addition, VNI2 integrates leaf senescence with abiotic stresses [25]. Thus, PhNAC089 and PhNAC106 may play roles in senescence.

PhNAC017 and PhNAC058 of cluster 1 (Fig. 6) are the putative orthologs of ORE1/ORS1/GhNAC12/SlORE1 and ANAC046, respectively. ORE1/ORS1/GhNAC12/SlORE1 promote leaf senescence in Arabidopsis, cotton, and tomato [20,21,31,45,93], while ANAC046 promotes the process in Arabidopsis [92] (Table 2). PhNAC017 and PhNAC058 were classified into adjacent groups (X and XI), but differed with respect to the presence of motif 16 in TAR (Supplementary Figs. S3, S4 and Table 2). This finding suggests that PhNAC017 and PhNAC058 may interact with distinct partner/s; therefore, regulating the expression of their targets differentially. *ORE1* expression is induced via EIN2 in the ethylene pathway [20,91]. Moreover, InEPH1 is a putative ortholog of ORE1, which promotes petal senescence in *Ipomoea*

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nil (Table 2) and currently represents the only NAC with a reported role in petal senescence [45]. Interestingly, InEPH1 has scant involvement in leaf senescence, and InEIN2 minimally affects the expression of InEPH1 during age-dependent petal senescence in *Ipomoea* [45]. Thus, similar NAC proteins may play different roles in senescence of different organs and species. Altogether, our results indicate that PhNAC058 and PhNAC017 may participate in senescence regulation in petunia.

Finally, PhNAC074 of cluster 1 (Fig. 6) is a putative ortholog of Arabidopsis ANAC021 (At1g56010) and of TaNAC-S, which is a negative regulator of leaf senescence in wheat [30]. Similar to the previously described PhNAC017 (group XI), ANAC021 and PhNAC074 (group VIII) possess a motif 16 in TAR (Supplementary Fig. S3 and Table 2). The putative orthologs of PhNAC017 increased their expression and positively regulated leaf senescence in different species (Table 2). Conversely, TaNAC-S, which lacks a motif 16 (Supplementary Fig. S3), decreases its expression to negatively regulate leaf senescence in wheat [30]. The negative function of TaNAC-S in senescence may be explained by the absence of motif 16. In different dicot species, this motif is present in known positive regulators of group XI (Supplementary Fig. S3 and Table 2). Therefore, motif 16 may enable PhNAC074 to positively regulate senescence (Supplementary Fig. S3). These observations suggest that proteins associated with this motif (groups VIII and XI) show divergent C-terminal regulation in monocots and dicots (Supplementary Fig. S3).

PhNAC033 and *PhNAC097*, which belong to adjacent phylogenetic groups (Table 2), were upregulated during natural leaf and petal senescence (cluster 3) (Fig.6). Arabidopsis ATAF1 is the putative ortholog of PhNAC033 and PhNAC097, which promote leaf senescence via ABA- and hydrogen peroxide-induced senescence (Table 2 and Supplementary Fig. S3) [23]. Homologs of PhNAC033 and PhNAC097 are upregulated during petal senescence in petunia [44,86], while virus-induced gene

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silencing (VIGS) of these genes slightly increases petal longevity [86]. The lack of consistent phenotypes can be explained by genetic redundancy, as is observed in ATAF subfamily members in Arabidopsis [94].

Genes predominantly upregulated during natural leaf senescence (Cluster 2; Fig. 6), such as *PhNAC051* and *PhNAC085* of group IX (Table 2), shared similar and consistent expression profiles (Fig. 6), suggesting the occurrence of genetic redundancy. Other genes in cluster 2 included *PhNAC096* and *PhNAC105*, which showed similar leaf expression profiles (Fig. 6). PhNAC096 and PhNAC105 were considered NTL4 and NAC016 orthologs, respectively (Table 2 and Supplementary Fig. S3). NAC016 promotes natural and dark-induced leaf senescence in Arabidopsis via regulation of several senescence-associated NACs [22]. Similarly to NTL4, NAC016 participates in regulation of senescence mediated by salt and oxidative stress signals [22]. Similarly, a putative ortholog of *PhNAC023*, NTL9, promotes leaf senescence in Arabidopsis and is induced by osmotic stress (Supplementary Fig. S3 and Table 2) [95]. These membrane-bound members of the NAC-b subfamily in Arabidopsis have been implicated in the regulation of leaf senescence that occurs in response to different abiotic and oxidative stresses. Therefore, these three petunia members may be involved in the regulation of senescence.

PhNAC082 of the NAC-c subfamily (group VII) and belonging to cluster 2 was early and strongly upregulated during leaf senescence in petunia (Fig. 6 and Supplementary Fig. S3). However, most genes in the NAC-c subfamily were downregulated during leaf senescence in Arabidopsis (Table 1). These contrasting findings suggest that PhNAC082 plays a different regulatory role in petunia. Other members of the NAC-c subfamily (group VII) in petunia, such as PhNAC052 and PhNAC053, which share identical motifs in TAR, were downregulated in the three types of senescence examined in this study (Supplementary Fig. S3, Fig. 6 and Table 1).

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Despite the absence of known senescence regulators, members of the NAC-c sub-family in Arabidopsis participate in xylem differentiation and programmed cell death [96], and physically interact with the negative senescence regulator VNI2 [25,97], indicating that they may be involved in senescence-associated processes. Interestingly, the opposing expression patterns observed in *PhNAC082* and *PhNAC052*/*PhNAC053* (Fig. 6) suggest that genetic compensation is involved in mediation of senescence processes.

Finally, *PhNAC004*, the only NAC-e subfamily member present in cluster 4, was downregulated in the three types of senescence (Fig. 6). *JUB1* and *GmNAC81*, which are the putative orthologs of *PhNAC004*, are upregulated in Arabidopsis and the soybean, respectively, but play opposite roles in senescence. JUB1 represses leaf senescence via inhibition of oxidative stress, and GmNAC81 promotes senescence via integration of osmotic stress and natural leaf senescence [24,32]. The opposing functions of JUB1 and GmNAC81, combined with transcriptional regulation of *PhNAC004*, indicate that members of the NAC-e subfamily may play diverse roles in transcriptional regulation and/or mediation of senescence across species.

In this study, we have shown that 10 to 15 PhNACs likely participate in the regulation of leaf and petal senescence. Because closely related PhNACs may play similar roles in senescence, a high degree of genetic redundancy is expected. Therefore, it may be necessary to downregulate more than one gene simultaneously to determine if signaling components controlling senescence in Arabidopsis and other species have an equivalent function in petunia. These studies will help to delay senescence via selective breeding in this ornamental crop.

Conflict of interest

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The authors have no conflict of interest to declare.

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References

[1] B. Rubinstein, Regulation of cell death in flower petals, Plant Mol. Biol. 44 (2000) 303–318. doi:10.1023/A:1026540524990.

[2] P.O. Lim, H.J. Kim, H. Gil Nam, Leaf Senescence, Annu. Rev. Plant Biol. 58 (2007) 115–136. doi:10.1146/annurev.arplant.57.032905.105316.

[3] M. Kusaba, A. Tanaka, R. Tanaka, Stay-green plants: What do they tell us about the molecular mechanism of leaf senescence, 117 (2013) 221–234. doi:10.1007/s11120-013-9862-x.

[4] J.H.M. Schippers, R. Schmidt, C. Wagstaff, H.-C. Jing, Living to Die and Dying to Live: The Survival Strategy behind Leaf Senescence, 169 (2015) 914–930. doi:10.1104/pp.15.00498.

[5] D. Podzimska-Sroka, C. O'Shea, P. Gregersen, K. Skriver, NAC Transcription Factors in Senescence: From Molecular Structure to Function in Crops, Plants (Basel) 4 (2015) 412-448. doi:10.3390/plants4030412.

[6] S. Gan, R.M. Amasino, Making Sense of Senescence (Molecular Genetic Regulation and Manipulation of Leaf Senescence), Plant Physiol. 113 (1997) 313–319. doi:10.1104/pp.113.2.313.

[7] S. Gepstein, G. Sabehi, M.J. Carp, T. Hajouj, M.F.O. Nesher, I. Yariv, et al., Large-scale identification of leaf senescence-associated genes, Plant J. 36 (2003) 629–642. doi:10.1046/j.1365-313X.2003.01908.x.

[8] V. Buchanan-Wollaston, S. Earl, E. Harrison, E. Mathas, S. Navabpour, T. Page, et al., The molecular analysis of leaf senescence-a genomics approach, Plant Biotechnol J. 1 (2003) 3–22. doi:10.1046/j.1467-7652.2003.00004.x.

[9] V. Buchanan-Wollaston, T. Page, E. Harrison, E. Breeze, O.L. Pyung, G.N. Hong, et al., Comparative transcriptome analysis reveals significant differences in gene expression and signalling pathways between developmental and dark/starvation-induced senescence in Arabidopsis, Plant J. 42 (2005) 567–585. doi:10.1111/j.1365-313X.2005.02399.x.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

[10] Y. Guo, Z. Cai, S. Gan, Transcriptome of Arabidopsis leaf senescence, Plant, Cell Environ. 27 (2004) 521–549. doi:10.1111/j.1365-3040.2003.01158.x.

[11] S. Balazadeh, D.M. Riaño-Pachón, B. Mueller-Roeber, Transcription factors regulating leaf senescence in *Arabidopsis thaliana*, Plant Biol. 10 (2008) 63–75. doi:10.1111/j.1438-8677.2008.00088.x.

[12] E. Breeze, E. Harrison, S. McHattie, L. Hughes, R. Hickman, C. Hill, S., et al., High-Resolution Temporal Profiling of Transcripts during Arabidopsis Leaf Senescence Reveals a Distinct Chronology of Processes and Regulation, 23 (2011) 873–894. doi:10.1105/tpc.111.083345.

[13] M.K. Jensen, T. Kjaersgaard, M.M. Nielsen, P. Galberg, K. Petersen, C. O'Shea, et al., The *Arabidopsis thaliana* NAC transcription factor family: structure–function relationships and determinants of ANAC019 stress signalling, Biochem. J. 426 (2010) 183–196. doi:10.1042/bj20091234.

[14] H. Ooka, K. Satoh, K. Doi, T. Nagata, Y. Otomo, K. Murakami, et al., Comprehensive Analysis of NAC Family Genes in *Oryza sativa* and *Arabidopsis thaliana*, DNA Res. 20 (2003) 239–247.

[15] A.N. Olsen, H.A. Ernst, L. Lo Leggio, K. Skriver, NAC transcription factors: Structurally distinct, functionally diverse, Trends Plant Sci. 10 (2005) 79–87. doi:10.1016/j.tplants.2004.12.010.

[16] A.K. Singh, V. Sharma, A.K. Pal, V. Acharya, P.S. Ahuja, Genome-wide organization and expression profiling of the NAC transcription factor family in potato (*Solanum tuberosum* L.), DNA Research 20 (2013) 403–423. doi:10.1093/dnares/dst019.

[17] T. Kjaersgaard, M.K. Jensen, M.W. Christiansen, P. Gregersen, B.B. Kragelund, K. Skriver, Senescence-associated barley NAC (NAM, ATAF1,2, CUC) transcription factor interacts with radical-induced cell death 1 through a disordered regulatory domain, J. Biol. Chem. 426 (2011) 183–196. doi:10.1074/jbc.M111.247221.

[18] S. Lindemose, M.K. Jensen, J. Van De Velde, C. O'Shea, K.S. Heyndrickx, C.T. Workman, et al., A DNA-binding-site landscape and regulatory network analysis for NAC transcription factors in *Arabidopsis thaliana*, Nucleic Acids Res. 42 (2014) 7681–7693. doi:10.1093/nar/gku502.

[19] Y. Guo, S. Gan, AtNAP, a NAC family transcription factor, has an important role in leaf senescence, Plant J. 46 (2006) 601–612. doi:10.1111/j.1365-313X.2006.02723.x.

[20] J.H. Kim, H.R. Woo, J. Kim, P.O. Lim, I.C. Lee, S.H. Choi, D. Hwang, et al., Trifurcate feed-forward regulation of age-dependent cell death involving miR164 in Arabidopsis, Science 323 (2009) 1053–1057. doi:10.1126/science.1166386.

[21] S. Balazadeh, M. Kwasniewski, C. Caldana, M. Mehrnia, M.I. Zanor, G.P. Xue, et al., ORS1, an H2O2-responsive NAC transcription factor, controls senescence in *Arabidopsis thaliana*, Mol. Plant 4 (2011) 346–360. doi:10.1093/mp/ssq080.

[22] Y.S. Kim, Y. Sakuraba, S.H. Han, S.C. Yoo, N.C. Paek, Mutation of the Arabidopsis NAC016 transcription factor delays leaf senescence, Plant Cell Physiol. 54 (2013) 1660–1672. doi:10.1093/pcp/pct113.

[23] P. Garapati, G.-P. Xue, S. Munné-Bosch, S. Balazadeh, Transcription Factor ATAF1 in Arabidopsis Promotes Senescence by Direct Regulation of Key Chloroplast Maintenance and Senescence Transcriptional Cascades, Plant Physiol. 168 (2015) 1122–1139. doi:10.1104/pp.15.00567.

[24] A. Wu, A.D. Allu, P. Garapati, H. Siddiqui, H. Dortay, M.-I. Zanor, et al., JUNGBRUNNEN1, a Reactive Oxygen Species-Responsive NAC Transcription Factor, Regulates Longevity in Arabidopsis, Plant Cell 24 (2012) 482–506. doi:10.1105/tpc.111.090894.

[25] S.-D. Yang, P.J. Seo, H.-K. Yoon, C.-M. Park, The Arabidopsis NAC Transcription Factor VNI2 Integrates Abscisic Acid Signals into Leaf Senescence via the COR / RD Genes, Plant Cell. 23 (2011) 2155–2168. doi:10.1105/tpc.111.084913.

[26] X. Chen, Y. Wang, B. Lv, J. Li, L. Luo, S. Lu, et al., The NAC family transcription factor OsNAP confers abiotic stress response through the ABA pathway, Plant Cell Physiol. 55 (2014) 604–619. doi:10.1093/pcp/pct204.

[27] C. Liang, Y. Wang, Y. Zhu, J. Tang, B. Hu, L. Liu, S. et al., OsNAP connects abscisic acid and leaf senescence by fine-tuning abscisic acid biosynthesis and directly targeting senescence-associated genes in rice, Proc. Natl. Acad. Sci. USA 111 (2014) 10013–10018. doi:10.1073/pnas.1321568111.

[28] K. Fan, N. Bibi, S. Gan, F. Li, S. Yuan, M. Ni, et al., A novel NAP member GhNAP is involved in leaf senescence in *Gossypium hirsutum*, J. Exp. Bot. 66 (2015) 4669–4682. doi:10.1093/jxb/erv240.

[29] Y. Sakuraba, W. Piao, J.H. Lim, S.H. Han, Y.S. Kim, G. An, et al., Rice ONAC106 Inhibits Leaf Senescence and Increases Salt Tolerance and Tiller Angle, Plant Cell Physiol. 56 (2015) 2325–2339. doi:10.1093/pcp/pcv144.

ACCEPTED MANUSCRIPT

[30] D. Zhao, A.P. Derkx, D.C. Liu, P. Buchner, M.J. Hawkesford, Overexpression of a NAC transcription factor delays leaf senescence and increases grain nitrogen concentration in wheat, Plant Biol. 17 (2015) 904–913. doi:10.1111/plb.12296.

[31] F. Zhao, J. Ma, L. Li, S. Fan, Y. Guo, M. Song, et al., GhNAC12, a neutral candidate gene, leads to early aging in cotton (*Gossypium hirsutum* L), Gene 576 (2016) 268–274. doi:10.1016/j.gene.2015.10.042.

[32] M.R. Pimenta, P.A. Silva, G.C. Mendes, J.R. Alves, H.D.N. Caetano, J.P.B. Machado, et al., The Stress-Induced Soybean NAC Transcription Factor GmNAC81 Plays a Positive Role in Developmentally Programmed Leaf Senescence, Plant Cell Physiol. 57 (2016) 1098–1114. doi:10.1093/pcp/pcw059.

[33] M.W. Christiansen, C. Matthewman, D. Podzimska-Sroka, C. O'Shea, S. Lindemose, N.E. Møllegaard, et al., Barley plants over-expressing the NAC transcription factor gene *HvNAC005* show stunting and delay in development combined with early senescence, J. Exp. Bot. 67 (2016) 5259–5273. doi:10.1093/jxb/erw286.

[34] H.J. Rogers, Programmed cell death in floral organs: How and why do flowers die?, Ann. Bot. 97 (2006) 309–315. doi:10.1093/aob/mcj051.

[35] W.G. Van Doorn, E.J. Woltering, Physiology and molecular biology of petal senescence, J. Exp. Bot. 59 (2008) 453–480. doi:10.1093/jxb/erm356.

[36] S.R. Broderick, S. Wijeratne, A.J. Wijeratn, L.J. Chapin, T. Meulia, M.L. Jones, RNA-sequencing reveals early, dynamic transcriptome changes in the corollas of pollinated petunias, BMC Plant Biol. 14 (2014) 307. doi:10.1186/s12870-014-0307-2.

[37] H. Thomas, H.J. Ougham, C. Wagstaff, A.D. Stead, Defining senescence and death, J. Exp. Bot. 54, 1127–1132. doi:10.1093/jxb/erg133.

[38] M.L. Jones, Changes in Gene Expression during Senescence, in: Plant Cell Death Process, Amsterdam Elsevier (2003) 51–71. doi:10.1016/B978-012520915-1/50007-2.

[39] H.J. Rogers, Is there an important role for reactive oxygen species and redox regulation during floral senescence?, Plant. Cell Environ. 35 (2012) 217–233. doi:10.1111/j.1365-3040.2011.02373.x.

[40] A.M. Price, D.F. Aros Orellana, F.M. Salleh, R. Stevens, R. Acock, V. Buchanan-Wollaston, et al., A Comparison of Leaf and Petal Senescence in Wallflower Reveals Common and Distinct Patterns of Gene Expression and Physiology, Plant Physiol. 147 (2008) 1898–1912. doi:10.1104/pp.108.120402.

[41] C. Wagstaff, T.J.W. Yang, A.D. Stead, V. Buchanan-Wollaston, J.A. Roberts, A molecular and structural characterization of senescing Arabidopsis siliques and comparison of transcriptional profiles with senescing petals and leaves, Plant J. 57 (2009) 690–705. doi:10.1111/j.1365-313X.2008.03722.x.

[42] S.D. O'Neill, Pollination regulation of flower development, Annu. Rev. Plant Physiol. Plant Mol. Biol. 48 (1997) 547-574. doi:10.1146/annurev.arplant.48.1.547.

[43] B.J. Langston, S. Bai, M.L. Jones, Increases in DNA fragmentation and induction of a senescence-specific nuclease are delayed during corolla senescence in ethylene-insensitive (*etr1-1*) transgenic petunias, J. Exp. Bot. 56 (2005) 15–23. doi:10.1093/jxb/eri002.

[44] H. Wang, G. Stier, J. Lin, G. Liu, Z. Zhang, Y. Changet al., Transcriptome Changes Associated with Delayed Flower Senescence on Transgenic Petunia by Inducing Expression of *etr1-1*, a Mutant Ethylene Receptor, PLoS One. 8 (2013) e65800. doi:10.1371/journal.pone.0065800.

[45] K. Shibuya, K. Shimizu, T. Niki, K. Ichimura, Identification of a NAC transcription factor, EPHEMERAL1, that controls petal senescence in Japanese morning glory, Plant J. 79 (2014) 1044–1051. doi:10.1111/tpj.12605.

[46] Y. Shinozaki, T. Tanaka, I. Ogiwara, M. Kanekatsu, W.G. Van Doorn, T. Yamada, Expression of an *AtNAP* gene homolog in senescing morning glory (*Ipomoea nil*) petals of two cultivars with a different flower life span, J. Plant Physiol. 171 (2014) 633–638. doi:10.1016/j.jplph.2014.01.008.

[47] S. Moschen, S.B. Luoni, N.B. Paniego, H.E. Hopp, G.A.A. Dosio, P. Fernandez, R.A. Heinz, Identification of candidate genes associated with leaf senescence in cultivated sunflower (Helianthus annuus L.), PLoS One 9 (2014) e1043792014. doi:10.1371/journal.pone.0104379.

[48] M.J. Moore, P.S. Soltis, C.D. Bell, J.G. Burleigh, D.E. Soltis, Phylogenetic analysis of 83 plastid genes further resolves the early diversification of eudicots, Proc. Natl. Acad. Sci. U.S.A. 107 (2010) 4623–4628. doi:10.1073/pnas.0907801107.

[49] M. Vandenbussche, P. Chambrier, S. Rodrigues Bento, P. Morel, Petunia, Your Next Supermodel?, Front. Plant Sci. 7 (2016) 1–11. doi:10.3389/fpls.2016.00072.

[50] J. Jin, H. Zhang, L. Kong, G. Gao, J. Luo, PlantTFDB 3.0: A portal for the functional and evolutionary study of plant transcription factors, Nucleic Acids Res. 42 (2014) 1182–1187. doi:10.1093/nar/gkt1016.

ACCEPTED MANUSCRIPT

[51] M.R. Hanlon, M. Vaughn, S. Mock, R. Dooley, W. Moreira, J. Stubbs, C. et al., Araport: An application platform for data discovery, Concurr. Comput. 27 (2015) 4412–4422. doi:10.1002/cpe.3542.

[52] X. Liu, Z. Li, Z. Jiang, Y. Zhao, J. Peng, J. Jin, et al., LSD: A leaf senescence database, Nucl. Acid Res. 39 (2011) 1103–1107. doi:10.1093/nar/gkq1169.

[53] D. Winter, B. Vinegar, H. Nahal, R. Ammar, G. V. Wilson, N.J. Provart, An "electronic fluorescent pictograph" Browser for exploring and analyzing large-scale biological data sets, PLoS One 2 (2007) e178. doi:10.1371/journal.pone.0000718.

[54] G.H. Villarino, A. Bombarely, J.J. Giovannoni, M.J. Scanlon, N.S. Mattson, Transcriptomic analysis of *Petunia hybrida* in response to salt stress using high throughput RNA sequencing, PLoS One 9 (2014) e94651. doi:10.1371/journal.pone.0094651.

[55] S. Gonzalez, B. Clavijo, M. Rivarola, P. Moreno, P. Fernandez, J. Dopazo, et al., ATGC transcriptomics: A web-based application to integrate, explore and analyze de novo transcriptomic data, BMC Bioinform. 18 (2017) 121. doi:10.1186/s12859-017-1494-2.

[56] A. Bombarely, M. Moser, A. Amrad, M. Bao, L. Bapaume, C.S. Barry, et al., Insight into the evolution of the Solanaceae from the parental genomes of *Petunia hybrida*, Nat Plants 2 (2016) 16074. doi:10.1038/nplants.2016.74.

[57] T.A. Hall, BIOEDIT: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/ NT., Nucleic Acids Symp. Ser. 41 (1999) 95–98.

[58] F. Abascal, R. Zardoya, D. Posada, ProtTest: Selection of best-fit models of protein evolution, Bioinformatics 21 (2005) 2104–2105. doi:10.1093/bioinformatics/bti263.

[59] S. Guindon, J.F. Dufayard, V. Lefort, M. Anisimova, W. Hordijk, O. Gascuel, New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0, Syst. Biol. 59 (2010) 307–321. doi:10.1093/sysbio/syq010.

[60] K. Tamura, D. Peterson, N. Peterson, G. Stecher, M. Nei, S. Kumar, MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods, Mol Biol Evol. 28 (2011) 2731–2739. doi:10.1093/molbev/msr121.

[61] T.L. Bailey, M. Boden, F.A. Buske, M. Frith, C.E. Grant, L. Clementi, et al., MEME Suite: Tools for motif discovery and searching, Nucleic Acids Res. 37 (2009) 202–208. doi:10.1093/nar/gkp335.

[62] J. You, L. Zhang, B. Song, X. Qi, Z. Chan, Systematic analysis and identification of stress-responsive genes of the NAC gene family in *Brachypodium distachyon*, PLoS One 10 (2015) e0122027. doi:10.1371/journal.pone.0122027.

[63] R. Moran, Formulae for determination of chlorophyllous pigments extracted with N,N-dimethylformamide, Plant Physiol. 69 (1982) 1376–1381.

[64] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.

[65] J.M. Ruijter, C. Ramakers, W.M.H. Hoogaars, Y. Karlen, O. Bakker, M.J.B. van den hoff, et al., Amplification efficiency: Linking baseline and bias in the analysis of quantitative PCR data, Nucleic Acids Res. 37 (2009) e45. doi:10.1093/nar/gkp045.

[66] Julio A. Di Rienzo, Statistical software for the analysis of experiments of functional genomics, 2009.

[67] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT–PCR, 29 (2001) e45.

[68] N.R. Pal, J.C. Bezdek, R.J. Hathaway, Sequential competitive learning and the fuzzy c-means clustering algorithms, Neural Netw. 9 (1996) 787–796.

[69] E. Dimitriadou, K. Hornik, F. Leisch, D. Meyer, A. Weingessel, e1071: Misc Functions of the Department of Statistics (e1071), TU Wien, 2006.

[70] T. Galili, A. O'Callaghan, J. Sidi, C. Sievert, Heatmaply: An R package for creating interactive cluster heatmaps for online publishing, Bioinformatics 34 (2018) 1600-1602. doi:10.1093/bioinformatics/btx657.

[71] H. Shen, Y. Yin, F. Chen, Y. Xu, R.A. Dixon, A bioinformatic analysis of *NAC* genes for plant cell wall development in relation to lignocellulosic bioenergy production, Bioenerg. Res. 2 (2009) 217–232. doi:10.1007/s12155-009-9047-9.

[72] K.N. Lohman, S. Gan, M.C. John, R.M. Amasino, Molecular analysis of natural leaf senescence in *Arabidopsis thaliana*, Physiol. Plant 92 (1994) 322–328. doi:10.1111/j.1399-3054.1994.tb05343.x.

[73] Y.S. Noh, R.M. Amasino, Identification of a promoter region responsible for the senescence-specific expression of *SAG12*, Plant Mol. Biol. 41 (1999) 181–194. doi:10.1023/A:1006342412688.

ACCEPTED MANUSCRIPT

[74] T. Czechowski, M. Stitt, T. Altmann, M.K. Udvardi, W.R Scheible, Genome-Wide Identification and Testing of Superior Reference Genes for Transcript Normalization in Arabidopsis, Plant Physiol. 139 (2005) 5–17. doi:10.1104/pp.105.063743.

[75] K.E. Reid, N. Olsson, J. Schlosser, F. Peng, S.T. Lund, An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development, BMC Plant Biol. 6 (2006) 27-37. doi:10.1186/1471-2229-6-27.

[76] T. Løvdal, C. Lillo, Reference gene selection for quantitative real-time PCR normalization in tomato subjected to nitrogen, cold, and light stress, Anal. Biochem. 387 (2009) 238–242. doi:10.1016/j.ab.2009.01.024.

[77] H. Liu, Y. Zhou, H. Li, T. Wang, J. Zhang, B. Ouyang, et al., Molecular and functional characterization of ShNAC1, an NAC transcription factor from *Solanum habrochaites*, Plant Sci. 271 (2018) 9–19. doi:10.1016/j.plantsci.2018.03.005.

[78] M.L. Jones, Ethylene signaling is required for pollination-accelerated corolla senescence in petunias, Plant Sci. 175 (2008) 190–196. doi:10.1016/j.plantsci.2008.03.011.

[79] A. Chiba, H. Ishida, N.K. Nishizawa, A. Makino, T. Mae, Exclusion of Ribulose-1,5-bisphosphate Carboxylase/oxygenase from Chloroplasts by Specific Bodies in Naturally Senescing Leaves of Wheat, Plant Cell Physiol. 44 (2003) 914–921. doi:10.1093/pcp/pcg118.

[80] D.E. Martínez, M.L. Costa, F.M. Gomez, M.S. Otegui, J.J. Guiamet, “Senescence-associated vacuoles” are involved in the degradation of chloroplast proteins in tobacco leaves, Plant J. 56 (2008) 196–206. doi:10.1111/j.1365-313X.2008.03585.x.

[81] H.R. Woo, J.H. Kim, J. Kim, J. Kim, U. Lee, I.J. Song, et al., The RAV1 transcription factor positively regulates leaf senescence in Arabidopsis, J. Exp. Bot. 61 (2010) 3947–3957. doi:10.1093/jxb/erq206.

[82] Y. Ono, S. Wada, M. Izumi, A. Makino, H. Ishida, Evidence for contribution of autophagy to Rubisco degradation during leaf senescence in *Arabidopsis thaliana*, Plant Cell Environ. 36 (2013) 1147–1159. doi:10.1111/pce.12049.

[83] W.G. Van Doorn, P.A. Balk, A.M. Van Houwelingen, F.A. Hoeberichts, R.D. Hall, O. Vorst, et al., Gene expression during anthesis and senescence in Iris flowers, Plant Mol. Biol. 53 (2003) 845–863. doi:10.1023/B:PLAN.0000023670.61059.1d.

[84] M.W. Christiansen, P.L. Gregersen, Members of the barley NAC transcription factor gene family show differential co-regulation with senescence-associated genes during senescence of flag leaves, J. Exp. Bot. 65 (2014) 4009–4022. doi:10.1093/jxb/eru046.

[85] G.F. Tsanakas, M.E. Manioudaki, A.S. Economou, P. Kalaitzis, De novo transcriptome analysis of petal senescence in *Gardenia jasminoides* Ellis, BMC Genomics 15, 554. doi:10.1186/1471-2164-15-554.

[86] H. Wang, X. Chang, J. Lin, Y. Chang, J.C. Chen, M.S. Reid, et al., Transcriptome profiling reveals regulatory mechanisms underlying corolla senescence in petunia, Hortic. Res. 5 (2018). doi:10.1038/s41438-018-0018-1.

[87] J. Guo, J. Liu, Q. Wei, R. Wang, W. Yang, Y. Ma, et al., Proteomes and Ubiquitylomes Analysis Reveals the Involvement of Ubiquitination in Protein Degradation in Petunias, Plant Physiol. 173 (2016) 668–687. doi:10.1104/pp.16.00795.

[88] L.J.W. Gilissen, F.A. Hoekstra, Pollination-Induced Corolla Wilting in *Petunia hybrida* Rapid Transfer through the Style of a Wilting-Inducing Substance, Plant Physiol. 75 (1984) 496–498. doi:10.1104/pp.75.2.496.

[89] F.A. Hoekstra, R. Weges, Lack of Control by Early Pistillate Ethylene of the Accelerated Wilting of *Petunia hybrida* Flowers, Plant Physiol. 80 (1986) 403–408. doi:10.1104/pp.80.2.403.

[90] X. Ma, Y. Zhang, V. Turečková, G.-P. Xue, A.R. Fernie, B. Mueller-Roeber, et al., The NAC Transcription Factor SlNAP2 Regulates Leaf Senescence and Fruit Yield in Tomato, Plant Physiol. 177 (2018) 1286–1302. doi:10.1104/pp.18.00292.

[91] H.J. Kim, S.H. Hong, Y.W. Kim, I.H. Lee, J.H. Jun, B.K. Phee, et al., Gene regulatory cascade of senescence-associated NAC transcription factors activated by ETHYLENE-INSENSITIVE2-mediated leaf senescence signalling in Arabidopsis, J. Exp. Bot. 65 (2014) 4023–4036. doi:10.1093/jxb/eru112.

[92] C. Oda-Yamamizo, N. Mitsuda, S. Sakamoto, D. Ogawa, M. Ohme-Takagi, A. Ohmiya, The NAC transcription factor ANAC046 is a positive regulator of chlorophyll degradation and senescence in Arabidopsis leaves, Nature Scientific Reports 6 (2016) 23609. doi:10.1038/srep23609.

[93] B.S. Lira, G. Gramegna, B.A. Trench, F.R.R. Alves, E.M. Silva, G.F.F. Silva, et al., Manipulation of a Senescence-Associated Gene Improves Fleshy Fruit Yield, Plant Physiol. 175 (2017) 77–91. doi:10.1104/pp.17.00452.

ACCEPTED MANUSCRIPT

[94] T. Kleinow, S. Himbert, B. Krenz, H. Jeske, C. Koncz, NAC domain transcription factor ATAF1 interacts with SNF1-related kinases and silencing of its subfamily causes severe developmental defects in Arabidopsis, Plant Sci. 177 (2009) 360–370. doi:10.1016/j.plantsci.2009.06.011.

[95] H.-K. Yoon, S.-G. Kim, S. Kim, C. Park, Molecules and Regulation of Leaf Senescence by NTL9-mediated Osmotic Stress Signaling in Arabidopsis, Mol. Cells 25 (2008), 438–445.

[96] J. Zhou, R. Zhong, Z.H. Ye, Arabidopsis NAC domain proteins, VND1 to VND5, are transcriptional regulators of secondary wall biosynthesis in vessels, PLoS One 9 (2014) e105726. doi:10.1371/journal.pone.0105726.

[97] M. Yamaguchi, M. Ohtani, N. Mitsuda, M. Kubo, M. Ohme-Takagi, H. Fukuda, et al., VND-INTERACTING2, a NAC Domain Transcription Factor, Negatively Regulates Xylem Vessel Formation in Arabidopsis, Plant Cell 22 (2010) 1249–1263. doi:10.1105/tpc.108.064048.

[98] R. Hickman, C. Hill, C.A. Penfold, E. Breeze, L. Bowden, J.D. Moore, et al., A local regulatory network around three NAC transcription factors in stress responses and senescence in Arabidopsis leaves, Plant J. 75 (2013) 26–39. doi:10.1111/tpj.12194.

[99] S. Lee, P.J. Seo, H.J. Lee, C.M. Park, A NAC transcription factor NTL4 promotes reactive oxygen species production during drought-induced leaf senescence in Arabidopsis, Plant J. 70 (2012) 831–844. doi:10.1111/j.1365-313X.2012.04932.x.

[100] S. Lee, H.J. Lee, S.U. Huh, K.H. Paek, J.H. Ha, C.M. Park, The Arabidopsis NAC transcription factor NTL4 participates in a positive feedback loop that induces programmed cell death under heat stress conditions, Plant Sci. 227 (2014) 76–83. doi:10.1016/j.plantsci.2014.07.003.

[101] S.T. Shah, C. Pang, S. Fan, M. Song, S. Arain, S. Yu, Isolation and expression profiling of GhNAC transcription factor genes in cotton (*Gossypium hirsutum* L.) during leaf senescence and in response to stresses, Gene 531 (2013) 220–234. doi:10.1016/j.gene.2013.09.007.

[102] C. Uauy, A. Distelfeld, T. Fahima, A. Blechl, J. Dubcovsky, A NAC gene regulating senescence improves grain protein, zinc, and iron content in wheat, Science 314 (2006) 1298-1301. doi:10.1126/science.1133649.

[103] R.A. Sperotto, F.K. Ricachenevsky, G.L. Duarte, T. Boff, K.L. Lopes, E.R. Sperb, et al., Identification of up-regulated genes in flag leaves during rice grain filling and characterization of OsNAC5, a new ABA-dependent transcription factor, Planta 230 (2009) 985-1002. doi:10.1007/s00425-009-1000-9.

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Figure legends

Fig. 1. Sequence alignment of PhNACs. ClustalW alignment of the highly conserved N-terminal residues spanning the NAC-binding domain in the 29 *P. hybrida* proteins and portion of the divergent C-terminal transcriptional activation region (TAR) identified in this study. ORE1 of Arabidopsis was used as reference. A phylogenetic tree was constructed from a complete alignment of the five subdomains (A-E). The nine identified clusters are represented in different colors. Highly conserved residues are depicted with black and grey backgrounds and define the five conserved subdomains. Dots indicate highly conserved amino acids in NAC of *A. thaliana* and rice [14]. Asterisks indicate four partial sequences that lacked the first portion of the N-terminal domain.

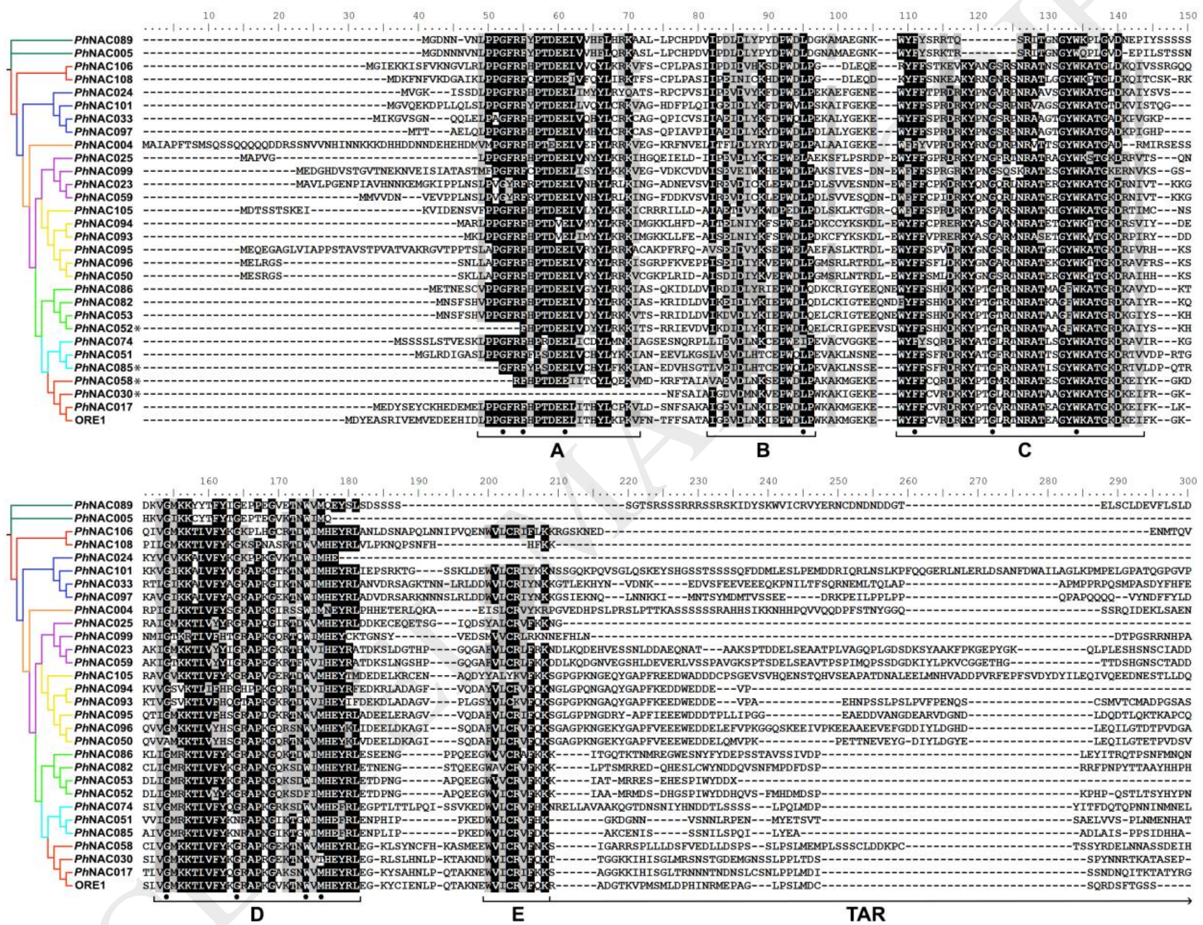

Fig. 2. Analysis of physiological and molecular parameters associated with the progression of natural leaf senescence in *P. hybrida*. (A) Content of chlorophyll and (B) total soluble proteins relative to fresh weight. (C) Relative transcript levels of *PhCAB2* and (D) *PhSAG12* are shown as the ratio (log2 scale) between each sampled point relative to the level at the first sampling point and expression of the reference gene *PhEf1a*. (E) Levels of the large subunit of Rubisco (RbcL), as analyzed by SDS-PAGE and stained with Coomassie blue. Three biological replicates were examined using three independent SDS-PAGEs; only the most representative replicate is shown. (F) *P. hybrida* leaves at days 4 and 33 after full leaf expansion. Asterisks denote significant differences among means between each sample point in relation to the first sampling point, as assessed using Tukey's post-test ($P < 0.05$). Error bars correspond to standard error means.

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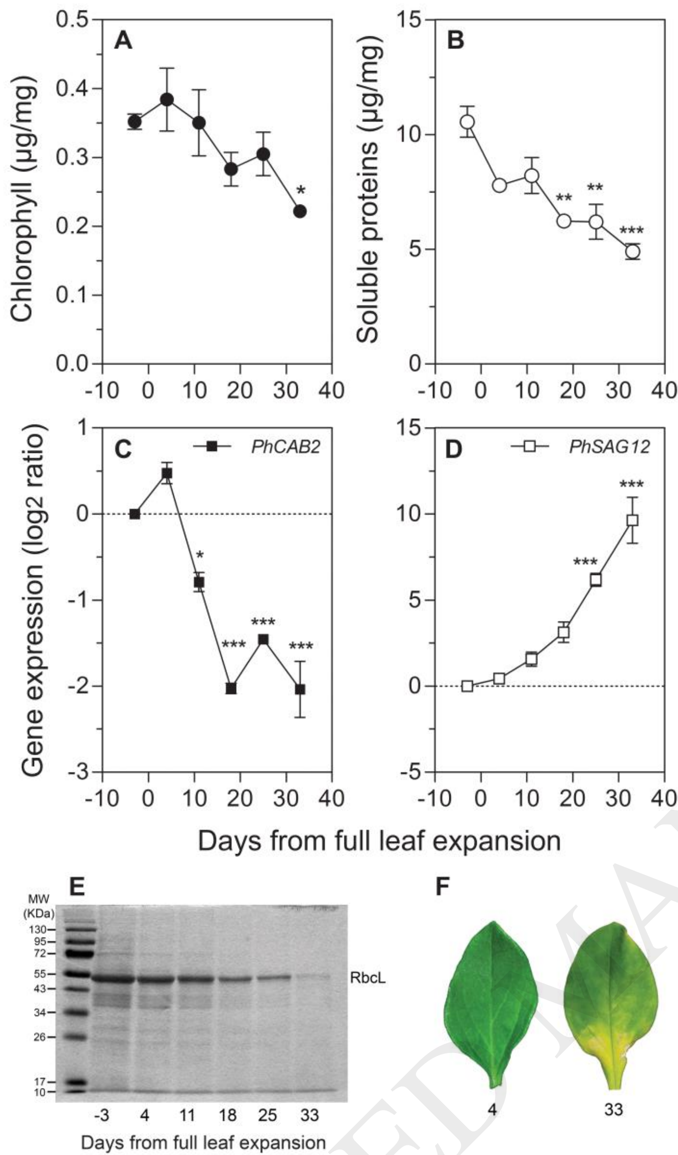

Fig. 3. Expression profiles of *PhNAC* during natural leaf senescence. A-D: Clustering of 23 differentially expressed *PhNACs*. Relative transcript levels are shown as the ratio (log2 scale) between each sampled point relative to the first sampling point and to the expression of the reference gene *PhEF1a*. No error bar is shown for better visualization.

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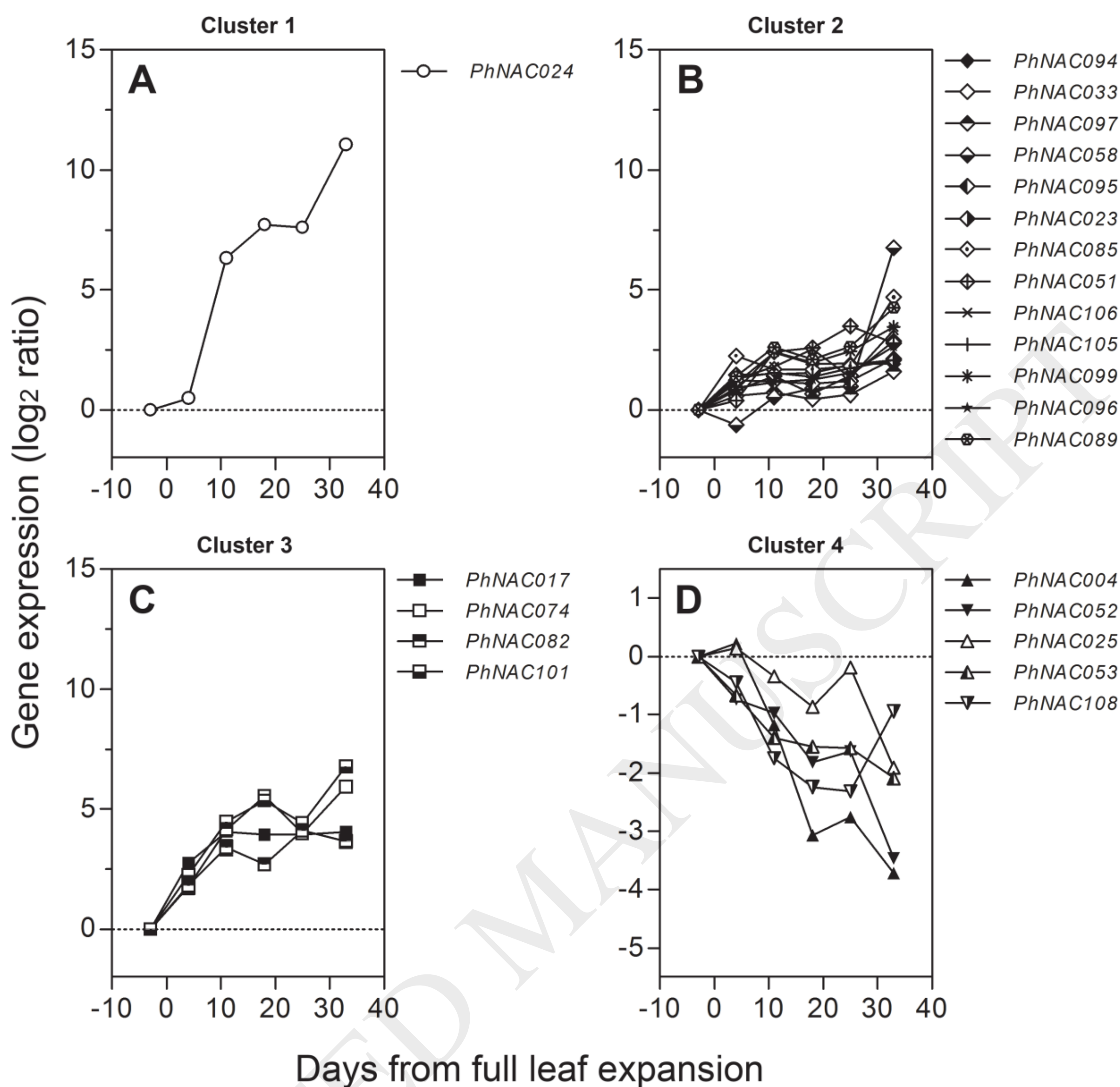

Fig. 4. Characterization of natural petal senescence and analysis of *PhNAC* expression. (A) Total soluble proteins (µg/mg) relative to petal fresh weight, and relative transcript levels of *PhSAG12*. (B-D) Clustering of gene expression patterns of 19 differentially expressed *PhNACs.* Relative transcript levels are shown as the ratio (log2 scale) between each sampled point relative to the level at first sampling point and to the expression of the reference gene *PhEF1a*. Asterisks denote significant differences among means relative to the first sampling point, as assessed using Tukey's post-test ($P < 0.05$). No error bar is shown for better visualization.

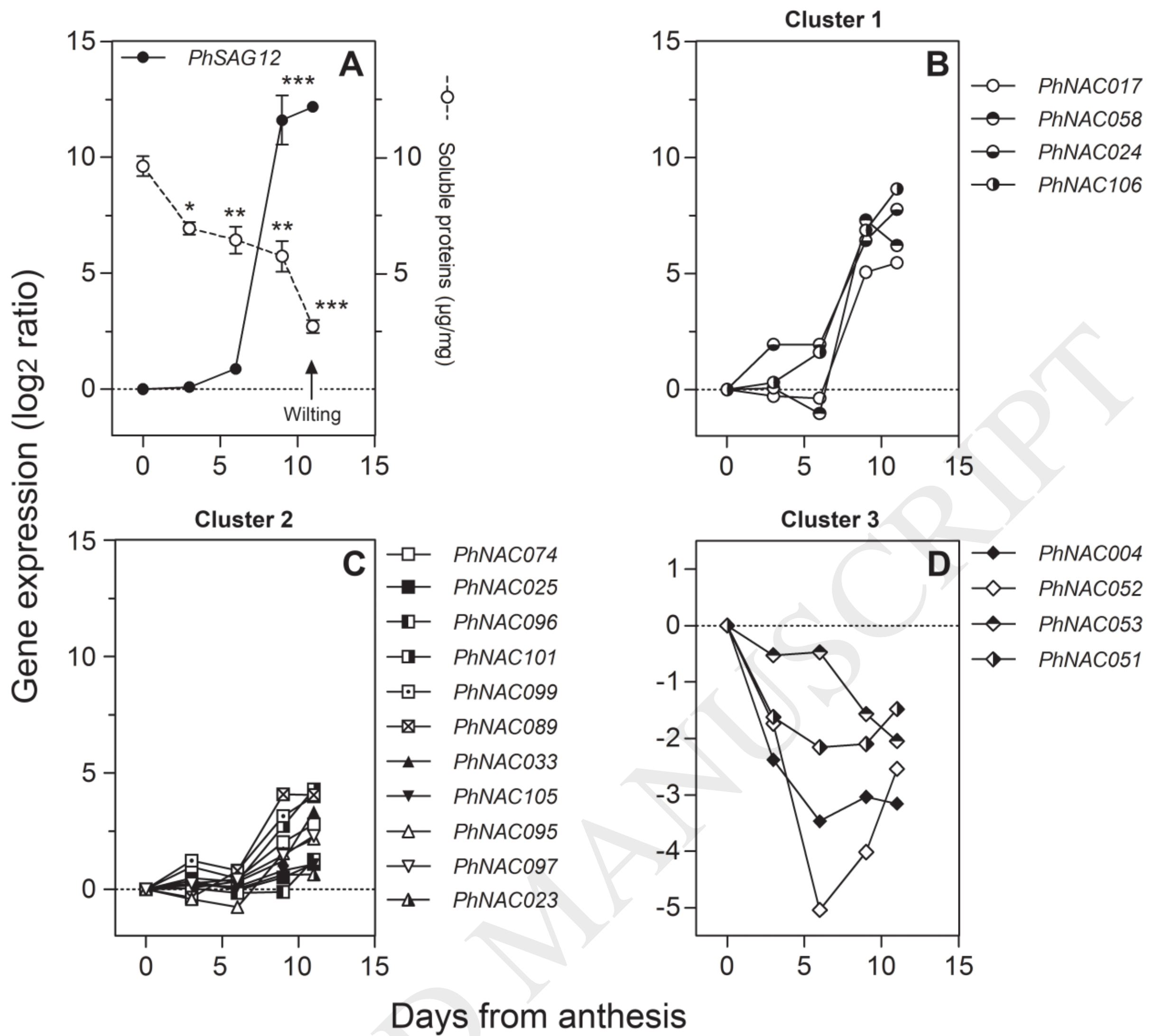

Fig. 5. Characterization of pollination-induced petal senescence and analysis of *PhNAC* expression. (A) Total soluble proteins (µg/mg) relative to petal fresh weight and relative transcript levels of *PhSAG12*. (B-D) Clustering of expression profiles of 18 differentially expressed genes. Relative transcript levels are shown (log2 scale) between each sampled point relative to the level at first sampling point (day 0) and to the expression of the reference gene *PhEF1a*. Asterisks denote significant differences among means in relation to the first sampling point, as assessed using Tukey's post-test ($P < 0.05$). No error bar is shown for better visualization.

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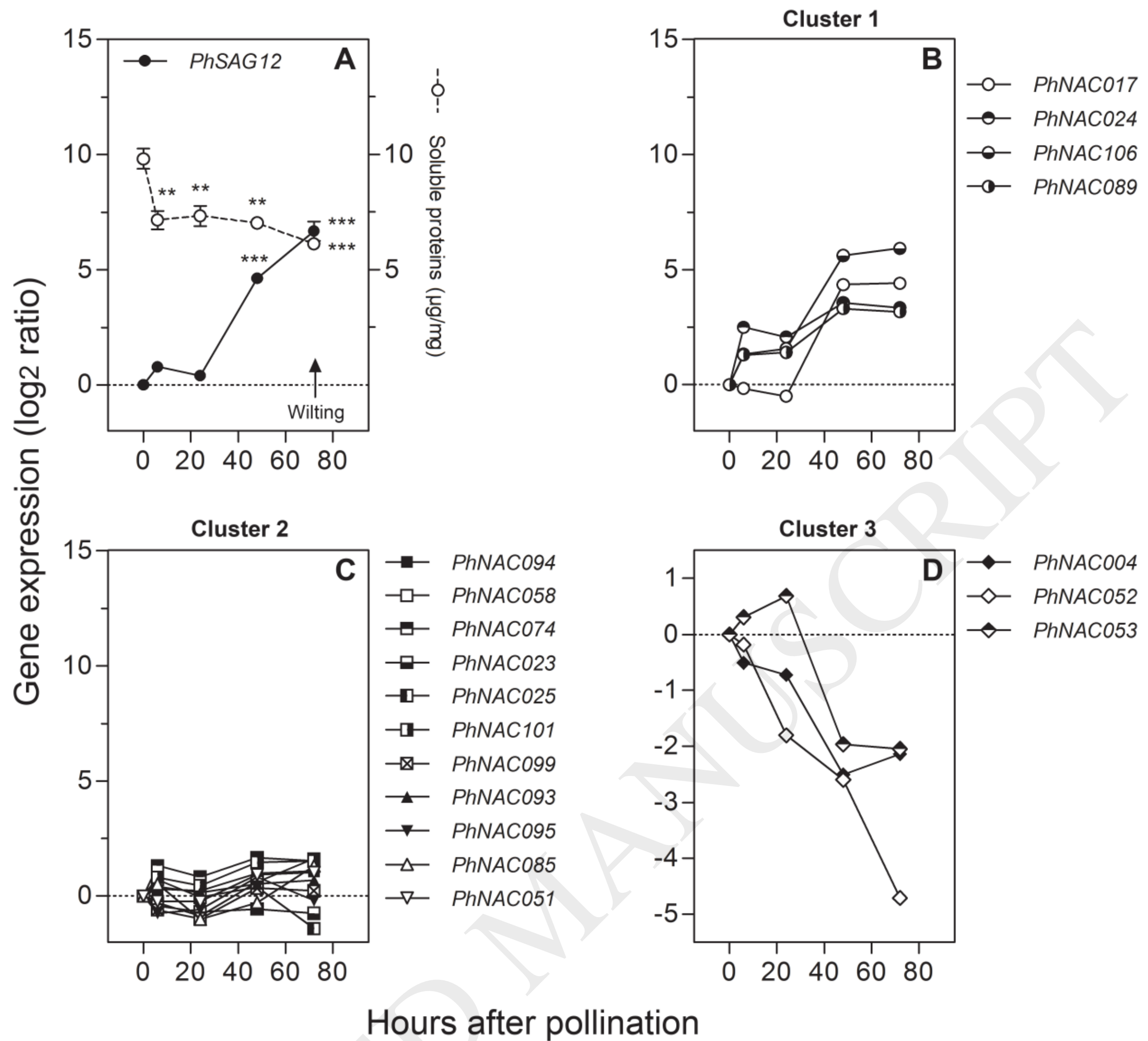

Fig. 6. Hierarchical clustering and heatmap of *PhNAC* expression profiles throughout the three types of senescence. Image shows relative transcript levels indicated by a color scale (log2 scale) between each sampled point relative to the level at the first sampling point and to the expression of the reference gene *PhEF1a*. Black dots indicate the time of initial significant change in relation to the first sampling point. E, M, and L indicate early, mid, and late stages of senescence, respectively, designated according to physiological and molecular parameters measured in leaves and petals. NS, non-significant. The corresponding phylogenetic group of each *PhNAC* based on Supplementary Fig. S3 is depicted in parenthesis.

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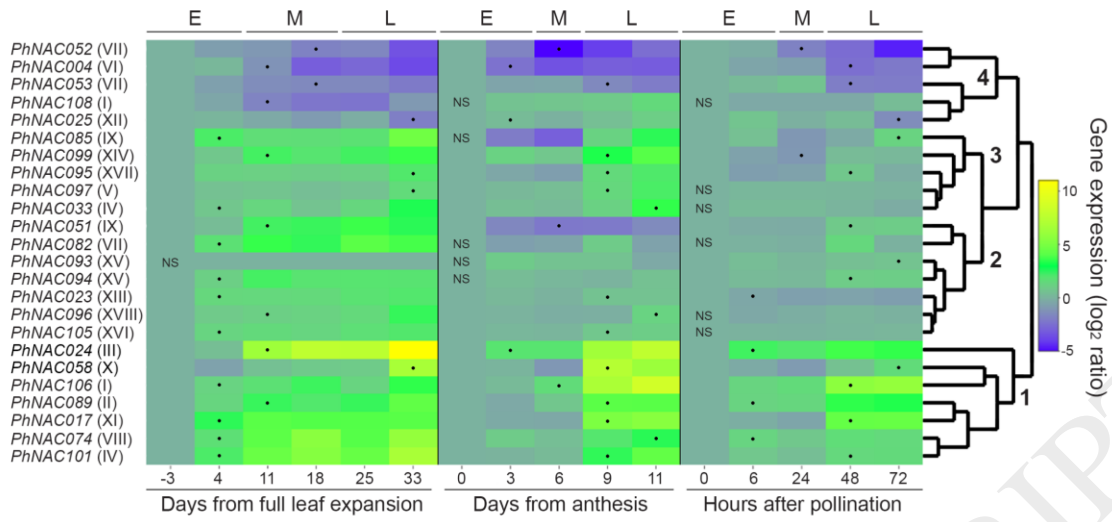

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Table legends

Table 1

Expression and functional annotation of *Arabidopsis SAG* and *SDG* genes of the NAC family, and putative *P. hybrida* orthologs, involved in leaf senescence. List of the 41 selected *A. thaliana* genes shows expression levels in leaves and petals and function in leaf senescence. Genes are classified into subfamilies and clades and ordered according to the Arabidopsis NAC tree (Supplementary Fig. S1). Putative orthologs of *P. hybrida* were obtained via tBLASTn using a transcriptomic leaf database [54]. Asterisks (*) indicate genes in Arabidopsis that play roles in leaf senescence. P indicates partial sequences of *P. hybrida* that were recovered from the database. VE, E, M, and L indicate very early, early, mid, or late changes in expression, respectively, according to Breeze et al. (2011) [12]. Plus (+ and ++) and minus signs (-) indicate marked changes in expression obtained from Arabidopsis eFP browser [53]. *AT3G04420*, *AT1G022020*, and *AT1G02250* genes lack orthologs in *P. hybrida.* AGI, Arabidopsis Genome Initiative; n/a, data not available.

Table 2

Summary of senescence expression categories, phylogenetic analysis, and comparison of *P. hybrida* candidates with putative orthologs in other species. Twenty-one *P. hybrida* genes were divided into four expression categories and classified into subfamilies and clusters. Descriptions of putative orthologs in other species include expression data and function in leaf senescence. PhNACs are displayed in ascending cluster order for each expression category. Asterisks indicate expression and function of InEPH1 in petal senescence.

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Table 1

Expression and functional annotation of *Arabidopsis SAG* and *SDG* genes of the NAC family, and putative *P. hybrida* orthologs, involved in leaf senescence. List of the 41 selected *A. thaliana* genes shows expression levels in leaves and petals and function in leaf senescence. Genes are classified into subfamilies and clades and ordered according to the Arabidopsis NAC tree (Supplementary Fig. S1). Putative orthologs of *P. hybrida* were obtained via tBLASTn using a transcriptomic leaf database [54]. Asterisks (*) indicate genes in Arabidopsis that play roles in leaf senescence. P indicates partial sequences of *P. hybrida* that were recovered from the database. VE, E, M, and L indicate very early, early, mid, or late changes in expression, respectively, according to Breeze et al. (2011) [12]. Plus (+ and ++) and minus signs (-) indicate marked changes in expression obtained from Arabidopsis eFP browser [53]. *AT3G04420*, *AT1G022020*, and *AT1G02250* genes lack orthologs in *P. hybrida*. AGI, Arabidopsis Genome Initiative; n/a, data not available.

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	Arabidopsis thaliana									Petunia hybrida			
AGI code	Synonyms	Subfamily	Clade	Leaf expression	References	Petal expression	References	Function in leaf senescence	References	Best accession	Name	Lower-order accession	Name
AT3G15500	ANAC055; NAC055; NAC3	NAC-a	AtNAC3	Increase (E) (++)	[9], [12], [53]	Increase (++)	[53], [41]	Unclear	[98]	comp33806_c0_seq1	PhNAC101		
AT1G52890	ANAC019; NAC019	NAC-a	AtNAC3	Increase (E) (++)	[9], [12], [53]	Increase (++)	[53], [41]	Unclear	[98]	comp33806_c0_seq1	PhNAC101		
AT4G27410	ANAC072; RD26	NAC-a	AtNAC3	Increase (E) (+)	[9], [12], [53]	Increase (+)	[53], [41]	Unclear	[98]	comp33806_c0_seq1	PhNAC101		
AT1G01720 (*)	ANAC002; ATAF1	NAC-a	ATAF1	Increase (VE) (++)	[9], [12], [53]	Unclear	[53]	Promote	[23]	comp30243_c0_seq1	PhNAC097		
AT1G77450	ANAC032; NAC032	NAC-a	ATAF1	Increase (E) (++)	[9], [12], [53]	Unclear	[53]	Unclear	n/a	comp30243_c0_seq1	PhNAC097		
AT5G63790	ANAC102; NAC102	NAC-a	ATAF1	Increase (E) (+)	[9], [12], [53]	Increase	[53]	Unclear	n/a	comp16927_c0_seq1	PhNAC033		
AT3G15510	ANAC056	NAC-a	NAP	Increase (E) (+)	[9], [12], [53]	Increase	[53], [41]	Unclear	n/a	comp16927_c0_seq1	PhNAC033		
AT1G52880	ANAC018; NAM	NAC-a	NAP	Increase (VE) (++)	[9], [12], [53]	Increase (+)	[53], [41]	Unclear	n/a	comp16927_c0_seq1	PhNAC033		
AT3G04070	ANAC047; NAC047	NAC-a	AtNAC3	Increase (E) (++)	[9], [12], [53]	Increase (++)	[53], [41]	Unclear	n/a	comp30243_c0_seq1	PhNAC097		
AT1G69490 (*)	ANAC029; NAP; ATNAP	NAC-a	NAP	Increase (E) (++)	[9], [12], [53]	Increase (++)	[53], [41]	Promote	[19]	comp559557_c0_seq1 (P)	PhNAC024		
AT5G13180 (*)	ANAC083; NAC083; VNI2	NAC-f	SENU5	Increase (E) (+)	[9], [12], [53]	Increase	[41]	Delay	[25]	comp11118_c0_seq1	PhNAC106	comp17685_c0_seq1 (P)	PhNAC108
AT2G33480	ANAC041; NAC041	NAC-f	SENU5	Increase (VE) (++)	[9], [12], [53]	Increase (+)	[53], [41]	Unclear	n/a	comp11118_c0_seq1	PhNAC106	comp17685_c0_seq1 (P)	PhNAC108
AT5G64530	ANAC104; XND1	NAC-a		Increase (+)	[9], [53]	Increase	[53], [41]	Unclear	n/a	comp24829_c0_seq2	PhNAC089	comp209662_c0_seq1 (P)	PhNAC005
AT2G43000 (*)	ANAC042; NAC042; JUB1	NAC-e	ONAC02	Increase (VE) (+)	[9], [12],	Unclear	[53]	Delay	[24]	comp27121_c0_seq1	PhNAC004		

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			2		[53]								
AT1G71930	ANAC030; VND7	NAC-c	OsNAC7	Decrease (E)	[12], [53]	Decrease (-)	[53], [41]	Unclear	n/a	comp102798_c0_seq1	PhNAC086		
AT2G18060	ANAC037; VND1	NAC-c	OsNAC7	Decrease	[52], [53]	Decrease	[53]	Unclear	n/a	comp102798_c0_seq1	PhNAC086		
AT1G62700	ANAC026; VND5	NAC-c	OsNAC7	Decrease	[52], [53]	Decrease	[53]	Unclear	n/a	comp22278_c0_seq3 (P)	PhNAC082		
AT1G12260	ANAC007; NAC07; VND4	NAC-c	OsNAC7	Decrease (-)	[52], [53]	Decrease	[53]	Unclear	n/a	comp17026_c0_seq1 (P)	PhNAC052	comp199967_c0_seq1 (P)	PhNAC053
AT4G28530	ANAC074; NAC074	NAC-d	NAC1	Increase (M)	[12]	Increase (++)	[53], [41]	Unclear	n/a	comp20998_c0_seq2 (P)	PhNAC051	comp20998_c0_seq1 (P)	PhNAC085
AT3G12977	n/a	NAC-d	NAC1	Increase (E)	[12]	n/a	n/a	Unclear	n/a	comp89070_c0_seq1	PhNAC074		
AT1G56010	ANAC021; ANAC022; NAC1	NAC-d	NAC1	Increase (VE) (++)	[12], [53]	Increase (++)	[53]	Unclear	n/a	comp89070_c0_seq1	PhNAC074		
AT5G18270	ANAC087	NAC-d	NAM	Increase (E) (++)	[9], [12], [53]	Increase	[53]	Unclear	n/a	comp9709_c0_seq1 (P)	PhNAC058	comp520856_c0_seq1 (P)	PhNAC030
AT3G04060 (*)	ANAC046; NAC046	NAC-d	NAM	Increase (VE) (++)	[9], [12], [53]	Increase (+)	[53], [41]	Promote	[92]	comp9709_c0_seq1 (P)	PhNAC058	comp520856_c0_seq1 (P)	PhNAC030
AT5G61430	ANAC100; NAC100	NAC-d	NAM	Increase (M) (+)	[9], [12], [53]	Increase	[53]	Unclear	n/a	comp22005_c0_seq3	PhNAC017		
AT5G39610 (*)	ANAC092; NAC2; ORE1	NAC-d	NAM	Increase (E) (++)	[9], [12], [53]	Increase (++)	[53], [41]	Promote	[20]	comp22005_c0_seq3	PhNAC017		
AT3G29035 (*)	ANAC059; ORS1	NAC-d	NAM	Increase (E) (++)	[9], [12], [53]	Increase (++)	[53], [41]	Promote	[21]	comp22005_c0_seq3	PhNAC017		
AT1G54330	ANAC020; NAC020	NAC-b	ANAC011	Increase	3	Decrease	[53]	Unclear	n/a	comp22839_c0_seq1	PhNAC025		
AT3G17730	ANAC057; NAC057	NAC-b	ANAC011	Decrease (-)	[52], [53]	Decrease (-)	[53]	Unclear	n/a	comp22839_c0_seq1	PhNAC025		
AT5G22290	ANAC089; NAC089; FSQ6	NAC-b	OsNAC8	Increase (E) (++)	[9], [12], [53]	Unclear	[53]	Unclear	n/a	comp18132_c0_seq1 (P)	PhNAC099		
AT5G64060	ANAC103; NAC103	NAC-b	OsNAC8	Increase (E)	[12]	Unclear	[53]	Unclear	n/a	comp12410_c0_seq1 (P)	PhNAC094	comp33776_c0_seq1 (P)	PhNAC093
AT1G32870	ANAC013; NAC13	NAC-b	NAC2	Increase (E)	[12], [53]	Unclear	[53]	Unclear	n/a	comp14368_c0_seq1	PhNAC105		

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AT1G34 190	ANAC017; NAC017; RAO2	NAC -b	NAC 2	Increase (E) (+)	[12], [53]	Unclear	[53]	Unclear	n/a	comp14368_c0_ seq1	PhNA C105		
AT1G34 180 (*)	ANAC016; NAC016	NAC -b	NAC 2	Increase (E) (+)	[9], [12], [53]	Decrease	[53]	Promote	[22]	comp14368_c0_ seq1	PhNA C105		
AT5G04 410	ANAC078	NAC -b	NAC 2	Increase (E)	[12], [53]	Decrease	[53]	Unclear	n/a	comp26387_c0_ seq2	PhNA C050	comp26387_c0 _seq6	PhNA C096
AT3G10 500 (*)	ANAC053; NAC053; NTL4	NAC -b	NAC 2	Increase (M) (+)	[9], [12], [53]	Unclear	[53]	Promote	[99], [100]	comp26387_c0_ seq2	PhNA C050	comp26387_c0 _seq6	PhNA C096
AT3G10 490	ANAC051; ANAC052	NAC -b	NAC 2	Increase (E) (+)	[12], [53]	Increase	[53]	Unclear	n/a	comp20693_c0_ seq1	PhNA C095		
AT4G35 580 (*)	CBNAC; NTL9	NAC -b	TIP	Increase (++)	[9], [53]	Increase	[53]	Promote	[95]	comp32221_c0_ seq1	PhNA C023	comp7749_c0_ seq1	PhNA C059
AT3G49 530	ANAC062; NAC062	NAC -b	TIP	Increase (E)	[12]	Decrease	[53]	Unclear	n/a	comp32221_c0_ seq1	PhNA C023	comp7749_c0_ seq1	PhNA C059
AT3G04 420	ANAC048	NAC -b	ANA C00 1	Increase (VE)	[12], [53]	Unclear	[53]	Unclear	n/a				
AT1G02 220	ANAC003; NAC003	NAC -b	ANA C00 1	Increase (L)	[9], [12], [53]	Unclear	[53]	Unclear	n/a				
AT1G02 250	ANAC005; BAC005	NAC -b	ANA C00 1	Increase (M)	[12]	Unclear	[53]	Unclear	n/a				

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Petunia hybrida				Putative othologs in other species			
Expression category	Gene	Group	Subfamily	Gene	Expression in leaf senescence	Function in senescence	References
Up-regulation in the three types of senescence (Cluster 1)	PhNAC106	I	NAC-f	VNI2	Increase in *A. thaliana*	Delay leaf senescence in *A. thaliana*	[25]
	PhNAC089	II	NAC-a	ONAC106; HvNAC026	Increase in rice and barley	Delay leaf senescence in rice	[29], [84]
	PhNAC024	III	NAC-a	AtNAP; OsNAP; BeNAC1; GhNAP; TtNAM-B1; SlNAP2	Increase in in *A. thaliana*, rice, bamboo, cotton, wheat and tomato	Promote leaf senescence in *A. thaliana*, rice, bamboo, cotton, wheat and tomato	[19], [27], [26], [28], [101], [102], [90]
	PhNAC101	IV	NAC-a	ANAC55; ANAC019; ANAC072	Increase in *A. thaliana*	Unclear	[98]
	PhNAC074	VIII	NAC-d	ANAC021; TaNAC-S	Increase in *A. thaliana* and decrease in wheat	TaNAC-s delay leaf senescence in wheat	[30]
	PhNAC058	X	NAC-d	ANAC046	Increase in *A. thaliana*	Promote leaf senescence in *A. thaliana*	[92]
	PhNAC017	XI	NAC-d	ORE1; ORS1; GhNAC12; SlORE1; InEPH1 (*)	Increase in *A. thaliana*, cotton, tomato and petals of *Ipomoea nil* (*)	Promote leaf senescence in *A. thaliana*, cotton and tomato, and petal senescence in *Ipomoea nil* (*)	[20], [21], [31], [45], [93]
Up-regulation in natural leaf senescence (Cluster 2)	PhNAC082	VII	NAC-c	-	-	-	-
	PhNAC051	IX	NAC-d	ANAC074;	Increase in *A. thaliana*	Unclear	[12]
	PhNAC085	IX	NAC-d				
	PhNAC023	XIII	NAC-b	NTL9	Increase in *A. thaliana*	Promote leaf senescence in *A. thaliana*	[95]
	PhNAC094	XV	NAC-b	-	-	-	-
	PhNAC105	XVI	NAC-b	NAC016	Increase in *A. thaliana*	Promote leaf senescence in *A. thaliana*	[22]
	PhNAC096	XVIII	NAC-b	NTL4; GhNAC13	Increase in *A. thaliana* and decrease in cotton	Promote drought- and heat- induced leaf senescence in *A. thaliana*	[99], [100], [101]
Up-regulation in natural leaf and petal senescence (Cluster 3)	PhNAC033	IV	NAC-a	ATAF1; OsNAC5	Increase in *A. thaliana* and rice	Promote leaf senescence in *A. thaliana*	[23], [103]
	PhNAC097	V	NAC-a				

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	PhNAC099	XIV	NAC-b	ANAC089	Increase in *A. thaliana*	-	-
	PhNAC095	XVII	NAC-b	GhNAC13	Decrease in cotton	-	[101]
Down-regulation in the three types of senescence (Cluster 4)	PhNAC004	VI	NAC-e	JUB1; GmNAC81	Increase in *A. thaliana* and soybean	Delay leaf senescence in *A. thaliana* and promote in soybean	[24], [32]
	PhNAC052	VII	NAC-c	ANAC030	Decrease in *A. thaliana*	-	-
	PhNAC053	VII	NAC-c	ANAC026; ANAC007	Decrease in *A. thaliana*	-	-

Table 2

Summary of senescence expression categories, phylogenetic analysis, and comparison of *P. hybrida* candidates with putative orthologs in other species. Twenty-one *P. hybrida* genes were divided into four expression categories and classified into subfamilies and clusters. Descriptions of putative orthologs in other species include expression data and function in leaf senescence. PhNACs are displayed in ascending cluster order for each expression category. Asterisks indicate expression and function of InEPH1 in petal senescence.

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