Identification of WRKY transcription factors associated with leaf and corolla senescence in *Petunia hybrida*

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Research Article

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Abstract

Several families of transcription factors (TFs) control the progression of senescence. Many key TFs belonging to the WRKY family have been described to play crucial roles in the regulation of leaf senescence, mainly in Arabidopsis. However, little is known about senescence-associated WRKY members in floricultural species. Delay of senescence in leaves and petals of *Petunia hybrida*, a worldwide ornamental crop are highly appreciated traits. In this work, starting from 28 differentially expressed *WRKY* genes of Arabidopsis during the progression of leaf senescence, we identified the orthologous in *P. hybrida* and explored the expression profiles of 20 *PhWRKY* genes during the progression of natural (age-related) leaf and corolla senescence as well as in the corollas of flowers undergoing pollination-induced senescence. Simultaneous visualization showed consistent and similar expression profiles of *PhWRKYs* during natural leaf and corolla senescence, although weak expression changes were observed during pollination-induced senescence. Comparable expression trends between *PhWRKYs* and the corresponding genes of Arabidopsis were observed during leaf senescence, although more divergences were found in petals of pollinated petunia flowers. Integration of expression data with phylogenetics, conserved motif and *cis*-regulatory element analyses were used to establish a list of solid candidates that could regulate more than one senescence process. Our results suggest that several members of the WRKY family of TFs are tightly linked to the regulation of senescence in *P. hybrida.*

Declarations

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Author contributions

SAT and PF designed the research; FHA, AHB, MCP and SAT performed most of the experiments and data analyses; MNG developed clustering analysis; VVL designed phylogenetic analyses; SG developed the Petunia Transcriptome Repository; SM, VCD, and RAH advised on experimental design and revised the paper. SAT, PF, and FHA wrote the manuscript. All authors revised and approved the final manuscript.

Compliance with ethical standards

Conflict of interest

The authors declare that they have no competing interests.

Introduction

Leaf senescence is the final stage of leaf development. It involves a type of programmed cell death through the execution of an orchestrated genetic program. During this program, nutrients and minerals generated from catabolism are remobilized to active growing organs such as meristems, young leaves, flowers and fruits (Butt et al. 1998; Quirino et al. 2000; Gepstein et al. 2003; Guo and Gan 2005). Leaf senescence can be induced by age, hormone signalling, or the onset of the reproductive phase,

although under adverse environmental conditions it can be triggered prematurely (Guo and Gan 2005; Griffiths et al. 2014; Jagadish et al. 2015; Woo et al. 2018). The process is characterized by the loss of photosynthetic activity, due to chloroplast protein breakdown, as well as a massive degradation of other macromolecules, including photosynthetic pigments, lipids, polysaccharides and finally nucleic acids. Dramatic changes in gene expression, hormone balance and metabolism have been observed during its progression (Kuai et al. 2018; Buet et al. 2019). Similarly, petal senescence represents the final stage of flower development, and it is characterized by the degradation of cellular components as well as remobilization of nutrients generated by catabolism (Langston et al. 2005; Jones 2013; Shibuya et al. 2016). Petal senescence can be influenced by endogenous age-related signals, although, compared to leaves, they are less influenced by environmental factors and involve a lower extent of nutrient remobilization (Jones 2004; Thomas et al. 2003; Rogers 2012). In most flowers the role of petals is to attract pollinators, although their maintenance is costly in terms of water loss and metabolic energy; therefore, in many species pollination activates or accelerates the senescence process (Van Doorn and Woltering 2008; Rogers 2013; Broderick et al. 2014). Hence, senescence processes occurring in leaves and petals are genetically coordinated and share the catabolism of cellular structures and nutrient remobilization to sink organs (Gregersen et al. 2013; Jones 2013; Ali et al. 2018). Genes that are upregulated or downregulated during senescence are generally termed as *senescence-associated genes* (*SAGs*). Several families of transcription factors (TFs) have been reported to change their expression during leaf and petal senescence (Guo et al. 2004; Buchanan-Wollaston et al. 2005; Balazadeh et al. 2008; Wagstaff et al. 2009; Breeze et al. 2011; Broderick et al. 2014; Tsanakas et al. 2014; Wang et al. 2018). Significantly, several WRKYs have been reported to regulate leaf senescence, mainly in Arabidopsis and rice (Miao et al. 2004; Ülker et al. 2007; Jing et al. 2009; Besseau et al. 2012; Li et al. 2012; Han et al. 2014; Chen et al. 2017b; Kim et al. 2019).

The WRKY family is one of the largest TF family in plants, and their members have been described in many plant species (Rushton et al. 2010; Huang et al. 2012; Tripathi et al. 2012; Wang et al. 2014; Yang et al. 2015; Cheng et al. 2016; Li et al. 2016). WRKY proteins are characterized by the presence of the WRKY domain, which consists of approximately 60 amino acids encompassing an almost invariant DNA-binding heptapeptide, WRKYGQK, and a zinc-finger binding motif that can be either $Cx_{4-5}Cx_{22-23}HxH$ or $Cx_7Cx_{23}HxC$ (Eulgem et al. 2000; Rushton et al. 2010). WRKY members are classified into three main groups. Group I contains two WRKY domains and two C_2H_2 zinc-finger motifs. Group II contains a single WRKY domain and a C_2H_2-type zinc-finger motif and is further divided into five subgroups (IIa, IIb, IIc, IId, and IIe). Group III contains a single WRKY domain and a C_2HC-type zinc finger motif (Xie et al. 2005; Eulgem and Somssich 2007). Besides senescence, WRKY proteins have been reported to regulate other biological processes, including plant growth and development (Bakshi and Oelmüller 2014; Ding et al. 2014; Raineri et al. 2016), diverse biotic and abiotic stress responses (Zou et al. 2010; Giacomelli et al. 2012; Phukan et al. 2016; Birkenbihl et al. 2018), and hormonal signalling (Jiang et al. 2014; Zhao et al. 2020).

In Arabidopsis, the WRKY family consists of 74 members (Rushton et al. 2010). Several genes change their expression during leaf senescence and some members have been described as positive (AtWRKY6, AtWRKY22, AtWRKY45, AtWRKY53 and AtWRKY75) or negative (AtWRKY25, AtWRKY54, and AtWRKY70) regulators of the process (Robatzek and Somssich 2001; Miao et al. 2004; Ülker et al. 2007; Zhou et al. 2011; Besseau et al. 2012; Chen et al. 2017b; Guo et al. 2017; Doll et al. 2020). It has been reported that members of the WRKY family can act redundantly and interact with each other, regulating the expression of other WRKY members presumably by binding to W-box sequences on their promoters (Zhou et al. 2011; Besseau et al. 2012). In rice, OsWRKY42 (Han et al. 2014) and OsWRKY5 (Kim et al. 2019) have been described to positively regulate leaf senescence. Overexpression of two wheat members in Arabidopsis, *TaWRKY7* and *TaWRKY40-D*, positively regulate senescence (Zhang et al. 2016b; Zhao et al. 2020). Similarly, members of cotton, *GhWRKY17*, *GhWRKY42,* and *GhWRKY27,* promote leaf senescence, whereas *GhWRKY91* represses the process when overexpressed in transgenic Arabidopsis lines (Gu et al. 2018a; Gu et al. 2018b; Gu et al. 2019a; Gu et al. 2019b). Finally, CpWRKY71, a WRKY member of the ornamental Wintersweet (*Chimonanthus praecox*) causes early leaf senescence when overexpressed in Arabidopsis (Huang et al. 2019). All of them increase their expression during the progression of leaf senescence. Overall, these evidences suggest that WRKYs play essential roles in the regulation of leaf senescence across monocot and dicot species.

Even though leaves and petals present different biological functions, global analysis of gene expression between both organs in Arabidopsis and the ornamental plant wallflower, show common and distinct patterns of expression and physiology (Price et al.

2008; Wagstaff et al. 2009). Several *WRKY* genes increase their expression during leaf and petal development, suggesting that regulation of gene expression may be conserved between both organs and species. Therefore, similarities in the signalling mechanisms triggering senescence in leaves and petals are expected (Price et al. 2008; Wagstaff et al. 2009). Although changes in gene expression of *WRKY*s have been described during the progression of petal senescence in several species, no members have been reported to regulate age-related or pollination-induced petal senescence yet (Price et al. 2008; Wagstaff et al. 2009; Broderick et al. 2014; Tsanakas et al. 2014; Trivellini et al. 2016; Chen et al. 2018; Wang et al. 2018; Wang et al. 2019; Ge et al. 2019; Wang et al. 2020).

Draft genome sequences have been recently published for various ornamental plants. However, comparison studies of senescence events between leaves and petals are scarce, and only minor efforts have been made to study co-regulation of *WRKY* genes between both organs. The identification of candidate genes that could simultaneously regulate different senescence processes would be of utmost importance for molecular breeding in ornamental plants (Broderick et al. 2014; Tsanakas et al. 2014; Trivellini et al. 2016; Chen et al. 2017a; Chen et al. 2018; Wang et al. 2018b; Wang et al. 2019; Ge et al. 2019; Wang et al. 2020).

Petunia hybrida is a popular bedding plant that has a long history as a genetic model system and represents one of the most valuable crops in the global floriculture market (Gerats and Vandenbussche 2005). In the USA, it ranks among the top annual bedding plants in terms of wholesale value (USDA 2019). Unlike Arabidopsis, petunia is an ideal model for studying similarities and differences between leaf and petal senescence processes (Gerats and Vandenbussche 2005; Gerats and Strommer 2009; Trupkin et al. 2019). The existing draft genomes of the parental species of *P. hybrida* (*P. axillaris* and *P. inflata*) (Bombarely et al. 2016), the well-established transformation techniques, and the recently published protocols for genome editing by CRISPR/Cas9 (Zhang et al. 2016a; Yu et al. 2020), makes petunia an excellent model to discover gene function (Vandenbussche et al. 2016; Druege and Franken 2019). Moreover, findings in petunia could be extrapolated to other related crops within the Solanaceae family (Gerats and Vandenbussche 2005; Vandenbussche et al. 2016). Here, we performed a detailed expression analysis of different *PhWRKY* genes during the progression of three senescence processes occurring in leaves and in the corollas. Together with phylogenetic, conserved motif and *cis*-regulatory element analyses, a valuable set of senescence-associated PhWRKY candidates was identified.

Materials And Methods

Plant material and sampling

Seedlings of *Petunia x hybrida* 'F1 Ultra™ White' (Syngenta Flowers, Inc., Gilroy, CA, USA) were grown in 10 cm diameter pots containing moistened Grow Mix soil (Terrafértil, Argentina). In all experiments, 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 $\mu molm^{-2}$ s^{-1}; TLD 36W/830, Philips, France). Plants were watered by sub-irrigation with a nutrient solution (Hakaphos® Rojo, COMPO, Spain) and regularly cycled to avoid positional effects. Natural (age-related) leaf senescence process was analyzed using samples of leaf 11 selected from a total of 14 rosette leaves at bolting time. Leaves were tagged when foliar primordia were approximately 70% of their final size; this was designated as the first sampling 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 was visible on day 33 after full leaf expansion. Under our conditions, maximum leaf area was reached approximately 8 d after primordia were visible (approximately 0.5 cm length). Leaves were harvested on each sampling day at 4 h into the light period, resulting in six-time points. At each time point, leaf 11 was sampled from ten randomly selected plants and divided into three biological replicates that contained 3 or 4 leaves each. Natural corolla senescence was assessed by tagging flowers at anthesis (day 0) and leaving them unpollinated to senesce naturally. Since the cultivar used has shorter stamens than pistils, auto-pollination was not possible in the absence of pollinators. This characteristic enables us to analyze both natural and pollination-induced senescence. In pollination-induced experiments, flowers were hand-pollinated by brushing pollen onto the stigma on the first day of anthesis (day 0). In both experiments, corollas were collected 8 h after the start of the light period at five different time points.

At each time point, 12 flowers were collected and divided into three biological replicates containing 4 corollas each. Harvested leaves and corollas were rapidly frozen in liquid nitrogen and stored at – 80 °C until use.

Identification of WRKY transcription factors in *P. hybrida*

The sequences of WRKY proteins of *A. thaliana* were retrieved from the Plant Transcription Factor Database (http://planttfdb.cbi.pku.edu.cn/). The dataset was screened to remove redundant and splicing isoforms and checked for the presence of WRKY domain (PF03106) using PFAM and Araport databases (Krishnakumar et al. 2015; El-Gebali et al. 2019). Twenty-eight *WRKY* genes of Arabidopsis were selected from published studies on the leaf transcriptome (Buchanan-Wollaston et al. 2005; Wagstaff et al. 2009; Breeze et al. 2011), public repositories including Leaf Senescence DataBase (Liu et al. 2011) and Arabidopsis eFP Browser (Winter et al. 2007). A leaf transcriptome dataset of *P. hybrida* (Villarino et al. 2014) was used to create a repository to run BLAST (Petunia Transcriptome Repository ATGC v1.0) (Gonzalez et al. 2017). Putative *P. hybrida* orthologs in this repository and the parental genomes of *P. hybrida* (*P. axillaris* and *P. inflata*) available at SOL Genomics Network (http:// solgenomics.net) (Bombarely et al. 2016) were searched by using tBLASTn. Predicted WRKY proteins were aligned and evaluated for the presence of the WRKY domain using ClustalW tool and BioEdit program (version 7.0.5.3) (Hall 1999). BLASTp identified putative orthologs of PhWRKY proteins in other species at the NCBI (https://www.ncbi.nlm.nih.gov/).

RNA extraction and expression analyses

High quality total RNA was isolated from leaf and corolla samples. Frozen tissues, conserved at – 80 °C, were grinded with liquid nitrogen and 150 mg of each sample were used to extract the RNA with TRIzol following manufacturer's instructions (Invitrogen, Argentina). DNase I (Invitrogen, Argentina) was used to eliminating genomic DNA. 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 of cDNA sample, and 6.25 µl of FastStart Universal SYBR Green Master (Roche Diagnostics, Mannheim, Germany). The assays included negative controls (no RT added) and non-template controls. A 96-well plate StepOne Plus cycler and software (Applied Biosystems, USA) was used to perform the reactions. The thermal profile setting was at 95 °C for 10 min and 40 cycles at 95 °C for 15 s; while hybridization temperature setting was at 60 °C for 1 min. Amplicon specificity was verified by melting curve analysis (60 to 95 °C) after 40 PCR cycles and products visualized using agarose gel electrophoresis. 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 LinReg PCR software (Ruijter et al. 2009). These profiles were estimated based on the first sampling and reference gene using fgStatistic software (Di Rienzo 2009), which has been built on the Pfaffl's algorithm (Pfaffl 2001). Final expression values were analyzed using one-way ANOVA followed by Bonferroni'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).

Gene clustering and heatmap analyses

Clustering was performed using cmeans function (Pal et al. 1996), a fuzzy clustering method included in the ‘e1071' R package et al. 2006) and R Core Team (https://www.R-project.org/). The heatmap was generated with ‘heatmaply’ R package (Galili et al. 2018).

Functional group classification of senescence-associated WRKY proteins by phylogenetic reconstruction and conserved motif analyses

WRKY proteins were classified into functional groups by using two approaches: phylogenetic reconstruction and by the identification of conserved motifs. For phylogenetic reconstruction, multiple sequence alignment of the conserved region containing the WRKY domains of 116 proteins from different species was performed using ClustalW tool, yielding a data matrix of 555 characters. The Jones, Taylor, and Thornton (JTT) model was selected as best-fitting amino-acid substitution model using ProtTest v3.4 software (Abascal et al. 2005). A neighbour-joining (NJ) tree was built using MEGA5 software (Tamura et al. 2011). Bootstrap values were calculated for 1000 iterations. The phylogenetic tree was visualized using Figtree v1.4.2 software (http://tree.bio.ed.ac.uk/ software/figtree/). The identification of conserved motifs and sequence logos using full-length amino-acid sequences of WRKY proteins was performed via MEME program (Bailey et al. 2009) and the parameters described by You et al. (2015).

Identification of senescence-associated *cis*-regulatory elements in the promoters of *WRKY* genes

To identify senescence-associated *cis*-regulatory elements in *WRKY* genes of petunia, the 2 kb (-2000 bp) promoter regions of the most similar parental homologs in *P. axillaris* or *P. inflata* of each *PhWRKY* gene were retrieved using the Genome Browser tool at Sol Genomics Network (http:// solgenomics.net). The PlantCARE database (Lescot et al. 2002) (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to analyze the promoters. Putative *cis*-regulatory elements associated with senescence were those reported in literature.

Results And Discussion

Identification of senescence-associated WRKY transcription factors in *P. hybrida* and in its parental species

To identify potential regulators of senescence in petunia, we searched for *WRKY* genes differentially expressed during the progression of leaf senescence in Arabidopsis (see Materials and Methods). Twenty-eight genes, representing approximately 36% of the total members in the family were selected by their consistent upregulation, seven of which were reported to regulate leaf senescence in Arabidopsis (Table 1). These proteins did not show a particular clustering and were distributed within phylogenetic groups I, IIb, IIc, IId, IIe, and III according to Eulgem et al. (2000) (Table 1). Thirteen genes (~47%) showed expression changes at early- (E) senescence, whereas six (~21%) and two (~7%) genes showed changes at mid- (M) and late- (L) senescence, respectively (Table 1). Seven members (~25%) increased their expression during senescence, although temporal expression changes data were not available.

In addition, we assessed the expression changes of selected *AtWRKY* genes in the petals at two different developmental stages; immediately before flower opening (stage 12), and in opened-pollinated flowers (stage 15) (Table 1). Of the 28 genes upregulated in the leaves, 23 genes (~82%) were also upregulated during petal development, and only five genes (~18%) did not

show clear expression changes or remained unaltered (Table 1). These observations indicate that most *AtWRKY* genes are upregulated during the early stages of leaf development and that almost all upregulated genes in the leaves were upregulated in the petals (Table 1).

In order to identify putative orthologs of these senescence-associated WRKYs in petunia, the predicted protein sequences of the *AtWRKY* genes were used to perform BLAST searches using a public transcriptomic leaf database of *Petunia x hybrida* cv. 'Mitchell Diploid' (Villarino et al. 2014) loaded into a web tool developed by Gonzalez et al. (2017). Of the 28 AtWRKY proteins, 20 cDNA sequences were obtained in *P. hybrida* (*PhWRKYs*) by using tBLASTn (Table 1 and Supplementary Table S3). In general, each AtWRKY protein had an equivalent member in *P. hybrida.* However, it was observed that a few different AtWRKY proteins showed the same PhWRKY equivalent, revealing a decrease in the total number of PhWRKYs identified (Table 1). This observation might be explained by the use of a transcriptomic leaf database instead of genome sequences to search the equivalent proteins in petunia. Since genomic sequences of *P. hybrida* were not available, the transcriptomic leaf database represented the best source of *P. hybrida* sequences (Villarino et al. 2014). Similar BLAST searches were conducted using the draft genomes of the parental species of *P. hybrida* (Supplementary Table S3).

Interestingly, the total number of WRKY members retrieved from *P. axillaris* and *P. inflata* were similar, 19 and 20, respectively (Supplementary Table S3). The recovered sequences from the parental species showed scores and e-values consistently similar to those obtained for *P. hybrida* sequences (Supplementary Table S3). The total number of WRKYs described among different species, including those within the Solanaceae family, is variable (Cheng et al., 2019). This fact could explain the reduction in the number of WRKYs identified in petunia when compared to Arabidopsis. In accordance, a total of 81 members were identified in tomato and potato, while a significantly lower number (65) was identified in pepper (Cheng et al. 2019). Moreover, the total number of WRKY members in the parental species of *P. hybrida* has not been assessed yet (Bombarely et al. 2016). Therefore, by using BLAST searches in transcriptome and genome databases, we identified several putative WRKY orthologs in petunia with identity values above 50% for most of the genes, suggesting a high level of conservation between Arabidopsis and petunia proteins (Table 1 and Supplementary Table S3).

Multiple sequence-alignments and phylogenetic classification of WRKY proteins in *P. hybrida*

To confirm that the recovered sequences of *P. hybrida* encode WRKY proteins, the predicted amino acid sequences were used to perform multiple sequence-alignments to investigate their phylogenetic relationships (Fig 1). The WRKY signature and zinc-finger structures were detected in all *P. hybrida* proteins. Based on previous classifications, proteins were classified into three main groups (Table 2) (Eulgem et al. 2000; Xie et al. 2005). Group I contained four members with two WRKY domains and the C_2H_2-type zinc-finger structure ($Cx_4Cx_{22}HxH/Cx_4Cx_{23}HxH$). Group II contained 10 members with a single WRKY domain and the C_2H_2-type zinc-finger structure ($Cx_{4\text{-}5}Cx_{23}HxH$). In this group, PhWRKYs were further divided into four subgroups: II-b (2), II-c (4), II-d (3), and II-e (1). Group III contained six members with a single WRKY domain and the C_2HC-type zinc-finger structure ($Cx_7Cx_{23}HxC$) (Table 2). Overall, these results show that all protein sequences retrieved from the transcriptomic leaf database are members of the WRKY family. However, half of the retrieved sequences were partial. Therefore, for better inference of amino acid sequences, PhWRKY proteins were further aligned and compared with their equivalents in *P. axillaris* and *P. inflata* (Supplementary Fig. S1). Findings in PhWRKYs were in accordance with previous works describing the WRKY family in different species of the Solanaceae family, including potato, pepper, eggplant, and tomato (Huang et al. 2012; Yang et al. 2015; Cheng et al. 2016; Zhang et al. 2017; Cheng et al. 2019) and species of other families like Arabidopsis, carrot, rice, and *Brachypodium distachyon* (Wu et al. 2005; Rushton et al. 2010; Tripathi et al. 2012; Li et al. 2016). Moreover, almost all senescence-associated PhWRKYs showed the most conserved WRKYGQK heptapeptide sequence, and only one member of the subgroup IIc showed a variation in this sequence (PhWRKY051). Accordingly, subgroup IIc has been described as the group with higher gene loss/gain variations in solanaceous species (Cheng et al. 2019).

Gene expression analysis of *PhWRKYs* during natural leaf senescence

In previous work, we characterized the progression of natural leaf and corolla senescence (age-related) and pollination-induced corolla senescence in order to study the expression dynamics of senescence-associated NAC TFs in *P. hybrida* (Trupkin et al. 2019). Here, we used those samples to evaluate the relative transcript levels of the identified *PhWRKYs* via qPCR (see Materials and Methods). Seventeen out of 20 genes were detected during the natural progression of leaf senescence, while three genes were undetected (*PhWRKY011*, *PhWRKY051* and *PhWRKY023*) (Fig. 2). The 17 genes detected in the leaves, were classified into six groups according to their expression patterns (Fig. 2). Group 1 contained seven genes, whose expression increased almost linearly from early stages and reached their highest levels during the late stages of senescence, representing the most interesting genes (Fig. 2a). Group 2 contained three genes that showed upregulation in early- senescence (day 4), a stable expression in mid- senescence (days 11 and 18) and higher expression in late- senescence (days 25 and 33) (Fig. 2b). Groups 3 (*PhWRKY024*) and 4 (*PhWRKY030* and *PhWRKY075*) showed expression profiles similar to those in group 2, although their members were significantly upregulated later (mid-and late- senescence) and reached higher expression values (Fig. 2c-d). Group 5 contained two genes (*PhWRKY015* and *PhWRKY035*) that showed weak upregulation in late- senescence (Fig. 2e). Finally, *PhWRKY007* and *PhWRKY004* in group 6, did not show significant changes in their expression (Fig. 2f). The expression of 15 *PhWRKYs* increased during leaf senescence. It revealed similar patterns when compared to their equivalents of Arabidopsis, although *PhWRKY072* (early) and *PhWRKY075* (late) showed putative orthologs with opposing expression patterns (Table 1, Supplementary Table S2 and Fig. 2).

Members of the WRKY family were associated with natural leaf senescence in many species, including rice (Han et al. 2014), wheat (Zhang et al. 2016b), cotton (Gu et al. 2019a,b), grapevine (Wang et al. 2014), sunflower (Moschen et al. 2019), alfalfa (Yuan et al. 2020), among others. Overall, evidence suggests a conserved role of this family in the regulation of natural leaf senescence, including monocot and dicot species. However, little is known about members of this family as potential regulators of leaf senescence in solanaceous species (Bai et al. 2018; Finatto et al. 2018; Tolosa and Zhang 2020). To our knowledge, the results presented here constitute the first report of WRKY TFs expressed during natural leaf senescence in *P. hybrida*.

Gene expression analysis of *PhWRKYs* during natural and pollination-induced corolla senescence

Analysis of senescence progression in petunia flowers, including gene expression analysis in petal organs, have been reported in *P. hybrida*. However, the participation of WRKY TFs as potential regulators of petal senescence was not specifically assessed, and so far, none of them has been reported to regulate corolla senescence (Jones et al. 2005; Langston et al. 2005; Jones 2013; Broderick and Jones 2014; Wang et al. 2018).

To investigate the participation of WRKY members during natural and pollination-induced corolla senescence, we measured the expression patterns of the 17 *PhWRKY* genes detected in the leaves (Fig. 3) (see Materials and Methods). During the progression of natural corolla senescence, two genes were not detected (*PhWRKY069* and *PhWRKY064*) and the remaining 15 genes were classified into six groups according to their expression patterns (Fig. 3). Groups 1 and 3 contained only one gene, PhWRKY030 and PhWRKY075, respectively. Both genes were upregulated in early- (*PhWRKY030*, day 3) and mid- (*PhWRKY075*, day 6) senescence, and showed very high expression changes in late- senescence (Fig. 3a, c). Group 2 represented the largest group, with eight genes upregulated in early- and mid- senescence. These genes showed a *plateau* in mid- senescence (day 6) and then increased their expression in late- senescence, reaching average values (Fig. 3b). Group 4 contained two genes, *PhWRKY024* and *PhWRKY072*, which were upregulated in late- senescence (days 9 and 11) (Fig. 3d), resembling the expression profile of *PhSAG12* gene (Trupkin et al. 2019). Group 5 only contained *PhWRKY004* gene, which showed weak upregulation in late-senescence (Fig. 3e). Finally, group 6 had *PhWRKY002* and *PhWRKY007* genes, which showed weak and unclear changes of expression (Fig. 3f).

During the progression of pollination-induced corolla senescence,12 of the 17 *PhWRKYs* detected in the leaves showed changes in their expression. The expression of *PhWRKY055*, *PhWRKY030*, *PhWRKY075*, *PhWRKY069* and *PhWRKY054* was not detected (Fig. 4 and Supplementary Table S2). Group 1 contained four genes, three of them were upregulated in mid-senescence (*PhWRKY033*, *PhWRKY028* and *PhWRKY006*) and the remaining gene was upregulated earlier at 6 hours after pollination (hap) (*PhWRKY072*). All these genes showed moderate changes in expression (Fig. 4a). Group 2 contained five genes with weakened upregulation during late senescence (Fig. 4b). Interestingly, group 3 contained three genes downregulated from an early stage (6 hap) (*PhWRKY070*, *PhWRKY024* and *PhWRKY053*) and maintained their expression in the subsequent time points (Fig. 4c). In Arabidopsis, pollination-induced petal senescence is the type of senescence that occurs in flowers (Wagstaff et al. 2009). Comparative expression analysis of *WRKY* genes between leaf and pollination-induced petal senescence revealed that the proportion of genes expressed in both organs was higher in Arabidopsis than in *P. hybrida* (Table 1; Figs. 2 and 4). Of the 28 *AtWRKYs* upregulated in the leaves, 23 genes were upregulated during pollination-induced petal senescence (82%), and only five genes did not change (18%). In *P. hybrida*, of the 15 *PhWRKYs* genes upregulated in the leaves, only seven genes were upregulated during pollination-induced senescence (47%), three genes were downregulated (20%), and five genes were undetected (33%) (Table 1; Figs. 2 and 4). Moreover, different expression profiles were observed for several *PhWRKYs* when compared to the corresponding Arabidopsis genes, suggesting discrepancies in signalling mechanisms between both species during pollination-induced senescence (Table 1; Figs. 2 and 4) (Wagstaff et al. 2009). In contrast, 12 of the 15 *PhWRKY* genes upregulated in the leaves were also upregulated during natural corolla senescence (80%), one was downregulated (*PhWRKY002*) and two were undetected (*PhWRKY054* and *PhWRKY069*) (Figs. 2 and 4). For example, several genes (*PhWRKY024*, *PhWRKY028*, *PhWRKY030*, *PhWRKY053*, *PhWRKY072*, and *PhWRKY075*) increased their expression during natural leaf and corolla senescence but not consistently during pollination-induced senescence. Moreover, a reduction in the total number of *PhWRKYs* and in their magnitude of expression changes were observed in the corollas of pollinated flowers. These results indicate that the majority of the senescence-associated PhWRKY members participate in the natural senescence processes occurring in leaves and petals and that both processes might be related, whereas pollination triggers a different senescence program in which PhWRKY TFs would have less participation (Langston et al. 2005; Broderick et al. 2014; Wang et al. 2018). Interestingly, Arabidopsis orthologs are consistently expressed in leaves and petals of flowers undergoing pollination (Table 1). In a similar way, some genes of petunia were leaf specific (for example PhWRKY054 and PhWRKY069) despite their putative orthologs in Arabidopsis increased their expression in petals (Table 1). Therefore, our findings suggest similarities but also discrepancies between *P. hybrida* and Arabidopsis in the regulation of senescence processes, mainly during pollination-induced senescence.

Members of the WRKY family have been reported to change their expression during petal senescence in different ornamental plants, including ethylene-sensitive and insensitive species, such as wallflower (Price et al. 2008), gardenia (Tsanakas et al. 2014), or astilbe (Yamazaki et al. 2020). Interestingly, in *Hibiscus rosa-sinensis*, an ornamental plant with ephemeral flowers, several members of the WRKY family are upregulated (Trivellini et al. 2016). Early transcriptome analysis in the corollas of pollinated petunia flowers (12-24 hap) identified 21 differentially expressed *WRKY* genes in petunia (Broderick et al. 2014). Three of these genes appear to be homologous to *PhWRKY002*, *PhWRKY006* and *PhWRKY007* of our expression analysis. Interestingly, the putative homologs of *PhWRKY002* and *PhWRKY006* are upregulated after pollination (Broderick et al. 2014), similarly to that here observed for *PhWRKY002* at 6 hap and *PhWRKY006* at 24 hap (Fig. 4). The putative homolog of *PhWRKY007* shows weak upregulation at 12-24 hap period (Broderick et al. 2014), whereas *PhWRKY007* showed no changes at 6-24 hap but increased later at 48 hap (Fig. 4). In a transcriptome analysis of natural corolla senescence in petunia, 13 *WRKY* genes showed to be differentially expressed for 0-7 days (Wang et al. 2018). Two of these genes are upregulated, eight are downregulated for the first two days after anthesis (early- senescence), and three are upregulated between the second and fourth day (mid-late- senescence) (Wang et al. 2018). In accordance with our results, the putative homologs of *PhWRKY007* and *PhWRKY024* decrease their expression after two days of anthesis (Wang et al. 2018). However, in the present study the expression of *PhWRKY024* increased during late stages of senescence (Fig. 3). Although previous transcriptome analyses identified some WRKY members with differential expression profiles during senescence, only a few matches with the *PhWRKYs* identified here. On the whole, our results and previous works suggest that WRKY members would have important roles in the regulation of petal senescence in different species.

Functional classification by hierarchical clustering and motif analyses

Gene expression patterns of the three types of senescence were visualized simultaneously by using a heatmap analysis. According to their expression profiles, four main clusters were retrieved (Fig. 5). Interestingly, most *PhWRKYs* were upregulated during early- and mid- natural leaf senescence, although a high proportion of genes was upregulated during late- natural corolla senescence, suggesting temporal expression differences between natural senescence occurring in leaves and petals (Fig. 5). visualization revealed that some *PhWRKYs* could act as regulatory factors in leaf and corolla senescence processes (Fig. 5). This trend was also observed in other species like Arabidopsis and wallflower, although in the majority of previous studies, leaf and petal senescence were analyzed separately (Price et al. 2008; Wagstaff et al. 2009; Tsanakas et al. 2014; Trivellini et al. 2016; Wang et al. 2018; Yamazaki et al. 2020).

To select the best candidates in *P. hybrida*, we constructed a senescence-associated phylogenetic tree using the conserved region. This phylogenetic tree spans the WRKY domain/s of the 28 selected proteins of Arabidopsis, the 20 proteins identified in *P. hybrida* and their putative orthologs in related Solanaceae species (tomato, potato, and *Nicotiana tomentosiformis*), and several WRKY members that were reported to regulate leaf senescence in different monocot and dicot species (Fig. 6). To strengthen our analysis, we conducted a conserved motif search using full-length amino acid sequences (Fig. 6 and Supplementary Fig. S2). Both analyses were complementary and helped to define seven major functional groups (I-VII) that contained WRKY members of the three subfamilies (Fig. 6). Interestingly, all functional groups possessed at least one WRKY member with a reported function in the regulation of leaf senescence (Fig. 6). Functional group Va did not contain Arabidopsis members and might be considered a solanaceous specific group. In addition, a search of *cis*-regulatory elements was performed in the promoter sequences of the best homologs in *P. axillaris* or *P. inflata* of each *PhWRKY* gene (Supplementary Table S4).

The six genes in cluster 1 were upregulated in the three types of senescence (Fig. 5), representing the most interesting genes characterized in this work (Fig. 5 and Table 3). *PhWRKY028*, *PhWRKY072*, and *PhWRKY033* were upregulated in early- and mid-leaf senescence and showed similar expression profiles in the three types of senescence. Notably, *PhWRKY033* and *PhWRKY072* showed the highest expression changes during natural leaf senescence (Figs. 2 and 5). The putative orthologs of these genes in Arabidopsis were simultaneously upregulated during leaf and petal development (Table 1), and a putative homolog of *PhWRKY033* tended to be upregulated in the corollas of petunia flowers at 24 hap (Broderick 2014), which is in agreement with our results (Figs. 4a and 5). The other genes in cluster 1, *PhWRKY063, PhWRKY006*, and *PhWRKY015* showed more attenuated expression changes in natural leaf and corolla senescence and similar expression in pollination-induced senescence (Fig. 5). Putative orthologs in Arabidopsis displayed similar expression profiles in both organs, although *AtWRKY63* showed unclear expression in pollinated flowers (Table 1). Moreover, putative homologs of *PhWRKY006* exhibit a moderately increase in their expression during mid-natural corolla senescence and during relatively early- pollination-induced corolla senescence, which coincides with our data (Fig. 5; Broderick et al. 2014; Wang et al. 2018). In addition, the search of *cis*-regulatory elements indicated the presence of three reported senescence-associated elements, W-box, G-box and ABREs, in the promoters of the parental equivalents of all PhWRKYs included in cluster 1 (Supplementary Table S4 and Fig. 5) (Zheng et al. 2005; Rinerson et al. 2015; Liu et al. 2016). Phylogenetic analysis showed that PhWRKY028 was closely related to CpWRKY71 in the functional group IVa (WRKY IIc subfamily) (Fig. 6). Interestingly, the expression of *CpWRKY71* increases during leaf senescence progression in Wintersweet, and its overexpression in transgenic Arabidopsis plants accelerates leaf senescence (Huang et al. 2019) (Table 3). PhWRKY033, classified into group III (WRKY I subfamily), is a putative ortholog of wheat TaWRKY7 that positively regulates leaf senescence when it is overexpressed in Arabidopsis (Fig. 6 and Table 3) (Zhang et al. 2016b; Doll et al. 2020). PhWRKY006 and PhWRKY072 were classified into functional group II (WRKY IIb subfamily) (Fig. 6). Both are putative orthologs of OsWRKY5, AtWRKY6, and are more distantly related to GhWRKY17. All these members increase their expression in rice, Arabidopsis, and cotton, respectively, and promote leaf senescence in rice and Arabidopsis. Moreover, heterologous expression of *GhWRKY17* in Arabidopsis promotes leaf senescence (Table 3) (Robatzek and Somssich 2001; Gu et al. 2018a; Kim et al. 2019). All these positive regulators of leaf senescence differed in regard to the presence of motifs 9 and/or

12 (Fig. 6), suggesting these motifs would not be important for the regulation of senescence. PhWRKY015 was classified into functional group I (WRKY IId subfamily), and it was closely related to GhWRKY42 and more distantly to OsWRKY42 (Fig. 6 and Table 3). OsWRKY42 is a positive regulator of leaf senescence in rice, whose expression increases during leaf development (Han et al. 2014); whereas GhWRKY42 increases its expression in cotton and promote leaf senescence in transgenic Arabidopsis plants (Gu et al. 2018b). PhWRKY063 was classified into functional group VI (WRKY III subfamily). It was closely related to the negative regulators of leaf senescence, AtWRKY54 and AtWRKY70, which increase their expression during the progression of senescence in leaves and petals and more distantly related to the positive regulator of wheat TaWRKY40-D. Thus, PhWRKY063 may function as a negative regulator of senescence in *P. hybrida* (Ülker et al. 2007; Besseau et al. 2012; Zhao et al. 2020) (Fig. 6 and Table 3). Taken together, our results show that PhWRKYs of cluster 1 are important candidates for the regulation of senescence in *P. hybrida*.

Genes in cluster 2, *PhWRKY075*, *PhWRKY030*, and *PhWRKY055*, were upregulated in natural leaf and corolla senescence (Fig. 5). Expression changes were observed relatively early during natural corolla senescence for the three genes, although in the leaves, each gene showed differences in the time of expression changes (Fig. 5). Expression profiles of *PhWRKY075* and *PhWRKY030* were strong, mainly in the corollas since they displayed the most significant changes, while the expression profiles of *PhWRKY055* did not stand out (Fig. 5). Promoter analysis in their parental equivalents showed they all contain the senescence-associated *cis*-elements mentioned previously (Supplementary Table S4). Interestingly, the homolog of *PhWRKY075* showed the maximum number of G-box and ABRE elements, which coincided with its highest expression in natural corolla senescence (Fig. 5). Moreover, the weaker expression profile of *PhWRKY055* coincided with the lower number of G-box and ABRE elements respect to *PhWRKY075* and *PhWRKY030* (Supplementary Table S4). PhWRKY075 was classified into functional group IVb (WRKY IIc subfamily) (Fig. 6). Their putative orthologs, *AtWRKY75* and *AtWRKY45*, increase their expression during senescence in leaves and petals (Table 1) and both promote leaf senescence in Arabidopsis (Table 3) (Li et al. 2012; Chen et al. 2017). The rice OsWRKY23 is another putative ortholog of PhWRKY075. Overexpression of OsWRKY23 in Arabidopsis promotes dark-induced leaf senescence (Jing et al. 2009). PhWRKY030 and PhWRKY055 were both classified into functional group V (WRKY III subfamily). PhWRKY030 was related to AtWRKY53 (subgroup Vb), whereas PhWRKY055 was more related to the cotton GhWRKY27 (subgroup Va) (Fig. 6 and Table 3). Expression of *AtWRKY53* and *GhWRKY27* increase during leaf senescence of Arabidopsis (Miao et al. 2004) and cotton (Gu et al. 2019a), respectively (Tables 1 and 3), and their overexpression in Arabidopsis promote leaf senescence (Miao et al. 2004; Gu et al. 2019a) (Table 3). This evidence suggests that members of cluster 2 are interesting candidates for the regulation of natural senescence processes in petunia. However, they do not seem to be involved in the regulation of pollination-induced senescence.

Cluster 3 was further divided into two subgroups. *PhWRKY007*, *PhWRKY004*, and *PhWRKY002* genes represented cluster 3a and *PhWRKY069* and *PhWRKY054* genes represented cluster 3b (Fig. 5). Genes in cluster 3a did not show consistent expression profiles in either of the three types of senescence studied (Fig. 5). Moreover, analysis of *cis*-elements in the promoters revealed the lack of W-boxes in the parental homologs of *PhWRKY007* and *PhWRKY004*, and G-boxes in the parental homolog of *PhWRKY002* (Supplementary Table S4), suggesting the importance of all senescence-associated regulatory elements (ABRE, W-box and G-box) for consistent expression profiles. A putative homolog of *PhWRKY007* shows weak expression changes in the corollas of pollinated petunia flowers (Broderick et al. 2014). Another putative homolog of *PhWRKY007* shows an erratic expression profile during natural corolla senescence (Wang et al. 2018). Putative homologs of *PhWRKY004* and *PhWRKY002* show weak and moderate upregulation in the corollas of pollinated petunia flowers, respectively, resembling our results (Fig. 5; Broderick et al. 2014). Interestingly, the putative orthologs of these genes in Arabidopsis were upregulated in both organs, suggesting a different type of regulation in petunia, specifically for *PhWRKY004* and *PhWRKY007* which did not show differential expression in the leaves (Table 1 and Fig. 5). PhWRKY002 and PhWRKY004 were classified into group III (WRKY I subfamily). PhWRKY002 is a putative ortholog of the negative regulator AtWRKY25, while PhWRKY004 is a putative ortholog of TaWRKY7 (Fig. 6 and Table 3) (Zhang et al. 2016b; Doll et al. 2020). PhWRKY007 was classified into group I (WRKY IId subfamily) along with the previously mentioned GhWRKY42 and OsWRKY42 (Fig. 6). Even though PhWRKYs of cluster 3a were associated with characterized regulators of senescence, their unstable expression profiles in the three types of senescence suggest they are not good candidates for senescence regulation in *P. hybrida* (Fig. 5). Genes in cluster 3b, *PhWRKY069* and *PhWRKY054*, were leaf specific and showed intermediate changes in expression in early- and mid- leaf senescence, respectively

(Fig. 5). Similarly, as the genes in cluster 3a, promoter analyses revealed that their homologs in the parental species did not show all the senescence-associated *cis*-regulatory elements (Supplementary Table S4). For example, the homolog of PhWRKY069 lacked ABRE elements but had W- and G- boxes, although the homolog of PhWRKY054 completely lacked the three types of elements. These results again indicate the importance of senescence-associated elements for the expression of *WRKY* genes in different types of senescence. Interestingly, the putative orthologs of PhWRKY069 and PhWRKY054 in Arabidopsis increased their expression during pollination-induced corolla senescence (Table 1 and Fig. 6), suggesting a different organ regulation for these WRKY members between the two species. PhWRKY054 and PhWRKY069 were classified into group VI (WRKY III subfamily) and group VII (WRKY IIe subfamily), respectively (Fig. 6). PhWRKY054 was closely related to the negative regulators AtWRKY54 and AtWRKY70 (Table 3) (Ülker et al. 2007; Besseau et al. 2012), and more distantly with the positive regulator TaWRKY40-D. PhWRKY069 was closely related to the negative regulator GhWRKY91, which represses leaf senescence in Arabidopsis (Gu et al. 2019b), and more distantly related to AtWRKY22, which regulates dark-induced leaf senescence in Arabidopsis (Zhou et al. 2011). Therefore, PhWRKY069 and PhWRKY054 can be considered candidates only for leaf senescence regulation in *P. hybrida*.

Finally, cluster 4 contained three genes, *PhWRKY070*, *PhWRKY024*, and *PhWRKY053*, which showed upregulation during natural leaf and corolla senescence and downregulation in a very early stage of pollination-induced corolla senescence (6 hap) (Figs. 5 and 6). Interestingly, a similar regulation was reported for the putative homolog of *PhWRKY053* in the corollas of pollinated petunia flowers (Broderick et al. 2014), and for the putative homolog of *PhWRKY024*, which decreases in early- natural corolla senescence, but increases later (Wang et al. 2018). Putative orthologs of these genes in Arabidopsis were upregulated during leaf senescence, although they also increased their expression in pollination-induced petal senescence (Table 1 and Fig. 6), suggesting a different regulation in this latter type of senescence between both species. Analysis of *cis*-regulatory elements revealed that the homologs of *PhWRKY024* and *PhWRKY070* displayed the three types of senescence-associated regulatory elements (ABRE, W- and G- boxes). However, the equivalent of PhWRKY053 did not show W-boxes, suggesting it might regulate senescence independently of WRKY regulation (Supplementary Table S4). Phylogenetic analysis showed that members of this cluster were related to positive and negative regulators of senescence (Fig. 6 and Table 3). PhWRKY053 was classified into group Va (WRKY III subfamily). This gene is a putative ortholog of the previously described GhWRKY27 (Fig. 6 and Table 3) (Gu et al. 2019a). PhWRKY070 was included into group VI (WRKY III subfamily) and related to the negative regulators AtWRKY54 and AtWRKY70 and more distantly with the positive regulator TaWRKY40-D (Fig. 6 and Table 3) (Ülker et al. 2007; Besseau et al. 2012; Zhao et al. 2020). PhWRKY024 was classified into group III (WRKY I subfamily) and shared the functional group with both positive (TaWRKY7) and negative (AtWRKY25) regulators (Fig. 6 and Table 3) (Zhang et al. 2016b; Doll et al. 2020).

WRKY TFs have been described to activate or repress expression of other members in the family and some of them show redundant functions (Zhou et al. 2011; Besseau et al. 2012; Potschin et al. 2014, Chen et al. 2018). Clustering analysis along the three types of senescence revealed groups of genes with similar expression profiles that also shared their functional groups, suggesting that these genes could have redundant roles (Table 3). For example, redundancy may be expected for PhWRKY006 and PhWRKY072 of functional group II, which showed similar expression profiles in the three types of senescence (Cluster 1). PhWRKY053 and PhWRKY055 of functional group Va, which showed consistent expression profiles in natural senescence processes; and PhWRKY070 and PhWRKY054 of functional group VI, which showed similar profiles during natural leaf senescence and are expected to negatively regulate senescence in *P. hybrida* (Figs. 5-6 and Table 3).

Conclusions

Even though genome-wide studies were reported for the WRKY family in different species, only a few works have associated WRKY members with leaf and flower senescence processes, mainly in ornamental plants. Here, we integrated expression profiles of PhWRKYs with phylogenetic analysis and identified at least eight strong candidates that may regulate more than one senescence process in *P. hybrida*. Functional analysis will be required to confirm whether WRKY TFs could act as regulators of senescence, which would help to delay senescence via molecular breeding.

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Tables

Table 1 Expression and function in leaf senescence of selected senescence-associated *WRKY* genes of Arabidopsis and identification of putative orthologs in *P. hybrida*

Arabidopsis thaliana								*Petunia hybrida*	
AGI code	Synonyms	Subfamily	Leaf expression	Petal expression	Source	Function in leaf senescence		Best hit accession	Name
AT5G24110	WRKY30	WRKY-III	Increase (M)	Increase	2, 4, 5	Unclear	n/a	comp730638_c0_seq1 (P)	PhWRKY030
AT1G66600	WRKY63	WRKY-III	Increase	Unclear	3	Unclear	n/a	comp21623_c0_seq1	PhWRKY063
AT2G03340	WRKY3	WRKY-I	Increase (E)	No change	2, 4	Unclear	n/a	comp22104_c1_seq3	PhWRKY004
AT1G13960	WRKY4	WRKY-I	Increase (E)	Increase	1, 2, 4, 5	Unclear	n/a	comp22104_c1_seq3	PhWRKY004
AT5G07100	WRKY26	WRKY-I	Increase (E)	Increase	1, 2, 4, 5	Unclear	n/a	comp30812_c0_seq1	PhWRKY024
AT4G01720	WRKY47	WRKY-IIb	Increase (E)	Increase	2, 4, 5	Unclear	n/a	comp6646_c0_seq1 (P)	PhWRKY006
AT1G29280	WRKY65	WRKY-IIe	Increase (E)	Increase	2, 4, 5	Unclear	n/a	comp18538_c0_seq1 (P)	PhWRKY069
AT2G23320	WRKY15	WRKY-IId	Increase (M)	Increase	2, 4, 5	Unclear	n/a	comp21369_c0_seq2	PhWRKY015
AT4G26440	WRKY34	WRKY-I	Increase (E)	Increase	2, 4, 5	Unclear	n/a	comp12645_c0_seq1	PhWRKY002
AT5G13080 (*)	WRKY75	WRKY-IIb	Increase (E)	Increase	1, 2, 4, 5	Promote	7	comp23620_c0_seq2	PhWRKY075
AT3G58710	WRKY69	WRKY-IIe	Increase (E)	Increase	2, 4, 5	Unclear	n/a	comp18538_c0_seq1 (P)	PhWRKY069
AT2G30250 (*)	WRKY25	WRKY-I	Increase (E)	Increase	2, 4, 5	Delay	11	comp30812_c0_seq1	PhWRKY024
AT5G15130	WRKY72	WRKY-IIb	Increase (L)	Increase	2, 4, 5	Unclear	n/a	comp16919_c0_seq1 (P)	PhWRKY072
AT1G18860	WRKY61	WRKY-IIb	Increase (M)	No change	2, 4	Unclear	n/a	comp6646_c0_seq1 (P)	PhWRKY006
AT1G62300 (*)	WRKY6	WRKY-IIb	Increase (L)	Increase	1, 2, 4, 5	Promote	8	comp6646_c0_seq1 (P)	PhWRKY006
AT5G64810	WRKY51	WRKY-IIc	Increase (M)	Unclear	2	Unclear	n/a	comp2525_c0_seq1 (P)	PhWRKY051
AT4G31550	WRKY11	WRKY-IId	Increase	No change	3, 4	Unclear	n/a	comp17118_c0_seq4	PhWRKY011
AT4G24240	WRKY7	WRKY-IId	Increase	Increase	3, 4, 5	Unclear	n/a	comp22664_c0_seq2	PhWRKY007
AT3G01080	WRKY58	WRKY-I	Increase	Increase	3, 4, 5	Unclear	n/a	comp22104_c1_seq3	PhWRKY004
AT4G18170	WRKY28	WRKY-IIc	Increase (E)	Increase	2, 4, 5	Unclear	n/a	comp325234_c0_seq1 (P)	PhWRKY028
AT2G40740	WRKY55	WRKY-III	Increase (M)	Increase	2, 4, 5	Unclear	n/a	comp15947_c0_seq2 (P)	PhWRKY055
AT2G38470	WRKY33	WRKY-I	Increase (M)	Increase	2, 4, 5	Unclear	n/a	comp23620_c0_seq3 (P)	PhWRKY033
AT4G23810 (*)	WRKY53	WRKY-III	Increase	Increase	4, 5	Promote	9	comp42882_c0_seq1	PhWRKY053
AT5G49520	WRKY48	WRKY-IIc	Increase (E)	Increase	2, 4, 5	Unclear	n/a	comp266655_c0_seq1 (P)	PhWRKY023
AT3G01970 (*)	WRKY45	WRKY-I	Increase (E)	Increase	1, 2, 4, 5	Promote	12	comp23620_c0_seq2	PhWRKY075
AT1G69810	WRKY36	WRKY-IIb	Increase (E)	Increase	2, 4, 5	Unclear	n/a	comp6646_c0_seq1 (P)	PhWRKY006
AT2G40750 (*)	WRKY54	WRKY-III	Increase	Increase	3, 4, 5	Delay	6	comp933645_c0_seq1	PhWRKY054
AT3G56400 (*)	WRKY70	WRKY-III	Increase	Increase	3, 4, 5	Delay	10	comp26279_c1_seq2	PhWRKY070

List of 28 Arabidopsis genes showing expression data in leaves and petals and function in leaf senescence. Genes were classified according to their subfamilies. Putative orthologs in *P. hybrida* were obtained through tBLASTn using a transcriptomic leaf database (Villarino et al. 2014). Asterisks (*) indicate genes of Arabidopsis with a reported function in leaf senescence. The letter P indicates partial sequences of *P. hybrida*. E, M and L indicate early, mid or late changes in expression, respectively, obtained from Breeze et al. (2011). The numbers 1 through 12 indicate the source from where expression and functional data were obtained: 1) Buchanan-Wollaston et al. 2005; 2) Breeze et al. 2011; 3) Leaf senescence database (Liu et al. 2011); 4) Arabidopsis eFP browser (Winter et al. 2007); 5) Wagstaff et al. 2009; 6) Besseau et al. 2012; 7) Li et al. 2012; 8) Robatzek and Somssich 2001; 9) Miao et al. 2004; 10) Ülker et al. 2007; 11) Doll et al. 2020; 12) Chen et al. 2017. Abbreviations: AGI, Arabidopsis Genome Initiative; n/a, data not available

Table 2 Summary of the 20 senescence-associated WRKY proteins identified in *P. hybrida* and the equivalents of *P. axillaris* and *P. inflata*. A variant of the conserved WRKYGQK heptapeptide is shown in italics

Name	Subfamily	WRKY domains	Conserved heptapeptide	Domain pattern	Zinc finger type	Accession in *P. axillaris*	Accession in *P. inflata*
PhWRKY002	I	2	WRKYGQK / WRKYGQK	Cx4Cx22HxH / Cx4Cx23HxH	C2H2	Peaxi162Scf00232g00810.1	Peinf101Scf00055g17013.1
PhWRKY004	I	2	WRKYGQK / WRKYGQK	Cx4Cx22HxH / Cx4Cx23HxH	C2H2	Peaxi162Scf00222g00117.1	Peinf101Scf00231g01029.1
PhWRKY024	I	2	WRKYGQK / WRKYGQK	Cx4Cx22HxH / Cx4Cx23HxH	C2H2	Peaxi162Scf00055g01910.1	Peinf101Scf00450g00007.1
PhWRKY033	I	2	WRKYGQK / WRKYGQK	Cx4Cx22HxH / Cx4Cx23HxH	C2H2	Peaxi162Scf00744g00220.1	Peinf101Scf00782g10028.1
PhWRKY006	IIb	1	WRKYGQK	Cx5Cx23HxH	C2H2	Peaxi162Scf00007g00315.1	Peinf101Scf01579g02011.1
PhWRKY072	IIb	1	WRKYGQK	Cx5Cx23HxH	C2H2	Peaxi162Scf00178g01110.1	Peinf101Scf01200g03007.1
PhWRKY023	IIc	1	WRKYGQK	Cx4Cx23HxH	C2H2	Peaxi162Scf00164g01010.1	Peinf101Scf00244g18025.1
PhWRKY028	IIc	1	WRKYGQK	Cx4Cx23HxH	C2H2	Peaxi162Scf01189g00009.1	Peinf101Scf00040g09006.1
PhWRKY051	IIc	1	*WRKYGKK*	Cx4Cx23HxH	C2H2	Peaxi162Scf00106g01616.1	Peinf101Scf00381g17007.1
PhWRKY075	IIc	1	WRKYGQK	Cx4Cx23HxH	C2H2	Peaxi162Scf00128g01541.1	Peinf101Scf00889g03041.1
PhWRKY007	IId	1	WRKYGQK	Cx5Cx23HxH	C2H2	Peaxi162Scf00121g00018.1	Peinf101Scf01179g02021.1
PhWRKY011	IId	1	WRKYGQK	Cx5Cx23HxH	C2H2	Peaxi162Scf00459g00841.1	Peinf101Scf00276g07026.1
PhWRKY015	IId	1	WRKYGQK	Cx5Cx23HxH	C2H2	Peaxi162Scf00549g00222.1	Peinf101Scf00887g05031.1
PhWRKY069	IIe	1	WRKYGQK	Cx5Cx23HxH	C2H2	Peaxi162Scf00469g00624.1	Peinf101Scf00442g03028.1
PhWRKY030	III	1	WRKYGQK	Cx7Cx23HxC	C2HC	Peaxi162Scf00904g00212.1	Peinf101Scf01632g03025.1
PhWRKY053	III	1	WRKYGQK	Cx7Cx23HxC	C2HC	Peaxi162Scf00102g01741.1	Peinf101Scf02382g03038.1
PhWRKY055	III	1	WRKYGQK	Cx7Cx23HxC	C2HC	Peaxi162Scf00102g01741.1	Peinf101Scf00962g23035.1
PhWRKY054	III	1	WRKYGQK	Cx7Cx23HxC	C2HC	Peaxi162Scf00304g00719.1	Peinf101Scf00339g02023.1
PhWRKY063	III	1	WRKYGQK	Cx7Cx23HxC	C2HC	Peaxi162Scf00732g00236.1	Peinf101Scf00782g02035.1
PhWRKY070	III	1	WRKYGQK	Cx7Cx23HxC	C2HC	Peaxi162Scf00073g02335.1	Peinf101Scf00191g46015.1

ımary of senescence-associated *PhWRKY* expression categories, functional group classification, and comparison with putative orthologs in other species

Petunia hybrida				Putative orthologs in other species			
Expression category	Gene	Functional group	Subfamily	Gene	Expression during leaf senescence	Function in leaf senescence	References
Upregulation in the three types of senescence (Cluster 1)	***PhWRKY033***	III	I	*TaWRKY7; AtWRKY25*	Increase expression in wheat and Arabidopsis	Overexpression of TaWRKY7 in Arabidopsis promotes leaf senescence AtWRKY25 delays leaf senescence in Arabidopsis	Zhang et al. 2016; Doll et al. 2020
	PhWRKY028	IVa	IIc	*CpWRKY71*	Increases expression in Wintersweet	Overexpression of *CpWRKY71* in Arabidopsis promotes leaf senescence	Huang et al. 2019
	PhWRKY072	II	IIb	*OsWRKY5; AtWRKY6; GhWRKY17*	Increase expression in rice, Arabidopsis, and cotton	OsWRKY5 promotes leaf senescence in rice AtWRKY6 promotes leaf senescence in Arabidopsis Overexpression of *GhWRKY17* in Arabidopsis promotes leaf senescence	Kim et al. 2019; Robatzek and Somssich 2001; Gu et al. 2018a
	PhWRKY006	II	IIb				
	PhWRKY063	VI	III	*AtWRKY54; AtWRKY70; TaWRKY40-D*	Increase expression in Arabidopsis and wheat	AtWRKY54 and AtWRKY70 delay leaf senescence in Arabidopsis TaWRKY40-D promotes leaf senescence in wheat	Ülker et al. 2007; Besseau et al. 2012; Zhao et al. 2020
	PhWRKY015	I	IId	*OsWRKY42; GhWRKY42*	Increase expression in rice and cotton	OsWRKY42 promotes leaf senescence in rice Overexpression of *GhWRKY42* in Arabidopsis promotes leaf senescence	Han et al. 2014; Gu et al. 2018b
Upregulation in natural leaf and petal senescence (Cluster 2)	***PhWRKY075***	IVb	IIc	*AtWRKY75; AtWRKY45; OsWRKY23*	Increase expression in Arabidopsis and rice	AtWRKY75 and AtWRKY45 promote leaf senescence in Arabidopsis OsWRKY23 promotes dark-induced leaf senescence in Arabidopsis	Chen et al. 2017; Li et al. 2012; Jing et al. 2009
	PhWRKY030	Vb	III	*AtWRKY53*	Increases expression in Arabidopsis	AtWRKY53 promotes leaf senescence in Arabidopsis	Miao et al. 2005
	PhWRKY055	Va	III	*GhWRKY27*	Increases expression in cotton	Overexpression of *GhWRKY27* in Arabidopsis promotes leaf senescence	Gu et al. 2019a
Unclear (Cluster 3a)	***PhWRKY007***	I	IId	*OsWRKY42; GhWRKY42*	Increase expression in rice and cotton	OsWRKY42 promotes leaf senescence in rice Overexpression of *GhWRKY42* in Arabidopsis promotes leaf senescence	Han et al. 2014; Gu et al. 2018b
	PhWRKY002	III	I	*TaWRKY7; AtWRKY25*	Increase expression in wheat and Arabidopsis	Overexpression of TaWRKY7 in Arabidopsis promotes leaf senescence AtWRKY25 delays leaf senescence in Arabidopsis	Zhang et al. 2016; Doll et al. 2020
	PhWRKY004	III	I				
Upregulation in natural leaf senescence (Cluster 3b)	***PhWRKY054***	VI	III	*AtWRKY54; AtWRKY70; TaWRKY40-D*	Increase expression in Arabidopsis and wheat	AtWRKY54 and AtWRKY70 delay leaf senescence in Arabidopsis TaWRKY40-D promotes leaf senescence in wheat	Ülker et al. 2007; Besseau et al. 2012; Zhao et al. 2020
	PhWRKY069	VII	IIe	*AtWRKY22; GhWRKY91*	Increase expression in Arabidopsis and cotton	AtWRKY22 promotes dark-induced leaf senescence in Arabidopsis Overexpression of *GhWRKY91* in Arabidopsis delays leaf senescence	Zhou et al. 2011; Gu et al. 2019b
Upregulation in natural leaf and petal senescence and down-regulation in pollination-induced petal senescence (Cluster 4)	***PhWRKY070***	VI	III	*AtWRKY54; AtWRKY70; TaWRKY40-D*	Increase expression in Arabidopsis and wheat	AtWRKY54 and AtWRKY70 delay leaf senescence in Arabidopsis TaWRKY40-D promotes leaf senescence in wheat	Ülker et al. 2007; Besseau et al. 2012; Zhao et al. 2020
	PhWRKY024	III	I	*TaWRKY7; AtWRKY25*	Increase expression in wheat and Arabidopsis	Overexpression of TaWRKY7 in Arabidopsis promotes leaf senescence AtWRKY25 delays leaf senescence in Arabidopsis	Zhang et al. 2016; Doll et al. 2020
	PhWRKY053	Va	III	*GhWRKY27*	Increases expression in cotton	Overexpression of *GhWRKY27* in Arabidopsis promotes leaf senescence	Gu et al. 2019a

PhWRKY genes were divided into four expression categories and classified in subfamilies and functional groups. Equivalent members of other species are
uding expression data, and reported functions in leaf senescence

Figures

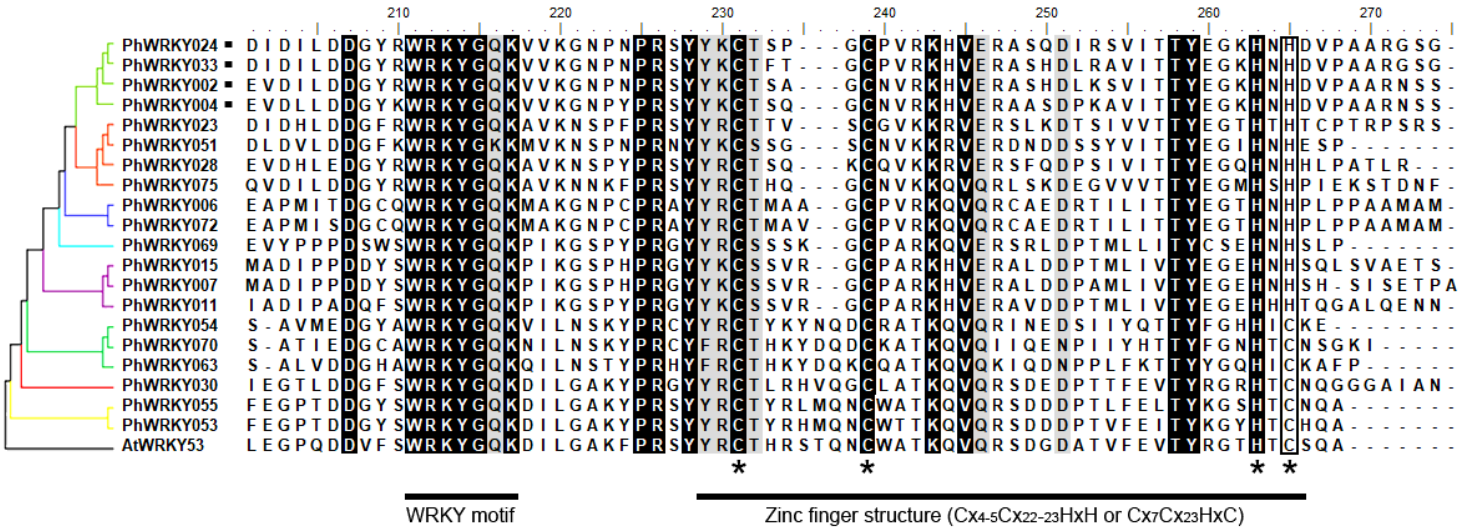

Figure 1

Sequence alignment of PhWRKYs. ClustalW alignment of the highly conserved residues spanning the WRKY domain in the 20 proteins of P. hybrida. Highly conserved residues are depicted with black and grey backgrounds. Asterisks (*) and the rectangle indicate residues in the WRKY motif and those that conformed the zinc-finger structure. Black points indicate four proteins of WRKY-I subfamily that contained a second WRKY domain that is not shown in the alignment. AtWRKY53 of Arabidopsis was included as a reference protein (Miao et al. 2004)

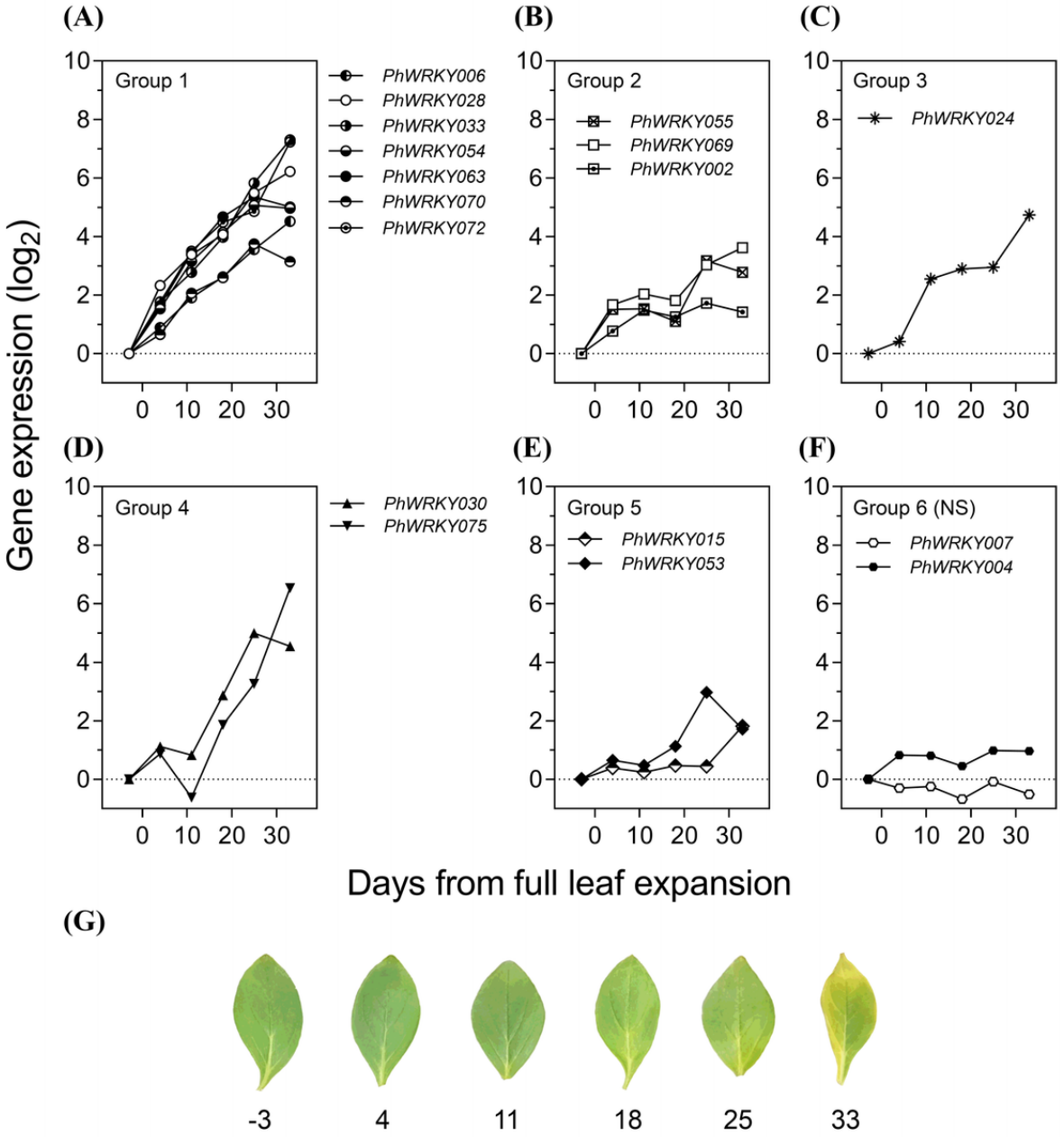

Figure 2

Expression profiles of PhWRKY genes during natural leaf senescence. Expression groups of 17 PhWRKYs (a-f) in the leaves at various times after full leaf expansion (g). Relative expression is shown as the ratio (log2 scale) between each sampled point relative to the first sampling point and to the expression of the reference PhEF1a gene. For better visualization error bars are not shown

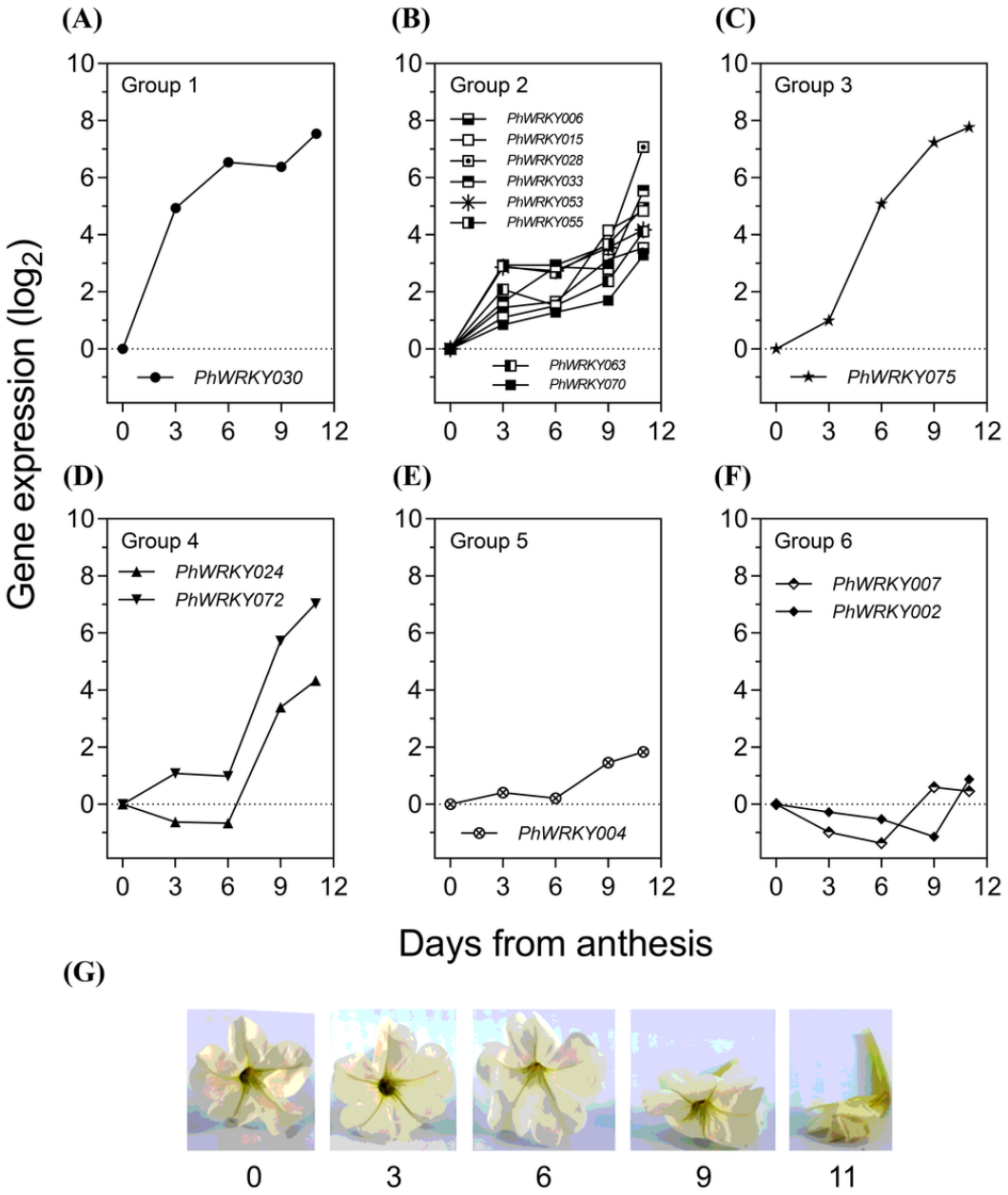

Figure 3

Expression profiles of PhWRKY genes during natural corolla senescence. Expression groups of 15 PhWRKYs (a-f) in unpollinated corollas at various times after anthesis (g). Relative expression is shown as the ratio (log2 scale) between each sampled point relative to the first sampling point and to the expression of the reference PhEF1a gene. For better visualization error bars are not shown

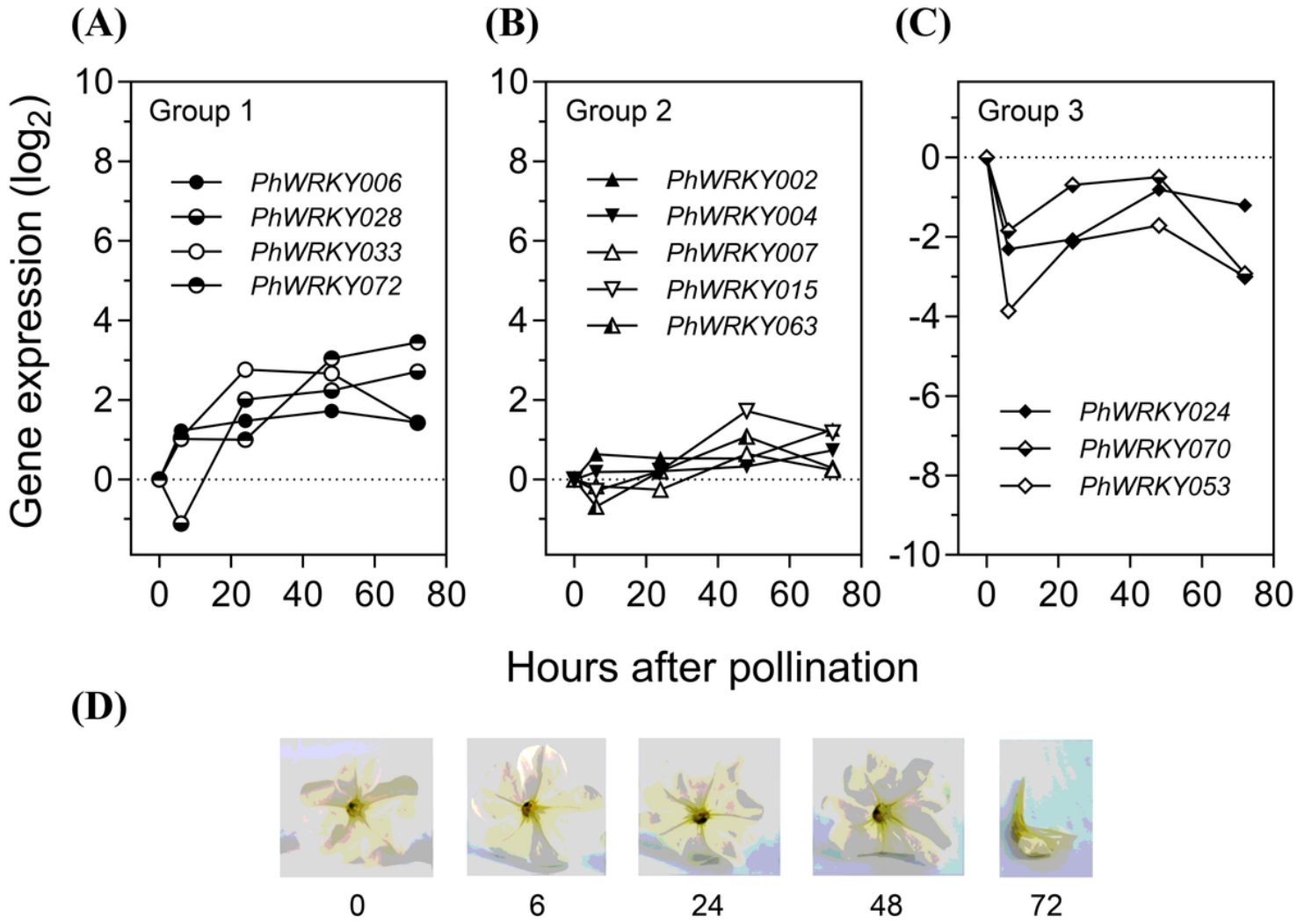

Figure 4

Expression profiles of PhWRKY genes during pollination-induced corolla senescence. Expression groups of 12 PhWRKYs (a-c) in corollas at various times after pollination (d). Relative expression is shown as the ratio (log2 scale) between each sampled point relative to the first sampling point and the expression of the reference PhEF1a gene. For better visualization error bars are not shown

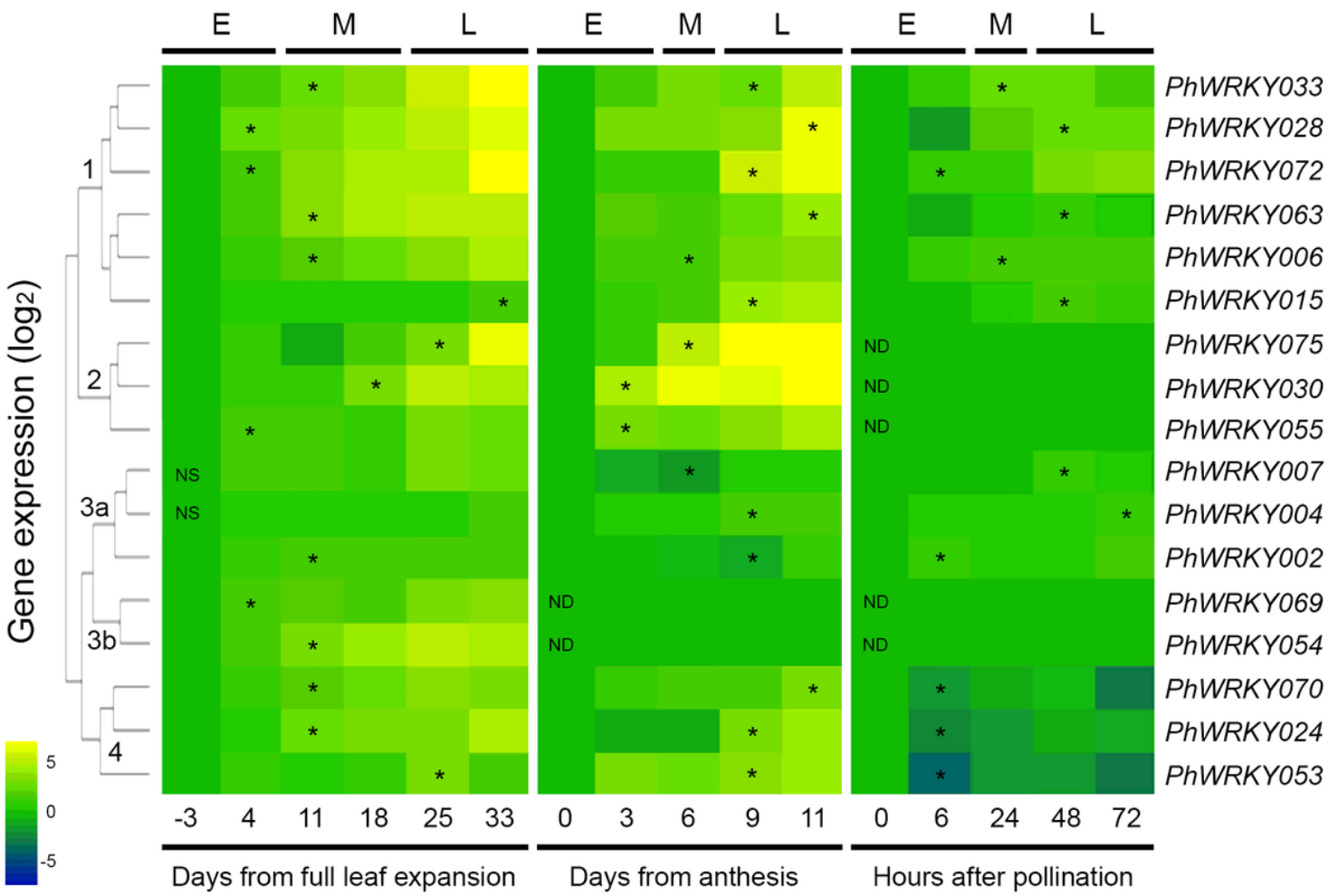

Figure 5

Heatmap analysis and hierarchical clustering of PhWRKY expression profiles throughout the three senescence processes. Relative transcript levels are indicated by a colour scale (log2 scale) between each sampled point relative to the level at the first sampling point and the expression of the reference gene PhEF1a. Asterisks (*) indicate the time of initial significant expression change respect to the first sampling point. E, M, and L indicate early-, mid-, and late- stages of senescence, respectively. NS, non-significant; ND, non-detected

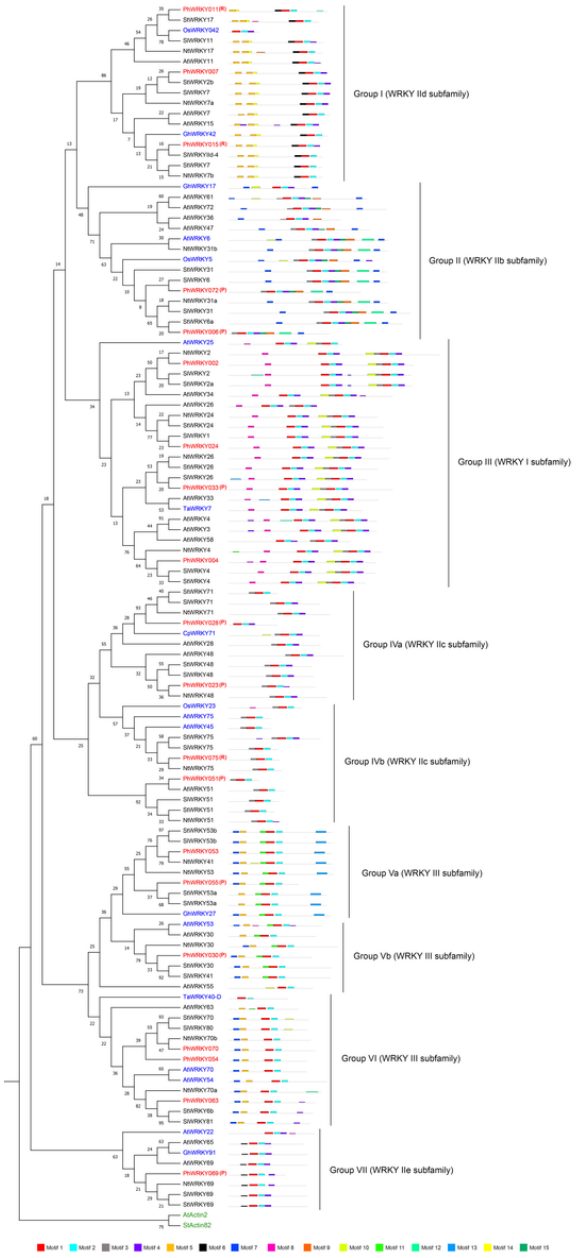

Figure 6

Phylogenetic analysis and motif composition of senescence-associated PhWRKYs and equivalent proteins of other species. Multiple sequence alignment of the conserved region of 117 proteins, spanning the WRKY amino-acid sequence, was done using ClustalW. The phylogenetic tree was constructed using MEGA5 by the Neighbor-joining method. Numbers at the nodes indicate the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates). Conserved motifs (15) were searched in whole sequences using the MEME program and represented as coloured boxes (Bailey et al. 2009). PhWRKYs are highlighted in red, proteins with a reported function in leaf senescence are highlighted in blue, while external group proteins are highlighted in green. Subfamily classification was based on the classification system proposed by Eulgem et al. 2000. Phylogenetic groups (I to VII) were defined by combining phylogenetic and motif analysis. (P) indicates partial PhWRKY sequences, whereas (R) indicates reconstructed sequences by overlapping with other contigs of the same gene from Villarino's database (Villarino et al. 2014). Accessions of Nicotiana tomentosiformis (Nt), Solanum lycopersicum (Sl) and S. tuberosum (St) were obtained from the NCBI (https://www.ncbi.nlm.nih.gov/). Accession codes of all WRKY proteins used are depicted in Supplementary Table S5

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFig.S1.pdf
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