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RESEARCH ARTICLE

Characterization and expression analysis of *WRKY* genes during leaf and corolla senescence of *Petunia hybrida* plants

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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 thaliana*. 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 *A. thaliana* 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 *A. thaliana* were observed during leaf senescence, although more divergence was 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 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.*

Keywords Leaf senescence · Corolla senescence · *WRKY* genes · Expression patterns · Phylogenetic analysis · *Petunia hybrida*

Introduction

The final stage of leaf development is called senescence. It involves execution of an orchestrated genetic program, including a type of programmed cell death, in which nutrients and minerals generated from catabolism are remobilized to active growing organs (Gepstein et al. 2003; Guo

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and Gan 2005). Leaf senescence can be induced by age, hormone signalling, or the onset of the reproductive phase, although under adverse conditions it can be triggered prematurely (Guo and Gan 2005; Jagadish et al. 2015). The process is characterized by the loss of photosynthetic activity, due in part to a massive degradation of organelles and macromolecules. Dramatic changes in gene expression, hormone balance and metabolism have been observed during its progression (Gregersen et al. 2013; Buet et al. 2019). Similarly, petal senescence represents the final stage of flower development, which involves remobilization of low-molecular weight components produced after the degradation of different cell constituents (Langston et al. 2005; Jones 2013; Shibuya et al. 2016). Petal senescence can be modulated by endogenous age-related signals, although, compared to leaves, it is poorly affected by external signals and the remobilization of minerals and nutrients is substantially reduced (Jones 2004; Thomas et al. 2003; Rogers 2013). In most flowers the role of petals is to attract pollinators, therefore, in many species pollination activates or accelerates petal senescence (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). A substantial number of genes exhibit up- and down- regulation of transcript abundance during senescence, they are commonly referred as *senescence-associated genes* or *SAGs*, and are responsible for senescence progression. Several families of transcription factors (TFs) have been reported to change their expression during leaf and petal senescence (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 WRKY TFs have been reported to regulate leaf senescence, mainly in *A. thaliana* and *Oryza sativa* (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).

In plants, WRKY proteins represent one of the most important family of TFs (Rushton et al. 2010). They are characterized by the presence of a 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 $Cx_{4\text{-}5}Cx_{22\text{-}23}HxH$ or $Cx_7Cx_{23}HxC$ (Eulgem et al. 2000; Rushton et al. 2010). The proteins of this family are classified into three main groups. Group I proteins possesses two WRKY domains and two C_2H_2 zinc-finger motifs. Group II proteins possesses a single WRKY domain and a C_2H_2-type zinc-finger motif. This group is divided into five subgroups (IIa-IId). Proteins in Group III possesses a 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, responses to diverse stresses and hormonal signalling (Bakshi and Oelmüller 2014; Giacomelli et al. 2012; Phukan et al. 2016; Zhao et al. 2020).

In *A. thaliana*, 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). WRKY members can act redundantly and interact with each other, regulating the expression of other *WRKY* genes presumably by binding to W-box sequences in their promoter region (Zhou et al. 2011; Besseau et al. 2012). In *O. sativa*, OsWRKY42 (Han et al. 2014) and OsWRKY5 (Kim et al. 2019) have been described to positively regulate leaf senescence. Overexpression of two *Triticum aestivum* members in *A. thaliana*, *TaWRKY7* and *TaWRKY40-D*, positively regulate senescence (Zhang et al. 2016; Zhao et al. 2020). Similarly, members of *Gossypium hirsutum*, *GhWRKY17*, *GhWRKY42,* and *GhWRKY27,* promote leaf senescence, whereas *GhWRKY91* represses the process when overexpressed in transgenic *A. thaliana* lines (Gu et al. 2018a, 2018b, 2019a, b). Finally, CpWRKY71 of the ornamental Wintersweet (*Chimonanthus praecox*), causes early leaf senescence when overexpressed in *A. thaliana* (Huang et al. 2019). All of them increase their expression during the progression of leaf senescence, suggesting 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 *A. thaliana* and wallflower, show different but also shared expression patterns 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 *WRKY* gene expression 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 (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, 2020; Ge et al. 2019).

Draft genomes have been recently published for various ornamental plants, including *Dianthus caryophyllus*, *Prunus*

mume, *Rosa chinensis*, *Chrysanthemum seticuspe*, among others (Zhang et al. 2012; Yagi et al. 2014; Hibrand Saint-Oyant et al. 2018; Song et al. 2018; Zheng et al. 2021). However, comparison studies of senescence programs between leaves and petals are scarce, and only minor efforts have been made to study any 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. 2018; Wang et al. 2014, 2018, 2020; Ge et al. 2019).

Petunia hybrida is one of the most popular ornamental crops in the floriculture market. It has been used for many years as a model plant for diverse genetic studies (Vandenbussche et al. 2016; USDA 2019). The recently published draft genomes of *P. axillaris* and *P. inflata*, the parental species of *P. hybrida*, makes petunia a renewed genus to study plant biology (Bombarely et al. 2016; Vandenbussche et al. 2016). Unlike Arabidopsis, petunia represents a more suitable model plant for studying similarities and differences between leaf and petal senescence processes. Here, we performed a detailed expression analysis of different *WRKY* genes in *P. hybrida* (*PhWRKYs*) during the progression of three senescence programs 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

Seeds of the cultivar 'F1 Ultra™ White' of *Petunia hybrida* (Syngenta Flowers Inc.) were germinated in petri dishes containing moistened paper filter. Seedlings were then transplanted into pots of 10 cm diameter (one seedling per pot) that contained a wet commercial substrate (Grow Mix, Terrafértil, Argentina). In all experiments, plants were grown under long-day conditions (16-h light and 8-h darkness) at 240 $\mu molm^{-2}\ s^{-1}$ of fluorescent white light (TLD 36 W/830, Philips, France) and constant temperature (20 °C). Fertilization was applied through sub-irrigation (Hakaphos® Rojo, COMPO, Spain). Positional effects inside the chamber were minimized by regularly changing the location of the pots. Leaves of the 11^{th} position on the shoot (counting from the base), of a total of 14 rosette leaves per plant in average, were used to analyze natural or age-related leaf senescence. When primordia of the leaf 11 of each plant reached a length of ~0.5 cm, we followed the evaluation of leaf area through image captures. Under our experimental conditions full leaf expansion was reached on an average on the 8th day. Leaves were tagged when they reached an approximated area of 70% (~3 days before full expansion). This stage of leaf development represented the first sampling point and was designated day -3. Leaf samples were collected at six different time points (4 h after the start of the light period). The last sampling point was performed at day 33, when 40% of the total leaf area showed signs of yellowing. Three biological replicates of leaf 11 were obtained from each sampling point. Each biological replicate consisted of 3 or 4 leaves, that were obtained from ten randomly selected plants. The experiment consisted of at least 60 plants and was repeated twice. Natural senescence in the corollas was evaluated in the absence of pollination, starting from anthesis (day 0) until they showed symptoms of wilting (day 11). Pollination-induced senescence was evaluated through hand-pollination, starting from anthesis (hour 0) until the corollas showed symptoms of wilting (hour 72). In both experiments, corolla samples were collected at five different time points (8 h after the start of the light period). At each time point, 12 flowers were collected and divided into three biological replicates containing 4 corollas each. Immediately after the harvest of leaf and corolla samples, they were harvested in liquid nitrogen and consequently stored at – 80 ºC.

Identification of *PhWRKY* genes

The sequences of WRKY proteins in *A. thaliana* were obtained from PlantTFDB (http://planttfdb.gao-lab.org/). Redundant and splicing forms were removed from the dataset and sequences were tested for the presence of the WRKY domain by using PFAM (http://pfam.xfam.org/) and Araport (https://www.araport.org/) databases. Twenty-eight *WRKY* genes from *A. thaliana* were selected from published studies on the leaf transcriptome (Buchanan-Wollaston et al. 2005; Wagstaff et al. 2009; Breeze et al. 2011), and public repositories including Leaf Senescence DataBase (https://ngdc.cncb.ac.cn/lsd/) and Arabidopsis eFP Browser (http://bar.utoronto.ca/). We generated a *P. hybrida* transcriptome repository to run BLAST using a previously developed platform (Gonzalez et al. 2017), in which a published leaf transcriptome dataset of *P. hybrida* was loaded (Villarino et al. 2014). By using tBLASTn, putative orthologs were searched in the *P. hybrida* platform and also in the genomes of *P. axillaris* and *P.inflata* at SOL Genomics Network (http://solgenomics.net) (Bombarely et al. 2016). Predicted WRKY proteins were aligned and evaluated for the presence of the WRKY domain using ClustalW tool and BioEdit program (Hall 1999). BLASTP

identified putative orthologs of PhWRKY proteins in other species at the NCBI (https://www.ncbi.nlm.nih.gov/).

Extraction of RNA and analysis of gene expression

Frozen leaf and corolla tissues were grinded with liquid nitrogen and 150 mg of each sample were used to extract total RNA using TRIzol reagent following manufacturer's instructions (Invitrogen, Argentina). The obtained high-quality RNA was treated with DNase I (Invitrogen, Argentina) to eliminate genomic DNA. The concentration of RNA was quantified using a spectrophotometer (Nanodrop ND-1000, NanoDrop Technologies, Wilmington, DE, USA). Agarose gel electrophoresis stained with ethidium bromide were used to determine RNA integrity and the ratio 260/280 nm was used to determine its purity. RNA samples (2 µg each) were reverse-transcribed via random hexamer primers and a commercial Superscript III first strand synthesis kit (Invitrogen, USA). Pairs of primers for quantitative real-time PCR (qPCR) were designed using Beacon designer 6.0 software (Premier Biosoft International, Palo Alto, CA, USA). The list of specific primers are shown in Table S1. Reactions of qPCR were performed in a final volume of 13 µl using a commercial SYBR green mix (Roche Diagnostics, Mannheim, Germany). Each reaction consisted of water (4.75 µl), primers (0.5 µl each at 200 nM), cDNA (1 µl) and FastStart Universal SYBR Green Master (Rox) (6.25 µl). As negative controls, reactions were performed without cDNA template and reverse transcriptase. All reactions were carried out in 96-well plates using a StepOne Plus cycler and v2.3 software (Applied Biosystems, USA). Thermal profile and gene expression analysis were performed as previously described (Trupkin et al., 2019). For each conditions, three biological and two technical replicates were used. *PhEF1a* gene was used as the reference gene since it showed stable and consistent expression throughout leaf and petal samples in *P. hybrida* (Trupkin et al., 2019). Relative expression is shown as the ratio ($\log_2$ scale) between each sampled point relative to the first sampling point and to the expression of the reference *PhEF1a* gene. Expression values were analyzed using one-way ANOVA at $P \leq 0.05$ and Bonferroni post-hoc tests (Table S2). Data was analyzed using Prism 5 software (GraphPad Software, La Jolla, CA, USA).

Clustering of gene expression profiles and heatmap analysis

The *cmeans* function was used to perform the clustering of gene expression profiles (Pal et al. 1996), which is included in the R package ('e1071') and R Core Team (https://www.R-project.org/). The 'heatmaply' R package was used to generate the heatmap (Galili et al. 2018).

Functional group classification of WRKY proteins

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 JTT model was selected as best-fitting amino-acid substitution model using ProtTest v3.4 software (Abascal et al. 2005). A neighbour-joining tree was constructed using MEGA5 software (https://www.megasoftware.net/). Bootstrap values were calculated for 1000 iterations. The phylogenetic tree was visualized using Figtree software (http://tree.bio.ed.ac.uk/). The identification of conserved motifs and sequence logos were performed using full-length amino-acid sequences of WRKY proteins via MEME program (https://meme-suite.org/meme/). Parameters used were as described by You et al. (2015).

Identification of senescence-associated *cis*-regulatory elements

The -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 (https://solgenomics.net/). The *cis*-regulatory elements in the promoters was analyzed using the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). Putative *cis*-regulatory elements associated with senescence were those reported in literature.

Results and discussion

Identification of senescence-associated *WRKY* genes in petunia

Differentially expressed *WRKY* genes of *A. thaliana* during the progression of leaf senescence were searched in order to identify potential regulators of senescence in *P. hybrida* (see Materials and Methods). Twenty-eight genes, representing approximately 36% of the total members in the family were selected for their consistent upregulation, seven of which were reported to regulate leaf senescence in *A. thaliana* (Table 1). These proteins did not show a particular clustering and were distributed within the phylogenetic groups I, IIb, IIc, IId, IIe, and III according to Eulgem et al. (2000) (Table 1). Thirteen genes (~47%) showed expression

Table 1 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, petals and function in leaf senescence. Classification of genes was based on their subfamily classification. Using a transcriptomic leaf dataset of *P. hybrida* (Villarino et al. 2014) loaded in a recently created platform (Gonzalez et al., 2017) putative orthologs of *A. thaliana* genes in *P. hybrida* (*PhWRKYs*) were identified via tBLASTn. Asterisks (*) indicate genes of Arabidopsis with a reported function in leaf senescence. Partial sequences of *P. hybrida* were indicated with the letter P. Early-, mid- or late-changes in expression were indicated with letters E, M and L, respectively. 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 (https://ngdc.cncb.ac.cn/lsd/); (4) Arabidopsis eFP browser (http://bar.utoronto.ca/); (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. 2017b. Abbreviations: Arabidopsis Genome Initiative (AGI), data not available (n/a)

changes at early- (E) senescence stage, 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 were not publicly available.

In addition, we assessed the expression changes of selected *A. thaliana WRKY* genes in the petals at two different developmental stages (Table 1): stage 12 just before flower opening and stage 15 when flowers are opened and pollinated. 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 any clear expression changes (Table 1). These observations indicate that most *AtWRKY* genes are upregulated during the early stages of leaf development and that almost all genes that were upregulated in the leaves were also upregulated in the corollas (Table 1).

To identify putative orthologs of these senescence-associated WRKYs in petunia, protein sequences of *AtWRKY* genes were used to perform BLAST searches using a public transcriptomic leaf database of *Petunia hybrida* (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 tBLASTn (Table 1, Table S3). In general, each AtWRKY 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 (Table 1). This observation might be explained by the use of transcriptomic leaf database instead of genome sequences to search the equivalent proteins in petunia. Since genomic sequences of *P. hybrida* were not available, the best source was represented by a public transcriptomic leaf database of *P. hybrida* published by Villarino et al. 2014. Similar BLAST searches were conducted using the draft genomes of the parental species of *P. hybrida* (Table S3). Interestingly, total number of WRKY members retrieved from *P. axillaris* and *P. inflata* were similar, 19 and 20, respectively (Table S3). The recovered sequences from these species showed similar scores and e-values to the obtained sequences of *P. hybrida* (Table S3). Total number of WRKYs among different species is notably variable, including those within the Solanaceae family (Cheng et al. 2019). This fact could be explained by reduced number of WRKYs identified in *P. hybrida* when compared to *A. thaliana*. In accordance, a total of 81 members were identified in *Solanum lycopersicum* and *Solanum tuberosum*, while a significant lower number (65) was identified in *Capsicum annuum* (Cheng et al. 2019). Moreover, 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 datasets and genome databases, we identified several putative WRKY orthologs in petunia. The identity values for most of the genes were above 50%, which suggest that proteins of *A. thaliana* and petunia possess a high level of conservation (Table 1, Table S3).

Phylogenetic classification of PhWRKY proteins

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

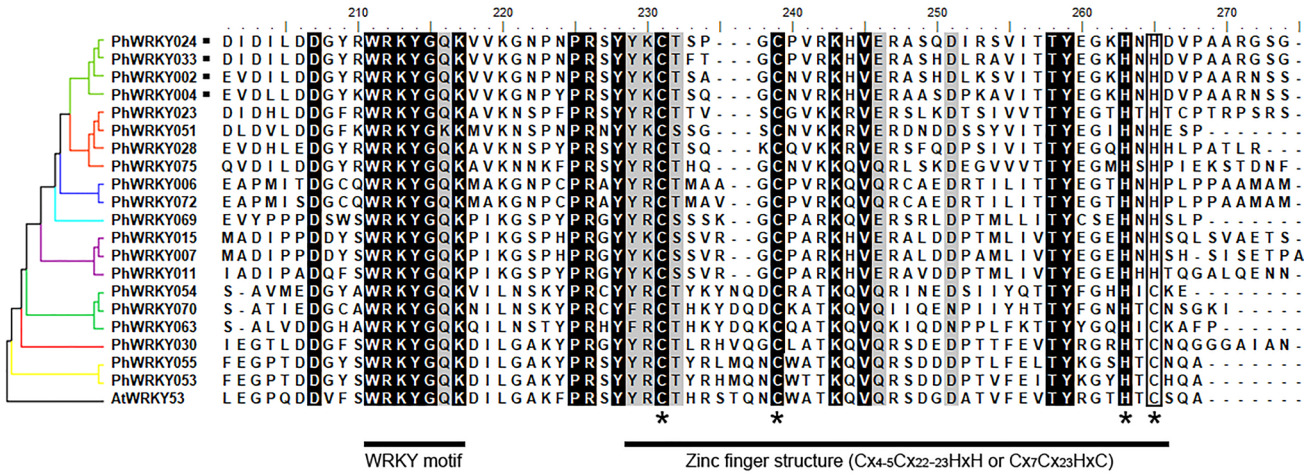

Fig. 1 Multiple sequence alignment of PhWRKY proteins. The highly conserved residues spanning the WRKY domain in the 20 proteins of *P. hybrida* were aligned using ClustalW tool. Highly conserved amino acid residues are showed with black and grey backgrounds. Asterisks (*) and the rectangle indicate 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 was included as a reference

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

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	Peaxi162S-cf00232g00810.1	Peinf-101Scf00055g17013.1
PhWRKY004	I	2	WRKYGQK/ WRKYGQK	Cx4Cx22HxH/ Cx4Cx23HxH	C2H2	Peaxi162S-cf00222g00117.1	Peinf-101Scf00231g01029.1
PhWRKY024	I	2	WRKYGQK/ WRKYGQK	Cx4Cx22HxH/ Cx4Cx23HxH	C2H2	Peaxi162S-cf00055g01910.1	Peinf-101Scf00450g00007.1
PhWRKY033	I	2	WRKYGQK/ WRKYGQK	Cx4Cx22HxH/ Cx4Cx23HxH	C2H2	Peaxi162S-cf00744g00220.1	Peinf-101Scf00782g10028.1
PhWRKY006	IIb	1	WRKYGQK	Cx5Cx23HxH	C2H2	Peaxi162S-cf00007g00315.1	Peinf-101Scf01579g02011.1
PhWRKY072	IIb	1	WRKYGQK	Cx5Cx23HxH	C2H2	Peaxi162S-cf00178g01110.1	Peinf-101Scf01200g03007.1
PhWRKY023	IIc	1	WRKYGQK	Cx4Cx23HxH	C2H2	Peaxi162S-cf00164g01010.1	Peinf-101Scf00244g18025.1
PhWRKY028	IIc	1	WRKYGQK	Cx4Cx23HxH	C2H2	Peaxi162S-cf01189g00009.1	Peinf-101Scf00040g09006.1
PhWRKY051	IIc	1	*WRKYGKK*	Cx4Cx23HxH	C2H2	Peaxi162S-cf00106g01616.1	Peinf-101Scf00381g17007.1
PhWRKY075	IIc	1	WRKYGQK	Cx4Cx23HxH	C2H2	Peaxi162S-cf00128g01541.1	Peinf-101Scf00889g03041.1
PhWRKY007	IId	1	WRKYGQK	Cx5Cx23HxH	C2H2	Peaxi162S-cf00121g00018.1	Peinf-101Scf01179g02021.1
PhWRKY011	IId	1	WRKYGQK	Cx5Cx23HxH	C2H2	Peaxi162S-cf00459g00841.1	Peinf-101Scf00276g07026.1
PhWRKY015	IId	1	WRKYGQK	Cx5Cx23HxH	C2H2	Peaxi162S-cf00549g00222.1	Peinf-101Scf00887g05031.1
PhWRKY069	IIe	1	WRKYGQK	Cx5Cx23HxH	C2H2	Peaxi162S-cf00469g00624.1	Peinf-101Scf00442g03028.1
PhWRKY030	III	1	WRKYGQK	Cx7Cx23HxC	C2HC	Peaxi162S-cf00904g00212.1	Peinf-101Scf01632g03025.1
PhWRKY053	III	1	WRKYGQK	Cx7Cx23HxC	C2HC	Peaxi162S-cf00102g01741.1	Peinf-101Scf02382g03038.1
PhWRKY055	III	1	WRKYGQK	Cx7Cx23HxC	C2HC	Peaxi162S-cf00102g01741.1	Peinf-101Scf00962g23035.1
PhWRKY054	III	1	WRKYGQK	Cx7Cx23HxC	C2HC	Peaxi162S-cf00304g00719.1	Peinf-101Scf00339g02023.1
PhWRKY063	III	1	WRKYGQK	Cx7Cx23HxC	C2HC	Peaxi162S-cf00732g00236.1	Peinf-101Scf00782g02035.1
PhWRKY070	III	1	WRKYGQK	Cx7Cx23HxC	C2HC	Peaxi162S-cf00073g02335.1	Peinf-101Scf00191g46015.1

investigate their phylogenetic relationships (Fig. 1). The conserved WRKY signatures and zinc-finger domains 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). The group I consisted of four proteins with two WRKY domains and the C_2H_2-type zinc-finger structure ($Cx_4Cx_{22}HxH/Cx_4Cx_{23}HxH$). The group II consisted of 10 proteins 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 divided into four subgroups: II-b (2), II-c (4), II-d (3), and II-e (1). The group III consisted of six proteins 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 WRKY members. 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* (Fig. S1). Findings in PhWRKYs

were in accordance with previous works describing the WRKY family in different species of the Solanaceae family, including *Solanum melongena*, *S. tuberosum*, *S. lycopersicum* and *C. annuum* (Huang et al. 2012; Yang et al. 2015; Cheng et al. 2016, 2019; Zhang et al. 2017) and species of other families like *A. thaliana*, *Daucus carota*, *O. sativa* 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 during natural leaf senescence identified fifteen differentially expressed *PhWRKYs*

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 Material and Methods). Seventeen out of 20 genes were detected during the natural progression of leaf senescence, while three genes were undetected (*PhWRKY011*, *PhWRKY023* and *PhWRKY051*) (Fig. 2). The 17 genes detected in the leaves, were classified into six groups according to their expression profiles (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 since they could regulate senescence from the very early stages (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

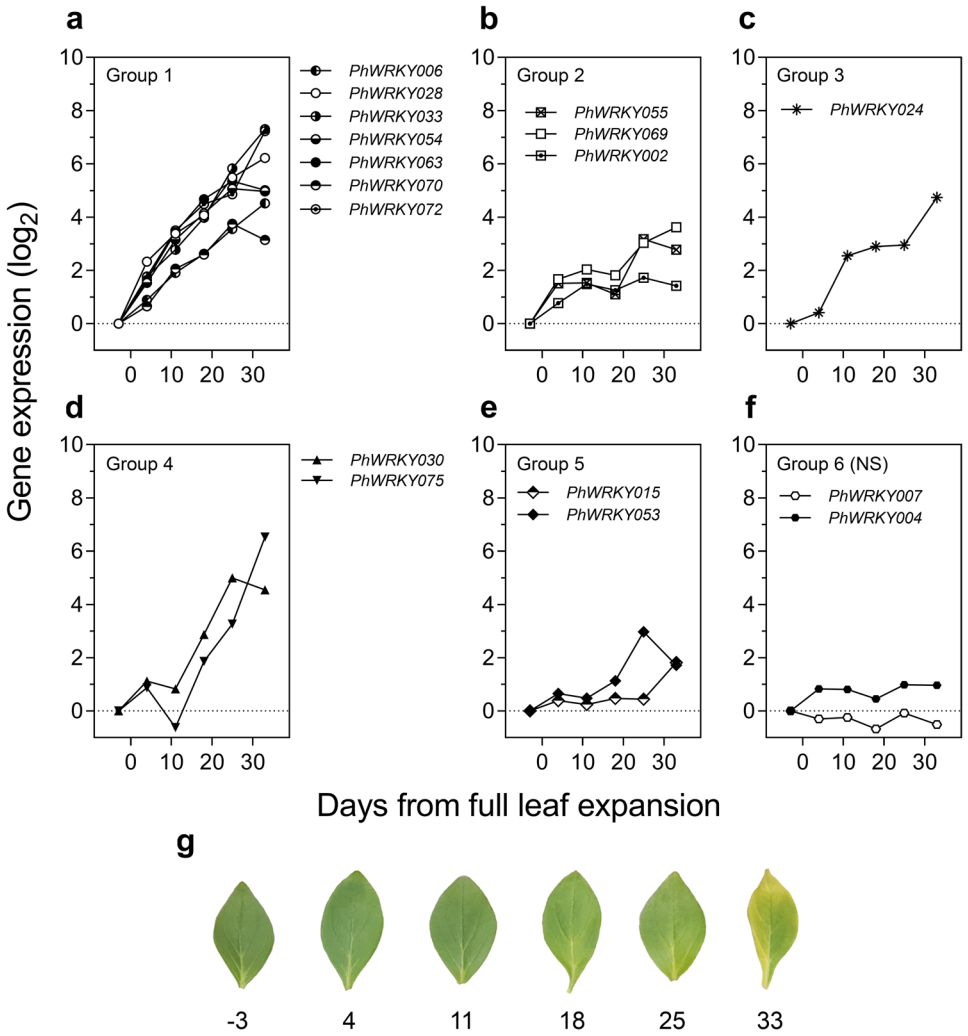

Fig. 2 Expression profiles of *PhWRKY* genes during natural leaf senescence. **a-f** Expression groups of 17 *PhWRKY* genes in the leaves at various times after full leaf expansion. **g** Representative images of the leaves at different time points (days from full leaf expansion). Expression values were analyzed using one-way ANOVA at $P \leq 0.05$ (Bonferroni post tests). For better visualization error bars are not shown

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, *PhWRKY004* and *PhWRKY007* in group 6, did not show significant changes in their expression (Fig. 2f). Genes of groups 1–4 represent very good candidates since they changed significantly their expression from early- and mid- senescence. The expression of 15 *PhWRKYs* increased during leaf senescence. It revealed similar profiles when compared to their equivalents of *A. thaliana*, although *PhWRKY072* (early) and *PhWRKY075* (late) showed putative orthologs with opposing expression profiles (Table 1, Table S2, Fig. 2).

Members of the WRKY family were associated with natural leaf senescence in many species, including *O. sativa* (Han et al. 2014), *T. aestivum* (Zhang et al. 2016), *G. hirsutum* (Gu et al. 2019a, b), *Helianthus annuus* (Moschen et al. 2019), *Vitis vinifera* (Wang et al. 2014), *Medicago sativa* (Yuan et al. 2020), among others. Putative orthologs of *PhWRKY030*, *PhWRKY063*, *PhWRKY070*, *PhWRKY054*, *PhWRKY072* and *PhWRKY006* are also upregulated during leaf senescence in *T. aestivum* (Zhang et al. 2016). In *H. annuus*, putative orthologs of *PhWRKY030*, *PhWRKY072* and *PhWRKY006* were similarly upregulated during leaf senescence in an early senescence line (R453), whereas putative orthologs of *PhWRKY070* and *PhWRKY033* were downregulated, showing opposing expression profiles during leaf senescence (Moschen et al. 2019). Moreover, the putative orthologs of *PhWRKY075* was also upregulated during natural leaf senescence in *V. vinifera* (Wang et al. 2014). Overall, evidence suggests a conserved role for this family in 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 during natural and pollination-induced corolla senescence identified fifteen and twelve differentially expressed *PhWRKYs*

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; Wang et al. 2018).

To investigate the participation of WRKY members during natural and pollination-induced corolla senescence, we measured in the petals the expression profiles of the 17 *PhWRKY* genes detected in leaves (Figs. 3 and 4). During the progression of natural corolla senescence, two genes were not detected (*PhWRKY064* and *PhWRKY069*) and the remaining 15 genes were classified into six groups according to their expression profiles (Fig. 3). Groups 1 and 3 contained only one gene each, *PhWRKY030*, 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). 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). Genes of groups 1 and 3 represent interesting candidates since they showed earliness and high values in their expression changes. Genes of group 2 are also well ranked due to their early expression changes and the maintenance of their expression during mid- senescence. Despite group 4 possesses two genes with late expression, they substantially changed the magnitude of expression and could be considered candidates (Fig. 3).

During the progression of pollination-induced corolla senescence,12 of the 17 *PhWRKYs* detected in the leaves showed changes in their gene expression. The expression of *PhWRKY030*, *PhWRKY054*, *PhWRKY055*, *PhWRKY069* and *PhWRKY075* was not detected (Fig. 4, Table S2). Group 1 contained four genes, three of them were upregulated in mid- senescence (*PhWRKY006*, *PhWRKY028* and *PhWRKY033*) and the remaining gene was upregulated earlier at 6 h after pollination (hap) (*PhWRKY072*). All these genes showed moderate changes in expression (Fig. 4a). Group 2 contained five genes with weak upregulation during late- senescence (Fig. 4b). Interestingly, group 3 contained three genes downregulated from an early stage (6 hap) (*PhWRKY024*, *PhWRKY053* and *PhWRKY070*), and maintained their expression in the subsequent time points (Fig. 4c). Overall, genes of group 1 represent the best candidates identified in pollination-induced corolla senescence. The genes of group 3 may be considered interesting candidates since they showed early and opposing expression profiles in comparison to those observed in the other two senescence process. In *A. thaliana*, the type of senescence occurring in the petals of the flowers is pollination-induced petal senescence (Wagstaff et al., 2009). Comparative expression analysis of *WRKY* genes between leaf and pollination-induced petal

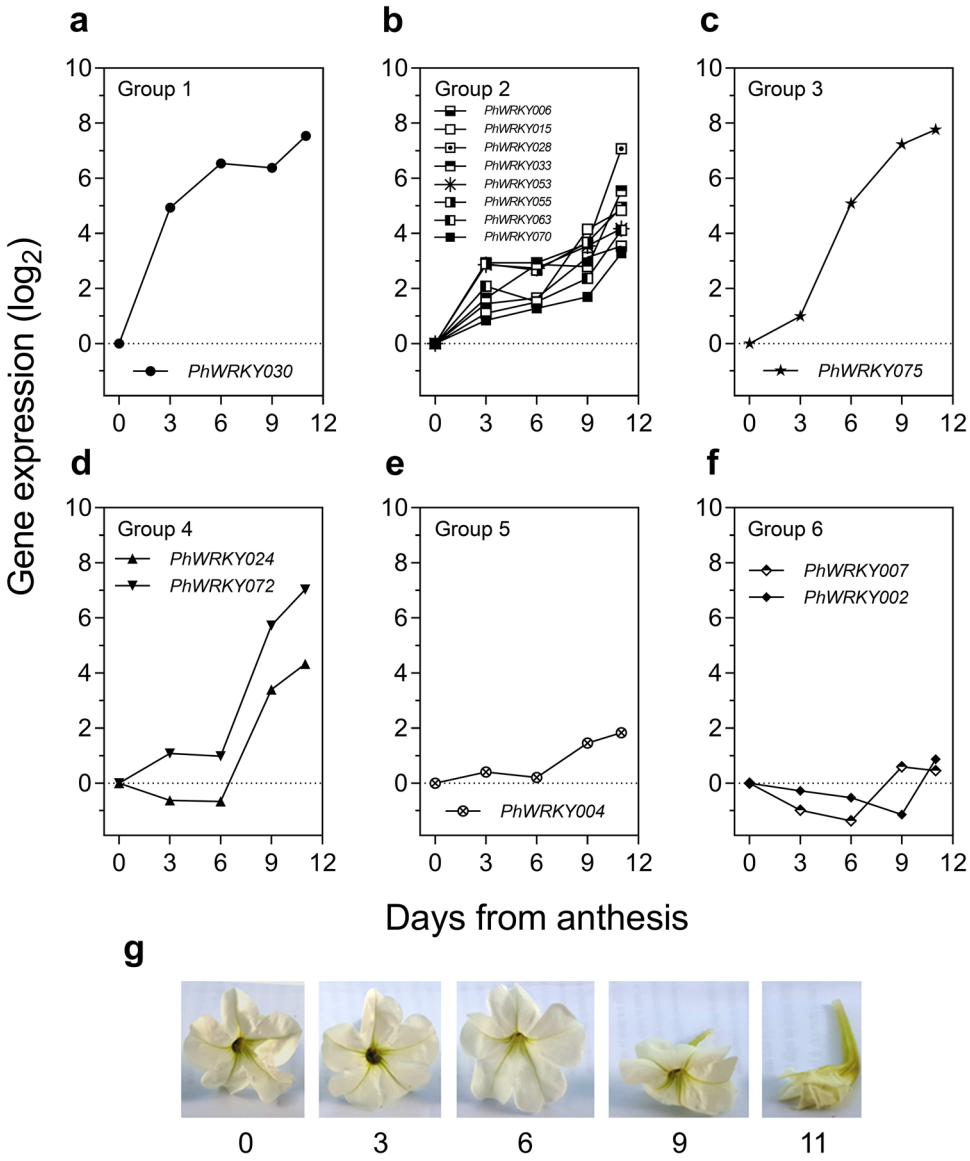

Fig. 3 Expression profiles of *PhWRKY* genes during natural corolla senescence. **a-f** Expression groups of 15 *PhWRKYs* in unpollinated corollas at various times after anthesis. **g** Representative images of the flowers at different time points (days from anthesis). Expression values were analyzed using one-way ANOVA at $P \leq 0.05$ (Bonferroni post tests). For better visualization error bars are not shown

senescence revealed that the proportion of genes expressed in both organs was higher in *A. thaliana* 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 show any significant change (18%). In *P. hybrida*, of the 15 *PhWRKY* genes upregulated in 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, 4). Moreover, different expression profiles were observed for several *PhWRKYs* when compared to the corresponding *A. thaliana* 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 3). For example, several genes (*PhWRKY024*, *PhWRKY028*, *PhWRKY030*, *PhWRKY053*, *PhWRKY072*, and *PhWRKY075*) showed increased 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 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* would have minor influence

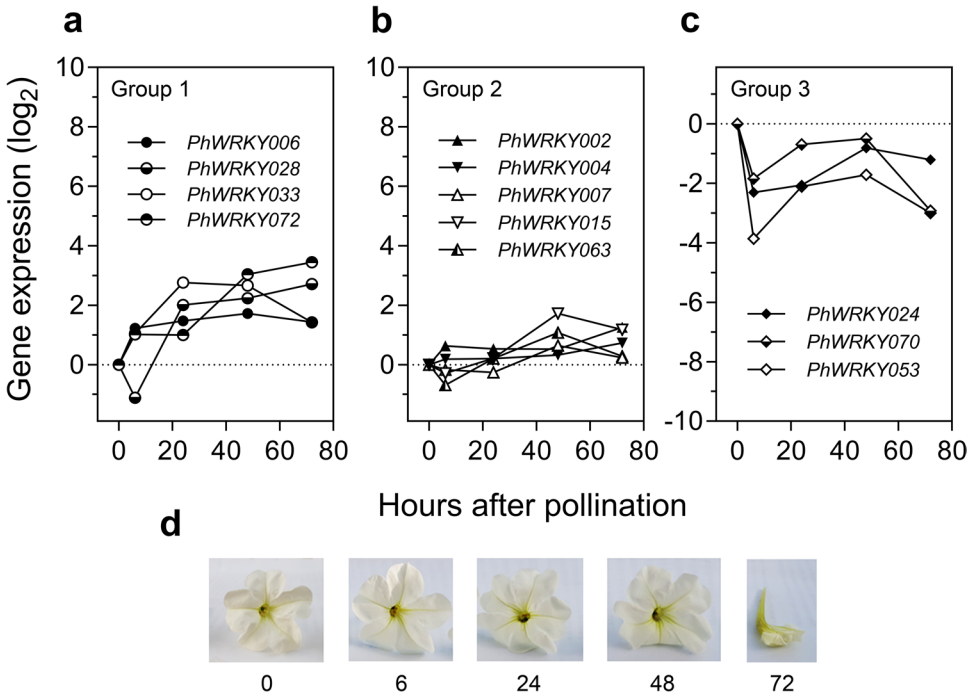

Fig. 4 Expression profiles of *PhWRKY* genes during pollination-induced corolla senescence. **a-c** Expression groups of 12 *PhWRKY* genes in corollas at various times after pollination (hours). **d** Representative images of the flowers at different time points. Expression values were analyzed using one-way ANOVA at $P \leq 0.05$ (Bonferroni post-hoc tests). For better visualization error bars are not shown

(Langston et al. 2005; Broderick et al. 2014; Wang et al. 2018). Interestingly, *A. thaliana* 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 *A. thaliana* increased their expression in petals (Table 1). Therefore, our findings suggest similarities but also discrepancies between *P. hybrida* and *A. thaliana* in the regulation of senescence processes, mainly during pollination-induced senescence.

WRKY members have been reported to change their expression during petal senescence in different ornamental plants, including ethylene-sensitive and insensitive species, such as *Erysimum linifolium* (Price et al. 2008), *Gardenia jasminoides* (Tsanakas et al. 2014), or *Astilbe* × *arendsii* Arends (Yamazaki et al. 2020). In *E. linifolium* two *WRKY* genes increase their expression in old petals (Price et al. 2008). These genes are putative orthologs of *PhWRKY015* and *PhWRKY075* genes, which increased considerably their expression during late- and mid- natural corolla senescence, respectively (Fig. 3). In *G. jasminoides* an ethylene-insensitive species, the WRKY family members showed a high number of members differentially expressed in the petals (Tsanakas et al. 2014). In *Astilbe* × *arendsii* Arends, another ethylene-insensitive species, *WRKY22* increased its expression in florets of cut inflorescences. This gene is a putative ortholog of *PhWRKY069*, although it was not detected in the corollas (Figs. 3 and 4). Interestingly, in *Hibiscus rosa-sinensis*, an ornamental plant with ephemeral flowers, five *WRKY* genes were upregulated in senescing petals (Trivellini et al. 2016). Three of them are related and are putative orthologs of *PhWRKY006* gene, which increased substantially its expression during natural and pollination-induced senescence (Figs. 3 and 4). One gene is a putative ortholog of *PhWRKY004* gene, which also increased its expression in both petal senescence processes (Figs. 3 and 4); and one gene did not match with any of the identified genes in *P. hybrida* (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 appeared to be homologous to *PhWRKY002*, *PhWRKY006* and *PhWRKY007* in our expression analysis. Interestingly, the putative homologs of *PhWRKY002* and *PhWRKY006* were 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 were upregulated, eight were downregulated for the first two days after anthesis (early- senescence), and three were upregulated between the second and fourth day (mid- and 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 matched with the *PhWRKYs* identified here. Our results and previous works suggest that WRKY members would have important roles in the regulation of petal senescence in *P. hybrida* and other species.

Functional WRKY classification and motif analyses

Gene expression profiles of the three senescence processes studied were analyzed simultaneously retrieving four main clusters (Fig. 5). Global visualization showed that some *PhWRKYs* could act as regulatory factors in both leaf and corolla senescence processes (Fig. 5). Expression of *WRKY* genes in both organs has also been observed in other species like *A. thaliana* and *E. linifolium*, 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). Moreover, most *PhWRKY* genes were upregulated during early- and mid- natural leaf senescence, while a high proportion of them were upregulated during late- natural corolla senescence, suggesting temporal expression differences between natural senescence processes (Fig. 5).

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 *A. thaliana*, the 20 proteins identified in *P. hybrida*, the putative orthologs of *P. hybrida* proteins in species of the Solanaceae family (*S. lycopersicum*, *S. tuberosum*, and *Nicotiana tomentosiformis*), and several proteins that were reported to regulate leaf senescence in various monocot and dicot species (Fig. 6). To strengthen our analysis, we conducted a conserved motif search using full-length amino acid sequences (Fig. 6, Fig. S2). Both analyses were complementary and helped to define seven major functional groups (I-VII) that contained members of the three WRKY subfamilies (Fig. 6). Interestingly, all functional groups possessed at least one member with a reported function in the regulation of leaf senescence (Fig. 6). Functional group Va did not contain *A. thaliana* members and might be considered a solanaceous specific group. In addition, *cis*-regulatory elements were identified in the promoter sequences of the best homologs in *P. axillaris* or *P. inflata* of each *PhWRKY* gene (Table S4).

In cluster 1, all the genes were upregulated in the three types of senescence (Fig. 5), representing the most interesting genes characterized in this work (Fig. 5, Table 3). *PhWRKY028* and *PhWRKY072* were upregulated in early- and *PhWRKY033* in mid- leaf senescence, and all 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 *A. thaliana* simultaneously increased their expression during leaf and petal development (Table 1), and a putative homolog of *PhWRKY033* showed an upregulation tendency in the corollas of petunia flowers at 24 hap (Broderick et al. 2014), which is in agreement with our results (Figs. 4a and 5). The other genes in cluster 1, *PhWRKY006*, *PhWRKY015* and

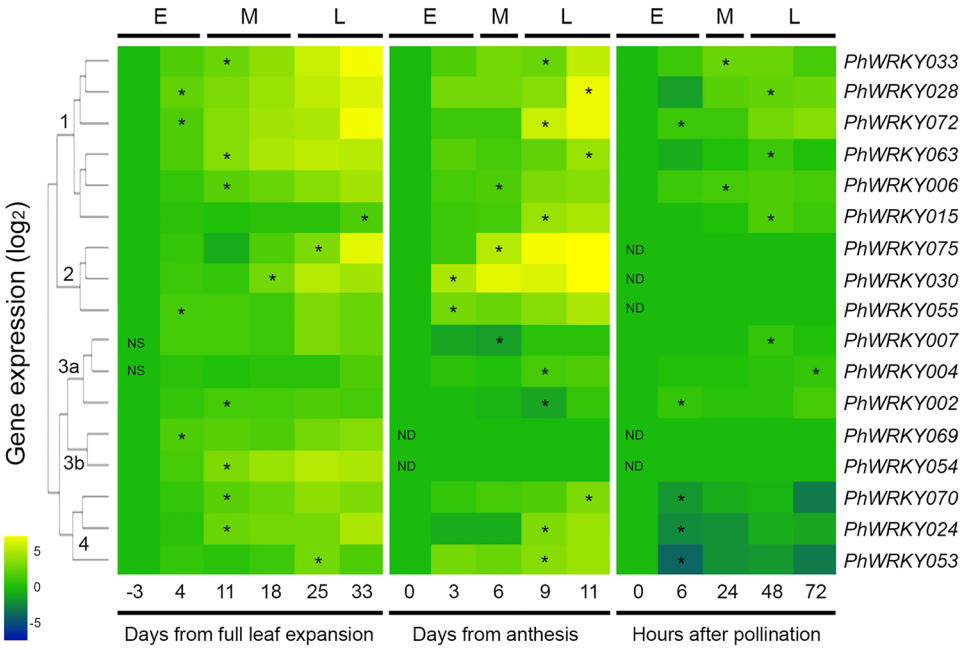

Fig. 5 Heatmap analysis and hierarchical clustering of *PhWRKY* expression profiles throughout the three senescence processes. The color scale indicates the relative transcript levels. Asterisks (*) indicate the time of initial significant expression change respect to the first sampling point. Early-, mid-, and late- stages of senescence are depicted with E, M, and L, respectively. NS, non-significant; ND, non-detected

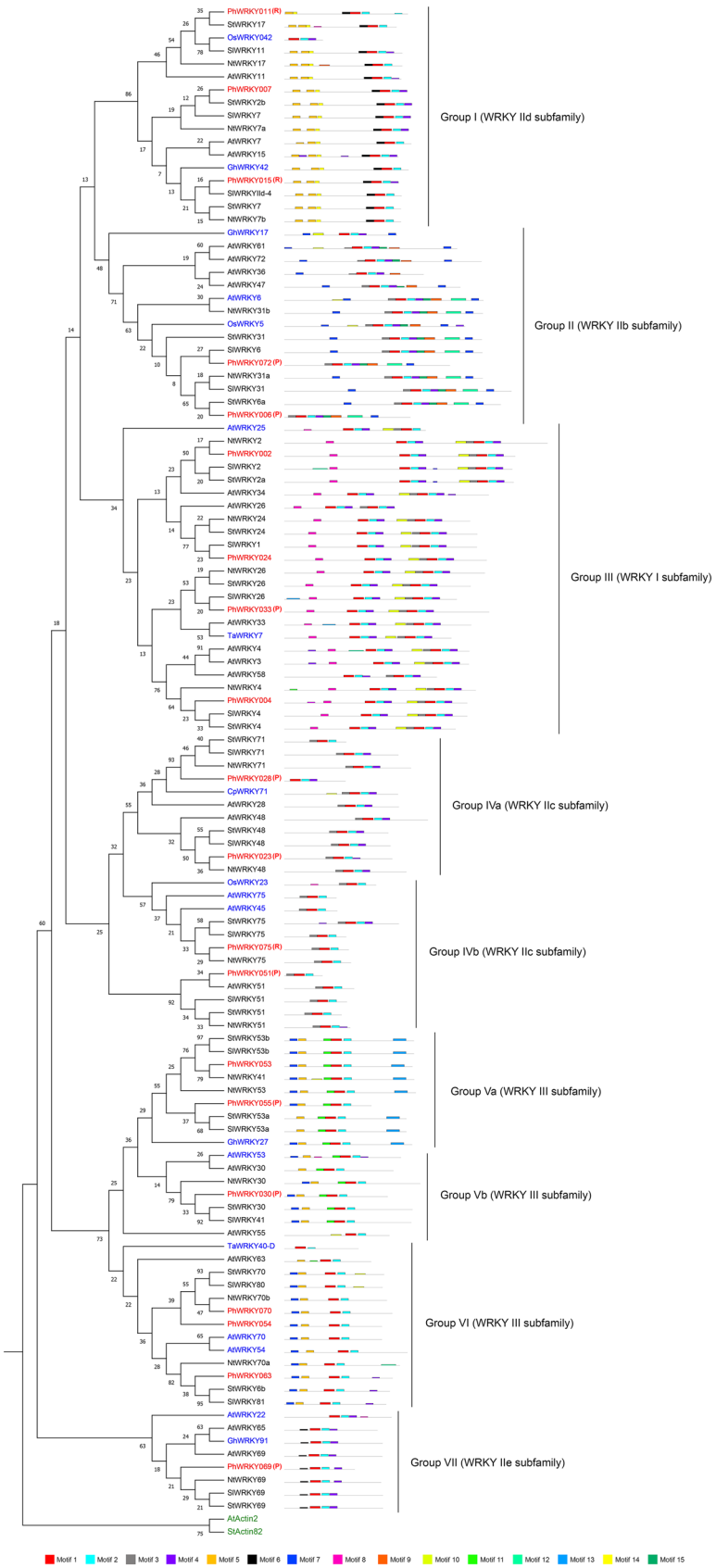

Fig. 6 Phylogenetic analysis and motif composition of PhWRKYs and homologs from other species. Multiple sequence alignment of the conserved region of 117 proteins, spanning the WRKY amino-acid sequence, was done using ClustalW. The phylogenetic tree was constructed by the Neighbor-joining method using MEGA5. 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 and represented as coloured boxes. 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. Classification in subfamilies was based on a previously proposed classification system (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 Table S5

Table 3 Summary 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*	Increased expression in *T. aestivum* and *A. thaliana*	Overexpression of *TaWRKY7* in *A. thaliana* promotes leaf senescence *AtWRKY25* delays leaf senescence in *A. thaliana*	Zhang et al. (2016) and Doll et al. (2020)
	PhWRKY028	IVa	IIc	*CpWRKY71*	Increased expression in *C. praecox*	Overexpression of *CpWRKY71* in *A. thaliana* promotes leaf senescence	Huang et al. (2019)
	PhWRKY072	II	IIb	*OsWRKY5*; *AtWRKY6*; *GhWRKY17*	Increased expression in *O. sativa*, *A. thaliana*, and *G. hirsutum*	OsWRKY5 promotes leaf senescence in *O. sativa* AtWRKY6 promotes leaf senescence in *A. thaliana* Overexpression of *GhWRKY17* in *A. thaliana* promotes leaf senescence	Kim et al. (2019), Robatzek and Somssich (2002) and Gu et al. (2018a)
	PhWRKY006	II	IIb				
	PhWRKY063	VI	III	*AtWRKY54*; *AtWRKY70*; *TaWRKY40-D*	Increased expression in *A. thaliana* and *T. aestivum*	AtWRKY54 and AtWRKY70 delay leaf senescence in *A. thaliana* TaWRKY40-D promotes leaf senescence in *T. aestivum*	Ülker et al. (2007), Besseau et al. (2012) Zhao et al. (2020)
	PhWRKY015	I	IId	*OsWRKY42*; *GhWRKY42*	Increased expression in *O. sativa* and *G. hirsutum*	OsWRKY42 promotes leaf senescence in *O. sativa* Overexpression of *GhWRKY42* in *A. thaliana* promotes leaf senescence	Han et al. (2014) and Gu et al. (2018b)
Upregulation in natural leaf and petal senescence (Cluster 2)	*PhWRKY075*	IVb	IIc	*AtWRKY75*; *AtWRKY45*; *OsWRKY23*	Increased expression in *A. thaliana* and *O. sativa*	AtWRKY75 and AtWRKY45 promote leaf senescence in *A. thaliana* OsWRKY23 promotes dark-induced leaf senescence in *A. thaliana*	Chen et al. (2017), Li et al. (2012) and Jing et al. (2009)
	PhWRKY030	Vb	III	*AtWRKY53*	Increased expression in *A. thaliana*	AtWRKY53 promotes leaf senescence in *A. thaliana*	Miao et al. (2004)
	PhWRKY055	Va	III	*GhWRKY27*	Increased expression in *G. hirsutum*	Overexpression of *GhWRKY27* in *A. thaliana* promotes leaf senescence	Gu et al. (2019a)

Table 3 (continued)

Petunia hybrida				Putative orthologs in other species			
Expression category	Gene	Functional group	Subfamily	Gene	Expression during leaf senescence	Function in leaf senescence	References
Unclear (Cluster 3a)	*PhWRKY007*	I	IId	*OsWRKY42*; *GhWRKY42*	Increased expression in *O. sativa* and *G. hirsutum*	OsWRKY42 promotes leaf senescence in *O. sativa* Overexpression of *GhWRKY42* in *A. thaliana* promotes leaf senescence	Han et al. (2014) and Gu et al. (2018b)
	PhWRKY002	III	I	*TaWRKY7*; *AtWRKY25*	Increased expression in *T. aestivum* and *A. thaliana*	Overexpression of *TaWRKY7* in *A. thaliana* promotes leaf senescence AtWRKY25 delays leaf senescence in *A. thaliana*	Zhang et al. (2016) and Doll et al. (2020)
	PhWRKY004	III	I				
Upregulation in natural leaf senescence (Cluster 3b)	*PhWRKY054*	VI	III	*AtWRKY54*; *AtWRKY70*; *TaWRKY40-D*	Increased expression in *A. thaliana* and *T. aestivum*	AtWRKY54 and AtWRKY70 delay leaf senescence in *A. thaliana* TaWRKY40-D promotes leaf senescence in *T. aestivum*	Ülker et al. (2007), Besseau et al. (2012), Zhao et al. (2020)
	PhWRKY069	VII	IIe	*AtWRKY22*; *GhWRKY91*	Increased expression in *A. thaliana* and *G. hirsutum*	AtWRKY22 promotes dark-induced leaf senescence in *A. thaliana* Overexpression of *GhWRKY91* in *A. thaliana* 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*	Increased expression in *A. thaliana* and *T. aestivum*	AtWRKY54 and AtWRKY70 delay leaf senescence in *A. thaliana* TaWRKY40-D promotes leaf senescence in *T. aestivum*	Ülker et al. (2007), Besseau et al. (2012)and Zhao et al. (2020)
	PhWRKY024	III	I	*TaWRKY7*; *AtWRKY25*	Increased expression in *T. aestivum* and *A. thaliana*	Overexpression of *TaWRKY7* in *A. thaliana* promotes leaf senescence AtWRKY25 delays leaf senescence in *A. thaliana*	Zhang et al. (2016) and Doll et al. (2020)
	PhWRKY053	Va	III	*GhWRKY27*	Increased expression in *G. hirsutum*	Overexpression of *GhWRKY27* in *A. thaliana* promotes leaf senescence	Gu et al. (2019a)

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

PhWRKY063 showed more attenuated expression changes in natural leaf and corolla senescence and similar expression in pollination-induced senescence (Fig. 5). Putative orthologs in *A. thaliana* 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 results (Fig. 5; Broderick et al. 2014; Wang et al. 2018). Phylogenetic analysis showed that PhWRKY028 was closely related to CpWRKY71 whose expression increased during leaf senescence progression in *C. praecox*, and its overexpression in transgenic *A. thaliana* plants accelerates leaf senescence (Fig. 6, Table 3) (Huang et al. 2019). PhWRKY033 is a putative ortholog of *T. aestivum* TaWRKY7 that positively regulate leaf senescence when it is overexpressed in *A. thaliana* (Fig. 6, Table 3) (Zhang et al. 2016; Doll et al. 2020). PhWRKY006 and PhWRKY072 are putative orthologs of OsWRKY5 and AtWRKY6, and are more distantly related to GhWRKY17. All these members increase their expression in *O. sativa*, *A. thaliana*, and *G. hirsutum*, respectively, and promote leaf senescence in *O. sativa* and *A. thaliana*. Moreover, heterologous expression of *GhWRKY17* in *A. thaliana* 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 regulation of senescence. PhWRKY015 was closely related to GhWRKY42 and more distantly to OsWRKY42 (Fig. 6, Table 3). OsWRKY42 is a positive regulator of leaf senescence in *O. sativa*, whose expression increases during leaf development (Han et al. 2014); whereas GhWRKY42 increases its expression in *G. hirsutum* and promote leaf senescence in transgenic *A. thaliana* plants (Gu et al. 2018b). PhWRKY063 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 of *A. thaliana*, and more distantly related to TaWRKY40-D, a positive regulator of leaf senescence in *T. aestivum*. Thus, PhWRKY063 may function as a repressor of senescence in *P. hybrida* (Ülker et al. 2007; Besseau et al. 2012; Zhao et al. 2020) (Fig. 6, Table 3). 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 (Table S4, Fig. 5) (Zheng et al. 2005; Rinerson et al. 2015; Liu et al. 2016). 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, *PhWRKY030*, *PhWRKY055* and *PhWRKY075*, were upregulated in natural senescence processes occurring in leaves and in the corollas (Fig. 5). Changes in expression were evident relatively early in naturally senescing corollas 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, while the expression profiles of *PhWRKY055* did not stand out (Fig. 5). *PhWRKY075* was classified as the putative ortholog of *AtWRKY45* and *AtWRKY75*, which increase their expression during senescence in leaves and petals (Table 1) and promote leaf senescence in *A. thaliana* (Table 3) (Li et al., 2012; Chen et al. 2017a, b). Another putative ortholog, OsWRKY23, promotes dark-induced leaf senescence when it is overexpressed in *A. thaliana* (Fig. 6, Table 3) (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 *G. hirsutum* GhWRKY27 (subgroup Va) (Fig. 6, Table 3). Expression of *AtWRKY53* and *GhWRKY27* increase during leaf senescence in *A. thaliana* (Miao et al. 2004) and *G. hirsutum* (Gu et al. 2019a), respectively (Tables 1, 3), and their overexpression in *A. thaliana* plants promote leaf senescence (Miao et al. 2004; Gu et al. 2019a) (Table 3). Promoter analysis of genes in cluster 2 showed that they all contain the senescence-associated *cis*-elements (Table S4). Interestingly, the homolog of *PhWRKY075* showed higher 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 with respect to *PhWRKY030* and *PhWRKY075* (Table S4). 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. *PhWRKY004*, *PhWRKY007* and *PhWRKY002* genes represented cluster 3a and *PhWRKY054* and *PhWRKY069* 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). Putative homologs of *PhWRKY007*, *PhWRKY002*, and *PhWRKY004* show weak or erratic expression profiles during corolla senescence in petunia, resembling our results (Fig. 5) (Broderick et al. 2014; Wang et al. 2018). Interestingly, the putative orthologs of these genes in *A. thaliana* 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, Fig. 5). Even though PhWRKYs of cluster 3a was

associated with characterized regulators of senescence, such as AtWRKY25, TaWRKY7, GhWRKY42 and OsWRKY42 (Fig. 6, Table 3), their unstable expression profiles in the three types of senescence suggest they are not good candidates for senescence regulation in petunia (Figs. 5 and 6). Genes in cluster 3b, PhWRKY054 and PhWRKY069, were leaf specific and showed intermediate changes in expression in mid- and early- leaf senescence, respectively (Fig. 5). Interestingly, the putative orthologs of PhWRKY054 and PhWRKY069 in *A. thaliana* increased their expression during pollination-induced corolla senescence (Table 1, Fig. 6), suggesting a different organ regulation for these members between the two species. PhWRKY054 was closely related to the negative regulators, AtWRKY54 and AtWRKY70 (Table 3) (Ülker et al. 2007; Besseau et al. 2012), and more distantly related with the positive regulator, TaWRKY40-D. PhWRKY069 was closely related to the negative regulator of *G. hirsutum*, GhWRKY91, which represses leaf senescence in *A. thaliana* when it is overexpressed (Gu et al. 2019b), and more distantly related to AtWRKY22, which regulates dark-induced leaf senescence in *A. thaliana* (Zhou et al. 2011). Analysis of *cis*-elements in the genes of cluster 3 revealed lack of W-boxes in the parental homologs of *PhWRKY069*, *PhWRKY004* and *PhWRKY007*, G-boxes in the parental homolog of *PhWRKY002* and the lack of three types of elements in the homolog of *PhWRKY054* (Table S4), suggesting the importance of all senescence-associated regulatory elements (ABRE, W-box and G-box) for consistent expression of *WRKY* genes in different senescence processes. Taken together, information suggests that, unlike genes in cluster 3a, *PhWRKY069* and *PhWRKY054* of cluster 3b could be considered candidates only for leaf senescence regulation in *P. hybrida*.

Finally, cluster 4 contained three genes, *PhWRKY024*, *PhWRKY053* and *PhWRKY070*, which showed upregulation during natural leaf and corolla senescence and downregulation starting from a very early stage in 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 decrease in early- natural corolla senescence, but increase later (Wang et al. 2018). Putative orthologs of these genes in *A. thaliana* were upregulated during leaf senescence, although they also increased their expression in pollination-induced petal senescence (Table 1, Fig. 6), suggesting a different regulation in this latter type of senescence between both species. Phylogenetic analysis showed that members of this cluster were related to positive and negative regulators of senescence (Fig. 6, Table 3). PhWRKY053 was classified as a putative ortholog of previously described GhWRKY27. PhWRKY070 was related to the negative regulators, AtWRKY54 and AtWRKY70 and more distantly with the positive regulator TaWRKY40-D (Fig. 6, Table 3) (Ülker et al. 2007; Besseau et al. 2012; Gu et al. 2019a; Zhao et al. 2020). Finally, PhWRKY024 shared the functional group with both positive (TaWRKY7) and negative (AtWRKY25) regulators (Fig. 6, Table 3) (Zhang et al. 2016; Doll et al. 2020). The putative homologs of *PhWRKY024* and *PhWRKY070* displayed the three types of senescence-associated regulatory elements. However, the equivalent of PhWRKY053 did not show W-boxes, suggesting that it might regulate senescence independently of WRKY (Table S4). Taken together, members of cluster 4 could be considered candidates for the regulation of the three types of senescence processes in *P. hybrida*, although to a lesser extent with respect to the members of cluster 1, possibly acting as positive and/or negative regulators.

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). The *PhWRKY* genes with early changes in expression might regulate other *PhWRKY* genes with mid to late expression changes in the senescence processes studied. In this sense, future research should be carried out to investigate protein-DNA interactions through the use of DPI-ELISA or ChIP sequencing techniques. 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 PhWRKY054 and PhWRKY070 of functional group VI, which showed similar profiles during natural leaf senescence and are expected to negatively regulate senescence in petunia (Figs. 5, 6, 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 detailed 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 these WRKY candidates could act as regulators

of senescence, which would help to delay senescence via molecular breeding.

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Author contribution 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.

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Declarations

Conflict of interest The authors declare they have no conflict of interest.

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