PLANT BREEDING, GENETIC AND GENETIC RESOURCES

RESEARCH PAPER

Aging gene pathway of microRNAs 156/157 and 172 is altered in juvenile and adult plants from *in vitro* propagated *Prunus* sp.

Adriana Bastías[1], Rubén Almada[2], Pamela Rojas[1], José Manuel Donoso[1], Patricio Hinrichsen[3], and Boris Sagredo[1]

[1]Instituto de Investigaciones Agropecuarias, INIA-Rayentué, Avenida Salamanca s/n, Sector Los Choapinos, Rengo, Chile.
[2]Centro de Estudios Avanzados en Fruticultura (CEAF), Avenida Salamanca s/n, Sector Los Choapinos, Rengo, Chile.
[3]Instituto de Investigaciones Agropecuarias, INIA-La Platina, Santa Rosa 11610, La Pintana, Santiago, Chile.

Abstract

A. Bastías, R. Almada, P. Rojas, J.M. Donoso, P. Hinrichsen, and B. Sagredo. 2016. Aging gene pathway of microRNAs 156/157 and 172 is altered in juvenile and adult plants from *in vitro* propagated *Prunus* sp. Cien. Inv. Agr. 43(3):429-441. *In vitro* culture is a very popular technique to mass propagate valuable plant genotypes, including *Prunus* sp. cultivars. Plants that undergo tissue culture processes often change their morphology and behavior due to the "rejuvenation" caused by the plant growth regulators included in the medium. To evaluate the effects of rejuvenation by tissue culture in *Prunus* sp., the expression patterns of the aging gene pathway described in plant models, which include the highly conserved microRNA (miRNA or miR) 156/157 and 172 families and several of their respective target genes, were analyzed in distinct *Prunus* sp. genotypes at different phases of maturity, including true seedling and tissue culture micropropagated plants. In genotypes from true seedling plants, the expression of miR156 and miR157 was higher in the leaves of juvenile plants (one year old) than in those of adult plants (six year old). The opposite pattern was observed with miRNA172 expression. Our results suggest that the aging gene pathway is relatively conserved in Prunus and likely plays a key role in vegetative phase change. However, *Prunus* sp. plants that were rejuvenated and propagated by *in vitro* methods showed more erratic behavior for miR156 and miR157 and their target genes, suggesting that tissue culture alters the normal control of the aging pathway.

Key words: development, *in vitro* culture, phase change, rejuvenation.

Introduction

Plants undergo several changes during their life cycle, including changes in vegetative morphology, reproductive potential, flowering, seed set and senescence. The shoot of a seedling begins its development in the juvenile phase, which is characterized by a variety of morphological traits including leaf shape and size and the insensitivity of the shoots to floral stimulus (Bergonzi and Albani, 2011). The transition to the adult phase, termed vegetative phase change, is marked by an increase in reproductive potential. Flower induc-

tion begins during the adult phase and is regulated by endogenous signals as well as environmental factors (Bergonzi and Albani, 2011).

Prunus is a commercially important tree genus that contains species with different juvenility periods where the juvenile-to-adult transition is an economically important trait because it directly affects the length of breeding cycles as well as the precocity of orchards. A very popular mass propagation technique for varieties of *Prunus* rootstocks is tissue culture (Alanagh *et al.*, 2014). Because not all the genotypes respond satisfactorily to similar *in vitro* propagation methods (even within the same species), there is ongoing extensive research to develop specific protocols for valuable genotypes (Alanagh *et al.*, 2014).

In vitro propagation of woody trees such as *Prunus* sp. has significant effects on the expression of juvenile or adult traits. A successful tissue culture protocol must reverse the tendency of aging of an adult plant by a process of rejuvenation (von Aderkas and Bonga, 2000). The reduced rooting ability of an adult plant is the main trait that has to be reversed, which is often achieved by the tissue culture of adult buds (Wendling *et al.*, 2014). However, it is still not clear whether these artificial methods generate true juvenile plants with reduced ontogenetic age or if the methods just produce plants with reduced physiological age (reinvigorated) by temporarily removing environmental and physiological constraints to growth (Wendling *et al.*, 2014).

At the molecular level, the microRNAs (miRNAs or miRs) 156/157 and 172 in angiosperms play a critical role in the regulation of juvenile-to-adult vegetative phase change and flowering (Wu *et al.*, 2009). This aging pathway seems to be conserved in fruit tree species, such as those belonging to the Prunus genus of the Rosaceae family (Xu *et al.*, 2015).

The miR156, probably together with the related miR157, regulates a family of transcription factors

called *Squamosa Promoter Binding Protein-Like* (SPL) (Bergonzi and Albani, 2011). SPL is a diverse family and contains an SBP (Squamosa binding protein) domain with two zinc-binding sites as well as a nuclear localization signal. This protein family is plant specific and is encoded by a large gene family (Cardon *et al.*, 1999). For instance, 16 and 15 *SPL* genes have been described in *A. thaliana* and *P. mume* Siebold & Zucc., respectively (Cardon *et al.*, 1999; Xu *et al.*, 2015).

In *A. thaliana*, miR156 regulates a subset of members of the SPL family post-transcriptionally and translationally (Bergonzi and Albani, 2011). During the juvenile phase, miR156 expression is high in leaves, whereas expression is lower in leaves produced during the adult phase in species such as *A. thaliana* (Wu *et al.*, 2009) and some trees such as *Populus × canadensis* Moench*, Acacia confuse* Merr. and *Eucalyptus globulus* Labill (Wang *et al.*, 2011). While expression of *SPLs* genes is low in the juvenile phase, it increases in the adult phase (Wang *et al.*, 2009). Plants with over-expression of miR156 flower late, whereas plants with inhibited miR156 activity flower early (Wang et al., 2009). Another miR involved in vegetative phase change is miR172, which is expressed in an opposite manner to miR156 (Aukerman and Sakai, 2003; Wu *et al.*, 2009). The over-expression of miR172 promotes adult leaf traits and flowering (Jung *et al.*, 2011). In *A. thaliana*, the targets of miR172 are the following six floral repressors: *Apetala 2* (*AP2*) and a group of *AP2-like* genes including *Target of Eat* (*TOE*) 1, 2, and 3, *Schlafmutze* (*SMZ*), and *Schnarchzapfen* (*SNZ*) (Aukerman and Sakai, 2003).

To increase our knowledge about the molecular components and the networks that regulate juvenile-to-adult vegetative and reproductive transitions in *Prunus* sp. and the effects of rejuvenation by tissue culture in *Prunus* sp. transitions, we characterized the expression patterns of mature miR156/157 and miR172 and their putative target genes in leaves of *Prunus* of several genotypes of different ages and developmental stages and compared plants

from true seeds with plants micropropagated by *in vitro* tissue culture.

Materials and methods

Plant material

Fully expanded leaf samples from four *P. avium* L. genotypes (C14H3P1, C14H8P1, C15H14P20 and C15H16P3) were obtained in the summer of 2016 from the INIA Cherry Breeding Program, Rengo, Chile. Samples were taken from among the central leaves of each tree. These genotypes are progeny of cv. 'Emperor Francis' as the mother in an open in-field pollination system. The younger genotypes, C15H14P20 and C15H16P3, were planted in 2015, while the older ones, C14H3P1 and C14H8P1, were planted in 2009. The latter genotypes bloomed for the first time during the 2012 season. Samples were collected, immediately frozen in liquid nitrogen, and then stored at -80 °C until RNA extraction and gene expression analyses.

Pathogen-free clonally *in vitro* propagated plants of *Prunus* hybrid genotypes were donated by Agromillora Sur nurseries (Chile). Plant material included the almond × peach hybrid (*P. amygdalus* (L.) Batsch × *P. persica* L.) 'Garnem' (G x N 15), the plum hybrid 'Mariana 2624' (*P. cerasifera* Ehrh. × *P. munsoniana* W. Wight & Hedrick), the sweet and sour cherry hybrid (*P. avium* × *P. pseudocerasus* Lindl.) 'Colt' and the sweet cherry Mazzard F12/1 (*P. avium*). Plants were transplanted in 2 L plastic pots with a mixture of sand:vermiculite:perlite (1:1:1) as substrate and kept in the field under a shade net (Raschel sun-shading net with 50% light transmittance) at the Instituto de Investigaciones Agropecuarias (INIA) Rayentué, Rengo, Chile (S34°19'16.8", W70°50'02.2"). Plants were watered three times a week with tap water and fertilized every two weeks with 1 g pot[-1] with N:P:K (25:10:10) (Ultrasol™, Soquimich, Chile). The above procedure was repeated during three growing seasons until obtaining one-, two- and three-year-old plants. Central

leaves from each tree of 'Garnem','Mariana2624', 'Colt' and 'Mazzard F12/1' genotypes were collected, immediately frozen in liquid nitrogen, and stored at -80 °C until RNA extraction and gene expression analyses. Three-year-old plants of the 'Garnem' and 'Mariana 2624' genotypes flowered, but 'Colt' and 'Mazzard F12/1' did not. The main characteristics of the plant genotypes used in this study are summarized in the Table 1.

Gene expression analysis

To analyze the expression of mature miR156 and miR172, miRNAs were isolated with the mirVana™ miRNA Isolation Kit according to the manufacturer's instructions (Ambion, Inc., Applied Biosystems, USA). TaqMan®MicroRNA Assays (Applied Biosystems, Life Technologies, USA) of *A. thaliana* were then used to assess miRNA expression. The probes were designed to detect and accurately quantify mature miRNAs 156, 157 and 172. Five ng of total RNA was used to detect miRNAs. The qPCR reactions included 1 µL of TaqMan® Small RNA Assay (20✕), 1.33 µL of product from reverse transcription (RT) reaction, 10 µL of TaqMan® Universal PCR Master Mix II (2✕), and nuclease-free water to reach a final volume of 20 µL. TaqMan® Small RNA Control (Applied Biosystems, Life technologies, US) of the sno41Y probe was used as predesigned assays for small, non-coding RNAs unrelated to miRNAs to normalize differences derived from RNA sample preparation.

Three technical replicates for each biological replicate (n=3) were performed in all the gene expression analyses in this study. In the case of vegetative propagated clones, the biological replicates consisted of different plants, but for seedling plants, the biological replicates were different leaves from the same plant.

To analyze the expression of *SPL* and *TOE* genes, total RNA was obtained from leaves of three plants according to Chang *et al.* (1993). Total RNA

Table 1. Summary of plants used in this study.

Name	Scientific name	Type	Propagation/origin	Age (year)	Flowered
C14H3P1	*P. avium*	Breeding clone	Seed	Six	Yes
C14H8P1	*P. avium*	Breeding clone	Seed	Six	Yes
C15H14P20	*P. avium*	Breeding clone	Seed	One	Not
C15H16P3	*P. avium*	Breeding clone	Seed	One	Not
'Garnem'	*P. amygdalus* × *P. persica*	Rootstock cultivar	*In vitro* culture	One two three	Not Not Yes
'Mariana 2624'	*P. cerasifera* × P. munsoniana	Rootstock cultivar	*In vitro* culture	One Two three	Not Not Yes
'Colt'	*P. avium* × *P. pseudocerasus*	Rootstock cultivar	*In vitro* culture	One Two three	Not Not Not
'Mazzard F12/1'	*P. avium*	Rootstock cultivar	*In vitro* culture	One Two three	Not Not Not

integrity and purity was verified by formaldehyde agarose gel electrophoresis and OD260/280 absorbance ratio, respectively. DNase treatment of total RNA and first-strand cDNA synthesis was carried out with 1 µg of total RNA for each sample using oligo (dT) primers according to the manufacturer's instructions (Invitrogen, USA). The quantitative real-time PCR reactions were performed with Brilliant SYBR Green Master Mix (Stratagene, USA) according to the manufacturer's instructions. The quantitative real-time PCR reactions included 2 µL Master Mix, 0.5 µL 250 nM of each primer, 1 µL diluted cDNA, and nuclease-free water to reach a final volume of 20 µL. Controls (without cDNA and RNA without RT) were included in each run. Amplification was followed by melting curve analysis. Expression was normalized against the *P. persica TEF2* gene (*translation elongation factor 2* gene, GenBank Database accession number TC3544). The primers used for each analyzed gene are shown in Table 2.

All the analyses of gene transcript levels were conducted with quantitative PCR using an Mx3000P QPCR System (Agilent Technologies, USA). Means, standard errors (SE) and ANOVA analysis were performed using Statistica v4.0 (StatSoft, Inc.) software at a significance level of 0.05.

Phylogenetic analysis

The nucleotide sequence of miR156/157 and miR172 and the deduced amino acid sequences of their target *SPL* and *TOE* genes from *A. thaliana* were used to find their orthologues in *P. persica* using a protein-protein BLAST from the following databases: miRBase (http://www.mirbase.org), Join Genome Institute (www.phytozome.org/peach) and GenBank (http://www.ncbi.nlm.nih.gov).

ClustalW was used to create multiple alignments in Bioedit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html). The resulting alignment was used to generate the phylogenetic tree by the neighbor joining method with MEGA software version 5.2 (http://www.megasoftware.net/).

Results

Identification of the aging gene pathway: miRs 156/157 and 172 and their putative targets in P. persica

To provide evidence of the homologous gene pathways related to vegetative phase change

in *P. persica*, the sequences of the *A. thaliana* miR 156 and miR172 and their putative target *SPL* and *TOE* genes were used to search for their orthologues in the peach genome from the International Peach Genome Initiative (www. phytozome.org/peach) using BLAST. We found that miR156 could be encoded by 9 (a-i) loci, while miR172 is encoded by 5 loci (a-e) (Figure 1), in agreement with the recent report of Zhu *et al.* (2012).

Figure 1 shows the high conservation in the mature sequences of miR 156/157 and miR172 families from *A. thaliana* and *P. persica*. In *A. thaliana*, miR156 and miR157 family members are encoded by the loci MIR156a-i and MIR157a-d, respectively. In peach, only the miR156 (a-i) isoforms have been described (Zhu *et al.*, 2012), but some isoforms of miR157 in Arabidopsis have been misclassified as MIR156 loci in *P. persica* (Figure 1).

The miR172 family of Arabidopsis is encoded by the MIR172a–e loci. There are also five isoforms of this microRNA in *P. persica,* but some sequences are slightly different from those described in Arabidopsis (Zhu *et al.*, 2012), (Figure 1B). In Arabidopsis, eight of the 17 genes that belong to the *SPL* family have been described as participating in vegetative phase change (Chen *et al.*, 2010). In addition, six of the 144 members of the Arabidopsis AP2 family are involved in vegetative phase change and juvenile and adult development (*TOE* genes). Based on the previous report described by Xu *et al.* (2015) and BLAST, we identified six *SPL*-like and four *TOE*-like genes in *P. persica*, with homology to Arabidopsis *SPL* and *TOE* genes involved in vegetative phase change. At the amino acid level, the putative members belonging to the SPL family have similitude percentages ranging from 43.6 to 55.7%, while the putative TOE proteins have amino acid similitude between 35.4 and 46.5%

Table 2. List of primers used in this study.

Name	Sequence (5'-3')
SPL_ppa012607-F	AAAGCAATACTATCGCCGCCATAAG
SPL_ppa012607-R	TCCGACGCCGCTCATTGTG
SPL_ppa022739-F	CCGCCGCCATAAGGTCTGTG
SPL_ppa022739-R	AGCCTTCTCCATAAGGTTCACCAG
SPL_ppa011968-F	GTTGTGCTTGTTTCTGGGCTGAG
SPL_ppa011968-R	ACGGAGTGCCTGTACCATTGC
SPL_ppa021582-F	GTTTCAAGGGAAATGAGGCTGGTAG
SPL_ppa021582-R	CTGCTGGTTGGTGGAGGAGTC
SPL_ppa005013-F	CAGGATTTTCATCGTGCTCTCTCTC
SPL_ppa005013-R	ATAGATGTTGAGTTTGCCAGTACCC
SPL_ppa007056-F	CCGATCACTCATGCTGGGACTG
SPL_ppa007056-R	GCTGCCTCCTGCTGCTCTG
TOE_ppa005230-F	AGAGCAACGGAAAAGAGAATGGATG
TOE_ppa005230-R	GGTGGTGGAGAATAGTTGTGTTAGG
TOE_ppa021782-F	AATGGAAAGAGGATGGAGCACAATG
TOE_ppa021782-R	TGCATTTGCCAAGCCCAGTTG
TOE_ppa003783-F	CCGCATCGGCAGTGATCTTTC
TOE_ppa003783-R	GCGTCTTGTGAGAGAGTGGAAAC
TOE_ppa018704-F	CCGAGATATACAAACTTGGCATGGC
TOE_ppa018704-R	CCGACGTTGTAGGAGGCAGAAG

(Figures S1-S2). The multiple alignments of SPL-like proteins from peach and Arabidopsis revealed a high degree of conservation in the SBP domains (Figure S1). Similar results were reported by Xu *et al.* (2015) in *P. mume*, while the TOE-like proteins with their AP2 domains had the same behavior.

We analyzed the expression patterns of mature miR156/157 and miR172 and their putative target genes in leaves of *Prunus* sp. of different genotypes, ages and developmental phases, assuming high synteny and homology throughout the *Prunus* species for SPL and TOE genes.

The expression of miR 156/157 and miR172 and their targets genes in P. avium genotypes from seedling plants is age-dependent

As a first approach to ascertain the role of miR 156/157 and miR172 in stone fruit tree vegetative and reproductive transitions, their expression patterns were analyzed in *P. avium* genotypes from true seedling plants of different ages, both unflowered juvenile and flowered adult genotypes (Table 1).

The expression of miR156 and miR157 was higher in the juvenile one-year-old C15H14P20 and C15H16P3 *P. avium* genotypes than in
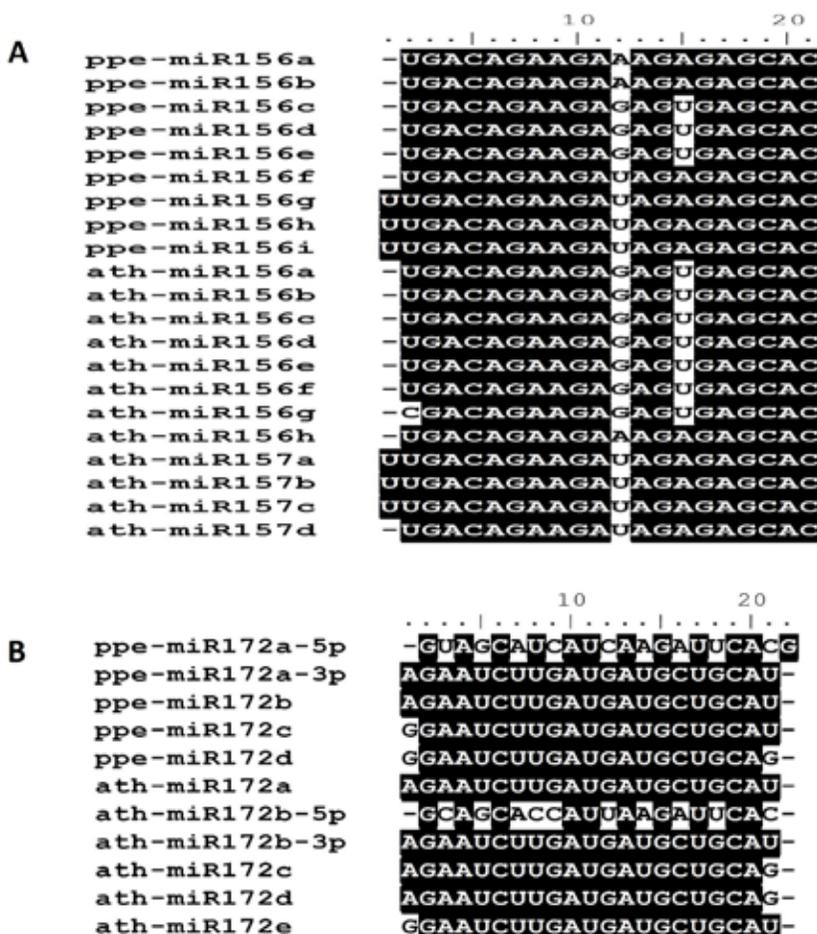

Figure 1. Alignment of mature sequences of *Prunus persica* miRNA156/157 and miRNA172 with homologues of *Arabidopsis thaliana*. A and B show the mature sequence of microRNAs 156/157 and 172, respectively. Identical nucleotides among sequences are shaded in black. Multiple sequence alignment was performed by Clustal W using Bioedit software.

the adult six-year-old C14H3P1 and C14H8P1 genotypes (Figure 2). The expression of miR172 was significantly higher in the older genotypes, C14H3P1 and C14H8P1, than in the younger ones, C15H14P20 and C15H16P3 (Figure 2).

Transcription profiles of the *SPL* and *TOE* genes, which are targets of miR156/157 and miR172, respectively, were also evaluated. A lower expression of the *SPL*-like genes ppa022739 and ppa021582 was observed in the older sweet cherry genotypes, C14H3P1 and C14H8P1 (Figure 3 B and E), while the expression of the other putative *SPL* genes did not show significant differences associated with age (Figure 3 A, C, D and F). In the case of putative *TOE* genes, the expression of the gene ppa021782 was significantly lower in the adult genotypes (Figure 3 J), while the expression of the other *TOE* genes did not show significant difference associated with age (Figure 3 G, H and I). In both groups of genes (*SPL* and *TOE*) that did not show a clear relationship with age, significant differences in the level of expression were associated with the genotype.

Expression of miRs 156/157 and 172 and their target genes in Prunus sp. that were propagated in vitro

Transcriptional profiles of mature miRs 156, 157 and 172 and their target genes in four different *Prunus* sp., which had been propagated by *in vitro* methods, were analyzed in one-, two- and three-year-old plants. We characterized genotypes with short 'Garnem' and 'Mariana 2624' and long 'Colt' and 'Mazzard F12/1' juvenile phases belonging to the subgenera *Amygdalus* and *Phrunophora*, respectively.

The expression of miR156 was higher in two-year-old than in three-year-old plants, except the 'Mazzard F12' genotype, but the results stated that miR156 expression is not significant in all genotypes (Figure 4). The expression of miR157 was also higher in two-year-old plants than in three-year-old plants in all analyzed genotypes (Figure 4).

On the other hand, in the 'Mariana 2624', 'Garnem' and 'Mazzard F12/1' genotypes, the expression level of miR172 was lower in one-year-old plants than in two- and three-year-old plants (Figure 4), while in 'Colt', a late flowering genotype, miR172 expression was higher in one-year-old plants than in two- and three-year-olds (Figure 4).

In general, the expression patterns of putative *SPL*-like genes differed according to the genotype analyzed. In the 'Garnem' genotype, the expression of putative *SPL* genes was lower in one-year-old plants than in three-year-old plants, with the exception of the gene ppa012607 (Figure 4). In the 'Mariana 2624' genotype, the expression of most putative *SPL* genes was higher in one-year-old plants than in older plants, with the exception of the *SPL*-like gene ppa005013, which does not significantly differ with the age of the plant and the *SPL* gene ppa007056, which has higher expression in two-year-old plants (Figure 4). In the 'Colt' genotype, the expression tendency of most putative *SPL* genes analyzed (ppa012607, ppa011968, ppa005013 and ppa021582) was higher in three-year-old plants (Figure 4) when the levels of miR156 and miR157 were low (Figure 4). Finally, in the 'Mazzard F12/1' genotype, there were neither significant differences nor any clear tendency in the expression of most *SPL* genes among the plants of different ages (Figure 4).

Putative *Prunus TOE* gene expression (ppa003783, ppa005230, ppa021782 and ppa018704) exhibited different behaviors among the analyzed genotypes, although the 'Garnem' and 'Colt genotypes seem to share a tendency. The expression of at least three putative *TOE* genes was higher in three-year-old plants (Figure 4). The exception was the *TOE* gene ppa003783, where its expression was not significantly different between the two ages in the 'Garnem' genotype. In the 'Mariana 2624' genotype, the expression of putative *TOE* genes ppa003783, ppa021782 and ppa005230 was higher in one-year-old plants (Figure 4), while in the 'Mazzard F12/1' genotype, the expression of three *TOE* genes (ppa018704, ppa005230 and
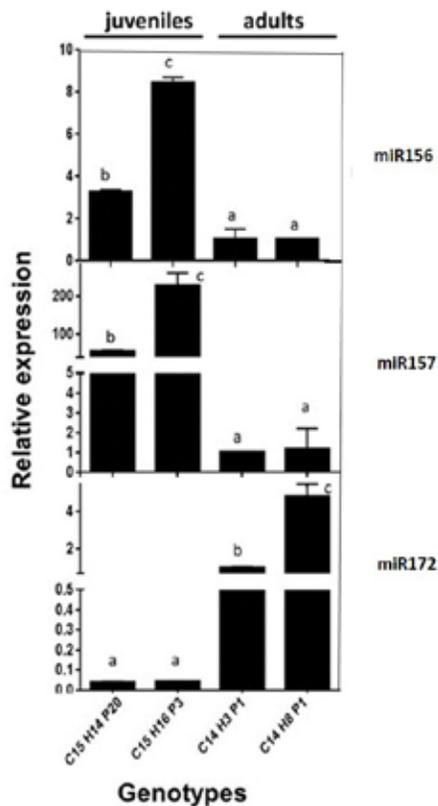
Figure 2. Expression profiles of mature microRNAs involved in developmental transitions in leaves of *P. avium* genotypes of different ages. Expression of mature microRNAs 156, 157 and 172 were examined by qRT-PCR using total RNA enriched in microRNAs and TaqMan® small RNA assays from leaves of one- (C15H14P20 and C15H16P3) and six-year-old (C14H3P1 and C14H8P1) *P. avium* genotypes. Bars indicate mean relative expression values +/- standard error (n=3), normalized with a snoR41Y Taqman ® small RNA control. The letters indicate significant differences.

ppa021782) was lower in three-year-old plants (Figure 4).

Discussion

Our results suggest that the role of the miR156/157-miR172 module and their target genes is conserved in *Prunus* sp. members of the Rosaceae family. These microRNA families are the main players in vegetative and reproductive transitions, regulators of developmental timing, and they are evolutionarily conserved in both annual herbaceous plants and perennial trees (Bergonzi and Albani, 2011) (Figure 1). They are abundant

with variable numbers of loci in Rosaceae plants (Barakat *et al.*, 2012). We searched the homologous genes of the aging pathway, miR-NAs 156/157 and 172 and their target genes in *P. persica* (Figure 1). Homologous genes of the aging pathway are found in the *P. persica* genome and are expressed in several *Prunus* species, suggesting that they play a role during developmental transitions in these perennial tree species (Xu *et al.*, 2015). The target genes of miR156 are the *SPL* genes that control aspects of plant growth and development, including vegetative phase change, flowering time, and the leaf initiation rate (Bergonzi and Albani, 2011). A bioinformatic analysis of putative *cis-*
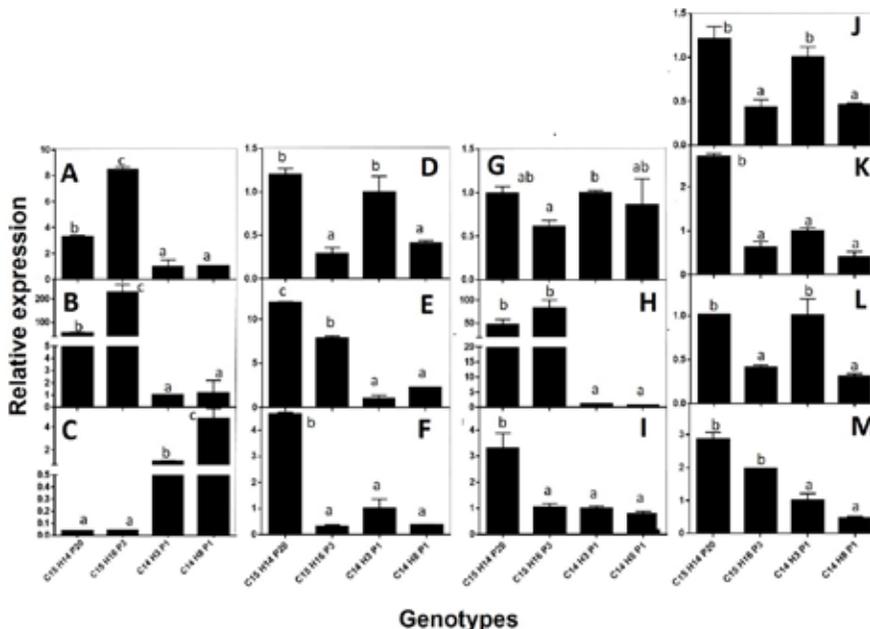
Figure 3. Expression profiles of *SPL* and *TOE*-like genes in leaves of *Prunus avium* genotypes of different ages. Relative gene expression analyses were examined by qRT-PCR using total RNA from leaves of one-(C15H14P20 and C15H16P3) and six-year-old (C14H3P1 and C14H8P1) *P. avium* genotypes. A, B, C, D, E, and F are the expressions of the *SPL* genes ppa012607, ppa022739, ppa007056, ppa011968, ppa021582, and ppa005013, respectively. G, H, I and J are the expressions of the *TOE*-like genes ppa003783, ppa018704, ppa005230 and ppa021782, respectively. Bars indicate mean relative expression values +/- standard error (n=3), normalized with *TEF2* as a constitutive expressed gene. The letters indicate significant differences.
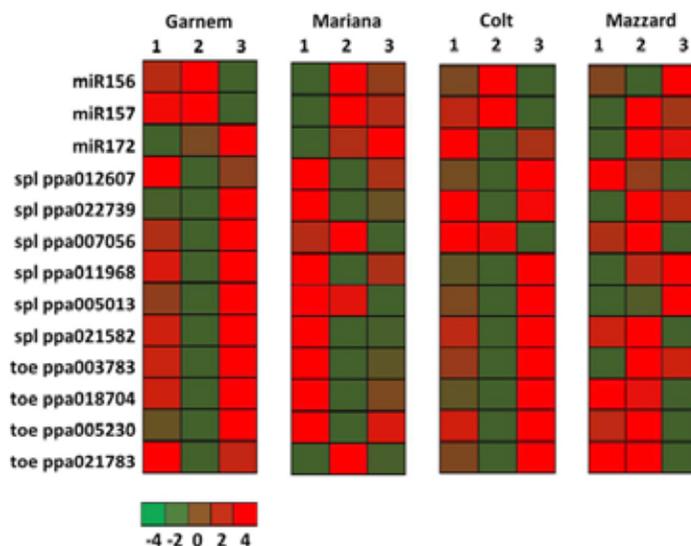

Figure 4. Expression profiles of aging gene pathway members in leaves of *Prunus* sp. of different ages. Heat map indicates the expression of microRNAs 156/157 and 172 and their putative targets, *SPL* and *TOE* genes, respectively, in one-, two- and three-year-old plants of the Mariana ('Mariana 2624'), Garnem, Colt and Mazzard ('Mazzard F12/1') genotypes. Color changes represent the ln (mean) of the expression of every gene in all the genotypes. The heat map color scale was adjusted at intervals of -4 minimum (green) and 4 maximum (red). Graphs with standard errors can be seen in the supplementary Figure S3.

regulatory elements in the promoter regions of *PpSPLs* and *PpTOEs* genes retrieved motifs that suggest their regulation by N metabolism, light, hormones and stress (Figure S4).

The expression profile of the miR156/157-miR172 module and their target genes was determined in different *Prunus* sp. plants at different phases of maturity (juvenile and adult) and from different origins (from true seedlings and tissue culture micropropagated plants). The first group of true seedling plants included four *P. avium* genotypes, two juvenile and two adults, at one- and six-years-old, respectively. The expression profiles of miR156/miR157 and miR172 were clearly associated with age (Figure 2). Transcription profiles of the *SPL* and *TOE* genes, which are targets of miR156/157 and miR172, respectively, were also evaluated. Contrary to what was expected, a low expression of the *SPL* genes ppa022739 and ppa021582 was observed in the older sweet cherry genotypes, C14H3P1 and C14H8P1 (Figure 3 B and E), while the expression of the other putative *SPL* genes did not show significant differences associated with the age of the analyzed genotypes (Figure 3 A, C, D and F). In the case of putative *TOE* genes, as was expected, the expression of gene ppa021782 was significantly lower in the adult genotypes (Figure 3 J), while the expression of the other *TOE* genes did not show significant difference associated with the age of the genotypes. In both groups of genes (*SPL* and *TOE*) that did not show a clear relation with age, significant differences in the level of expression were associated with the genotype effect. In addition, there are others regulators of these genes. For example, in Arabidopsis, under short day conditions, all three SPL genes are positively regulated by SUPPRESSION OF OVEREXPRESSION OF CONSTANS 1 (SOC1) through the gibberellin pathway. In contrast, under long day conditions, SOC1, FLOWERING LOCUS T (FT), and FLOWERING LOCUS D (FD) positively

regulated AtSPL3, AtSPL4, and AtSPL5 in leaves in response to photoperiod signals (Jung *et al.*, 2012).

The relationship between the flowering time and the adult phase in plants has been well described (Bergonzi and Albani, 2011). The expression of miR156 and miR172 in sweet cherry (Figure 2) correlates with observations in other species such as Arabidopsis, where miR156 expression is high in leaves during the juvenile phase and declines after the phase change, while miR172 expression has the opposite behavior (Wang *et al.*, 2011; Wu *et al.*, 2009). Our results suggest that in sweet cherry, the miRNA156/172 module plays a role in controlling juvenility and emerges as a candidate for developing strategies to shorten the juvenile phase in this economically important tree species. Its direct use as a potential biomarker of the adult phase in the management of breeding populations should be evaluated in large populations.

The expression of the miR156/miR172 module is affected not only by age but also by nutrients, stress and epigenetic factors, among others (Kim *et al.*, 2015; Stief *et al.*, 2014; Yu *et al.*, 2013). However, we do not rule out that other miRNA families also have roles in developmental timing (Li *et al.*, 2013; Xu *et al.*, 2014).

Regarding the *Prunus* sp. from *in vitro* propagated plants, which include two known precocious genotypes, 'Garnem' and 'Mariana 2624', and two genotypes with longer juvenility, 'Colt' and 'Mazzard F12/1', the expression profiles were different for each genotype. Because all the genotypes were clonally propagated, age was the only factor that affected expression profiles. Therefore, a good correlation was expected between the age pathway model for both miRNA (miR156/157 and miR172) and their target genes. However, the expression profiles of these genes did not show clear tendencies. Our results suggest that

the miR156/172 module in *Prunus* sp. plants from *in vitro* culture is deregulated (Figure 4). However, the accumulation of miR172 in *in vitro* derived plants seems to occur before that in seed derived plants. This level of accumulation would be different in each genotype (data not shown).

The specific relationships among members of the miR156/miR172 pathway can be complex. In the flowers of *P. mume*, the miR156 targets presented two opposite expression patterns. In contrast, the targets of miR172 were upregulated to different extents in the flowering stage (Wang *et al.*, 2014). Different pathways participated in developmental transitions apart from the aging pathway. In Arabidopsis, SPL proteins positively regulated floral integrators and floral meristem identity genes (Wang *et al.*, 2009; Wu *et al.*, 2009). Other players are also involved in the miR156/miR172 module (Lal *et al.*, 2011).

It is known that *in vitro* culture induces rejuvenation in woody species (Read and Bavougian, 2013), but it is not clear if this artificial method generates truly juvenile plants with reduced ontogenetic age or just plants with reduced physiological age (reinvigorated) by temporarily removing environmental and physiological constraints to growth. Our molecular analysis suggests that such plants do not have normal juvenile stages and/or transitions to adulthood, as do true seed plants. This supports the hypothesis that rejuvenated *in vitro* plants are reinvigorated plants with reduced physiological age. This may also explain the reason *in vitro* rejuvenated and micropropagated plants of mature origin may regain their real age after they are removed from the *in vitro* culture (Nas *et al.*, 2003). The organogenesis *in vitro* system was used to micropropagate the *Prunus* sp. rootstock plants in this study (Agromillora S.A, personal communication), which means that buds of an adult plant were rejuvenated by tissue culture in special medium, thus avoiding callus formation and somatic embryogenesis. The latter is often associated with somaclonal variation, which is undesirable for micropropagation of valuable genotypes (Rodriguez-Enriquez *et al.*, 2011).

The differential expression of miRNAs between conventional and micropropagated plants has been reported in strawberry, and during *in vitro* culture, miR156 was highly expressed and was inversely proportional to the expressions of its target gene *SPL9* and miR172 (Li *et al.*, 2012). Additionally, there is evidence about the role of miR156 in redifferentiation during the induction of organogenesis (Liu *et al.*, 2014) and somatic embryo induction (Wu *et al.*, 2011). Moreover, it is well documented that prolonged cultivation of plant cells *in vitro* induces single nucleotide substitutions and increases the global DNA methylation level in a time-dependent fashion, which is associated with aging in plants (Dubrovina and Kiselev, 2016).

These results demonstrate that the analysis of the expression of the aging gene pathway of miRNAs and its components are useful as molecular markers to study developmental transitions and rejuvenation of plants under *in vitro* culture or other treatments. In the future, these markers might help to understand the response of each genotype to different *in vitro* mediums and treatments and help to develop new protocols for propagating recalcitrant *Prunus* sp.

Resumen

A. Bastías, R. Almada, P. Rojas, J.M. Donoso, P. Hinrichsen y B. Sagredo. 2016. La vía génica del envejecimiento de los microARNs 156/157 and 172 es alterada en plantas juveniles y adultas de *Prunus* sp. que provienen de propagación *in vitro*. Cien. Inv. Agr. 43(3):429-441. El cultivo *in vitro* es una técnica muy popular para propagar en masa genotipos de plantas valiosas, incluyendo cultivares de *Prunus* sp. Las plantas que se someten a procesos de cultivo de tejidos a menudo cambian su morfología y comportamiento debido a la "rejuvenilización" causada por los reguladores de crecimiento de planta incluidos en el medio. Para evaluar el efecto de la rejuvenilización por cultivo de tejido en *Prunus* sp, los patrones de expresión de la vía génica del envejecimiento descrita en plantas modelos, la cual incluye a las familias altamente conservadas de microRNAs (miR) 156/157 y miR172 y varios de sus genes blancos respectivos, fueron analizados en diferentes genotipos de *Prunus* sp. en fases de madurez distintas, incluyendo tanto plantas provenientes de semilla verdadera como micropropagadas mediante cultivo de tejidos. En los genotipos de plantas que provienen de semilla verdadera, la expresión de microR156 and miR157 fue más alta en hojas de plantas juveniles que en plantas adultas. El patrón opuesto fue observado con el patrón de expresión de miR172. Nuestros resultados sugieren que la vía génica del envejecimiento es conservada en *Prunus* y probablemente juega una función en el cambio de fase vegetativo. Sin embargo, plantas de *Prunus* sp. que fueron rejuvenecidas y propagadas por métodos *in vitro* mostraron un comportamiento más errático para los mi156 y miR157 y sus genes blancos, sugiriendo que el cultivo de tejidos altera el control normal de la vía del envejecimiento.

Palabras clave: cambio de fase, cultivo *in vitro*, desarrollo, rejuvenilización.

References

Alanagh, E.N., G.-a. Garoosi, R. Haddad, S. Maleki, M. Landín, and P.P. Gallego. 2014. Design of tissue culture media for efficient Prunus rootstock micropropagation using artificial intelligence models. Plant Cell, Tissue and Organ Culture (PCTOC) 117:349-359.

Aukerman, M.J., and H. Sakai. 2003. Regulation of flowering time and floral organ identity by a MicroRNA and its APETALA2 -Like target genes. The Plant Cell 15:2730-2741.

Barakat, A., A. Sriram, J. Park, T. Zhebentyayeva, D. Main, and A. Abbott. 2012. Genome wide identification of chilling responsive microRNAs in *Prunus persica*. BMC Genomics 13:481-492.

Bergonzi, S., and M.C. Albani. 2011. Reproductive competence from an annual and a perennial perspective. Journal of Experimental Botany 62:4415-4422.

Cardon, G., S. Hohmann, J. Klein, K. Nettesheim, H. Saedler, and P. Huijser. 1999. Molecular characterisation of the Arabidopsis SBP-box genes. Gene 237:91-104.

Chang, S., J. Puryear, and J. Cairney. 1993. A simple and efficient method for isolating RNA from pine trees. Plant Mol. Biol. Rep. 11:113-116.

Chen, X., Z. Zhang, D. Liu, K. Zhang, A. Li, and L. Mao. 2010. SQUAMOSA promoter-binding protein-like transcription factors: Star players for plant growth and development. Journal of Integrative Plant Biology 52:946-951.

Dubrovina, A.S., and K.V. Kiselev. 2016. Age-associated alterations in the somatic mutation and DNA methylation levels in plants. Plant Biology 18:185-196.

Jung, J.-H., P.J. Seo, S.K. Kang, and C.-M. Park. 2011. miR172 signals are incorporated into the miR156 signaling pathway at the SPL3/4/5 genes in Arabidopsis developmental transitions. Plant Molecular Biology 76:35-45.

Jung J.-H., Y. Ju, P.J. Seo, J.-H. Lee, and C.-M. Park. 2012. The SOC1-SPL module integrates photoperiod and gibberellic acid signals to control flowering time in Arabidopsis. Plant J. 69:577-588.

Kim, J.-Y., J.E. Oh, Y.-S. Noh, and B. Noh. 2015. Epigenetic control of juvenile-to-adult phase transition by the Arabidopsis SAGA-like complex. The Plant Journal 83:537-545.

Lal, S., L.B. Pacis, and H.M.S. Smith. 2011. Regulation of the Squamosa Promoter-Binding Protein-Like genes/microRNA156 module by the homeodomain proteins Pennywise and Pound-Foolish in Arabidopsis. Molecular Plant 4:1123-1132.

Li, H., X. Zhao, H. Dai, W. Wu, W. Mao, and Z. Zhang. 2012. Tissue culture responsive MicroRNAs in strawberry. Plant Mol. Biol. Rep. 30:1047-1054.

Li, X., H. Bian, D. Song, S. Ma, N. Han, J. Wang, and M. Zhu. 2013. Flowering time control in ornamental gloxinia (*Sinningia speciosa*) by manipulation of miR159 expression. Annals of Botany 111:791-799.

Liu, W., W. Yu, L. Hou, X. Wang, F. Zheng, W. Wang, D. Liang, H. Yang, Y. Jin, and X. Xie. 2014. Analysis of miRNAs and their targets during adventitious shoot organogenesis of *Acacia crassicarpa*. PLoS ONE 9: e93438.

Read, P.E., and C.M. Bavougian. 2013. *In vitro* rejuvenation of woody species. Methods Mol Biol. 994:383-395.

Rodriguez-Enriquez, J., H.G. Dickinson, and R.T. Grant-Downton. 2011. MicroRNA misregulation: an overlooked factor generating somaclonal variation? Trends in Plant Science 16:242-248.

Stief, A., S. Altmann, K. Hoffmann, B.D. Pant, W.-R. Scheible, and I. Bäurlea. 2014. Arabidopsis miR156 regulates tolerance to recurring environmental stress through SPL transcription factors. The Plant Cell 26:1792-1807.

von Aderkas, P., and J.M. Bonga. 2000. Influencing micropropagation and somatic embryogenesis in mature trees by manipulation of phase change, stress and culture environment. Tree Physiology 20:921-928.

Wang, J.-W., B. Czech, and D. Weigel. 2009. miR156-Regulated SPL transcription factors define an endogenous flowering pathway in *Arabidopsis thaliana*. Cell 138:738-749.

Wang, J.-W., M.Y. Park, L.-J. Wang, Y. Koo, X.-Y. Chen, D. Weigel, and R.S. Poethig. 2011. MiRNA control of vegetative phase change in trees. PLoS Genetics 7: e1002012.

Wang, T., H. Pan, J. Wang, W. Yang, T. Cheng, and Q. Zhang. 2014. Identification and profiling of novel and conserved microRNAs during the flower opening process in Prunus mume via deep sequencing. Molecular Genetics and Genomics 289:169-183.

Wendling, I., S.J. Trueman, and A. Xavier. 2014. Maturation and related aspects in clonal forestry–part II: reinvigoration, rejuvenation and juvenility maintenance. New Forests 45:473-486.

Wu, G., M.Y. Park, S.R. Conway, J.-W. Wang, D. Weigel, and R.S. Poethig. 2009. The sequential action of miR156 and miR172 regulates developmental timing in Arabidopsis. Cell 138:750-759.

Wu, X.M., M.Y. Liu, X.X. Ge, Q. Xu, and W.W., Guo. 2011. Stage and tissue-specific modulation of ten conserved miRNAs and their targets during somatic embryogenesis of Valencia sweet orange. Planta 233:495-505.

Xu, M.Y., L. Zhang, W.W. Li, X.L. Hu, M.-B. Wang, Y.L. Fan, C.Y. Zhang, and L. Wang. 2014. Stress-induced early flowering is mediated by miR169 in *Arabidopsis thaliana*. J. Exp. Bot. 65:89-101.

Xu, Z., L. Sun, Y. Zhou, W. Yang, T. Cheng, J. Wang, and Q. Zhang. 2015. Identification and expression analysis of the Squamosa promoter−binding protein (SBP)−box gene family in *Prunus mume*. Mol Genet Genomics 290:1701-1715.

Yu, S., L. Cao, C.-M. Zhou, T.-Q. Zhang, H. Lian, Y. Sun, J. Wu, J. Huang, G. Wang, and J. Wang. 2013. Sugar is an endogenous cue for juvenile-to-adult phase transition in plants. eLife 2: e00269.

Zhu, H., R. Xia, B. Zhao, Y.-q. An, C.D. Dardick, A.M. Callahan, and Z. Liu. 2012. Unique expression, processing regulation, and regulatory network of peach (*Prunus persica*) miRNAs. BMC Molecular Biology 12:149-167.