Changes in micro RNA expression in a wild tuber-bearing Solanum *species induced by 5-Azacytidine treatment*

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ORIGINAL PAPER

Changes in micro RNA expression in a wild tuber-bearing *Solanum* species induced by 5-Azacytidine treatment

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Abstract Phenotypic plasticity is often postulated as a principal characteristic of tuber-bearing wild *Solanum* species. The hypotheses to explore this observation have been developed based on the presence of genetic variation. In this context, evolutionary changes and adaptation are impossible without genetic variation. However, epigenetic effects, which include DNA methylation and microRNAs expression control, could be another source of phenotypic variation in ecologically relevant traits. To achieve a detailed mechanistic understanding of these processes, it is necessary to separate epigenetic from DNA sequence-based effects and to evaluate their relative importance on phenotypic variability. We explored the potential relevance of epigenetic effects in individuals with the same genotype. For this purpose, a clone of the wild potato *Solanum ruiz-lealii*, a non-model species in which natural methylation variability has been demonstrated, was selected and its DNA methylation was manipulated applying 5-Azacytidine (AzaC), a demethylating agent. The AzaC treatment induced early flowering and changes in leaf morphology. Using quantitative real-time PCR, we identified four miRNAs up-regulated in the AzaC-treated plants. One of them, *miRNA172*, could play a role on the early flowering phenotype. In this work, we showed that the treatment with AzaC could provide meaningful results allowing to study both the phenotypic plasticity in tuber-bearing *Solanum* species and the inter-relation between DNA methylation and miRNA accumulations in a wide range of species.

Keywords DNA methylation · Epigenetics · Micro RNAs · Phenotypic plasticity · Wild potato species

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Introduction

Potatoes, *Solanum* L. section *Petota* Dumortier, comprise more than 200 species, which possess an extensive range of biological diversity and a wide geographical distribution in America. The interspecific relationships are yet unclear in spite of the efforts of taxonomist to clarify it. The difficulties in the classification arise due to the similarity in morphology between many of the described species. These morphological similarities may be explained by the phenotypic plasticity observed when the same species are grown in different environments, and also by the high degree of interspecific hybridization that exists among them (Alvarez et al. 2008;

Bedogni and Camadro 2009; Erazzú et al. 2009; Masuelli et al. 2009). Even though molecular analyses have determined relationships among species, they are incongruent, especially to resolve the South American species. There are several examples in which morphologically related species show no such proximity at molecular level or, conversely, molecular data that group together species showing stark morphological differences (Spooner and Castillo 1997; Jacobs et al. 2008). The development of molecular markers has allowed surveying the genetic base of the morphological variability naturally found in wild potato populations. However, the results obtained working with different species and with a wide number and types of molecular markers have shown lack of correlations between morphological and genetic data (Miller and Spooner 1999; Spooner et al. 2005; Solis et al. 2007; Alvarez et al. 2008; Bedogni and Camadro 2009).

Classically, several hypotheses have been developed on the basis that the aptitude of plant populations to adapt to local habitat conditions or environmental changes depend critically on the presence of genetic variation; without genetic variation, evolutionary change and adaptation are impossible. However, in potato there would be other mechanisms operating in the generation of the extensive phenotypic variability in addition to the genetic variability. In this sense, our group performed a pioneer study to understand the possible role of epigenetic information in the development and evolution of wild potato species. Analyzing the methylation status of the natural homoploid hybrid *Solanum ruiz-lealii* Brüch. ($2n = 2x = 24$), we found an association between methylation patterns and abnormal flower phenotypes and demonstrated that the epigenetic variability (DNA methylation) was higher than the genetic variability (Marfil et al. 2009).

The phenotypic plasticity in plants may involve epigenetic regulation; that is, DNA methylation, miRNA expression, and chromatin organization could be responsible for establishing and/or stabilizing changes in gene expression during plant development. The methylation naturally found on gene sequences can affect its expression. The variation among individuals in the methylation level of a gene (epialleles) produces new phenotypes that are heritable through generations. Up to now, in plants, epialleles of genes with ecological importance, which affect development, floral morphology, pigmentation of seed, and resistance to pathogens have been characterized (for review see Kalisz and Purugganan 2004).

There are studies that have demonstrated phenotypic effects of epigenetic variation describing naturally occurring epialleles (Cubas et al. 1999; Cocciolone et al. 2001). On the other hand, several stable epialleles have been found as by-products of artificially induced mutagenesis screens (Jacobsen and Meyerowitz 1997; Soppe et al. 2000; Stokes et al. 2002). A related technique is the treatment of plant and animal cells with 5-Azacytidine (AzaC), which causes partial demethylation of the DNA by inhibiting the enzyme methyltransferase (Stresemann and Lyko 2008). In plants, phenotypic changes induced by AzaC have been reported in *Arabidopsis thaliana* and *Thlaspi arvense* (Burn et al. 1993), *Brassica oleracea* (King 1995), *Linum usitatissimum* (flax) (Fieldes and Amyot 1999), *Melandrium album* (Janoušek et al. 1996), *Nicotiana tabaccum* (Vyskot et al. 1995), and *Oryza sativa* (rice) (Sano et al. 1990). Flowering time is the most recurrent and studied change; this process is accelerated in plants treated with AzaC, which flower earlier in time compared with the controls in *Linum usitatissimum* (Fieldes and Amyot 1999; Fieldes et al. 2005; Brown et al. 2008), *Perilla frutescens* (Kondo et al. 2010), *Pharbitis nil* (Kondo et al. 2007; Iwase et al. 2010), and *Silene armeria* (Kondo et al. 2007, 2010). In a recent publication, Bossdorf et al. (2010) have demonstrated that experimental alteration of DNA methylation with AzaC affects the phenotypic plasticity of ecologically relevant traits in *A. thaliana*. Altogether, these works describe several phenotypes and associate them with global changes in DNA methylation. Nevertheless, the molecular mechanisms that participate in the phenotypic novelties have not been described.

In plants, there are genes encoding microRNAs (miRNAs), non-coding sequences that are transcribed and naturally form hairpin double-stranded RNA, which are then enzymatically processed by the enzyme DCL1 in the nucleus to form corresponding small RNAs of around 21 nucleotides (Bartel 2004). In plants, it has been shown that small RNAs mediate in many processes of gene regulation, including plant development, signal transduction, protein degradation, response to environmental stress, and pathogen invasion, among others (Jones-Rhoades et al. 2006; Bazzini et al. 2009). Differential expressions of miRNAs could participate in the origin of the phenotypic plasticity in potatoes. In this sense, it has been proposed that the regulation mediated by miRNAs is a mechanism that can explain phenomena in which there is no correlation between genotype and phenotype such as incomplete penetrance and variable expressivity (Ahluwalia et al. 2009). In *A. thaliana*, it has been shown that the abundance of miRNAs regulates the phenotypic plasticity in the development of adventitious roots (Gutierrez et al. 2009) and that *miRNA172* regulates flowering time through silencing of its target gene *APETALA2* (Aukerman and Sakai 2003). The mechanisms of the regulation of miRNA production and the maintenance of their homeostasis are still unclear in both plants and animals. The contribution of miRNA species to DNA methylation has become an area of recent interest. In animals, miRNAs have been implicated in de novo DNA methylation (Benetti et al. 2008;

Sinkkonen et al. 2008), whereas there are some other evidence that strongly indicated that DNA methylation silencing involves miRNA genes expression (Sato et al. 2011). In plants, a miRNA pathway that mediates DNA methylation has been described using monocot *O. sativa* as a model (Wu et al. 2010). However, currently, there is no published evidence about changes in miRNA expression produced by altering DNA methylation patterns in plants.

We considered relevant the characterization of wild germplasm related to edible plant crop of great economic importance such as potato. In addition, epigenetic studies performed in non-model organisms will contribute to a greater understanding of the evolutionary importance of epigenetic mechanisms. The hypothesis of the present work is that in *S. ruiz-lealii* some plant traits with ecological and taxonomic importance are controlled in part by an epigenetic component. We used AzaC to change the DNA methylation patterns in a selected clone of *S. ruiz-lealii* and examined the consequences of these modifications for plant traits. Specifically, we posed the following questions: (1) how does experimental alteration of methylation patterns by application of AzaC affect the means of ecologically and taxonomically important phenotypic traits in *S. ruiz-lealii*? (2) Do experimental changes in DNA methylation alter the expression of a group of miRNAs? Here, we show that experimental alteration of DNA methylation with AzaC induces early flowering and changes in leaf morphology. In addition, we demonstrated that the AzaC treatment significantly affects the expression of, at least, four miRNA genes and that the up-regulation of *miRNA172* could be responsible for the early flowering phenotype.

Materials and methods

Plant material and 5-Azacytidine treatment

The clone 13.4 of *Solanum ruiz-lealii*, described by Raimondi et al. (2005), was treated with the demethylating agent AzaC in the 2009 (2009 experiment). Eight treated and eight untreated control plants were grown to flowering and compared phenotypically. Sprouting tubers were placed in plastic pots with sterile substrate in a chamber at 24 °C with a 16/8 h L:D photoperiod supplied by cool white fluorescent tubes with a light intensity of 130 μmol m^{-2} s^{-1}, at pot level. Tubers between 2.2 and 3.3 g were selected and drops of AzaC 40 μM solution (Sigma-Aldrich) were applied to the leaves of the shoot meristems during the dark period. This process was repeated for 30 days. Control tubers of plant 13.4 were treated similarly, but water drops were placed instead of AzaC solution. All plants were grown in the same conditions, in a randomized arrangement, and watered with constant volumes of inorganic nutrient solutions starting 7 days after sowing. Leaf characters were measured at bloom on the sixth and seventh true leaves from the base of each plant. The number of day from sowing to anthesis of the first flower was computed in each plant. A one-way ANOVA combined with a Tukey's multiple comparison tests was performed to examine the differences between the control and AzaC-treated plants using the software InfoStat version 1.1. The standard error (SE) bars shown on bar graphs were obtained from the individual data ($n = 8$) for each mean. DNA and RNA were extracted from the apical leaf clusters of 30-day-old plants. This procedure was repeated in a second independent experiment (2010 experiment): tubers produced by the control plants were harvested and stored at 4 °C for 3 months. When tubers started to sprout, the AzaC treatment was performed with the same growing conditions as in the 2009 assay, phenotypic characterization was recorded, and nucleic acid extraction was repeated as described previously. In addition, the number of flowers at the first inflorescence and the number of leaves per plant at flowering were computed in the 2010 experiment.

Methylation-sensitive amplification polymorphism (MSAP) analysis

Methylation-sensitive amplification polymorphism analysis was performed to confirm methylation changes in the AzaC-treated plants with respect to the untreated control plants. DNA extraction and the protocol for restriction, ligation, amplification, electrophoresis, and staining were performed as previously described (Marfil et al. 2009).

Analysis of mRNA and microRNA levels

In order to quantify miRNAs and their target genes, quantitative PCR detection and analysis procedure were performed as in Bazzini et al. (2011). Briefly, total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). The integrity of total RNA was assessed on a denaturing agarose gel stained with ethidium bromide. Measurement of RNA concentration was performed in a NanoDropTechnologies spectrophotometer. To remove genomic DNA, 800 ng of 100 ng μl^{-1} RNA was treated with DNase I (Invitrogen, USA). The reaction was performed in 10 μl of final volume and incubated at room temperature for 15 min and then heated at 65 °C for 10 min. Absence of genomic DNA contamination was verified by PCR using *EF1α* primers (online resource, Supplementary Table 1). Two kinds of first-strand cDNA were synthesized using Superscript III (Invitrogen, USA) according to Superscript manufacturer's instructions: (i) for mRNA quantification, oligo $d(T)_{20}$ and a total of 800 ng of 100 ng μl^{-1} DNase-treated RNA were

used, and (ii) the cDNAs of miRNAs were synthesized with 100 ng of 100 ng μl^{-1} DNase-treated RNA and using a pulsed reverse transcription reaction and specific stem-loop primers for each miRNA (online resource, Supplementary Table 2). The groups of 100 ng μl^{-1} DNase-treated RNA were put together with primers [oligo $d(T)_{20}$ or miRNA specific primers]. The reaction tubes were heated at 80 °C for 5 min to denature RNA and cooled rapidly on ice. They were then mixed with a reaction mixture consisting of buffer, dNTPs, RNase inhibitor, and 200 U of Superscript III in a final volume of 20 µl. The reaction to obtain cDNA using oligo $d(T)_{20}$ was incubated 10 min at 25 °C and then 60 min at 50 °C. To obtain the cDNA to quantify miRNA expression (pulsed reverse transcription), the reaction was allowed to proceed for 30 min at 16 °C, followed by 60 cycles at 20 °C for 30 s, 42 °C for 30 s, and 50 °C for 1 s. Finally, in both kinds of reaction, the reagent was treated at 85 °C for 5 min to inactivate the reverse transcriptase. The oligonucleotide primer sets used for miRNA real-time qPCR analysis and the PCR cycle used are listed in Supplementary Table 2 (online resource). To determine the implications of changes in miRNA accumulations, sets of primers were designed to quantify, in the same sample, the levels of their target genes (online resource, Supplementary Table 1). Some sequences were selected from potential targeted genes reported by Zhang et al. (2009). Moreover, an in silico analysis was performed to select additional sequences: the presence of conserved miRNA-target recognition sites was investigated in potato genes that were orthologous to known miRNA-targets in *A. thaliana* and in tomato for a set of miRNAs detected previously on this work (online resource, Supplementary Table 1). Additionally, the expression patterns of the flowering-time genes *FT* and *LFY*, and the gene *DCL1*, required for miRNA biogenesis were studied in the same samples. Experiments were carried out using eight biological replicates in an Applied Biosystems 7500 equipment. *Solanum tuberosum elongation factor-1α* (*EF1α*) was used as a reference gene. Results presented by Nicot et al. (2005), obtained from three experimental conditions, indicated that *EF1α* was the most stable among seven tested reference genes. In addition, in this work we confirmed that there was no difference in *EF1α* expression level between the control and AzaC-treated plants in the two independent experiments (online resource, Supplementary Fig. 1). Two technical repeats were performed on each cDNA sample. The specificity of PCR was checked with dissociation curve analysis and a no-template control was included to ensure that the results were not influenced by primer-dimer formation or DNA contamination. The fragment length of each sample was confirmed by agarose gel electrophoresis and PCR-efficiency was calculated using the LinReg method (Ramakers et al. 2003) (online resource, Supplementary Tables 1 and 2). Relative expression ratios and statistical analysis were performed using fgStatistics interface (Di Rienzo 2009). This software makes use of an algorithm reported by Pfaffl et al. (2002) which uses the Pair Wise Fixed Reallocation Randomisation Test© to compare differences in expression across treatments.

Results

During two consecutive years, 2009 and 2010, two independent experiments were performed to investigate the effect of AzaC on genomic methylation, phenotypic traits, and patterns of gene expression in a selected clone of *S. ruiz-lealii*. Eight biological replicates, clonally propagated by tubers, were evaluated in each experiment and two kinds of plants were compared: (i) AzaC-treated plants, in which drops of a solution of AzaC (40 µM) were applied to the leaves of the shoot meristem for 30 days and (ii) control plants, in which water drops were applied instead of AzaC solution.

Methylation changes induced by AzaC

To test if genome-wide methylation changes occur in the plants treated with AzaC, we analyzed the broad methylation status of these plants in comparison with the control plants. MSAP analysis was used to detect genome-wide methylation changes. *Hpa*II and *Msp*I are a pair of isoschizomers that recognize the same restriction site (5′-CCGG), but posses differential sensitivity to the methylation status of the cytosines: *Hpa*II does not cut if either of the cytosines is fully (double-strand) methylated, whereas *Msp*I does not cut if the external cytosine is fully or hemi-(single-strand) methylated (McClelland et al. 1994). Thus, for a given DNA sample, the full methylation of the internal cytosine, or hemi-methylation of the external cytosine, at the assayed 5′-CCGG sites, can be revealed as absence in *Hpa*II-digest versus presence in *Msp*I-digest and absence in *Msp*I-digest versus presence in *Hpa*II digest, of the specific band, respectively, in the MSAP profiles.

Ninety-nine MSAP fragments were analyzed using four primer combinations. Thirty-two point 3 % of the fragments showed the same pattern in the control and in AzaC-treated plants while 64.7 % showed altered profiles induced by the AzaC treatment (differentially methylated polymorphic fragments), as shown in Fig. 1 (patterns I and II). Thus, this chemical strongly affects the methylation state of 5′-CCGG sites. Fifty-one percent of the differentially methylated polymorphic fragments observed between the control and AzaC-treated plants corresponded to new restriction fragments in the latter, which were computed as demethylations (Fig. 1, pattern I-A and I-B). The other 13 % corresponded to fragments present in the control

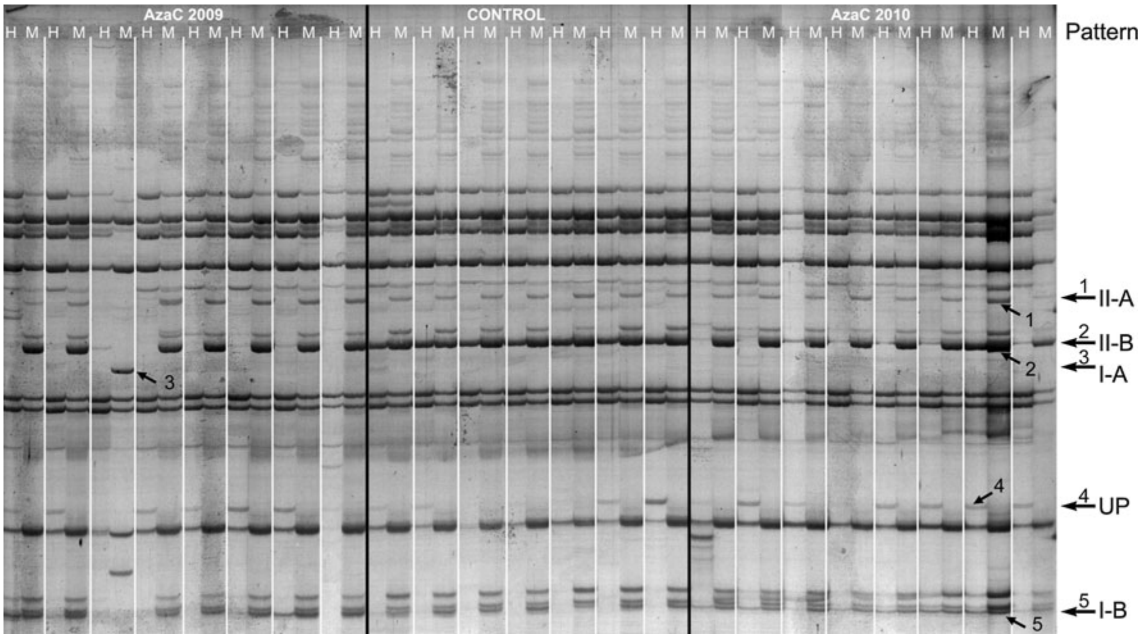

Fig. 1 Changes in cytosine methylation in *Solanum ruiz-lealii* associated with 5-Azacytidine treatment. Representative MSAP profiles of two *Eco*RI/*Hpa*II (H) and *Eco*RI/*Msp*I (M) digestions of DNA extracted from seven control plants (CONTROL) and sixteen 5-Azacytidine-treated plants, eight from the 2009 experiment (AzaC 2009) and eight from the 2010 experiment (AzaC 2010). The *arrows* indicate examples of the fragments corresponding to the five analyzed patterns: *UP* unstable patterns; *I-A* and *I-B* particular and general demethylations, respectively, observed in the AzaC-treated plants; *II-A* and *II-B*, particular and general hypermethylations, respectively, observed in the AzaC-treated plants

plants that disappeared in the AzaC-treated plants and were computed as hipermethylations (Fig. 1, pattern II-A and II-B). The AzaC treatment created different epigenetic variants of the same genotypes. When the methylation changes affected <25 % of the AzaC-treated plants, they were classified as "particular changes" (both demethylations, pattern I-A, and hypermethylations, pattern II-A), whereas, when the same methylation change affected more than 50 % of the AzaC-treated plants, they were classified as "general changes" (demethylations, pattern I-B, and hypermethylations, pattern II-B) (Table 1). Additionally, 3 % of the fragments were variable among control plants (Unstable Patterns-UP, Fig. 1).

Morphological and phenological analyses

Experimental treatment with AzaC strongly affected leaf morphology (Fig. 2a; Table 2). Morphological characters measured in the sixth and seventh leaves in both experiments (2009 and 2010) showed significant differences, making the AzaC-treated plants distinguishable from the control plants. Those differences included reduced area of terminal leaflet (Fig. 2b), increased number of lateral, and intercalar leaflets (Fig. 2c) and larger raquis (Fig. 2d). The total area of the leaves was not affected by treatment with AzaC (Fig. 2b). The AzaC treatment significantly altered the flowering time in both independent experiments (Fig. 3a). On average, plants treated with AzaC flowered 13 days earlier than control plants (Table 2). As index of vegetative growth, the number of leaves per plant at flowering were computed in the experiment of year 2010: the AzaC-treated plants were significantly smaller at time of flowering than control plants and, in addition, developed more flowers than control plants on the first inflorescence (Table 2; Fig. 3b).

Detection and quantification of microRNA in *Solanum ruiz-lealii*

To determine whether the AzaC treatment altered miRNA accumulation, the expression of miRNAs was measured by

Table 1 Methylation changes induced by 5-Azacytidine in *Solanum ruiz-lealii*

	Monomorphic patterns	Unstable patterns in control plants	General demethylations	Particular demethylations	General hypermethylations	Particular hypermethylations	Total number of analyzed fragments
	32 (32.3 %)	3 (3.0 %)	17 (17.2 %)	34 (34.3%)	6 (6.1 %)	7 (7.1 %)	99 (100 %)
Pattern	M	UP	I-B	I-A	II-B	II-A	

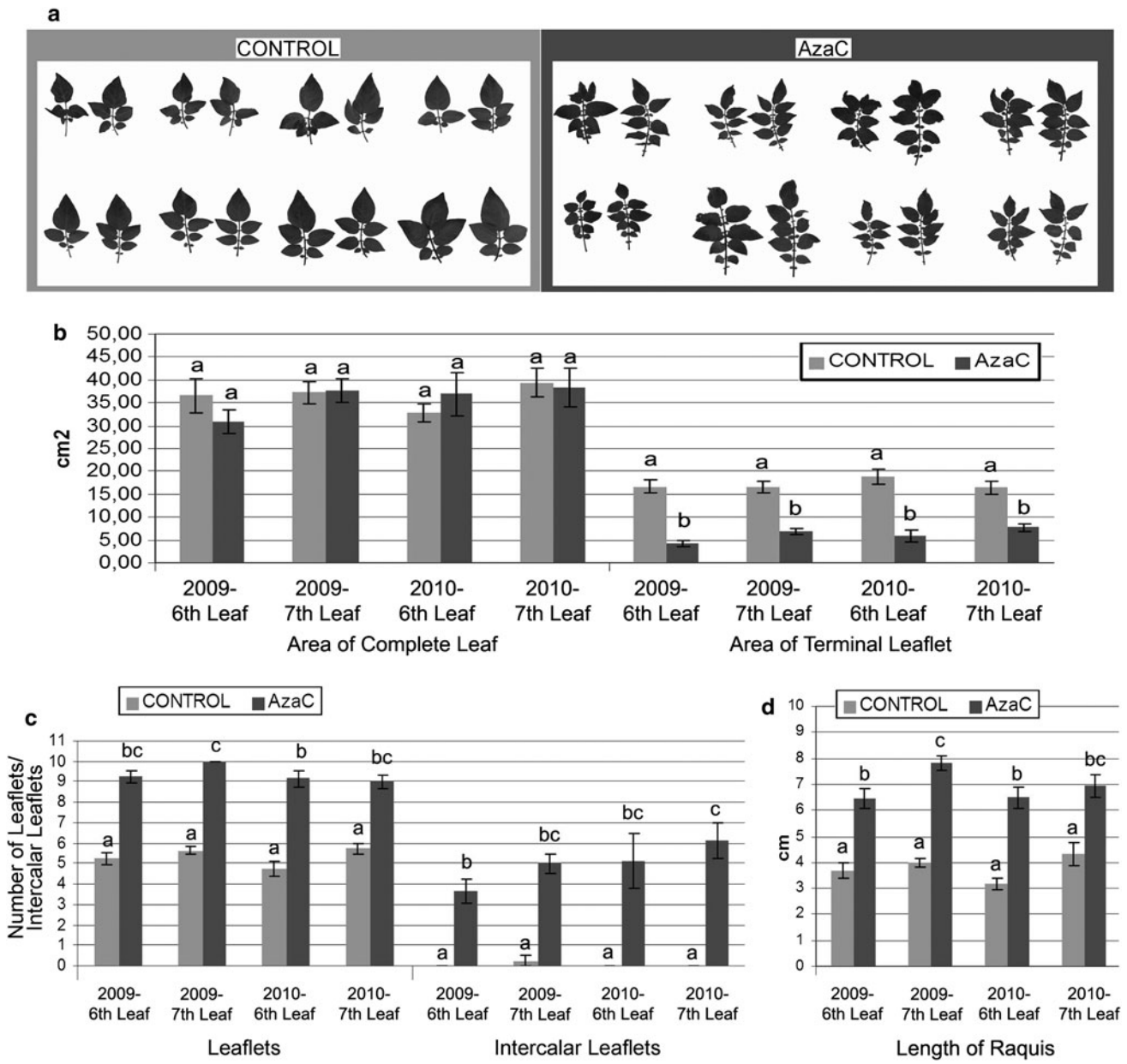

Fig. 2 Changes in leaf morphology induced by 5-Azacytidine treatment in *Solanum ruiz-lealii*. **a** Representative leaf morphology of eight control plants (CONTROL) and eight 5-Azacytidine-treated (AzaC) plants evaluated in the experiment performed in 2009. *Top* photography of the sixth leaf of each plant. *Bottom* photography of the seventh leaf of each plant. **b** Full leaf area (*left*) and full terminal leaflet area (*right*). **c** Number of leaflets (*left*) and number of intercalary leaflet (*right*). **d** Length of raquis. From **b** to **d**: all parameters evaluated in the sixth and seventh leaves in two independent experiments (2009 and 2010); *error bars* show SE; *different letters* indicate statistically significant difference at $P < 0.01$

quantitative PCR. At 30 days after the start of the treatment, total RNA was isolated from leaf on the apical meristem of each plant and accumulation of miRNAs was analyzed using stem–loop RT followed by qPCR analysis. A selection of miRNAs that are involved in plant development and that could play a role in the production of the observed phenotypes was studied using miRNA sequences from *A. thaliana*. As control of a general miRNA pathway alteration, *miRNA168* (which has an essential role on the regulation of AGO1) was also tested. Figure 4 shows the accumulation of miRNAs in two independent experiments. The amount of miRNA species in the AzaC-treated plants were computed relative to the control plants, which was arbitrarily set at 1.0. Of particular interest were *miRNA156*, *miRNA164*, *miRNA167,* and *miRNA172* that were significantly over expressed in the AzaC-treated plants with respect to the control plants in both experiments (2009 and 2010). The expression of *miRNA319* increased in the

Table 2 Phenotypic changes observed in *Solanum ruiz-lealii* plants treated with 5-Azacytidine with respect to control plants

	2009 Experiment[a]			2010 Experiment[a]		
	Control	AzaC	% Change	Control	AzaC	% Change
Number of lateral leaflets/6th leaf	5.25 ± 0.44	9.25 ± 0.44	+76	4.75 ± 0.51	9.14 ± 0.53	+92
Number of lateral leaflets/7th leaf	5.63 ± 0.25	10.00 ± 0	+77	5.75 ± 0.35	9.00 ± 0.46	+56
Number of intercalar leaflets/6th leaf	0	3.62 ± 0.84	–	0	5.13 ± 1.87	–
Number of intercalar leaflets/7th leaf	0.25 ± 0.35	5.00 ± 0.70	–	0	6.13 ± 1.20	–
Terminal leaflet area/6th leaf (cm^2)	16.68 ± 1.92	4.19 ± 1.02	−75	18.78 ± 2.28	5.84 ± 1.67	−69
Terminal leaflet area/7th leaf (cm^2)	16.58 ± 1.58	6.78 ± 0.75	−60	16.42 ± 2.26	7.64 ± 1.13	−54
Flowering time (d)	38.50 ± 0.75	25.00 ± 0.92	−35	39.25 ± 2.10	25.75 ± 0.83	−35
Number of leaves at flowering	ND[b]	ND[b]	ND[b]	15.13 ± 0.49	8.13 ± 0.32	−47
Number of flowers on first inflorescence	ND[b]	ND[b]	ND[b]	6.62 ± 1.03	18.5 ± 2.17	+179

The values are means ± standard errors from the raw data

[a] Two independent experiments were performed

[b] Not determined

AzaC-treated plants in relation to the control plants in both experiments; however, the differences were significant only in the experiment of year 2009. The expression of *miRNA165*, *miRNA168,* and *miRNA393* did not show significant differences between the AzaC and control plants in the two independent experiments. In addition, we studied the expression of *Dicer-like1* (*DCL1*) which mediates miRNA metabolism. The expression of this gene was not altered by the AzaC treatment in either of the independent experiments (Fig. 4).

Detection and quantification of microRNA target genes in *Solanum ruiz-lealii*

In order to detect only uncut mRNA targets, the accumulation of putative miRNA target sequences was analyzed by qRT-PCR using sets of primers annealing at both sides of the miRNA recognition sites. The PCR conditions were adjusted using both genomic DNA (positive control) and cDNA as templates. The set of miRNA targets assayed corresponding to *miRNA164* and *miRNA167* could not be quantified. No amplification was observed using the miRNA167 target primers; and for the two *miRNA164* targets, the amplifications were successful only using genomic DNA as template (Additional Table 2). No significant differences between the control and AzaC-treated plants were observed in the expression of *miRNA165/166* targets. The *miRNA319* target *LANCEOLATE* decreased its expression in the AzaC-treated plants in both experiments (Fig. 5); however, the differences were not statistically significant. The two *miRNA172* targets studied, *TOE1* and *TOE3*, were significantly down regulated in the AzaC-treated plants in relation to the control plants in the experiments (Fig. 5). *TOE1* and *TOE3* transcript levels show an inverse correlation with *miRNA172* accumulation, suggesting that this miRNA could target *TOE1* and *TOE3* mRNAs for degradation.

Early flowering phenotype induced by AzaC treatment

Results reported in arabidopsis, cucurbita, and tobacco have established that the FT protein and its orthologs are components of the long-range signals for flowering (Lin et al. 2007; Li et al. 2009; Wellmer and Riechmann 2010). In order to obtain more evidence about the early flowering phenotype exhibited by the AzaC-treated plants, transcript levels of *FT* gene were analyzed. The expression of this gene was statistically increased in the AzaC-treated plants with respect to the control plants in both independent experiments (Fig. 6). In the cultivated potato, *S. tuberosum*, *miRNA172* accelerates flowering by negatively regulating *RAP1*, an *AP2*-like *FT* repressor (Martin et al. 2009), whereas, in *A. thaliana*, *miRNA172* promotes flowering by negatively regulating *AP2*-like flowering repressors, such as *TOE1*, *TOE2*, *TOE3*, *SMZ,* and *SNZ* (Aukerman and Sakai 2003; Schmid et al. 2003; Jung et al. 2007; Mathieu et al. 2009). In the present work, *FT* transcript levels show a positive correlation with *miRNA172* accumulation and an inverse correlation with *TOE1* and *TOE3* accumulation. Thus, in *S. ruiz-lealii*, *miRNA172* may regulate flowering by negatively regulating *TOE1* and *TOE3*, which would be additional floral repressors regulating negatively *FT* expression in potatoes.

Phytohormone gibberellin (GA) could have a role in the early flowering phenotype observed in the AzaC-treated plants. In *A. thaliana*, flower initiation under short-day conditions is dependent on the biosynthesis of GA. This dependency can be explained by GA upregulation at the transcriptional level of the flower meristem identity gene *LEAFY* (*LFY*) (Eriksson et al. 2006). The expression of

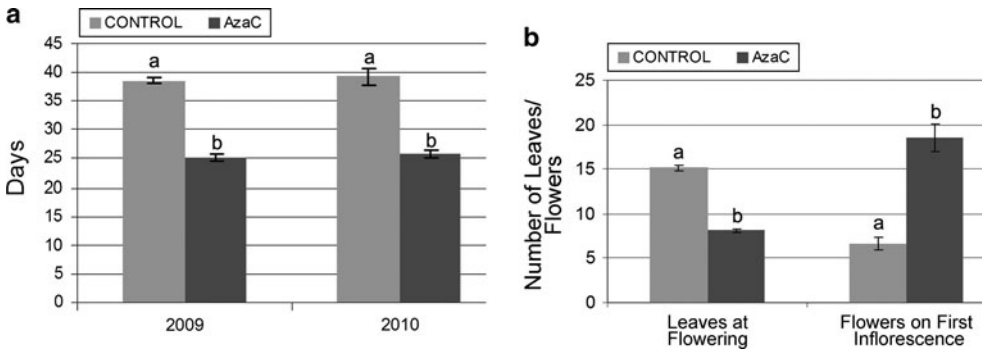

Fig. 3 Changes in flowering time induced by 5-Azacytidine treatment in *Solanum ruiz-lealii*. **a** Time to flowering of the control and 5-Azacytidine (AzaC) treated plants in two independent experiments (2009 and 2010). **b** As the index of vegetative growth, the number of leaf at flowering and the numbers of flower on first inflorescence were computed in the experiment performed in 2010. *Error bars* show SE; *different letters* indicate statistically significant difference at $P < 0.01$

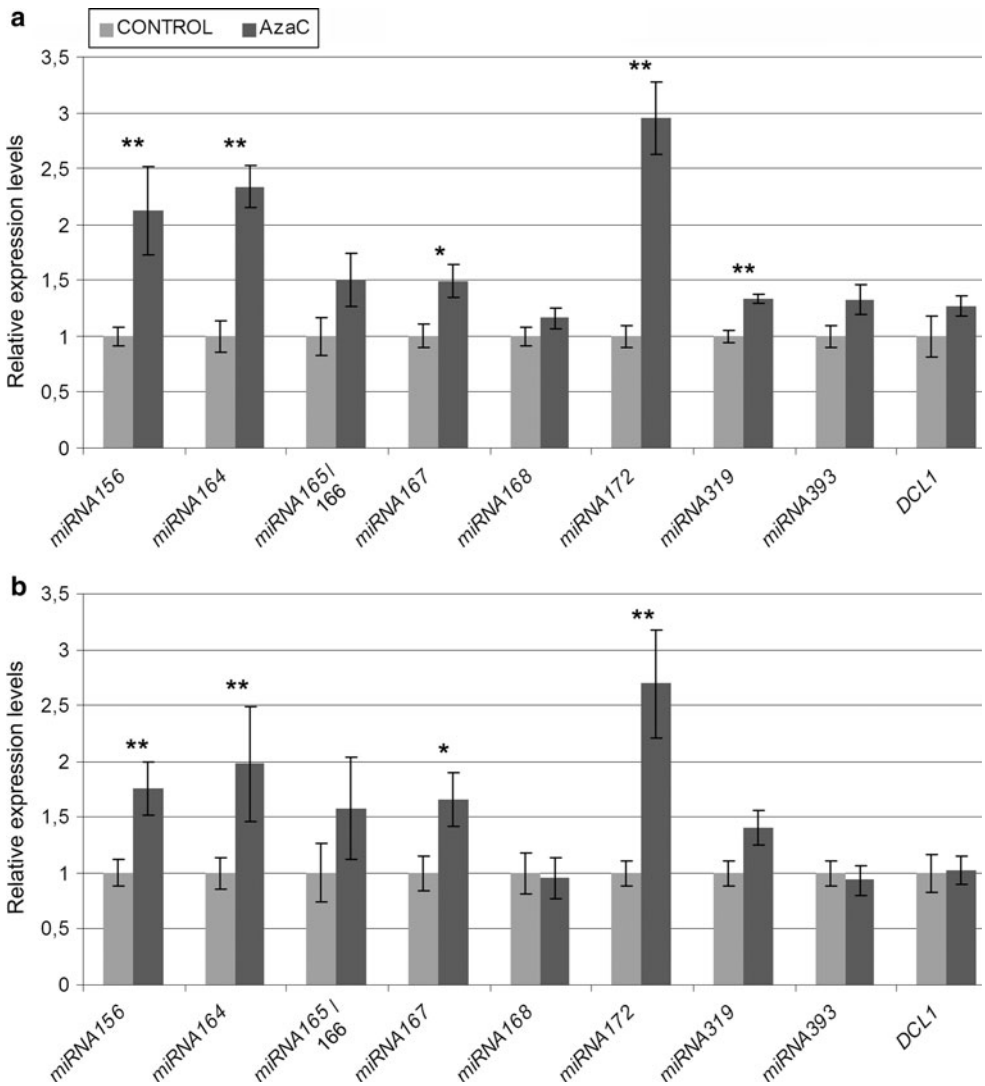

Fig. 4 Effects of 5-Azacytidine treatment on expression levels of a group of miRNA genes and *DCL1*. *Bar charts* showing the results of relative qRT-PCR experiments of the control (CONTROL) and 5-Azacytidine treated plants (AzaC). Results were normalized using *Ef1α* transcript levels. *Bars* represent the SE of the measurements. *Asterisks* indicate *P* values of permutation test: $*P < 0.05$, $**P < 0.01$. Two independent experiments were performed: 2009 experiment (**a**) and 2010 experiment (**b**)

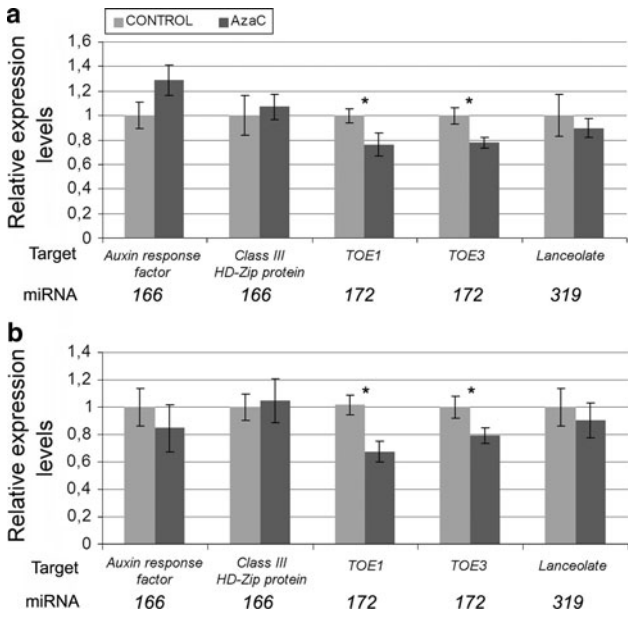

Fig. 5 Target genes of *miRNA172* are downregulated in plants of *Solanum ruiz-lealii* treated with 5-Azacytidine. The target genes and the corresponding miRNA genes are indicated. *Bar charts* showing the results of relative qRT-PCR experiments of control (CONTROL) and 5-Azacytidine treated plants (AzaC). Results were normalized using *Ef1α* transcript levels. *Bars* represent the SE of the measurements. *Asterisk* indicates *P* values <0.05 in permutation test. Two independent experiments were performed: 2009 experiment (**a**) and 2010 experiment (**b**)

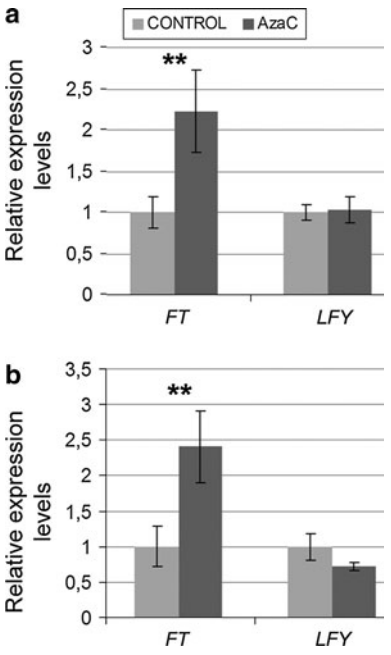

Fig. 6 Expression patterns of the flowering-time genes *FT* and *LFY* in plants of *Solanum ruiz-lealii* treated with 5-Azacytidine. *Bar charts* showing the results of relative qRT-PCR experiments of control (CONTROL) and 5-Azacytidine treated plants (AzaC). Results were normalized using *Ef1α* transcript levels. *Bars* represent the SE of the measurements. *Asterisks* indicate *P* values <0.01 in permutation test. Two independent experiments were performed: 2009 experiment (**a**) and 2010 experiment (**b**)

LFY was not affected by the AzaC treatment in both experiments (Fig. 6).

Discussion

This study surveys the effects of changes in DNA methylation on plant phenotypes and miRNA expression. Working with a selected clone of *S. ruiz-lealii* in two independent experiments and comparing plants treated with a demethylating agent AzaC (AzaC plants) versus the untreated control plants (control plants), we analyzed changes in DNA methylation at 5′-CCGG sites, in leaf morphology and flowering time, and in a selected set of miRNA expression. In our study, we used the MSAP analysis to verify methylation changes in the AzaC-treated plants. Previous studies have demonstrated the demethylating effect of AzaC (Burn et al. 1993; Fieldes et al. 2005). We observed both demethylations and hipermethylations in the AzaC plants with respect to the control plants. Only about 3 % of the analyzed MSAP patterns were not repeatable in the eight biological replicates of the control plants. This data can be used as a quantification of the experimental error, intrinsic to the MSAP technique, and the additional variability in the methylation patterns between the AzaC and control plants can be attributed undoubtedly to the AzaC treatment. Thirty-two percent of the fragments showed the same pattern in the AzaC and control plants. On the other hand, MSAP patterns induced by AzaC were observed. The most frequent change was demethylations in the AzaC plants (51.5 %, of the analyzed MSAP patterns, considering both, general and particular demethylations) (Fig. 1; Table 1). On the other hand, 13.2 % of the MSAP patterns were hypermethylations in the AzaC plants. These hypermethylations (methylation of genome sequences originally unmethylated in the control plants) may be explained by the presence of a de novo methylation system in *S. ruiz-lealii*: the non-specific demethylating activity of AzaC may be sensed, activating in turn this system which could be activated, even in the regions that were not originally programmed to be methylated. miRNAs could participate in the regulation of this system: results reported in mouse embryonic stem cells indicate that de novo DNA methylation is controlled by miRNAs (Sinkkonen et al. 2008). Moreover, in rice, an miRNA pathway that directs DNA methylation at the same loci in which they are produced as well as in *trans* at their target genes and which, in addition, participates in gene regulation has been described (Wu et al. 2010).

The phenotypic plasticity of the wild potatoes was shown very effectively by Correll (1962) who illustrated three quite different morphological variants of the hexaploid wild species *Solanum demissum*. At the present, this phenomenon is considered as a factor that makes the potato taxonomy difficult (Hijmans et al. 2002). However, no experimental approaches are performed for studying the genetic and molecular bases and the ecological consequences of the phenotypic plasticity in potatoes. Bossdorf et al. (2010) have demonstrated that an experimental alteration of DNA methylation with AzaC affects the phenotypic plasticity of ecologically relevant traits in *A. thaliana*. In the present work, we showed that epigenetic modifications may have a significant participation in phenotypic plasticity in *S. ruiz-lealii*. Methylation changes induced by AzaC altered significantly leaf morphology and flowering time. For example, we demonstrated that the presence and number of intercalar leaflet, a morphological character used in the potato taxonomy, are affected by changes in DNA methylation. In addition, an ecologically important trait such as flowering time was affected by changes in DNA methylation.

Although AzaC has a non-specific demethylating activity, our results indicate that the treatment with this chemical did not induce a generalized change in gene expression. Out of 17 genes evaluated (including the reference gene *EF1α*), 10 (59 %) were not affected by the AzaC treatment, and 7 (41 %) showed significant changes in transcript accumulation between the AzaC and control plants. Within the altered group, four were miRNAs (*miRNA156*, *miRNA164*, *miRNA167*, and *miRNA172*) and the remaining three were primary (*TOE1* and *TOE3*) and secondary targets of *miRNA172* (*FT*). Of particular interest resulted the change in the accumulation of *miRNA164* and *miRNA172* induced by AzaC. Berger et al. (2009) have demonstrated that *miRNA164* affects leaflet separation in *Solanum lycopersicum* and in *Cardamine hirsuta*, species with complex leaves. In *S. ruiz-lealii*, changes in *miRNA164* accumulation induced by the AzaC treatment may participate in the described changes in leaf morphology. In order to obtain more evidence, we designed primers to analyze two predicted target genes (*NAM like protein* and *salicylic acid-induced protein*) of this miRNA, but no detectable quantities of these transcripts were observed on the assayed conditions. An updating in the database would be required to explore the expression of additional putative targets of *miRNA164* in *S. ruiz-lealii*. It will be interesting to learn if *miRNA164* controls variation in leaf morphology in different species. On the other hand, the AzaC treatment did not induce changes in leaf size, a trait that could have important consequences for ecological and evolutionary responses in wild potato species. Our results indicate that, in addition to the leaf morphology, the leaf size must be considered to study the species boundary in section *Petota*.

Although many functions of microRNAs in plants and animals have been revealed in recent years, the regulation of their expression is still poorly understood. In humans, six miRNA genes (*miRNA124a-1*, *miRNA124a-2*, *miRNA124a-3*, *miRNA137*, *miRNA193a* and *miRNA127*) have been reported to be silenced by promoter methylation and to have tumor-suppressor functions in colon, bladder, oral, and gastric cancers (for review see Sato et al. 2011). *miRNA127* was found to be remarkably up-regulated in cancer cell lines after the treatment with AzaC and 4-phenylbutyric acid (PBA), a histone deacetylase inhibitor. Together, AzaC and PBA lead to reduced DNA methylation levels and more open chromatin structures, and therefore, they induce the re-expression of genes that had been silenced epigenetically (Egger et al. 2004; Sato et al. 2011). In *S. ruiz-lealii*, the expression of *DCL1* was not altered by the AzaC treatment in the two independent experiments, which may indicate that the steady increase of *miRNA156*, *miRNA164*, *miRNA167,* and *miRNA172* abundance could be governed at the transcriptional level rather than be regulated by miRNA metabolism. It is possible that the expression of *miRNA156*, *miRNA164*, *miRNA167*, and *miRNA172* can be regulated by the methylation of its promoter sequences. Another possibility could be that the methylation changes induced by AzaC could activate a transcription factor regulated by methylation and that this putative protein recognizes conserved motifs in the promoters of these four miRNAs. Exploring the results obtained by Megraw et al. (2006) in *Arabidopsis*, we found that these four miRNA promoters share five putative transcription factor binding sites and that another four binding sites are present at least in three of the four mentioned miRNAs (online resource, Supplementary Table 3). Furthermore, a third hypothesis could be that methylation changes trigger a stress response that in turn alters the miRNA level as it has been observed in other stresses (Khraiwesh et al. 2012). The extension of these approaches to explore the genomic shock induced by interspecific hybridization holds exciting potential. Ha et al. (2009) have shown that small RNAs serve as a genetic buffer against genomic shock in *Arabidopsis* interspecific hybrids. Moreover, Masuelli et al. (2009) have proposed that homoploid hybridization is the main mechanism involved in the origin and evolution of diploid potato species.

After the discovery of the induction of flowering by the AzaC treatment in vernalization-requiring ecotypes and mutants of *A. thaliana* and the related species *Thlaspi arvense* (Burn et al. 1993), this field has rapidly progressed and this experimental approach has been used in several species. However, flowering regulatory genes that are activated by changes in DNA demethylation have not been

identified. The results presented here suggest that in *S. ruiz-lealii miRNA172* is the molecule that might act as an effector of the flowering state change induced by AzaC. Jung et al. (2007) have shown that the *miRNA172* regulation of *TOE1* exerts its role by inducing *FT* through a genetic pathway, other than the autonomous and GA pathways, and concluded that *miRNA172* and its targets constitute a unique genetic pathway that regulates flowering time by mediating *FT* expression in response to day-length changes. In *S. ruiz-lealii*, the expression of *miRNA172* and their target genes *TOE1* and *TOE3* are inversely correlated in two independent experiments, which suggests that this pathway is influencing *FT* expression. The plant hormone GA strongly affects flowering time, which is achieved in part by up-regulating *LFY*. We found no discernible changes in *LFY* abundance in the AzaC plants. This is consistent with the unique genetic pathway regulating flowering proposed by Jung et al. (2007). However, in addition to the photoperiodic control of this way, our results might indicate that the DNA methylation participates in its regulation.

The *miRNA172* regulation of flowering time has also been demonstrated in *Nicotiana benthamiana* and in the cultivated potato (*S. tuberosum*). Transgenic *N. benthamiana* lines overexpressing the *Arabidopsis miRNA172* gene exhibit early flowering (Mlotshwa et al. 2006). Overexpression of this microRNA in potato promotes flowering, accelerates tuberization under moderately inductive photoperiods, and triggers tuber formation under long days (Martin et al. 2009). These observations and the reported by us in *S. ruiz-lealii* suggest that *miRNA172* activity is conserved at least in these plant species, and the upregulation of *miRNA172* induced by the AzaC treatment could be the explanation for the early flower phenotypes reported in several species (Burn et al. 1993; Fieldes and Amyot 1999, 2005; Kondo et al. 2007, 2010; Brown et al. 2008; Iwase et al. 2010). The participation of epigenetic mechanism regulating this development stage would be important in the microevolutionary process in the section *Petota*. The wild potato species are genetically similar and have incomplete crossing barriers resulting in a swamp of true species, hybrids, and introgressed forms growing in the same niche. Environmental and/or hybridization stresses could alter the methylation pattern, in the same manner that it was shown here with chemical treatment, altering, in turn, time of flowering. This phenomenon could have important ecological consequences, for example, isolating recently formed hybrids from the parental species that differ in the flowering time. Other interesting point is that the epigenetic changes could be reversible giving the possibility to the hybrids to revert to the parental epigenetic state making them compatible again.

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