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Three-year study of DNA cytosine methylation dynamics in transplanted Malbec grapevines

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Abstract
DNA cytosine methylation, an epigenetic mechanism involved in gene regulation and genome stability, remains poorly understood in terms of its role under changing environmental conditions. Previous research using methylation-sensitive amplified polymorphism (MSAP) markers in a *Vitis vinifera* L. cv. Malbec clone showed vineyard-specific DNA methylation polymorphism, but no change in overall methylation levels. To complement these findings, the present study investigates the intra-seasonal epigenetic dynamics between genetically identical plants grown in different vineyards through a *transplanting experiment*. Cuttings of the same clone, showing differential methylation patterns imposed by the vineyard of origin (Agrelo and Gualtallary), were cultivated in a common vineyard (Lunlunta). Using high-performance liquid chromatography-ultraviolet detection, the quantification of global DNA 5-methylcytosine (5-mC) levels revealed relatively low overall 5-mC percentages in grapevines, with higher levels in Agrelo (5.8%) compared to Gualtallary plants (3.7%). The transplanted plants maintained the 5-mC levels differences between vineyards (9.8% vs 6.2%), which equalized in subsequent seasons (7.5% vs 7%). Additionally, the study examined 5-mC polymorphism using MSAP markers in Lunlunta transplanted plants over three seasons. The observed differences between vineyards in MSAP patterns during the initial growing season gradually diminished, suggesting a reprogramming of the hemimethylated pattern following implantation in the common vineyard. In contrast, the non-methylated pattern exhibited greater stability, indicating a potential memory effect. Overall, this study provides valuable insights into the dynamic nature of DNA methylation in grapevines under changing environmental conditions, with potential implications for crop management and breeding strategies.

Keywords
Acclimation mechanisms, asexual reproduction, epigenetic memory, natural environment, phenotypic plasticity, *Vitis vinifera* L.

1. Introduction

Grapevine is a perennial plant traditionally reproduced by clonal propagation using cuttings. In this process, sections of shoots are excised and transplanted to establish new plants. This method offers

several benefits, such as the fixation of agronomically valuable genotypes, adaptation to local environmental conditions, and the potential transmission of epigenetic traits through mitotic inheritance. Research by McKey et al. (2010) suggest that such transmission could significantly contribute to heritable phenotypic variations.

Epigenetics refer to heritable changes in genome functioning that are not mediated by DNA sequence variations. Dynamics of DNA cytosine methylation refers to changes over time in overall methylation levels and methylation patterns. It has become a topic of intense research in recent years due to its potential role in gene regulation, development, adaptation to the environment and stress response in plants (Guarino et al., 2022). Understanding the impact of DNA methylation on gene expression is challenging due to its semi-stable and semi-labile nature (Ito et al., 2019). Moreover, the dynamics of DNA methylation can vary across different model species, tissues and under diverse conditions.

Distinct reproductive strategies can influence the DNA methylation memory in plants (review by Ibañez et al. 2023). The methylomes of offspring are crafted during gametogenesis and embryogenesis, wherein DNA methylation marks are reinforced, functioning as a corrective mechanism for epigenetic changes. In contrast, it is suggested that plants propagated clonally, bypassing the processes of meiosis and gametogenesis, are expected to exhibit hypomethylation likely perpetuating accidental epigenetic defects and augmenting the pool of epialleles within populations. That is, through multiple cycles of vegetative propagations, there is a gradual and possibly irreversible loss of DNA methylation.

Significant studies have evaluated DNA methylation dynamics in natural conditions, including research conducted on *Arabidopsis halleri* and wild potato (*Solanum kurtzianum*). The clonal propagation and perennial life-cycle of *Arabidopsis halleri* enabled researchers to sample leaves throughout the year from a single clonal individual, whereas wild potato was studied over three seasons. Whole-genome bisulfite sequencing of *Arabidopsis halleri* leaves collected at eight time points, revealed that 0.5% of total cytosines exhibited seasonal 5-methylcytosine (5-mC) modifications, and that DNA methylation changes occurred in a context-dependent manner (CG, CHG or CHH). They also found that 5-mC levels and the distribution of methylated sites remained stable over the year, being necessary for stable gene expression (Ito et al., 2019). In the case of three wild potato clones grown for three seasons in two experimental gardens with an altitude difference of approximately 1000 m, mitotic reprogramming of the epigenetic differences between the experimental gardens was observed. This reprogramming was primarily associated with each growing season and it is believed to contribute to the asexual propagation mode of potato while potentially serving as an adaptive response to stressful environmental conditions (Ibañez et al., 2021). Furthermore, studies have explored the role of epigenetic variations in priming or stress memory effects, where plants exhibited a quicker or more efficient response to drought or heat stress (Kumar and Mohapatra, 2021; Varotto et al., 2020). There is evidence of the maintenance of stress-induced epigenetic imprints in clonally generated progeny of white clover (*Trifolium repens*; Rendina González et al., 2018), dandelion (*Taraxacum officinale*; Verhoeven et al., 2010) and strawberry (*Fragaria vesca*; Xu et al., 2016). In a research by Crisp et al. (2020), non-methylated regions in various monocots were examined as markers of accessible chromatin regions. The authors propose that these stable non-methylated regions have the potential to serve as effective filters for condensing large genomes into a smaller fraction of putative functional genes and regulatory elements, thus facilitating the assembly and annotation of functional genome elements. These findings underscore the significance of exploring epigenetic persistence and inheritance in nonmodel organisms to enhance our broader understanding of plant epigenetics. Evidence suggests the involvement of epigenetic mechanisms in the development and responses to environmental challenges in grapevines (Berger et al., 2023). Previews studies of methylation dynamics over time, conducted in "Kyoho" grapes (*Vitis vinifera* × *Vitis labrusca*), showed that treatment with 5-azacytidine, which induces demethylation, delayed berry coloring, softening, and aroma accumulation during ripening (Jia et al., 2020). However, our current understanding of seasonal DNA methylation dynamics in *V. vinifera* under natural conditions remains limited.

DNA global methylation level refers to the relative percentage of 5-mC compared to the total cytosine content in the genome. It is maintained through a dynamic balance between DNA methylation

and demethylation processes. The level of DNA 5-mC varies significantly across species, ranging from 5.3% in *Arabidopsis thaliana* to 39.2% in *Narcissus nevadensis* (Alonso et al., 2015), as well as during different developmental stages, such as fruit growth and ripening (Chen et al., 2019; Huang et al., 2019; Jia et al., 2020) and among tissues of the same plant, e.g. from 19.4% in *Camellia sinensis* L. capillary root to 38.3% in tender leaf (Gao et al., 2019). Two commonly used techniques for analyzing DNA 5-mC level are methylation-sensitive amplified polymorphism (MSAP) and high-performance liquid chromatography coupled to diode-array detection (HPLC-DAD). MSAP provides estimates of 5-mC levels but has limitations as it is restricted to the 5' CCGG cleavage sites of the endonuclease enzymes (Fraga and Esteller, 2002). In contrast, HPLC-DAD is a reproducible and precise technique to quantify global DNA methylation (Alonso et al., 2015; Gao et al., 2019; Wagner and Capesius, 1981). In this technique, DNA is hydrolyzed into deoxyribonucleosides and individual nucleosides are identified by HPLC-DAD. While there may be discrepancies between the results obtained by MSAP and HPLC techniques (Salmon et al., 2008), using both techniques in a complementary manner can provide a general idea of the methylation status (Alonso et al., 2016; Smyda-Dajmund et al., 2021).

A previous study conducted on three *Vitis vinifera* L. cv. Malbec clones during the 2016 season, which had been cultivated for 20 years in two natural contrasting vineyards of Mendoza, Argentina (Agrelo and Gualtallary), showed that epigenetic modulation is clone-dependent. Also, methylation polymorphism of clone 10 (MB10) was associated with each vineyard, although no significant changes were observed in the overall 5-mC level (Varela et al., 2020). In our present study we extend these findings by studying the epigenetic dynamics in a *transplanting experiment*. Cuttings of the same MB10 clone, with distinct methylation patterns imposed by their respective vineyards of origin (Agrelo and Gualtallary), were cultivated in a common vineyard (Lunlunta) and evaluated the intra-seasonal methylation differences among plants of different origins over a period of three years. In conjunction with this epigenetic analysis, we conducted a complementary phenotypic study to gain a general overview of how the vineyard of origin influences the acclimation of the clone to the new environment. This decision is led by findings in our earlier investigation, where distinct Malbec clones exhibited varying levels of phenotypic plasticity (Varela et al., 2020). Also, because of the potential role of epigenetic memories in mediating acclimation in clonal populations with limited genetic diversity (Berger et al., 2023). We **hypothesized** that MB10 shows mitotic stability in the preservation of its differential epigenetic marks over time.

2. Materials and methods

2.1 Plant material and experimental design

In 2015, cuttings were collected from 5 plants of *Vitis vinifera* cv. Malbec clone 10 (MB10; Catena Institute of Wine, Bodega Catena Zapata) grown in Agrelo (68°54′40″ W, 33°09′58″ S) and 5 plants grown in Gualtallary (69°14′54″ W, 33°23′42″ S), Mendoza, Argentina. The plants were selected from different panels and based on their trunk diameter homogeneity. A prior genetic analysis confirmed that the selected plants were genetically identical (Varela et al., 2020). Cuttings were labeled as LA (originating from Agrelo) and LG (originating from Gualtallary), and transplanted onto their own roots in a common vineyard located in Lunlunta (68°50'56.5" W, 33°02'43.3" S), located 15 km from Agrelo and 54 km from Gualtallary (**Fig. 1a)**. No variations in rooting efficiency were observed and the vines were trained on a vertical trellis system, cane pruned to 14–16 buds, arranged in north–south oriented rows (2 m row spacing and 1.25 m between plants), and maintained with a drip irrigation system.

For evaluating epigenetic changes environmentally induced during one season and mitotically inherited in the next vintage, shoot tips in active cell division and differentiation, situated at the apical region of healthy grapevine shoots, were meticulously excised and subsequently deposited within an Eppendorf tube (stage 19, Coombe 1995, **Fig. 2**). Shoot tips contain the vine's past environmental experiences since grapevine fruit production extends over two years: buds formed in the first year give rise to shoots bearing fruit in the second year (Keller, 2020). Samples from Agrelo and Gualtallary were collected during the 2016-2017 (2016) growing season, and for the Lunlunta transplanted grapevines (LA and LG), samples were collected in the following growing seasons: 2016, 2017-2018 (2017) and 2018-2019 (2018). The exact dates of sample collection can be found in Table 1. Samples were

immediately ice-cooled in the vineyard, subsequently frozen with liquid nitrogen in the laboratory, and stored at -80° C until DNA extraction procedures and subsequent molecular analyses.

Tubers of potato (*Solanum tuberosum*) var. Spunta and seeds of *A. thaliana* ecotype Columbia of the Instituto de Biología Agrícola de Mendoza (IBAM) germplasm collection were used to generate the control plants of the HPLC-DAD quantification, which were cultivated in a grow chamber with a photoperiod of 16 h, a constant temperature of 25 °C and a white fluorescent daylight (PAR 133.8 µmol m^{-2} s^{-1} and UV-B 0.13 µW cm^{-2}). For DNA extraction, potato shoot tips were sampled in anthesis and rosettes of *A. thaliana* were collected after 5 weeks of germination.

2.2 Sample preparation

For the DNA 5-methylcytosine (5-mC) quantification, DNA was extracted from three biological replicates of Agrelo, Gualtallary and Lunlunta (LA and LG) during the 2016 season, and from LA and LG during the 2017 season, as reported in Varela et al. (2020). To assess the precision and accuracy of the measurements, for each biological replicate two technical replicates were used whenever possible. To validate the measurements of the method, we chose as positive control two technical replicates of *A. thaliana* ecotype Columbia and two of *S. tuberosum* var. Spunta, whose methylation has already been measured by HPLC technique (Rozhon et al., 2008; Alonso et al., 2015). The DNA samples (20 µg) were hydrolyzed with 30 U of DNAse I (Sigma Aldrich, Missouri, USA) in a final volume of 40 µL, incubated at 37º C for 3 h followed by a thermal inactivation of the enzymatic reaction at 90º C for 2 min. The samples were kept on ice until the second hydrolysis was carried out, where the previous 40 µL digestions were added 8 µL ZnSO4 (10mM), 5 µL Nuclease P1 200U.ml^{-1} (Sigma Aldrich) and brought to a final volume of 65 µL for later incubation at 37º C for 12 h. The samples fully hydrolyzed in their individual nucleotides were afterward dephosphorylated adding 8 µL buffer AP 10X (Promega, Wisconsin, USA), 1.5 U of alkaline phosphatase (Promega) to the 65 µL of hydrolyzate and brought to a final volume of 80 µL. The mix was incubated at 37º C for 5 h and all samples were stored at −20º C until analysis.

MSAP analysis was performed in 5 biological replicates in the LA and LG samples during the three growing seasons. For molecular analysis, genomic DNA was extracted from shoot tips as described in Varela et al. (2020) and treated according to the MSAP protocol described by Cara et al. (2013). A selective *Eco*RI primer labeled with FAM fluorophore (*ACG) was combined with two *Hpa*II/*Msp*I primers (ATC and AAT). To identify the similarity or difference in epigenetic patterns after one year of changing cultivation site, the samples from LA and LG in the 2016 were enzymatically digested, together with samples from the vineyards of origin, one from Agrelo and one from Gualtallary.

2.3 Quantification of nucleosides by HPLC-DAD

Chromatographic separation was achieved with water (A), 50 mM diammonium phosphate [$(NH_4)_2HPO_4$] pH 3.8 (B), and methanol (C). The samples were brought to a final 100 µL with the initial phase of the chromatographic method, which consisted of a mixture of A/B/C (80/15/5, v/v/v), and were placed in a 200 µL vial., For the separation and quantification of nucleosides, 20 µL were injected into a Dionex Ultimate 3000 (Dionex Softron GmbH, Thermo Fisher Scientific Inc, Germering, Germany) equipped with a vacuum degasser unit, an autosampler, a quaternary pump, a chromatographic oven and a diode-array detector (Dionex DAD-3000). The column used was a Kinetex XB-C_{18} (4.6 mm × 150 mm, 5 µm) Phenomenex (Torrance, CA, USA) with a guard column of the same material., Ultra-purified water was obtained with the Milli-Q system (Millipore, Billerica, USA). HPLC grade methanol was obtained from Mallinckrodt Baker Inc. (Phillipsburg, NJ, USA). HPLC grade diammonium phosphate salt was used to prepare the buffer and the pH was adjusted with pure phosphoric acid from Cicarelli (San Lorenzo, Santa Fe, Argentina). Nucleosides were separated using a constant proportion of 15% B throughout the chromatographic separation and gradient: 0 to 1 min, 5% C; 1 to 2 min, 30% C; 2 to 4 min, 5% C. The column temperature was maintained at 30° C. Chromatographic separations were achieved at a flow rate of 1.5 mL.min^{-1}. The UV absorbance detector was set at 280 nm. Chromatograms were analyzed using Chromeleon 7.1 console software. Nucleosides identity was obtained by comparing the retention times and absorbance values against a standard solution of pure compounds. For the quantification of nucleotides, an external calibration was done using pure standards of the five nucleotide

bases: adenine (A), guanine (G), thymine (T), cytosine (C) and 5-mC (Sigma Aldrich, Missouri, USA). The nucleotides were dephosphorylated into nucleosides with the same technique as performed on the samples. Then, a mix of 1000 $mg.L^{-1}$ of the nucleosides was prepared, followed by subsequent dilutions to achieve 10 calibration points between 0.5 to 200 $mg.L^{-1}$. Each dilution was run on the equipment and the value of the area under the detected peaks was used to obtain the equation of the regression line. The percentage of global cytosine methylation was determined for each sample as 100 × 5-mC/(5-mC + C), where 5-mC and C are concentrations ($ng.mL^{-1}$) obtained from the regression of equations.

2.4 Methylation-sensitive amplified polymorphism (MSAP) analysis

The amplification products were electrophoretically separated using the LIZ500 marker (GeneScanTM) and the ABI PRISM 3130xl genetic analyzer (Applied Biosystems, Foster City, CA, USA) of the Genomics Unit/Genomics Platform Node CATG of the National Institute of Agricultural Technology (INTA), Buenos Aires, Argentina. In the 2017 and 2018 seasons, only LA and LG samples were analyzed in order to observe the epigenetic dynamics as compared to what was seen in 2016. Samples from both seasons were digested simultaneously and the amplification products were separated electrophoretically using the LIZ500 marker (GeneScanTM) and the ABI PRISM 3500 genetic analyzer (Applied Biosystems, Foster City, CA, USA) of the Genomics Unit/Genomics Platform Node CATG of the INTA (Buenos Aires, Argentina). To compare the LA and LG results to those observed in our previous research, a reanalysis of the MSAP data from Varela et al. (2020) was done using only the two primer combinations used in this study. Electropherograms and the presence/absence of MSAP fragments were analyzed as described in Varela et al. (2020)**.**

2.5 Meteorological data

The daily mean air temperature and daily precipitation were monitored in Lunlunta during the 2016, 2017 and 2018 growing seasons (**Supplementary Data S1)**. The collection of meteorological data followed the same methodology as described in Varela et al. (2020).

2.6 Phenotypic analysis

During the 2016 growing season, all the LA and LG plants developed flowers after transplantation, and they were thinned to promote optimal growth since they were in the formation stage. No phenotypic characterization was performed during this stage. In the 2017 and 2018 seasons, the LA and LG plants were fully formed and when the berries were ripe, a fruit shoot per experimental unit was selected. The basal bunch along with its corresponding leaf on the opposite side were collected and used for a phenotypic characterization (exact dates are in **Table 1**). Samples were placed in nylon bags, kept on ice and carried to the laboratory where bunch and berry fresh weight were determined. The berries were then stored at −20° C for further analyses. In the 2017 and 2018 seasons, a total of 17 phenotypic traits were measured for LA and LG. These included trunk diameter (measured at 65 cm from ground level), bunch fresh weight (FW), number of berries (# berries) per bunch, berry FW, berry dry weight (DW, (drying at 60° C to constant weight), berry water content (estimated as berry FW minus berry DW), berry total soluble solids in concentration (TSS conc) and on a per berry basis (TSS abs), number of seeds (# seeds) per berry, berry skin DW, berry skin total anthocyanins (TA conc and TA abs), berry skin polyphenolic content (TP conc and TP abs), specific leaf area (SLA), and leaf DW. The measurement methods were detailed in Varela et al. (2020). In the present work, an additional variable, the number of bunch (# bunch) was included (**Supplementary Data S2**).

2.7 Statistical analyses

To compare the 5-mC level among vineyards by HPLC technique, the global 5-mC percentages of each sample were subjected to a two-way ANOVA. Post-hoc LSD Fisher's test was then used to compare multiple means using the *agricolae* R package (version 1.3.5). *P* values ≤ 0.05 were considered significant.

For the analysis of the phenotypic variable, the methodology described in Varela et al., (2020) was followed. Principal Components Analysis (PCA) together with its biplot graph were performed with

InfoStat software (InfoStat version 2009, Grupo InfoStat, Córdoba, Argentina). Additionally, to quantify the phenotypic plasticity of each variable, the Relative Distance Plasticity Index (RDPI) was calculated according to Valladares et al. (2006). RDPI represents the absolute value obtained from the difference between the measured values in plants of each origin (LA and LG) divided by the sum of both values. The RDPI index ranges between 0 and 1, indicating the absence or maximum plasticity, respectively. An ANOVA of the RDPI values was performed, and the LSD Fisher comparison test ($P \leq 0.05$) was used with the *agricolae* R package (version 1.3.5).

For the MSAP analysis, four datasets were analyzed: i. 2016 season with Agrelo and Gualtallary plants (re-analysis form Varela et al., 2020 data), ii. 2016 season with LA and LG Lunlunta plants together with the two reference samples, one from Agrelo and other from Gualtallary, iii. Season 2017 with LA and LG Lunlunta plants and iv. 2018 with LA and LG plants (**Fig. 1b, Supplementary Data S3**). Principal Coordinate Analysis (PCoA) and methylation levels were analyzed as described in Varela et al. (2020). A UPGMA hierarchical clustering tree of epiloci together with the bootstrap was carried out in R with the BinMat library, with 1000 permutations and Jaccard's distance.

3. Results

3.1 Climatic variability between seasons and vineyards

Fig. 3 shows the climatic variability in Lunlunta throughout the three seasons from 2016 to 2018, along with the corresponding records for Agrelo and Gualtallary during the 2016 season. The results reveal significant differences in the number of days with temperatures above 33ºC (**Fig. 3b**) and the monthly rainfall (**Fig. 3c**). In terms of rainfall, the 2016 season was wetter than historical records, with an excess of 380 mm and 124 mm, respectively. Conversely, the 2017 and 2018 seasons were drier, with deficits of 33 and 69 mm, respectively. Moreover, the 2016 and 2018 seasons had 8 and 14 more days with temperatures surpassing 33º C compared to the historical data. However, the mean temperature exhibited minimal variation across the seasons (**Fig. 3d**). While the mean annual temperature in the 2016 and 2017 seasons aligned closely with the historical average, the 2018 season decreased 0.4ºC. Notably, the absolute minimum temperature followed a similar pattern throughout the 3 seasons, with minimum temperatures below normal in the 2018 season, particularly in August 2018 and January 2019. Furthermore, 2018 had 8 additional days with temperatures below freezing compared to historical records (see **Supplementary Data S1**).

When comparing the climatic records of the 2016 season among the three vineyards, Lunlunta exhibited a similar trend to Agrelo regarding the number of days with temperatures above 33ºC, monthly precipitation mean temperatures (**Fig. 3b**, 3**c** and 3**d**, respectively). However, Lunlunta experienced fewer days with temperatures below 0ºC compared to Agrelo and Gualtallary (**Fig. 3a**). In both Agrelo and Gualtallary, the recorded rainfall exceeded historic records, with an increase of 176 mm and 122 mm, respectively. The mean annual temperature in Agrelo was 0.1ºC higher than the historical average, whereas Gualtallary exhibited a 0.5ºC decrease. Finally, differences were observed in the number of days with temperatures above 33°C. From May to April, Agrelo had 15 more days than historical records (23 days), while Gualtallary had two fewer days compared to historical data (10 days).

3.2 Loss of phenotypic differences between the vineyards of origins after the transplantation

To establish the phenotypic variability 17 variables were measured, 11 showed significant effects attributed to the season, vineyard of origin, or the interaction between both factors (**Fig. 4**). Particularly, the interaction between season and vineyard was evident in the TSS concentration, with LG plants displaying the highest values in 2018. There was a significant reduction in the variation of trunk diameter between plants from different origins (**Fig. 4a**). Furthermore, a decrease in leaf DW, # seeds per berry, SLA and TA conc were observed in 2018 (**Fig. 4b**). In contrast, increase in the yield variables (# bunches, berry water content and berry FW) was observed, along with the quality variables of TSS abs and skin DW (**Fig. 4c**). Principal component analysis (PCA) did not differentiate LA plants and LG plants (**Fig. 5a**), and there was a considerable increase in data dispersion among LG plants. The PCA of the two variables shown in **Fig. 4a** displayed some separation between LA and LG in 2017, which was lost in 2018 (**Fig. 5b**).

The phenotypic plasticity index, measured as RDPI, ranged between 0.03 and 0.31. Six variables showed significantly different RDPI values between seasons, while the mean value of MB10 remained consistent across seasons. Trunk diameter and TSS conc (°Brix) demonstrated lower plasticity between plants from different origins, while the yield variables of bunch FW, # bunch and # berries, as well as the quality variables of TP conc and TA conc, exhibited higher levels of plasticity (**Table 2**).

3.3 Differences in DNA 5-methylcytosine levels between Agrelo and Gualtallary grapevines persist even one growing season after the transplantation

The global 5-methylcytosine (5-mC) level in MB10 plants grown in Agrelo and Gualtallary was quantified using HPLC technique (**Supplementary Data S4**). The regression lines for cytosine ($y = 0.0353x + 0.0288$) and 5-mC ($y = 0.0445x + 0.0141$) exhibited a high coefficient of determination ($R^2 = 0.9999$).

In the 2016 season, the statistical analysis using ANOVA showed a significant difference in the percentage of 5-mC explained by the vineyard site ($P = 0.005$, **Table 3**). The 5-mC levels were found to be higher in Agrelo compared to Gualtallary (5.7% vs. 3.7%; **Fig. 6**). Furthermore, during the 2016 season, the transplanted plants exhibited a marked increase in 5-mC levels. The LA plants consistently had higher 5-mC levels compared to those LG (9.3% vs. 6.2%). However, these intra-vineyard differences in methylation were not evident in the subsequent 2017 season as plants from different vineyards of origin showed comparable 5-mC levels (7.5% in LA plants vs. 7% in LG plants).

3.4 Significant differences in MSAP patterns were observed between the Agrelo and Gualtallary vineyards

Four datasets were generated for comparison: Agrelo-Gualtallary 2016, Lunlunta 2016, Lunlunta 2017, and Lunlunta 2018, using the primers *AAG-ATC and *AAG-AAT, respectively. Each dataset had a comparable number of MSAP fragments and polymorphic loci analyzed (**Table 4**). On average, the primer combination *AAG-ATC generated 121 fragments, while the combination *AAG-AAT generated 111 fragments. PCoA re-analysis of 149 polymorphic epiloci from the Agrelo-Gualtallary 2016 dataset showed a clear separation between Agrelo and Gualtallary. The first two components explained 54.2%, 43.9% and 55.4% of the differences for the non-methylated, hemimethylated and methylated patterns, respectively (**Fig. 7a**). No differences in 5-mC level were observed with MSAP markers between A and G (**Fig. 7b**).

3.5 The differences in MSAP patterns between the vineyards gradually reduced but were not completely lost after changing the cultivation site

The PCoA of non-methylated and hemimethylated MSAP patterns for the Lunlunta 2016 dataset demonstrated sample grouping according to the vineyard of origin, with a slight overlap between LA and LG observed in the methylated pattern (**Fig. 8a, upper panel**). The non-methylated and methylated patterns of the Agrelo and Gualtallary reference plants fell within the confidence ellipses of LA and LG, while in the hemimethylated pattern, both reference plants were grouped within the LG confidence ellipse. Over the next two seasons (2017 and 2018), the LA and LG groups observed in Lunlunta 2016 gradually approached each other, accompanied by an increase in dispersion (**Fig. 8a, middle and lower panels**). Throughout the three-years analysis, the LA and LG groups did not overlap in the non-methylated pattern. No significant differences in 5-mC levels were observed between LA and LG (**Fig. 8.4, right panel**). However, in the 2016 season, differences in non-methylated and methylated levels were observed between LA and LG (**Fig. 8a.4**). The cluster analysis of polymorphic epiloci showed clear differentiation by vineyard of origin in 2016, along with the Agrelo and Gualtallary reference plants. In 2017, the Agrelo plants continued to cluster, while statistical support was lost in Lunlunta 2018 (**Fig. 9**).

4. Discussion and conclusion

In a previous study, phenotypic and epigenetic differences induced by the environment were observed in the *V. vinifera* cv. Malbec clone MB10, which had been cultivated for 20 years in the Agrelo and Gualtallary vineyards in Mendoza, Argentina. This current study aims to build upon those previous findings by investigating the stability of these vineyard differences through a transplanting experiment. Genetically identical plants (Varela et al., 2020) showing different phenotypes and methylation patterns associated with each vineyard of origin were transplanted and cultivated in a common environment in Lunlunta, Mendoza, Argentina. The objective was to determine whether the initially observed epigenetic differences in the MB10 clone between its original vineyards persists or undergo reconfiguration and lose their differences when cultivated in a common vineyard over three seasons.

The climatic records obtained in this study align with those reported in Mendoza by Urvieta et al., (2021), indicating that the 2016 season was particularly wet, while the 2018 season exhibited the most extreme temperatures among the three seasons analyzed, with higher temperatures and less rainfall compared to the 2017 season. Additionally, the closer geographical proximity of Lunlunta to Agrelo contributes to a greater similarity in the climatic variables observed.

The phenotypic traits of MB10 exhibit a high degree of dynamism, and a rapid homogenization (loss of differences) was observed following the transplantation. The phenotypic plasticity indices (RDPI) between the vineyards of origin and the common vineyard revealed the following insights: i. the mean phenotypic plasticity value of MB10 remained consistent at 0.135; ii. the least plastic trait was Ø trunk, presenting significant differences in 2017 that were lost as the cuttings continued growing in 2018; iii. the variables Bunch FW and # Berries (per bunch) exhibited higher plasticity compared to Berry FW, Berry water and # Seeds (per berry). The loss of phenotypic differences observed in the new location could be attributed to the influence of environmental conditions on the expression of phenotypic traits. Environmental factors such as temperature and water availability have been shown to have a significant impact on anthocyanin accumulation. The concentration of total anthocyanins can vary between clones, seasons and production areas (Ortega-Regules et al., 2008; Pagliarani et al., 2019), and under high temperatures (> 35ºC) the accumulation of anthocyanins tends to slow down (Keller, 2020). This could explain the decrease in TA conc observed in 2018. Also, the temperatures registered in the 2018 season can affect the berry TSS conc and TSS abs by improving photosynthetic partitioning (Suter et al., 2021). The number of flower primordia and the percentage of fruit set on the grapevines are strongly modulated by environmental conditions (Keller, 2010), which could account for the high plasticity observed in the number of bunches. Also, the age of the plants may have played an important role in these traits, because 2017 was the first harvest for the evaluated plants. Despite previous studies suggesting that the number of flowers per inflorescence is a trait with high plasticity (Keller, 2010), our findings indicate that the number of berries per bunch remained unaffected by the season, indicating a lower level of plasticity for this particular trait. It is worth noting that significant variations in Berry FW have been observed among *V. vinifera* cultivars, clones of the same cultivar, and even between berries of the same bunch, where berry weight can vary more than double (Keller, 2020). This variation can be attributed to the influence of environmental factors on cell expansion. Typically, there is a positive correlation between # Seeds (per berry) and Berry FW (Keller, 2020); however, in this study, fewer # Seeds were observed while higher Berry FW was found in LA and LG plants during the 2018 season. Overall, these findings highlight the complex interplay between genetic factors and environmental conditions in shaping the phenotypic characteristics of grapevines.

In the 2016 season, we quantified the 5-mC level of MB10 using the HPLC technique in plants cultivated in the vineyards of origin and in the common vineyard. Concentration values were used for 5-mC quantification instead of areas under the curve, facilitating results comparison with other analytical procedures that measure absorbance at different wavelengths. We used *A. thaliana* and *S. tuberosum* DNA as control with low and high 5-mC content, respectively, as recommended by Ramsahoye (2002). The results obtained in the *A. thaliana* and *S. tuberosum* control samples align with previously reported measurements using HPLC, such as those reported by Alonso et al. (2015; 5.3% vs. 24.6%) and Alonso et al. (2016; 4.6% for *A. thaliana*). Higher levels of 5m-C were observed in Agrelo (5.8%) compared to Gualtallary (3.7%) vineyards of origin. To our knowledge, there is a lack of quantification for global DNA methylation in *V. vinifera* shoot tips using HPLC. Alonso et al. (2019) employed HPLC-derived

methylation measurements, reporting a value of 9.98 % of 5-mC, although the authors performed the study with only two individuals, did not specify the grapevine cultivar and used total expanded leaf instead of shoot tip. Alternative methods have been employed to measure global DNA methylation values in grapevines. Vidalis et al. (2016) show in their Figure S1 a methylation level of 6-7% measured by whole-genome bisulfite sequencing. Also, Gao et al. (2020) shows a Merlot grape berry methylation level of 7.8% obtained by single-base DNA methylation bisulfite sequencing. Previous findings reported by Varela et al. (2020) used the Reduced Representation Bisulfite Sequencing tecniche on the same plant material and the same tissue and found 7.4% of methylation in CpG context, while 3.2% and 0.5% were methylated in the CpHpG and CpHpH contexts, respectively, regardless of the vineyard of origin. The higher methylation in Agrelo was unexpected since previous evidence suggested that higher levels of UV-B radiation, characteristic of high-altitude locations like Gualtallary, are associated with increased cytosine methylation (Marfil et al., 2019; Xie et al., 2017). During the 2016 season, Lunlunta plants derived from cuttings obtained from Agrelo consistently showed higher 5-mC levels compared to those from Gualtallary (9.3% vs. 6.2%). However, these intra-vineyard differences in methylation were not evident in the subsequent 2017 season, in which the evaluated samples showed on average 7.5% and 7% for LA and LG plants. Significant variations in DNA methylation levels environmentally induced were assessed in chicory (*Cichorium intybus* L.) after a cold treatment across root and shoot apex tissues using HPLC, as reported by Demeulemeester et al. (1999). During the growing season, root tissue before cold treatment exhibited a minimum of 8.9% 5-mC measurement which even up to 20% after cold treatment. A substantial decrease in methylation levels after cold treatment (14% to 4% 5-mC) was also observed for the last two harvest dates. Likewise, statistically significant differences in DNA methylation before and after cold treatment were observed in shoot apices. In a more recent investigation, diverse levels of global DNA methylation in garden cress (*Lepidium sativum*) were identified using HPLC technique (Yanez Barrientos et al., 2013). The study subjected hydroponically grown plants to varying concentrations of Selenium and Cadmium. In control plants, DNA methylation was recorded at 14.43% ± 0.15%, while in cultures exposed to the elements, the 5-mC content was elevated to 18%. It is crucial to note that our objective was not to analyze methylation differences between old plants and freshly established cuttings, but to identify stable differences among plants from different vineyard of origins in field conditions. This is because the observed changes in global DNA methylation levels among seasons could be related to several factors. One possibility is the structural differences in shoot tips across different seasons. Factors such as varying leaf, tendril, or bud quantities on the shoot tip could contribute to these fluctuations. Due to the absence of specific tissue dissections across seasons, the focus of our work is on intra-seasonal differences rather than inter-season. Also, DNA methylation is a crucial mechanism involved in plant stress responses, and it is well established that the cutting and transplanting process can induce various abiotic stresses on the plant. Therefore, it is reasonable to speculate that the observed increase in DNA 5-mC levels after transplantation may be a response to the stress imposed by the new environmental conditions. The significant increases in DNA methylation levels between the vineyards of origin and Lunlunta may be associated with gene-poor heterochromatic regions. This aligns with findings by Niederhuth, et al. (2016) in *V. vinifera*, where a negative correlation was observed between DNA methylation levels in the three methylation contexts (mCG, mCHG, mCHH) and gene numbers. While the functional consequences of this increment could be for the maintenance of the genome architecture and integrity, additional focused studies are required. If the observed methylation increments predominantly occurred in mCG regions, it could potentially impact gene expression since genes that are only mCG methylated within the gene body are often constitutively expressed genes (Niederhuth et al., 2016). Also, to further understand the molecular mechanism underlying the different 5-mC levels, future research should investigate the expression of methyltransferases and demethylases in both sites.

A comprehensive 3-year study (2016, 2017 and 2018 growing seasons) was conducted to analyze the 5-mC polymorphism in MB10 using MSAP markers. While MSAP markers are not precise for 5-mC quantification, they are valuable for observing methylation changes in controlled assays (Alonso et al., 2016). After transplantation, no significant changes in the 5-mC level, as measured by MSAP markers, were observed between plants of different origin. The discrepancy between the results

obtained using MSAP and HPLC is expected due to the inherent differences in their respective quantification methods. MSAP markers have limitations in representing the overall 5-mC level of the entire genome, as they depend on the distribution of CCGG cutting sites and the relative frequency of different methylation patterns, such as CG, CHG and CHH (Alonso et al., 2016). Furthermore, MSAP fragments cannot distinguish methylated cytosines in CHH contex, fully methylated cytosines in the CCGG context, or cytosines from the CTG or CAG contexts. In contrast, the HPLC technique enables the effective separation of C and 5-mC from other nucleosides. The chromatogram demonstrated that deoxycytidine and 5-methyl-deoxycytidine appear first due to their high hydrophilicity, while deoxyadenosine, which has a higher affinity for the stationary phase, appears last (Rozhon et al., 2008). Based on these findings, we conclude that MSAP markers do not provide informative data regarding changes in 5-mC levels. It is essential to consider the limitations and differences of each technique when interpreting results related to DNA methylation.

Differences in DNA methylation patterns between LA and LG plants were evident through the use of MSAP markers. The similarity of the MSAP markers obtained from Agrelo and Gualtallary reference plants to those of LA and LG plants, respectively, led us to infer that the Lunlunta plants in 2016 exhibited epigenetic similarities to their respective vineyards of origin. Interestingly, the hemimethylated pattern displayed higher sensitivity to the change of vineyard, while the non-methylated pattern was the least sensitive. To investigate the potential involvement of an active demethylation mechanism in the loss of differences in methylated and hemimethylated patterns, it would be valuable to analyze the expression enzymes such as ROS1, DME, DML2 and DML3 (Lucibelli et al., 2022). Furthermore, future studies could consider selecting a common vineyard that is completely distinct from the vineyards of origin to further explore the observed phenomena. Notably, transgenerational epigenetic memory of stable epigenetic marks has been observed in "climate-smart" crops in response to factors such as thermal increases, UV radiation and pathogen attack, indicating the potential for enhancing crop resistance to specific stress conditions (Varotto et al., 2020). Moreover, in rice (*Oriza sativa* L.), approximately 29% of the methylation changes induced by drought conditions were maintained even after the removal of water stress (Wang et al., 2011). However, contrasting results have been reported in alligator weed (*Alternanthera philoxeroides*) and potatoes, where epigenetic reprogramming was observed from one year to the next (Gao et al., 2010; Ibañez et al., 2021). These findings highlight the complex nature of epigenetic regulation and suggest the existence of dynamic mechanisms that can both maintain and modify epigenetic marks in response to environmental cues. Further investigation into these mechanisms can provide valuable insights for crop improvement and stress adaptation strategies.

Although MSAP methods are unable to reveal causality between epigenetic and phenotypic variation, by analyzing the clone's phenotype we aim to compare trends in DNA methylation and phenotypic dynamics between cuttings of different vineyards of origin. A similar approach using MSAP markers was performed in *Solanum kurtzianum* (Ibañez et al., 2021) and in natural populations of clonal plants of *Hydrocotyle vulgaris L* (Wang et al., 2020). We did not find correlation between epigenetic and phenotypic variation (data not shown), probably because of the temporal disparity in shoot tips collection and the moment of phenotypic measurements. Although the mechanisms governing epigenetic regulation of gene expression are unclear, increasing evidence suggests the involvement of DNA methylation patterns (Niederhuth and Schmitz, 2017). To explore the association between DNA methylation and phenotypic plasticity, an alternative approach could be considered: i. identifying contrasting phenotypes in plants of the same clone cultivated in different environments, ii. selecting candidate genes for expression analyzes, and iii. examining the methylation of differentially expressed genes.

In **conclusion**, two different approaches confirm that a gradual decrease in environmentally induced epigenetic differences occurs after changing cultivation site for MB10 plants in Agrelo and Gualtallary. Although the different 5-mC levels are lost within two years after transplantation, the distinct methylation patterns observed in non-methylated MSAP markers persist even after 3 years. This persistence suggests a certain mitotic stability in the non-methylated cytosines pattern specific to each vineyard, indicating it could serve as a distinct epigenetic marker in grapevine plants.

Authors' contributions

AV: Data curation, methodology, investigation, formal analysis, writing - original draft, writing - review and editing. CFM: Conceptualization, project administration, funding acquisition, methodology, formal analysis, supervision, writing - review and editing. SGT: Laboratory facilities, contributed to the experimental execution. AF: HPLC technique, formal analysis of quantitative chromatographic results, writing - review and editing. SA: Funding acquisition, formal analysis. FB: Funding acquisition, plant resources, development of Lunlunta experimental plot. FJB: Conceptualization, project administration, funding acquisition, methodology, formal analysis, supervision, writing - review and editing.

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Figures

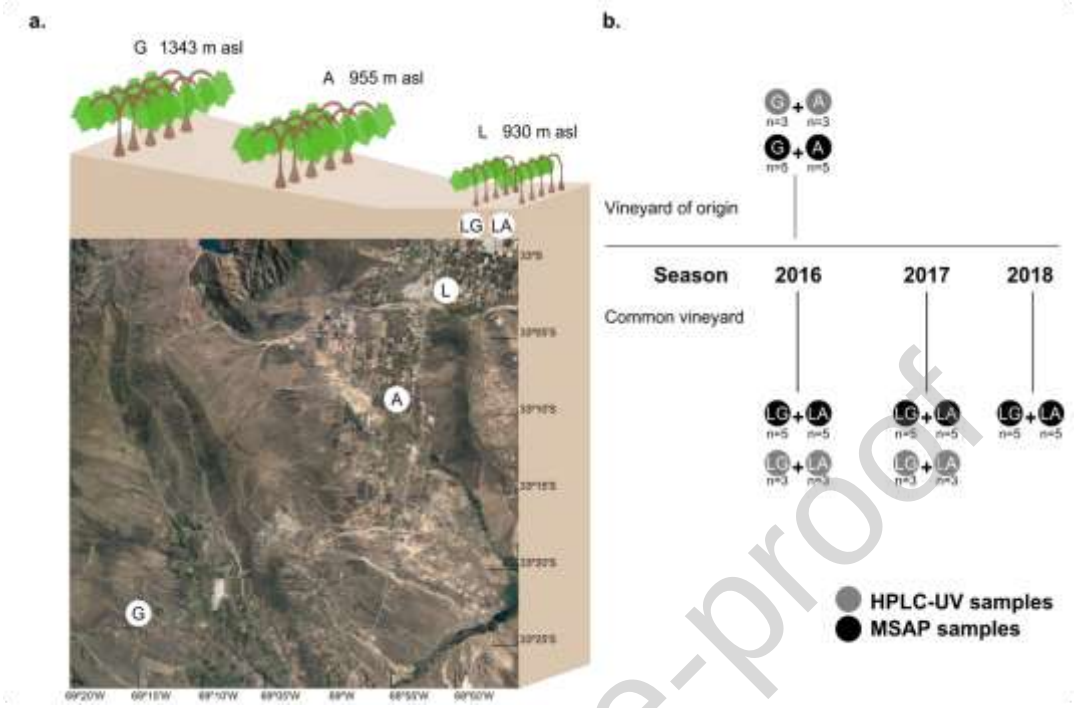

Fig. 1 Overview of the experimental approach. Site locations and altitudes of the vineyards of origin (A, G) and the common vineyard (L) in Mendoza province, Argentina, planted with *Vitis vinifera* cv. Malbec clone MB10 (**a**). Groups of samples used for evaluating the dynamics of DNA methylation by HPLC-DAD and MSAP analyses (**b**). A, Agrelo; G, Gualtallary; LA, plants implanted and cultivated in Lunlunta and derived of cuttings obtained from Agrelo in 2015; LG, plants implanted and cultivated in Lunlunta and derived of cuttings obtained from Gualtallary in 2015. n= number of independent plants analyzed in each group of samples.

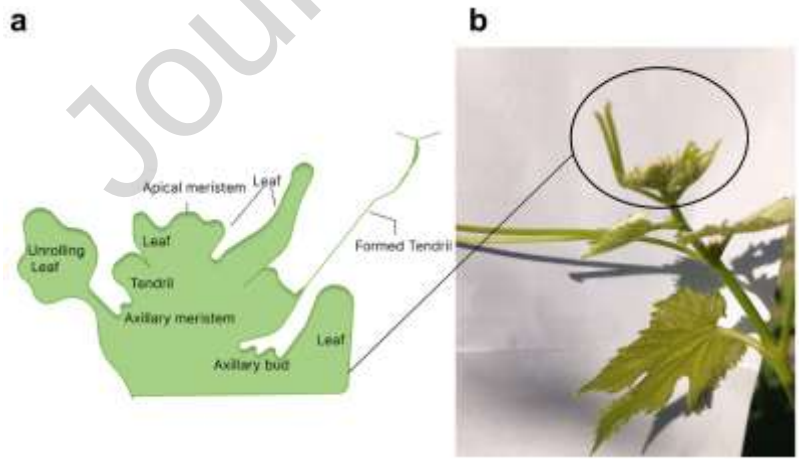

Fig. 2 Malbec shoot tip sampled for DNA extraction. Diagrammatic longitudinal section of the sampled shoot tip (a). Shoot tip photo (b). Diagram inspired by Keller's "Science of grapevine" book (2020). Photo by Anabella Varela taken in November 2018.

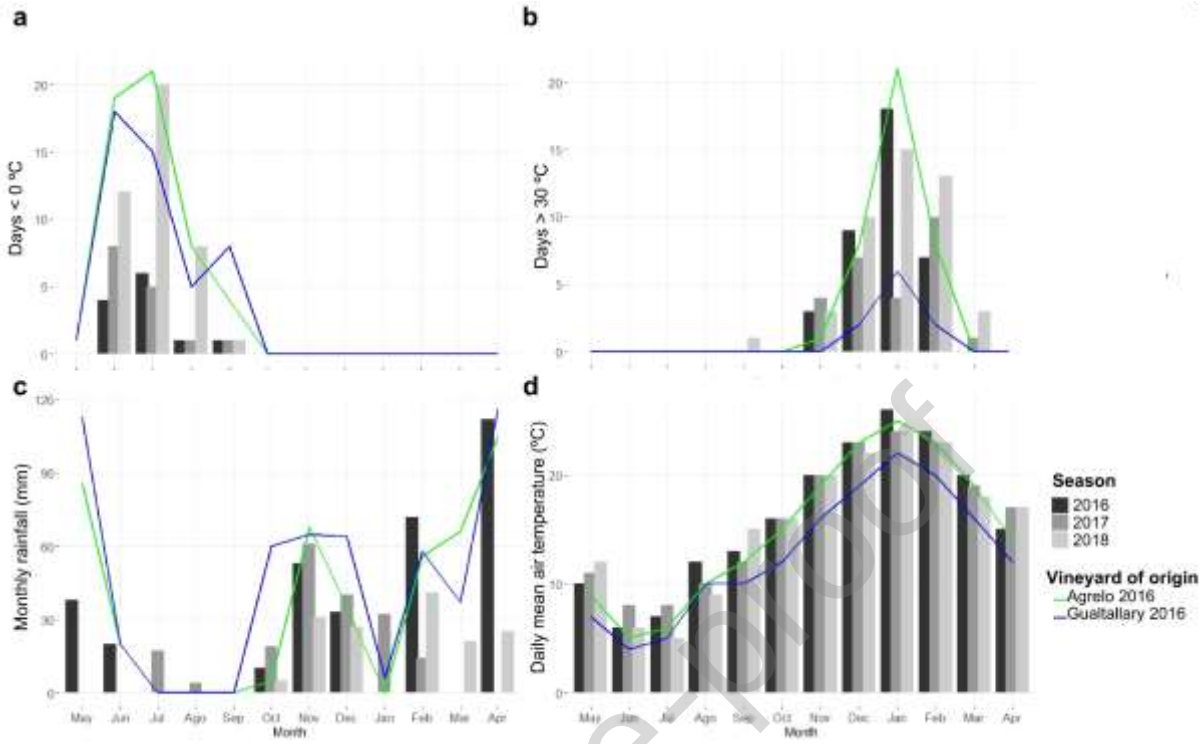

Fig. 3 Graphic visualization of four climatic parameters measured from May to April during the 2016, 2017 and 2018 seasons in Lunlunta (**gray bars**) and during the 2016 season in Agrelo and Gualtallary in the province of Mendoza, Argentina. Days with temperatures below 0º C (**a**), days with temperatures above 33º C (**b**), monthly rainfall (**c**), daily mean air temperature (**d**).

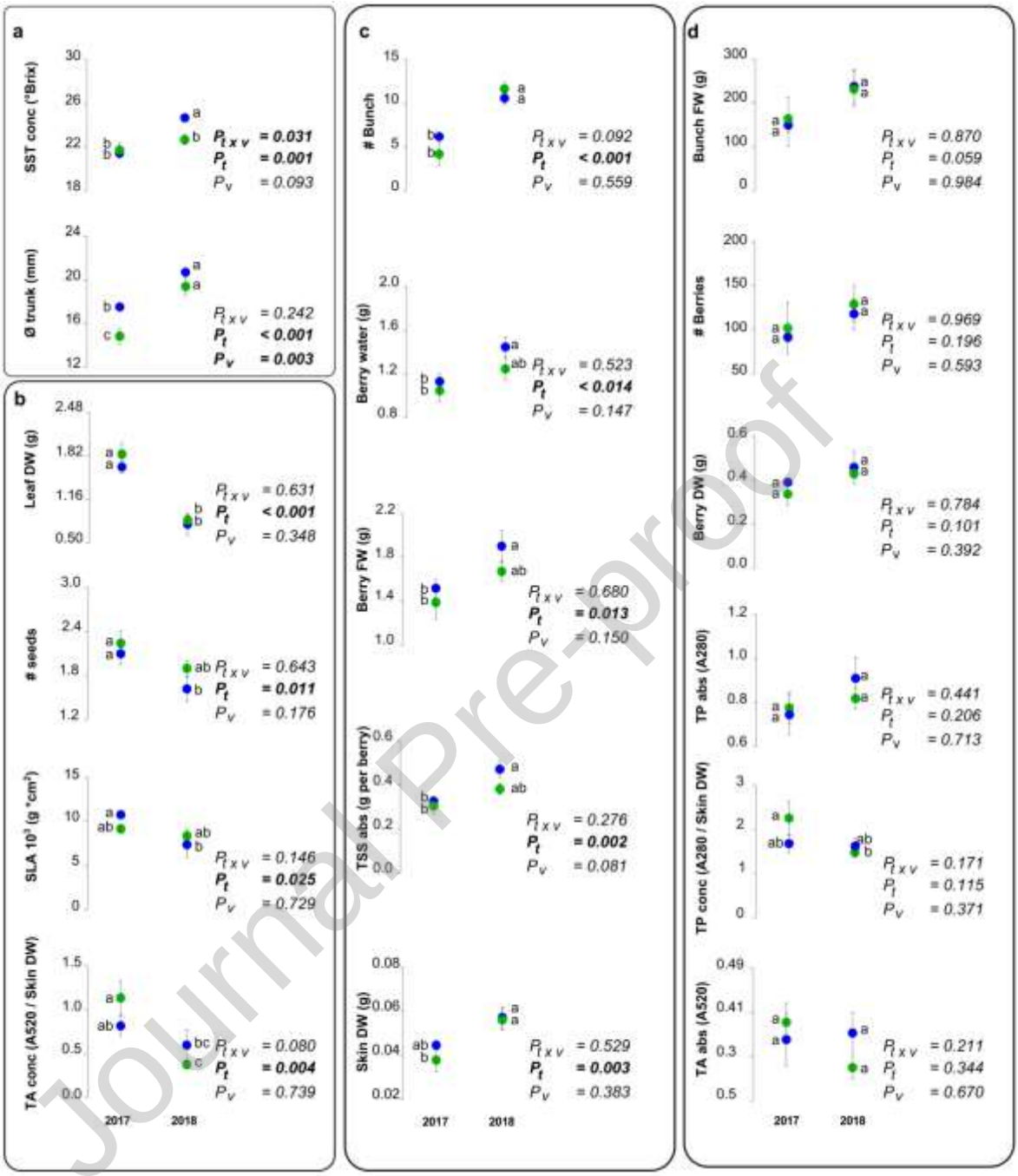

Fig. 4 Multifactorial analysis of 17 phenotypic variables of *Vitis vinifera* cv. Malbec clone MB10 cultivated in Lunlunta (Mendoza, Argentina) during two seasons (2017 and 2018). The evaluated plants were implanted in Lunlunta in 2015 and derived from cuttings obtained from two contrasting vineyards of origin: Agrelo and Gualtallary (LA and LG, respectively). Values are means (n = 5) for LA (green) and LG (blue) and season ± standard error. Different letters indicate significant differences (Fisher's LSD, $P \leq 0.05$). Variables with effects on the phenotype due to the interaction season × vineyard of origin ($P_{s \times v}$), to the season (P_s) or to the vineyard (P_v; **a**); variables with significantly decreased effects on the phenotype between seasons (**b**); variables with significantly increased effects on the phenotype between seasons (**c**) and variables without significant effect (**d**). Ø trunk, trunk diameter; # berries, number of berries; FW, fresh weight; DW, dry weight; TSS, total soluble solids; TA, total anthocyanins; TP, total polyphenols; conc, concentration; abs, absolute amounts.

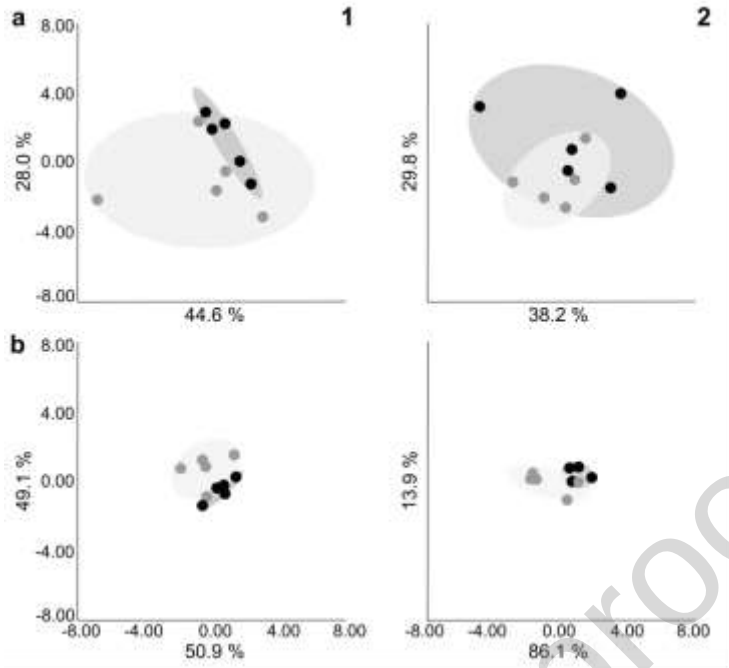

Fig. 5 Biplot visualization and 0.95 confidence ellipses of the Principal Component Analysis of phenotypic traits in *Vitis vinifera* cv. Malbec clone MB10 transplanted in LA (gray) and LG (black) for the 2017 (**1**) and 2018 (**2**) season. Analysis of 17 phenotypic traits (**a**) and of the 2 variables that had an effect on the phenotype due to the vineyard of origin (trunk diameter) or the vineyard x season interaction (**b**).

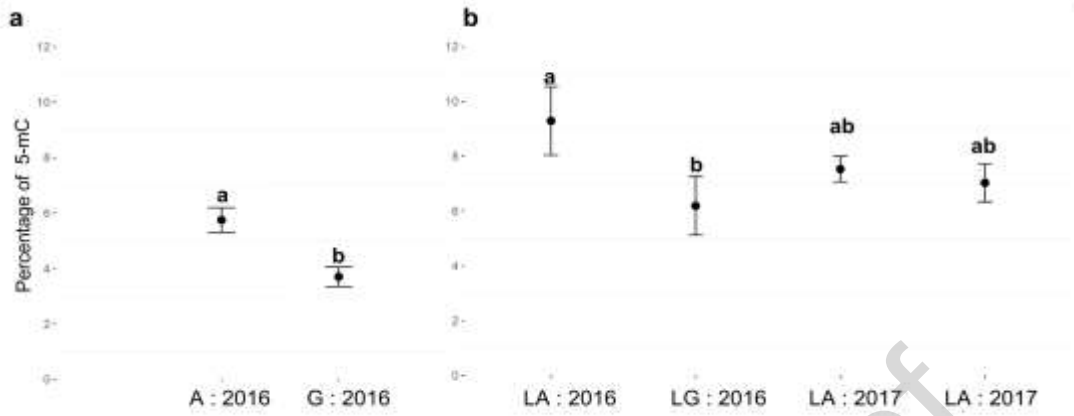

Fig. 6 Average percentage of 5-methylcytosine (5-mC) in plants of *Vitis vinifera* cv. Malbec clone MB10 in the vineyards of origin Agrelo and Gualtallary (**a**) and transplanted in Lunlunta (**b**) in Mendoza province, Argentina, analyzed by HPLC-DAD. A, Agrelo; G, Gualtallary; LA, plants implanted and cultivated in Lunlunta and derived from cuttings obtained from Agrelo; LG, plants implanted and cultivated in Lunlunta and derived from cuttings obtained from Gualtallary. Error bars indicate standard error. Different letters indicate significant differences (Fisher's LSD $P \leq 0.05$).

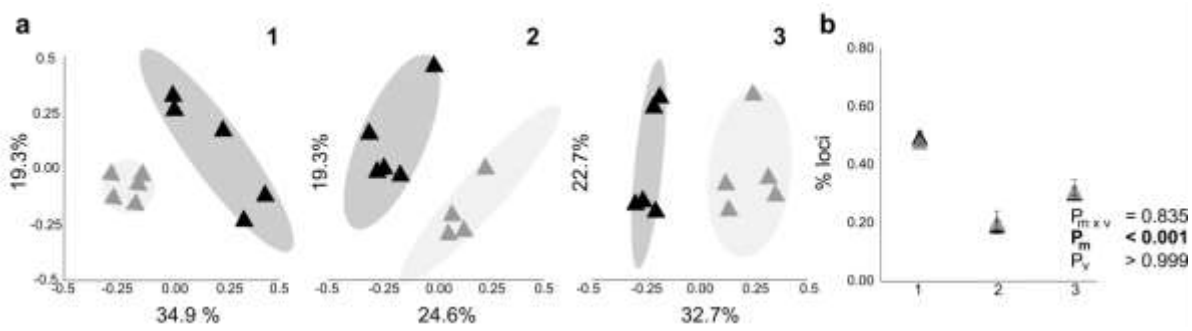

Fig. 7 Epigenetic variability estimated with a re-analysis of methylation sensitive amplified polymorphism markers (MSAP) obtained in Varela *et al.*, (2020) in *Vitis vinifera* cv. Malbec clone MB10 grown in Agrelo (gray) and Gualtallary (black) in the 2016 season using only two primer combinations (in the original article, three primer combinations were used, with similar results). Biplot visualization and 0.95 confidence ellipses of the PCoA analyzing non-methylated (**a1**), hemimethylated (**a2**), methylated (**a3**) patterns. Methylation levels of non-methylated (**b1**), hemimethylated (**b2**) and methylated (**b3**) patterns. *P*-values of the effects in the level of methylation due to the interaction methylation pattern × vineyard of origin ($P_{m\,x\,v}$), to the methylation pattern (P_m) or to the vineyard (P_v) are indicated.

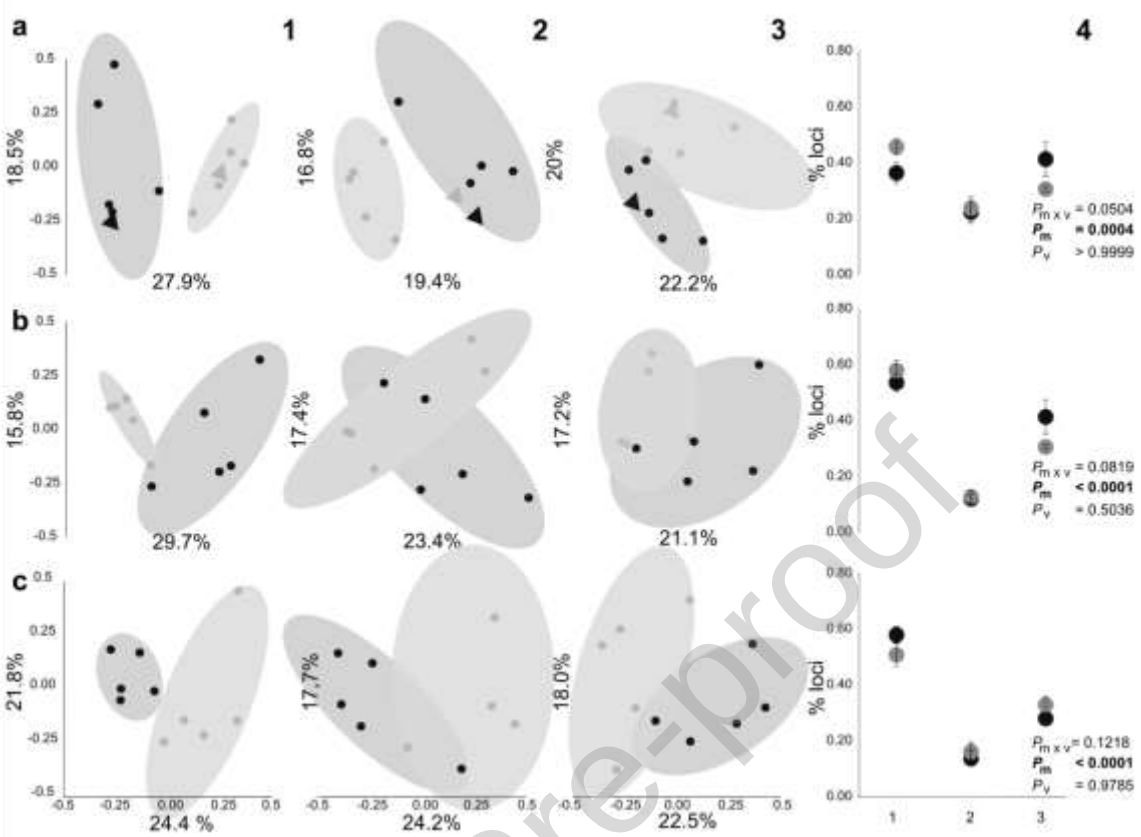

Fig. 8 Epigenetic variability estimated with methylation sensitive amplified polymorphism markers (MSAP) in *Vitis vinifera* cv. Malbec clone MB10 transplanted in Lunlunta during three seasons (2016, 2017 and 2018). The evaluated plants were implanted in Lunlunta in 2015 and derived from cuttings obtained from two contrasting vineyards of origin: Agrelo (LA, gray circle) and Gualtallary (LG, black circle). Biplot visualization and 0.95 confidence ellipses of PCoA for seasons 2016 (**a**), 2017 (**b**) and 2018 (**c**) analyzing only the non-methylated (**1**), hemimethylated (**2**) and methylated (**3**) patterns. Methylation levels for patterns 1, 2 and 3 (**4**). *P*-values of the effects in the level of methylation due to the interaction methylation pattern × vineyard of origin ($P_{m \times v}$), to the methylation pattern (P_m) or to the vineyard (P_v) are indicated. Reference plant from Agrelo (gray triangle); reference plant from Gualtallary (black triangle).

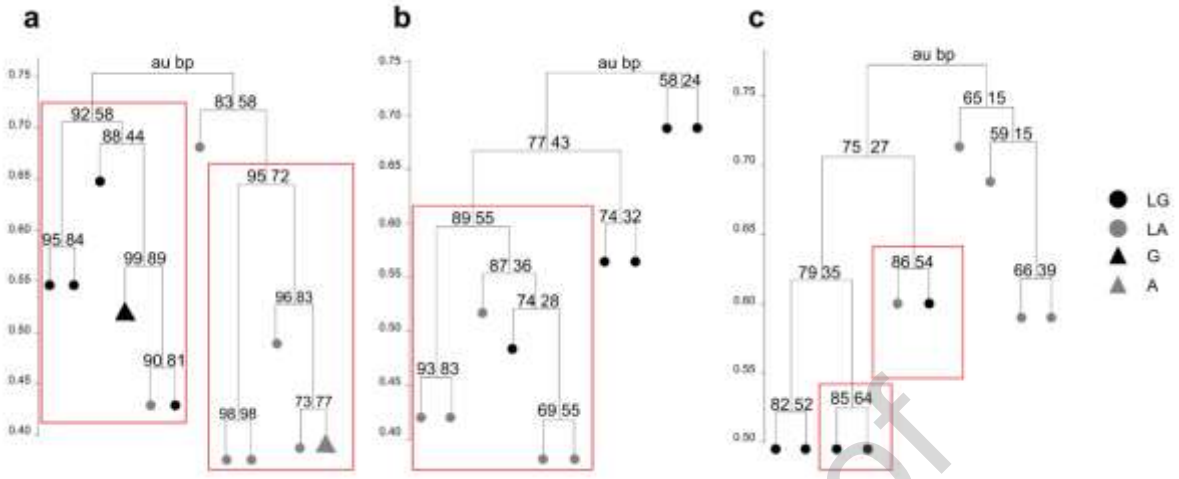

Fig. 9 UPGMA hierarchical clustering tree derived from methylation sensitive amplified polymorphism markers (MSAP) in *Vitis vinifera* cv. Malbec clone MB10 cultivated in Lunlunta during three seasons Lunlunta 2016 (**a**), Lunlunta 2017 (**b**) and Lunlunta 2018 (**c**). The analysis was performed with 1000 bootstrap repetitions and two types of *P*-values are presented: approximately unbiased (au) and bootstrap probability (bp). au ≥ 85% is considered as evidence of clustering and highlighted with a red rectangle. A, reference plant from Agrelo; G, reference plant from Gualtallary; LA, plants implanted and cultivated in Lunlunta and derived of cuttings obtained from Agrelo in 2015; LG, plants implanted and cultivated in Lunlunta and derived of cuttings obtained from Gualtallary in 2015

Tables

Table 1 Date of shoot tips sampling, phenotypic measurements and molecular measurements performed in *Vitis vinifera* cv. Malbec clone MB10 implanted and cultivated in Lunlunta common vineyard. yyyy/mm/dd format. NM, not measured.

Season	Shoot tip sampling	Phenotypic measurements	Molecular analyses
2016	2016/11/01	NM	HPLC, MSAP
2017	2017/11/11	2018/02/28	HPLC, MSAP
2018	2018/11/22	2019/03/15	MSAP

Table 2 Relative Distance Plasticity Index (RDPI) of *Vitis vinifera* cv. Malbec clone MB10 implanted and cultivated in Lunlunta and derived from cuttings obtained from Agrelo and Gualtallary. RDPI values of 17 phenotypic traits are means ± standard error and obtained by comparing Lunlunta plants of different vineyards of origin. The list of phenotypic traits is highlighted on a color scale from red to green for maximum to minimum values and it is presented in decreasing order according to the trait average. *P* - values ≤ 0.05 are indicated in bold. Ø trunk, trunk diameter; # berries, number of berries; FW, fresh weight; DW, dry weight; TSS, total soluble solids; TA, total anthocyanins; TP, total polyphenols; conc, concentration; abs, absolute amounts.

Season	2017			2018			Trait average	*P* - value
Bunch FW (g)	0.27	±	0.03	0.21	±	0.03	0.24	0.187
TP conc	0.20	±	0.03	0.25	±	0.03	0.23	0.267
# Bunch	0.31	±	0.04	0.09	±	0.04	0.20	**<0.001**
# Berries (per bunch)	0.21	±	0.03	0.19	±	0.03	0.20	0.612
TA conc (A520 / Skin DW)	0.18	±	0.02	0.12	±	0.02	0.15	**0.047**
Leaf DW (g)	0.10	±	0.02	0.21	±	0.02	0.16	**0.002**
SLA (g/cm2)	0.10	±	0.03	0.18	±	0.03	0.14	**0.047**
Berry DW (g)	0.14	±	0.02	0.13	±	0.02	0.14	0.812
TP abs (A280)	0.14	±	0.02	0.12	±	0.02	0.13	0.405
TSS abs (g per berrie)	0.11	±	0.02	0.12	±	0.02	0.12	0.714
Skin DW (g)	0.13	±	0.02	0.10	±	0.02	0.12	0.359
TA abs (A520)	0.12	±	0.02	0.10	±	0.02	0.11	0.260
Berry water (g)	0.10	±	0.02	0.11	±	0.02	0.11	0.774
# Seeds (per berry)	0.09	±	0.02	0.11	±	0.02	0.10	0.293
Berry FW (g)	0.10	±	0.02	0.09	±	0.02	0.10	0.810
Ø Trunk (mm)	0.08	±	0.01	0.05	±	0.01	0.07	**0.002**
TSS conc (°Brix)	0.03	±	0.01	0.04	±	0.01	0.04	**0.038**
Season average	0.14			0.13				

Table 3 Methylation level differences among vineyards quantified by HPLC-DAD, analyzed by a two way Analysis of Variance (ANOVA) for the effect of Site and Season, as well as their interaction, on the 5-methylcytosine percentage of the total cytosines (5mC%) in *Vitis vinifera* cv Malbec clone MB10. The analysis was conducted separately for two sets of sites: Agrelo-Gualtallary, and Lunlunta vineyards. A, Agrelo; G, Gualtallary; LA, plants implanted and cultivated in Lunlunta and derived of cuttings obtained from Agrelo; LG, plants implanted and cultivated in Lunlunta and derived of cuttings obtained from Gualtallary, *df*, degrees of freedom. *P* - values ≤ 0.05 are indicated in bold.

Site:Season	Source of variation	*df*	Sums of squares	*F* - value	*P* - value
A-G:2016	Site	1	12.722	12.92	**0.0049**
	Residuals	10	9.849		
LA-LG:2016-2017	Site of origin	1	21.26	3.9030	0.0637
	Season	1	0.54	0.1000	0.7560
	Site:Season	1	8.96	1.6450	0.2160
	Residuals	18	98.03		

Table 4 Methylation sensitive amplified polymorphism (MSAP) fragments obtained in *Vitis vinifera* cv. Malbec clone MB10 implanted in Lunlunta vineyard for the methylation dynamic analysis. For each data series are indicated. i. the number and percentage of MSAP fragments obtained for each primer combination, ii. classification of the total MSAP fragments based on their methylation patterns and iii. the epiloci classification as unique, monomorphic and polymorphic. Only polymorphic epiloci were used in the statistical analyses.

	Season : Vineyard			
i. Raw data	2016 : A-G	2016 : L	2017 : L	2018 : L
*AAG-ATC fragments	122 (52%)	161 (62%)	83 (48%)	67 (42%)
*AAG-AAT fragments	114 (48%)	99 (38%)	90 (52%)	95 (59%)
Total of fragments	236 (100%)	260 (100%)	173 (100%)	162 (100%)
ii. Fragment classification				
Genetic loci	52 (22%)	22 (8%)	35 (20%)	28 (17%)
Epiloci	184 (78%)	238 (92%)	138 (80%)	134 (83%)
iii. Epiloci classification				
Unique	103 (38%)	189 (52%)	120 (58%)	118 (57%)
Monomorphic	23 (8%)	3 (1%)	5 (2%)	6 (3%)
Polymorphic	149 (54%)	174 (47%)	82 (40%)	82 (40%)
Total	275 (100%)	366 (100%)	207 (100%)	206 (100%)

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Highlights

- The environment plays a role in shaping the epigenetic landscape of *Vitis vinifera*
- Phenotypic and epigenetic dynamics was studied in a *transplanting experiment*
- Original differences in DNA methylation levels are lost within two years of transplantation
- Hemimethylated pattern are reprogramed after transplantation
- Non-methylated pattern exhibited greater stability, indicating a potential memory