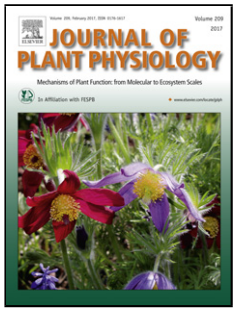

Title: Changes in DNA methylation pattern of apple long-term *in vitro* shoot culture and acclimatized plants

Authors: Andrea Gulyás, Judit Dobránszki, Erzsébet Kiss, Jaime A. Teixeira da Silva, Katalin Posta, Norbert Hidvégi

PII: S0176-1617(19)30070-7
DOI: https://doi.org/10.1016/j.jplph.2019.05.007
Reference: JPLPH 52980

To appear in:

Received date: 27 November 2018
Revised date: 17 April 2019
Accepted date: 13 May 2019

Please cite this article as: Gulyás A, Dobránszki J, Kiss E, da Silva JAT, Posta K, Hidvégi N, Changes in DNA methylation pattern of apple long-term *in vitro* shoot culture and acclimatized plants, *Journal of Plant Physiology* (2019), https://doi.org/10.1016/j.jplph.2019.05.007

Changes in DNA methylation pattern of apple long-term *in vitro* shoot culture and acclimatized plants

Andrea Gulyás[1*], Judit Dobránszki[1], Erzsébet Kiss[2], Jaime A. Teixeira da Silva[1], Katalin Posta[2], Norbert Hidvégi[1]

[1] Research Institute of Nyíregyháza, IAREF, University of Debrecen, Nyíregyháza, P.O. Box 12, H-4400, Hungary; gulyas.andrea@agr.unideb.hu, dobranszki@freemail.hu, jaimetex@yahoo.com, hidvegi.norbert@agr.unideb.hu

[2] Institute of Genetics, Microbiology and Biotechnology, Faculty of Agricultural and Environmental Sciences, Szent István University, Gödöllő, Páter Károly u.1., H-2100, Hungary; kiss.erzsebet@mkk.szie.hu, posta.katalin@mkk.szie.hu

Abstract

DNA methylation is a process of epigenetic modification that can alter the functionality of a genome. Using whole-genome bisulfite sequencing, this study quantify the level of DNA methylation in the epigenomes of two diploid apple (*Malus x domestica*) scion cultivars ('McIntosh' and 'Húsvéti rozmaring') derived from three environmental conditions: *in vivo* mother plants in an orchard, *in vitro* culture, and acclimatized *in vitro* plants. The global DNA methylation levels were not dependent on the source of plant material, and the average level of DNA methylation was 49.77%, 34.65% and 8.77% in CpG, CHG and CHH contexts, respectively. Significant differences in DNA methylation were identified in 586 (specifically 334, 201 and 131 in CpG, CHG and CHH contexts, respectively) out of 45,116 genes, including promoter and coding sequences. These were classified as differentially methylated genes (DMGs). This is a 1.3% difference in the level of DNA methylation of genes in response to a change in the environment. Differential methylation was visualised by MA plots and functional genomic maps were established for biological processes, molecular functions and cellular components. When the DMGs were considered, *in vitro* tissue culture resulted in the highest level of methylation, but it was lower in acclimatized *in vitro* plants which was similar to that in the mother

tree. Methylation patterns of the two scions differed, indicating cultivar-specific epigenetic regulation of gene expression during adaptation to various environments. After selecting genes that displayed differences larger than ±10% in CpG and CHG contexts, or larger than ±1.35% in the CHH context from among the DMGs, they were annotated in Blast2GO v5.1.12 for Gene Ontology. DMGs identified as MD07G1113000 (protein transport), MD08G1041600 (extracellular space), MD09G1054800 (phosphatidic acid binding), and MD10G1265800 (not annotated) were methylated in all three contexts in *in vitro* shoots. These DNA methylation results suggest that epigenetic changes may contribute to the adaptation of apple to environmental changes by modifying the epigenome and thereby gene expression.

Keywords: epigenetic; *Malus* sp.; tissue culture; whole-genome bisulfite sequencing

Introduction

The firstly published high-quality draft genome sequence of domesticated apple (*Malus x domestica* Borkh.) was by Velasco et al. (2010), who used Sanger sequencing and 454 pyrosequences. The domesticated apple genotype is highly heterozygous, while sequencing and assembly is a technical challenge. Velasco et al. (2010) described 57,386 putative genes, 31,678 transposable element (TE)-related open reading frames (ORFs), 11,444 apple-specific genes and 4,021 transcription factor genes. Daccord et al. (2017) resequenced the *M. x domestica* genome with the latest sequencing and optical mapping technologies. They reduced the estimated number of annotated genes in apple to 42,140 and the genome size to 649.7 Mb.

Apart from changes to DNA sequences, various epigenetic modifications in the genome, including DNA methylation, chromatin methylation and acetylation, as well as changes to siRNA, contribute to the actual operation and functionality of a genome due to modifications in gene expression (Probst and Scheid, 2015; Hewezi, 2018; Lee and Seo, 2018). A DNA methylation pattern can be inherited over several generations, and the molecular pathways responsible for this pattern may induce natural variation (Niederhuth et al., 2016). When methylation occurs, the level of methylated cytosine (5mC) varies by as much as 30% in plants (Cokus et al., 2008).

In plants, C methylation may or may not be linked, as CpG, CHG and CHH, where G is guanine, and H may represent any other nucleotide other than G. While CHG is symmetric, CHH is an asymmetric sequence (Gouil and Baulcombe, 2016). In plants, CpG, CHG and CHH play important roles, and CpG is the most methylated area, CHG is moderately methylated, while CHH is the least methylated region. In plants, CG methylation is maintained by DNA methyltransferase 1 (MET1), which is a homologue of the conserved mammalian DNA methyltransferase 1 (DNMT1) (Cao et al., 2000). A high level of CHG context in *Arabidopsis thaliana* L. Heynh. (thale cress) is maintained by a plant-specific CHROMOMETHYLASE 3 (CMT3), whereas CHH methylation and to some extent CHG methylation are generally maintained by domains rearranged methyltransferases (DRMs) and CMT2 methyltransferase (Zemach et al., 2013). Cytosine methylation is frequently found in transposons, TEs and other repeated sequences in a wide range of plant species (Zemach et al., 2010). DNA methylation can deactivate TEs, whose reactivation is prevented while methylated (Tirado-Magallanes et al., 2017). In the model plant *A. thaliana*, the methylation of CpG, CHG and CHH amounts to 24%, 6.7% and 1.7%, respectively (Cokus et al., 2008), while in maize (*Zea mays* L.), these values are 86.4%, 70.9%, and 1.2%, respectively (West et al., 2014). In both plant species, methylated and highly methylated levels (80-100%) in CpG regions, non-methylated and partially methylated levels (20-100%) in CHG regions and non-methylated and weakly methylated levels (~ 10%) in CHH regions have been observed (Cokus et al., 2008; Lister et al., 2008). A green alga, *Chlamydomonas reinhardtii*, has the lowest level of DNA methylation among plants, with 5.4%, 2.6% and 2.5% CpG, CHG and CHH contexts, respectively (Bartels, 2018). Rice (*Oryza sativa* L.) leaves have 58.4%, 31.0% and 5.1% of CpG, CHG and CHH sites methylated, respectively (Niederhuth et al., 2016). In *Capsicum annuum* L. (pepper), the DNA in 19.9%, 30.5% and 49.6% of CpG, CHG and CHH contexts, respectively is methylated (Rawoof et al., 2019). These values are very different from other plants, but roughly similar to tomato (*Solanum lycopersicum* L.) (Zhong et al., 2013). The findings by Rawoof et al. (2019) indicate that *C. annuum* has the highest recorded global cytosine methylation level among different methylation contexts compared to potato (*Solanum tuberosum* L.) and maize (Wang et al., 2018), soybean (*Glycine max* (L.) Merr.) (An et al., 2017) and field mustard (*Sinapis arvensis* L.) (Chen et al., 2015; Liu et al., 2018). The average global DNA methylation level

in black cottonwood (*Populus trichocarpa* Torr. & A.Gray ex. Hook.) at CG, CHG and CHH sites was 43.99%, 29.84% and 11.57%, respectively (Liang et al., 2019). In different *Poaceae* species the methylation of CHH occurred mainly in short, highly methylated regions, while in soybean it occurred mainly in long regions with lower levels of methylation (Niederhuth et al., 2016). DNA methylation and the silencing of repetitive DNA sequences in the genome are closely associated, and this influences the epigenome (Bewick and Schmitz, 2017).

Larkin and Scowcroft (1981) were among the first scientists to discover phenotypic and genotypic differences among independent individuals of the same clone raised from *in vitro* plant cultures. These differences, which they referred to as somaclonal variation, are well characterised in certain tissue-cultured plants (Miguel and Marum, 2011; Neelakandan and Wang, 2012). *In vitro* maize plants showed a close correlation between the levels of DNA methylation and gene expression, with significantly higher levels of methylation relative to non-micropropagated plants (Brown et al., 1991; Kaeppler, 1992, 1993). *In vitro* rice tissue cultures activated epimutation (i.e., differences in DNA methylation between plants *in vitro* and control mother plants), and induced changes in chromatin structure (Brown et al., 1990; Müller et al., 1990). Genomic stability is not a literal stable or default state of a plant's genome, but is an active process that includes continuous maintenance, control and balance (Pardue, 1991). DNA hypomethylation was induced during plant tissue culture with a 15% increase in methylation observed in *in vivo* plants relative to *in vitro* plants, and molecular structural changes were induced as a result of the stress caused by tissue culture, primarily by the application of different plant growth regulators (PGRs) that are added to the culture medium, such as auxin (Kumar and Van Staden, 2017). These early studies confirmed Pardue's hypothesis (1991) that maintenance of a continuous balance in a plant's genome can be overturned or altered by *in vitro* tissue culture. Machczyńska et al. (2014) used RP-HPLC to determine the genomic DNA methylation of double haploid winter triticale (*Triticosecale*) cultured in different *in vitro* propagation environments, using different *in vitro* culture methods and resulting in regenerated progenies of regenerants. They noted that the genomic DNA methylation of *in vitro* regenerants depended on both the regeneration method and genotype of donor plants because they found differences in DNA methylation between different genotypes and regeneration methods (mean values were 25.39±0.14 for donor plants, 24.68±0.41 for

shed microspore culture, 24.43±0.47 for anther culture, and 23.19±0.44 for immature zygotic culture). Using plants that had been regenerated *in vitro* using meristem culture, Kitimu et al. (2015) analyzed the genome-wide changes in DNA methylation between different varieties of cassava (*Manihot esculenta* Crantz) with methylation-sensitive amplified polymorphism (MSAP) and sequencing-based methylation sensitive genotyping. They found that the differences in DNA methylation patterns that were detected by MSAP were not random but were instead induced by micropropagation, and were thus likely associated with cell and tissue differentiation. In addition, Kitimu et al. (2015) determined 105 unique sequences with different levels of DNA methylation between propagation systems with sequencing-based methylation sensitive genotyping. Rathore et al. (2015) used MSAP markers to asses DNA methylation and detect methylation polymorphism in *Salvadora persica* L. (mustard tree), *Commiphora wightii* (Arn.) Bhandari (Indian bdellium-tree), *Simmondsia chinensis* (Link) C. K. Schneid. (jojoba), *Jatropha curcas* L. (physic nut), and *Withania coagulans* (Stocks) Dunal (paneer booti) leaf tissues growing *in vivo* and *in vitro*, determining that the percentage of the polymorphism in methylated DNA was 8.71–13.98%. Goyali et al. (2018) used MSAP to detect DNA methylation patterns in lowbush blueberry (*Vaccinium angustifolium* Aiton) grown using conventional vegetative propagation, softwood cuttings, and *in vitro* tissue culture environments. They found 106 and 107 amplified DNA fragments in QB9C and Fundy plants, respectively that were derived from softwood cuttings, but 105 and 109 DNA fragments in tissue cultured QB9C and Fundy plants, respectively.

Whole genome bisulfite sequencing (WGBS) generates unbiased genome-wide DNA methylation profiles and has been successfully applied in several studies to find different patterns and functional effects on DNA methylation (Feng et al., 2010a, 2010b; Zemach et al., 2010) in different plant species, such as *A. thaliana* (Xu et al., 2018; Zhou et al., 2019), rice (Li et al., 2012), tomato (Zhong et al., 2013), field mustard (Liu et al., 2018), cabbage (*Brassica oleracea* L.) (Parkin et al., 2014), and black cottonwood (Liang et al., 2019).

The aim of this study was to examine the epigenomes of two diploid apple scion cultivars from three distinct environments: 1) *in vitro* shoots maintained for 16 years in tissue culture; 2) *in vivo* mother trees (20 years old); 3) acclimatized *in vitro* plants (one year after acclimatization). Using WGBS, the level of DNA methylation, as well as the DNA methylation pattern, was measured in these

three biological samples to determine whether an epigenetic footprint was left within the epigenome of apple due to different environments (*in vivo* mother tree vs. *in vitro*) or a change in the environment (*in vitro* culture to acclimatized stage).

Methods

Sources and sampling of plant materials

Plant material was collected from three sources. The first source was *in vitro* leaves from four-week-old *in vitro* shoot cultures of two *Malus x domestica* Borkh. scion cultivars 'McIntosh' and 'Húsvéti rozmaring'. Shoot cultures were maintained for 16 years and subcultured monthly on Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) supplemented with 4.4 µM 6-benzyladenine (BA; Sigma-Aldrich, Budapest, Hungary) and 1.48 µM indole-3-butyric acid (IBA; Sigma-Aldrich) under a 16-h photoperiod, a photosynthetic photon flux density of 105 µM s^{-1} m^{-2} and at 22±2°C. The second source was *in vivo* leaves collected from *in vivo* mother trees of both cultivars from which *in vitro* cultures had been originally established 16 years earlier. The third source was leaves collected randomly from rooted *in vitro* apple shoots (three leaves/tree; three trees sampled) one year after acclimatization. For acclimatization, we collected *in vitro* shoots from the proliferation media which we described above. Four-week-old and 45-50 mm long *in vitro* shoots were placed vertically into root induction medium (MS basal medium at half strength with 4.9 µM IBA). Cultures were incubated at 26°C in the dark for one week before transferring to PGR-free root elongation medium. After two weeks, rooted shoots were rinsed gently in tap water to remove any attached medium and then planted in Jiffy-7® pellets (Magyar-Tábori et al., 2009). Before planting, pellets were soaked with sterilized MS salt solution (0.1× strength) supplemented with 0.15% Previcur® (Bayer, Leverkusen, Germany) and then were incubated in the light in growth chambers under the same conditions described for *in vitro* shoot cultures. After 2-3 weeks, when roots began emerge from the pellets, each plant was gently removed and transferred to a plastic pot (8 cm in diameter) filled with black mould and placed in growth chambers at 70-80% relative humidity at 22±2°C under cool white fluorescent tube lights (16-h photoperiod, 50-60 µM s^{-1} m^{-2}), as was suggested by Bolar et al. (1998).

Sample preparation and WGBS

DNA was extracted and purified from all three samples of each cultivar with a NucleoSpin plant II DNA extraction kit (Macherey-Nagel, Düren, Germany), following the manufacturer's instructions. We used three biological replicates and three technical replicates for the *in vitro* shoots, *in vivo* mother plants and one-year acclimatized plants, which were pooled for DNA extraction. Bisulfite was applied to determine the status of cytosine methylation with the Pico MethylSeq Library Prep kit (Zymo Research, Irvine, CA, USA) using 100 ng of genomic DNA based on the user manual. WGBS was performed on a Illumina HiSeq 2500 (Illumina, San Diego, CA, USA) with Illumina paired-end (PE) reads, and differential methylation analysis between the three treatment groups.

Whole genome assembly and methylation extraction

The quality of DNA reads obtained from WGBS sequencing was assessed using FastQC v0.11.5 (https://github.com/s-andrews/FastQC). Illumina adapter sequences left in reads were removed using cutadapt v1.15 (Martin, 2011). Low quality reads at the 3′ and 5′ ends of the reads were trimmed with Trimmomatic v0.36 (Bolger et al., 2014). After sequence quality control, an average of 9.7 Gb/sample of Illumina PE reads (approximate sequencing depth = 17x) were separately assembled using Bowtie v2.3.4 (Langmead et al., 2009) based on the *Malus x domestica* GDDH13 Whole Genome v1.1 apple genome data (Daccord et al., 2017). DNA methylation analysis and gene clustering analysis to assess methylation patterns were performed with Bismark v0.17.0 (Krueger and Andrews, 2011).

Statistical analysis and evaluation

Differential methylation, statistical analysis in log fold change (LFC), DNA methylation distribution plots and gene clustering were performed with SeqMonk v1.41.0 (https://github.com/s-andrews/SeqMonk), using a bisulphite pipeline over sets of 50 CpGs, CHGs and CHHs. Unreplicated differential methylation was performed with a χ^2 test ($P < 0.05$), based on the generated LFC values. Genes that displayed significant differences in DNA methylation in either their promoter or coding regions according to the χ^2 test were classified as differentially methylated genes (DMGs). The χ^2 test was visualised with a SeqMonk-generated MA plot (Bland-Altman plot) in which the differences in

measurements between any of the three samples in all permutations were assessed by transforming, using SeqMonk, the data onto M (log ratio) and A (mean average) scales, then plotting these values (Bland and Altman, 1999).

Function and GO annotation

All assembled DMGs (based on the χ^2 test) were considered for functional mapping of biological processes, molecular functions and cellular compounds. The functions of all DMGs in the three environments were determined by Gene Ontology (GO) annotation, focusing on biological processes, molecular functions and cellular components of green plants (Viridiplantae), as these might have important roles during *in vitro* culture. GO annotation was performed with Blast2GO v5.1.12 (Conesa and Götz, 2008) based on the *Malus x domestica* GDDH13 Whole Genome v1.1 mRNA sequences (Daccord et al., 2017). DMGs were then selected for GO annotation when differences were larger than ±10.00% in CpG and CHG, or larger than the average change in methylation (±1.35%) in CHH, based on the χ^2 results for differences in DNA methylation patterns and their comparisons. For functional mapping and GO annotation, several databases were used [NCBI Nr: non-redundant protein database (ftp://ftp.ncbi.nlm.nih.gov/blast/db/); Swissprot-Uniprot database (https://www.uniprot.org/); Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.genome.jp/kegg/); GO; InterproScan; https://www.ebi.ac.uk/interpro/] by BlastX-fast with the E-value cut-off set to 10^{-3}.

Results and discussion

In plants, gene regulation is also related to the level of DNA methylation, and this epigenetic mechanism is intricately linked to growth and development, including *in vitro* such as in somatic embryogenesis (Kumar and Van Staden, 2017). Studies on pineapple and cocoa (Scherer et al., 2015; Quinga et al., 2017) showed significant differences in the level of global genomic DNA methylation between *in vitro* and mother plants. In contrast, in the present study on apple, when studying the level of global DNA methylation, no significant differences were found in in the degree of methylated cytosine positions (CpG, CHG and CHH) between apple scion cultivars ('McIntosh' and 'Húsvéti rozmaring') or between three tested environments (acclimatized, *in vitro* tissue-cultured, and mother

plant) (Fig. 1). Average levels of DNA methylation in the examined scions and their environments were 49.77%, 34.65% and 8.77% in CpG, CHG, and CHH contexts, respectively (Fig. 1A). These levels were similar to those recorded by Daccord et al. (2017) (49%, 39%, and 12%, respectively) for dihaploid apple genotypes when leaves and young fruits were analyzed. As far as the authors are aware, no other studies in plant tissue culture have assessed the relative changes in patterns of DNA methylation between *in vitro* plants and their acclimatized counterparts by using WGBS.

Analysis of DNA methylation at the level of the entire genome showed significant differences in C methylation between some genes in either their promoters or coding regions. A total of 45,116 genes, including their promoters and coding regions, were studied (Suppl. Tables 1-3). Significant differences in DNA methylation were identified in 586 genes, i.e. DMGs (Fig. 1B), specifically 334, 201, and 131 in CpG, CHG and CHH contexts, respectively, 446 of which were found in the Blas2GO database (Suppl. Tables 4-6). This indicates a 1.3% difference in the level of DNA methylation of genes in response to a change in environment, i.e. *in vivo* vs. *in vitro*, or after replacing the *in vitro* environment with an *in vivo* environment. Considering these three contexts, 72 DMGs were identical in CHG and CpG, five in CHG and CHH, and seven in CpG and CHH. Moreover, four DMGs were identical in all three contexts while 259, 128 and 123 DMGs were specifically linked to CpG, CHG and CHH contexts, respectively (Fig. 1B). DMGs identified as MD07G1113000 (protein transport), MD08G1041600 (extracellular space), MD09G1054800 (phosphatidic acid binding), and MD10G1265800 (not annotated) were methylated in all three contexts in *in vitro* shoots (Suppl. Tables 4-6). *In vitro* tissue culture had the highest level of methylated DMGs. Some DMGs that participate in oxidation-reduction processes, metabolism and biosynthesis, and that are not essential during *in vitro* culture (Kawakatsu et al., 2017; Bouyer et al., 2017), were primarily methylated. The level of DNA methylation of DMGs was lower in 'McIntosh' than in 'Húsvéti rozmaring' (Suppl. Tables 4-6; Suppl. Fig. 4). The level of methylation in DMGs decreased after acclimatization.

According to GO annotation of all DMGs, a total of 235, 310 and 189 DMGs play important roles in biological processes (Fig. 1C), molecular functions (Fig. 1D), and cellular components (Fig. 1E).

Comparison of differences in DNA methylation patterns

Four MA plot (Bland-Altman plot) analyses were applied to determine and compare the DNA methylation patterns of samples from different environments in both scion cultivars. Paired comparisons in DNA methylation patterns in CpG, CHG and CHH contexts were made between acclimatized and *in vitro* plants, acclimatized and mother plants, and *in vitro* and mother plants of each cultivar separately, in 'McIntosh' (Fig. 2) and 'Húsvéti rozmaring' (Fig. 3), and by considering the average of both cultivars (Fig. 4). Inter-cultivar DNA methylation patterns were also compared between *in vitro*, acclimatized and mother plants of 'McIntosh' and 'Húsvéti rozmaring' (Fig. 5). Considering DMGs, functional genomic maps were established for their biological processes (Suppl. Fig. 1), molecular functions (Suppl. Fig. 2) and cellular components (Suppl. Fig. 3). DMGs were annotated by GO where the difference in DNA methylation was larger than ±10.00% in CpG and CHG contexts, or larger than ±1.35% in the CHH context (Suppl. Fig. 4).

When comparing the DNA methylation patterns of 'McIntosh', DMGs were downregulated in *in vitro* plants compared to acclimatized plants (Fig. 2A, 2D, 2G). The level of methylation was higher in *in vitro* plants than in mother plants, i.e., DMGs were upregulated causing the level of methylation to be lower in the mother plant (Fig. 2C, 2F, 2I). No considerable directional changes were detected in the MA plots when acclimatized and mother plants were compared (Fig. 2B, 2E, 2H) indicating that after acclimatization the DNA methylation pattern became similar to that of the mother plant. According to the GO annotation function of the DMGs, DMGs most frequently encode: i) metabolic processes, cellular, phosphorylation, and biosynthetic processes among their biological functions; ii) regulatory sequences for catalytic, kinase and transferase activity among their molecular functions; and (iii) sequences characteristic of membrane components, intracellular parts and cell parts among their cellular components (Suppl. Fig. 4). When the differences in DNA methylation patterns of 'Húsvéti rozmaring' were compared, a similar tendency was observed in all three contexts (CpG, Fig. 3A, 3B, 3C; CHG, Fig. 3D, 3E, 3F; CHH, Fig. 3G, 3H, 3I), as for 'McIntosh'. According to the GO annotation function and taking all contexts (CpG, CHG and CHH) into consideration, the genes involved most frequently encode: i) metabolic, phosphorylation, and biosynthetic processes, and biological regulation among their biological functions; ii) regulatory sequences for catalytic, kinase and transferase activity among their molecular functions; and iii) sequences characteristic of

membrane components and cell parts among the cellular components (Suppl. Fig. 4).

We identified several DMGs between three different environmental conditions, which confirm the findings in a series of studies that have also implicated DNA methylation in the regulation of genes controlling pathways in plant developmental progression or tissue differentiation, during embryogenesis, apical dominance regulation and leaf formation under *in vitro* propagation (Us-Camas et al., 2014). DNA methylation plays an important role in gene expression and plant development under stress conditions (Crisp et al., 2016; Lämke and Bäurle, 2017). The *in vitro* environment can constitute a stressful abiotic environment for explants in *in vitro* tissue culture, as was shown for potato nodal explants that had been cut, which, after transcriptomic analyses, demonstrated the up- and down-regulation of several differentially expressed genes related to abiotic stress (Teixeira da Silva et al., 2019).

When the two scion cultivars, 'McIntosh' and 'Húsvéti rozmaring', were compared, both up- and downregulation of DMGs were observed in all three contexts (CpG, Fig. 4A, 4B, 4C; CHG, Fig. 4D, 4E, 4F; CHH, Fig. 4G, 4H, 4F), independent of the environment. Establishment of GO annotation functions based on CpG, CHG and CHH contexts showed that the most frequent genes encode: i) metabolic cellular, biosynthetic and transfer processes, as well as phosphorylation among their biological functions; ii) catalytic, transferase, kinase and hydrolase activities among their molecular functions; iii) membrane compounds, cell parts, intracellular parts and cytoplasmic parts among their cellular components (Suppl. Fig. 4). Methylation patterns of the two scion cultivars differed, indicating different and cultivar-specific regulation of the epigenome during the adaptation of apple to various environments (Suppl. Tables 4-6; Suppl. Fig. 4). Our results suggest that the potential role of cultivar-specific DNA methylation patterns constitute an important regulatory mechanism for sensing and responding to stress conditions by regulating stress-responsive gene expression, such as occurs during drought and salinity (Rajkumar et al., 2019), as well as the response and adaptation to other biotic and abiotic stresses (Viggiano and de Pinto, 2017).

In general, significant differences in DNA methylation pattern were detected in both cultivars and in all three contexts (CpG, CHG and CHH). The following trends were observed: i) *in vitro* plants were highly methylated compared to acclimatized plants, i.e. *in vitro* plants were downregulated (Fig.

5A, 5D, 5G); ii) *in vitro* plants were downregulated relative to mother plants (Fig. 5C, 5F, 5I); iii) DNA methylation patterns were similar when acclimatized and mother plants were compared, in both scion cultivars (Fig. 5B, 5E, 5H). These trends were similar to those observed in separate MA plots for each cultivar (Fig. 2, 3). Our study fortifies the notion that *in vitro* propagation constitutes a stress for plants, which have to adapt from the cut mother plant to the *in vitro* environment, as was also shown for potato (Teixeira da Silva et al., 2019), and then again to the acclimatized *ex vitro* state. Very importantly, the genotype of the donor plant influences the level of DNA methylation and pattern of regenerants and the ability to adapt to the *in vitro* environment (Machczyńska et al., 2014). Other studies have shown that DNA methylation levels during *in vitro* propagation are related to the donor tissue in *Clivia miniata* (Lindl.) Bosse (Natal lily) plantlets with MSAP (Wang et al., 2012), to the length of culture in *Corylus avellana* L. (common hazel) with methyl-sensitive restriction endonuclease (Diaz-Sala et al., 1995), in *Theobroma cacao* L. (cocoa tree) with MSAP (Rodríguez López et al., 2010a, 2010b) and the media components in *Daucus carota* L. (wild carrot) with HPLC and methyl-sensitive restriction endonuclease (LoSchiavo et al., 1989; Arnholdt-Schmitt, 1993).

Methylation in plant in vitro culture studies

Using bisulfite sequencing (BS-seq), Stroud et al. (2013) investigated differences in DNA methylation levels of *in vitro* regenerated and wild-type rice plants, observing that DNA hypermethylation in CHH was eliminated during *in vitro* regeneration because it occurs exclusively in dedifferentiated cells, i.e. in callus only. Furthermore, in CpG, CHG, and CHH, there was a decrease in DNA methylation of plants regenerated from *in vitro* culture compared to wild type, non-tissue cultured plants, which affected the expression of certain genes. The longer the period of *in vitro* tissue culture, the greater the number of epigenetic footprints that are left in a plant's epigenome, which may explain somaclonal variation. Stelpflug et al. (2014) analysed *in vitro* maize immature embryo tissue (10-12 days after pollination) using meDIP-ChIP epigenome profiling, methylation-sensitive qPCR and RNA-seq, and found that the combined level of DNA methylation was not affected by tissue culture but rather by variation in the levels of methylation in some DNA components. Rathore et al. (2015) found that DNA methylation ranged between 9% and 14% among *in vitro* and *in vivo* plants of several plant species

(*Salvadora persica*, *Commiphora wightii*, *Simmondsia chinensis*, *Jatropha curcas*, and *Withania coagulans*) using MSAP. Using MSAP, Scherer et al. (2015) observed small differences between *in vitro* (33.66%) and *in vivo* (30.82%) pineapple (*Ananas comosus* var. *comosus*) when grown under different environments. Using HPLC, Quinga et al. (2017) detected global changes in DNA methylation during cocoa (*Theobroma cacao* L.) somatic embryogenesis, detecting 17.49% and 27.06% DNA methylation in somatic embryos that were 12 and 36 months old, respectively. Their findings confirmed those by Stroud et al. (2013), namely that a longer period of *in vitro* tissue culture results in a stable epigenetic footprint in a plant's epigenome over multiple generations and may partially explain somaclonal variation.

Our experiments show that the levels of global genomic DNA methylation in apple were steady, independent of the cultivar or growth environment. However, analyses of the methylation pattern in the entire genome confirmed that individual genes display constantly changing levels of methylation. The dynamic changes in their methylation levels might regulate responses and adaptation to a changing environment (*in vitro* environment or recovery to the *in vivo* environment). After acclimatization, the pattern of DNA methylation in the two apple cultivars were similar to the methylation pattern of the mother plant.

Additional detailed studies are necessary to clarify how these changes in DNA methylation levels of identified DMRs are connected to changes in gene expression and if they play any role in the adaptation of apple to *in vitro* versus *ex vitro* environments. Such knowledge will allow for the development of a technology for apple that would allow for the production of high-quality material in desired quantities, or at specific stages of development.

Author contributions

A.G., E.K. and J.D. designed the research; A.G. N.H. and K.P. performed the library preparation for WGBS; J.T.dS. and E.K. performed statistical analysis; A.G. and N.H. performed the bioinformatics analysis; A.G., J.D., J.T.dS. and N.H. wrote the manuscript.

Conflicts of interest

The authors declare no financil and non-financial conflicts of interest.

Acknowledgement

The research was financed by the Higher Education Institutional Excellence Programme of the Ministry of Human Capacities in Hungary, within the framework of the Biotechnology thematic programme of the University of Debrecen (20428-3/2018/FEKUTSTRAT), and that of Szent Istvan University (1783-3/2018/FEKUTSTART).

Data availability: The raw Illumina WGBS sequences were submitted to the NCBI under BioProject ID PRJNA480249, and the processed data were deposited under Gene Expression Omnibus ID GSE116995.

References

An, Y. Q. C., Goettel, W., Han, Q., Bartels, A., Liu, Z., Xiao, W., 2017. Dynamic changes of genome-wide DNA methylation during soybean seed development. Scientific Reports 7.1, 12263. https://doi.org/10.1038/s41598-017-12510-4

Arnholdt-Schmitt, B., 1993. Rapid changes in amplification and methylation pattern of genomic DNA in cultured carrot root explants (*Daucus carota* L.). Theoretical and Applied Genetics 85.6-7, 793-800. https://doi.org/10.1007/BF00225021

Bartels, A., Han, Q., Nair, P., Stacey, L., Gaynier, H., Mosley, M., Huang, Q., Pearson, J., Hsieh, T.F., An, Y.Q., Xiao, W., 2018. Dynamic DNA methylation in plant growth and development. International Journal of Molecular Sciences 19, 2144. https://doi.org/10.3390/ijms19072144

Bewick, A. J., Schmitz, R. J., 2017. Gene body DNA methylation in plants. Current Opinion in Plant Biology 36, 103-110. https://doi.org/10.1016/j.pbi.2016.12.007

Bland, J. M., Altman, D. G., 1999. Measuring agreement in method comparison studies. Statistical Methods in Medical Research 8.2, 135-160. https://doi.org/10.1177/096228029900800204

Bolar, J. P., Norelli, J. L., Aldwinckle, H. S., Hanke, V., 1998. An efficient method for rooting and acclimation of micropropagated apple cultivars. HortScience 33.7, 1251-1252. https://doi.org/10.21273/HORTSCI.33.7.1251

Bolger, A. M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30.15, 2114-2120. https://doi.org/10.1093/bioinformatics/btu170

Bouyer, D., Kramdi, A., Kassam, M., Heese, M., Schnittger, A., Roudier, F., Colot, V., 2017. DNA methylation dynamics during early plant life. Genome Biology 18.1, 179. https://doi.org/10.1186/s13059-017-1313-0

Brown, P. T. H., Kyozuka, J., Sukekiyo, Y., Kimura, Y., Shimamoto, K., Lörz, H., 1990. Molecular changes in protoplast-derived rice plants. Molecular and General Genetics 223, 324-328. https://doi.org/10.1007/BF00265070

Brown, P. T. H., Gobel, E., Lorz, H., 1991. RFLP analysis of *Zea mays* callus cultures and their regenerated plants. Theoretical and Applied Genetics 81.2, 227-232. https://doi.org/10.1007/BF00215727

Cao, X., Springer, N. M., Muszynski, M. G., Phillips, R. L., Kaeppler, S., Jacobsen, S. E., 2000. Conserved plant genes with similarity to mammalian *de novo* DNA methyltransferases. Proceedings of the National Academy of Sciences USA 97.9, 4979-4984. https://doi.org/10.1073/pnas.97.9.4979

Chen, X., Ge, X., Wang, J., Tan, C., King, G. J., Liu, K. 2015. Genome-wide DNA methylation profiling by modified reduced representation bisulfite sequencing in *Brassica rapa* suggests that epigenetic modifications play a key role in polyploid genome evolution. Frontiers in Plant Science 6, 836. https://doi.org/10.3389/fpls.2015.00836

Cokus, S.J., Feng, S., Zhang, X., Chen. Z., Merriman, B., Haudenschild, C.D., Pradhan S., Nelson, S.F., Pellegrini, M., Jacobsen, S.E., 2008. Shotgun bisulphite sequencing of the *Arabidopsis* genome reveals DNA methylation patterning. Nature 452.7184, 215. https://doi.org/10.1038/nature06745

Conesa, A., Götz, S., 2008. Blast2GO: A comprehensive suite for functional analysis in plant genomics. International Journal of Plant Genomics 2008, 619832. http://dx.doi.org/10.1155/2008/619832

Crisp, P. A., Ganguly, D., Eichten, S. R., Borevitz, J. O., Pogson, B. J., 2016. Reconsidering plant memory: intersections between stress recovery, RNA turnover, and epigenetics. Science Advances 2.2, e1501340. https://doi.org/10.1126/sciadv.1501340

Daccord, N., Celton, J. M., Linsmith, G., Becker, C., Choisne, N., Schijlen, E., van de Geest, H., Bianco, L., Micheletti, D., Velasco, R., Di Pierro, E. A., Gouzy, J., Rees, D. J. G., Guérif, P., Muranty, H., Durel, C. E., Laurens, F., Lespinasse, Y., Gaillard, S., Aubourg, S., Quesneville, H., Weigel, D., van de Weg, E., Troggio, M., Bucher, E., 2017. High-quality *de novo* assembly of the apple genome and methylome dynamics of early fruit development. Nature Genetics 49.7, 1099. https://doi.org/10.1038/ng.3886

Diaz-Sala, C., Rey, M., Boronat, A., Besford, R., Rodriguez, R., 1995. Variations in the DNA methylation and polypeptide patterns of adult hazel (*Corylus avellana* L.) associated with sequential *in vitro* subcultures. Plant Cell Reports 15, 218–221. https://doi.org/10.1007/BF00193723

Feng, S., Cokus, S. J., Zhang, X., Chen, P.-Y., Bostick, M., Goll, M. G., Hetzel, J., Jain, J., Strauss, S. H., Halpern, M. E., Ukomadu,C., Sadler, K.C., Pradhan, S., Pellegrini, M., Steven E. Jacobsen, S. E., 2010a. Conservation and divergence of methylation patterning in plants and animals. Proceedings of the National Academy of Sciences USA 107, 8689–8694. https://doi.org/10.1073/pnas.1002720107

Feng, S., Jacobsen, S. E., Reik, W., 2010b. Epigenetic reprogramming in plant and animal development. Science 330.6004, 622-627. https://doi.org/10.1126/science.1190614

Gouil, Q., Baulcombe, D. C., 2016. DNA methylation signatures of the plant chromomethyltransferases. PLoS Genetics 12.12, e1006526. https://doi.org/10.1371/journal.pgen.1006526

Goyali, J. C., Igamberdiev, A. U., Debnath, S. C., 2018. DNA methylation in lowbush blueberry (*Vaccinium angustifolium Ait.*) propagated by softwood cutting and tissue culture. Canadian Journal of Plant Science 98.5, 1035-1044. https://doi.org/10.1139/cjps-2017-0297

Hewezi, T., 2018. Epigenetic regulation of plant development and stress responses. Springer 37.1, 1–2. https://doi.org/10.1007/s00299-017-2233-x

Kaeppler, S. M., 1992. Molecular and genetic studies of tissue culture-induced variation in maize. PhD Thesis (St. Paul: Univ. Minnesota).

Kaeppler, S. M., Phillips, R. L.,1993. Tissue culture-induced DNA methylation variation in maize. Proceedings of the National Academy of Sciences USA 90.19, 8773-8776. https://doi.org/10.1073/pnas.90.19.8773

Kawakatsu, T., Nery, J. R., Castanon, R., Ecker, J. R., 2017. Dynamic DNA methylation reconfiguration during seed development and germination. Genome Biology 18.1, 171. https://doi.org/10.1186/s13059-017-1251-x

Kitimu, S. R., Taylor, J., March, T. J., Tairo, F., Wilkinson, M. J., Rodríguez López, C. M., 2015. Meristem micropropagation of cassava (*Manihot esculenta*) evokes genome-wide changes in DNA methylation. Frontiers in Plant Science 6, 590. https://doi.org/10.3389/fpls.2015.00590

Krueger, F., Andrews, S. R., 2011. Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. Bioinformatics 27.11, 1571-1572. https://doi.org/10.1093/bioinformatics/btr167

Kumar, V., Van Staden, J., 2017. New insights into plant somatic embryogenesis: an epigenetic view. Acta Physiologiae Plantarum 39.9, 194. https://doi.org/10.1007/s11738-017-2487-5

Lämke, J., Bäurle, I., 2017. Epigenetic and chromatin-based mechanisms in environmental stress adaptation and stress memory in plants. Genome Biology 18.1, 124. https://doi.org/10.1186/s13059-017-1263-6

Langmead, B., Trapnell, C., Pop, M., Salzberg, S. L., 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biology 10.3, R25. https://doi.org/10.1186/gb-2009-10-3-r25

Larkin, P. J., Scowcroft, W. R., 1981. Somaclonal variation—a novel source of variability from cell cultures for plant improvement. Theoretical and Applied Genetics 60.4, 197-214. https://doi.org/10.1007/BF02342540

Lee, K., Seo, P. J., 2018. Dynamic epigenetic changes during plant regeneration. Trends in Plant Science 23, 235-247. https://doi.org/10.1016/j.tplants.2017.11.009

Li, X.; Zhu, J.; Hu, F.; Ge, S.; Ye, M.; Xiang, H.; Zhang, G.; Zheng, X.; Zhang, H.; Zhang, S., Li, Q., Luo, R., Yu, C., Yu, J., Sun, J., Zou, X., 2012. Single-base resolution maps of cultivated and wild rice methylomes and regulatory roles of DNA methylation in plant gene expression. BMC Genomics 13.1, 300. https://doi.org/10.1186/1471-2164-13-300

Liang, L., Chang, Y., Lu, J., Wu, X., Liu, Q., Zhang, W., Su., X., Zhang, B., 2019. Global methylomic and transcriptomic analyses reveal the broad participation of DNA methylation in daily gene expression regulation of *Populus trichocarpa*. Frontiers in Plant Science 10. https://doi.org/10.3389/fpls.2019.00243

Lister, R., O'Malley, R. C., Tonti-Filippini, J., Gregory, B. D., Berry, C. C., Millar, A. H., Ecker, J. R., 2008. Highly integrated single-base resolution maps of the epigenome in *Arabidopsis*. Cell 133.3, 523-536. https://doi.org/10.1016/j.cell.2008.03.029

Liu, G., Xia, Y., Liu, T., Dai, S., Hou, X., 2018. The DNA methylome and association of differentially methylated regions with differential gene expression during heat stress in *Brassica rapa*. International Journal of Molecular Sciences 19.5, 1414. https://doi.org/10.3390/ijms19051414

LoSchiavo, F., Pitto, L., Giuliano, G., Torti, G., Nuti-Ronchi, V., Marazziti, D., Vergara, R., Orselli, S., Terzi, M., 1989. DNA methylation of embryogenic carrot cell cultures and its variations as caused by mutation, differentiation, hormones and hypomethylating drugs. Theoretical and Applied Genetics 77, 325–331. https://doi.org/10.1007/BF00305823

Machczyńska, J., Orłowska, R., Mańkowski, D. R., Zimny, J., Bednarek, P. T., 2014. DNA methylation changes in triticale due to *in vitro* culture plant regeneration and consecutive reproduction. Plant Cell, Tissue and Organ Culture 119.2, 289-299. https://doi.org/10.1007/s11240-014-0533-1

Magyar-Tábori, K., Dobránszki, J., Hudák, I., 2009. Factors affecting the rooting ability of *in vitro* apple shoots. Hungarian Agricultural Research 18.10, 4.

Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet. Journal 17.1, 10. https://doi.org/10.14806/ej.17.1.200

Miguel, C., Marum, L., 2011. An epigenetic view of plant cells cultured *in vitro*: somaclonal variation and beyond. Journal of Experimental Botany 62.11, 3713-3725. https://doi.org/10.1093/jxb/err155

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiologia Plantarum 15.3, 473-497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Müller, E., Brown, P. T. H., Hartke, S., Lörz, H., 1990. DNA variation in tissue-culture-derived rice plants. Theoretical and Applied Genetics 80.5, 673-679. https://doi.org/10.1007/BF00224228

Neelakandan, A. K., Wang, K., 2012. Recent progress in the understanding of tissue culture-induced genome level changes in plants and potential applications. Plant Cell Reports 31.4, 597-620. https://doi.org/10.1007/s00299-011-1202-z

Niederhuth, C. E., Bewick, A. J., Ji, L., Alabady, M., Do Kim, K., Page, J.T., Li, Q., Rohr, N. A., Rambani,A., Burke, J. M., Udall, J.A, Egesi, C., Schmutz,J., Grimwood, J., Jackson, S. A., Springer, N. M., Schmitz, R. J., 2016. Widespread natural variation of DNA methylation within angiosperms. Genome Biology 17.1, 194. https://doi.org/10.1186/s13059-016-1059-0

Pardue, M. L., 1991. Dynamic instability of chromosomes and genomes. Cell 66.3, 427-431.

Parkin, I. A., Koh, C., Tang, H., Robinson, S. J., Kagale, S., Clarke, W. E., Town, C. D., Nixon, J., Krishnakumar, V., Bidwell, S. L., Denoeud, F., Belcram H., Links, M. G., Just, J., Clarke, C., Bender, T., Huebert, T., Mason, A. S., Pires, J. C., Barker, G., Moore, J., Walley, P. G., Manoli, S., Batley, J., Edwards, D., Nelson, M. N., Wang, X., Paterson, A. H., King, G., Bancroft, I., Chalhoub, B., Sharpe, A. G., 2014. Transcriptome and methylome profiling reveals relics of genome dominance in the mesopolyploid *Brassica oleracea*. Genome Biol. 15, R77 https://doi.org/10.1186/gb-2014-15-6-r77

Probst, A. V., Scheid, O. M., 2015. Stress-induced structural changes in plant chromatin. Current Opinion in Plant Biology 27, 8-16. https://doi.org/10.1016/j.pbi.2015.05.011

Quinga, L. A. P., de Freitas Fraga, H. P., do Nascimento Vieira, L., Guerra, M. P., 2017. Epigenetics of long-term somatic embryogenesis in *Theobroma cacao* L.: DNA methylation and recovery of embryogenic potential. Plant Cell, Tissue and Organ Culture 131.2, 295-305. https://doi.org/10.1007/s11240-017-1284-6

Rajkumar, M. S., Shankar, R., Garg, R., Jain, M., 2019. Role of DNA methylation dynamics in desiccation and salinity stress responses in rice cultivars. BioRxiv, 558064. https://doi.org/10.1101/558064

Rathore, M. S., Mastan, S. G., Agarwal, P. K., 2015. Evaluation of DNA methylation using methylation-sensitive amplification polymorphism in plant tissues grown *in vivo* and *in vitro*. Plant Growth Regulation 75.1, 11-19. https://doi.org/10.1007/s10725-014-9926-8

Rawoof, A., Ahmad, I., Ramchiary, N., 2019. Epigenome landscape in *Capsicum* genome. In: The *Capsicum* Genome, Springer, Cham. pp. 187-199 https://doi.org/10.1007/978-3-319-97217-6_11

Rodríguez López, C. M., Wetten, A. C., Wilkinson, M. J., 2010a. Progressive erosion of genetic and epigenetic variation in callus-derived cocoa (*Theobroma cacao*) plants. New Phytologist 186, 856–868. https://doi.org/10.1111/j.1469-8137.2010.03242.x

Rodríguez López, C. M., Sicilia Bravo, H., Wetten, A. C., Wilkinson, M. J., 2010b. Detection of somaclonal variation during cocoa somatic embryogenesis characterised using cleaved amplified polymorphic sequence and the new freeware Artbio. Molecular Breeding 25, 501–516. https://doi.org/10.1007/s11032-009-9348-x

Scherer, R. F., de Freitas Fraga, H. P., Klabunde, G. F., da Silva, D. A., Guerra, M. P., 2015. Global DNA methylation levels during the development of nodule cluster cultures and assessment of genetic fidelity of *in vitro*-regenerated pineapple plants (*Ananas comosus* var. *comosus*). Journal of Plant Growth Regulation 34.3, 677-683. https://doi.org/10.1007/s00344-015-9493-x

Stelpflug, S. C., Eichten, S. R., Hermanson, P. J., Springer, N. M., Kaeppler, S. M., 2014. Consistent and heritable alterations of DNA methylation are induced by tissue culture in maize. Genetics 198.1, 209-218. https://doi.org/10.1534/genetics.114.165480

Stroud, H., Ding,B., Simon, S. A., Feng, S., Bellizzi,M., Pellegrini, M., Wang, G-L., Meyers,B. C., Steven E Jacobsen, S. E., 2013. Plants regenerated from tissue culture contain stable epigenome changes in rice. eLife 2, e00354. https://doi.org/10.7554/eLife.00354.001

Teixeira da Silva, J.A., Hidvégi, N.T., Gulyás, A., Dobránszki, J. (2019) mRNA transcription profile of potato (*Solanum tuberosum* L.) in response to explant cutting. Plant Cell, Tissue and Organ Culture (in press) https://doi.org/10.1007/s11240-019-01613-7

Tirado-Magallanes, R., Rebbani, K., Lim, R., Pradhan, S., Benoukraf, T., 2017. Whole genome DNA methylation: beyond genes silencing. Oncotarget 8.3, 5629. https://doi.org/10.18632/oncotarget.13562

Us-Camas, R., Rivera-Solís, G., Duarte-Aké, F., De-la-Pena, C., 2014. *In vitro* culture: an epigenetic challenge for plants. Plant Cell, Tissue and Organ Culture 118.2, 187-201. https://doi.org/10.1007/s11240-014-0482-8

Velasco, R., Zharkikh, A., Affourtit, J., Dhingra, A., Cestaro, A., Kalyanaraman, A., Fontana, P., Bhatnagar, S. K., Troggio, M., Pruss, D., Salvi,S., Pindo, M., Baldi, P., Castelletti, S., Cavaiuolo, M., Coppola, G., Costa, F., Cova, V., Dal Ri, A., Goremykin,V., Komjanc, M., Longhi, S., Magnago, P., Malacarne, G., Malnoy, M., Micheletti, D., Moretto, M., Perazzolli, M., Si-Ammour, A., Vezzulli, S., Zini,E., Eldredge, G., Fitzgerald, L. M., Gutin,N., Lanchbury, J., Macalma, T., Mitchell, J. T., Reid, J., Wardell, B., Kodira, C., Chen, Z., Desany, B., Niazi, F., Palmer, M., Koepke, T., Jiwan, D., Schaeffer, S., Krishnan, V., Wu, C., Chu, V. T., King, S. T., Vick, J., Tao, Q., Mraz, A., Stormo, A., Stormo, K., Bogden, R., Ederle, D., Stella, A., Vecchietti, A., Kater, M. M., Masiero, S., Lasserre, P., Lespinasse, Y., Allan, A. C., Bus, V., Chagné, D.,

Crowhurst, R. N., Gleave, A. P., Lavezzo, E., Fawcett, J. A., Proost, S., Rouzé, P., Sterck, L., Toppo, S., Lazzari, B., Hellens, R. P., Durel, C-E., Gutin, A., Bumgarner, R. E., Gardiner, S. E., Skolnick, M., Egholm, M., Van de Peer, Y., Salamini, F., Viola, R., 2010. The genome of the domesticated apple (*Malus*× *domestica* Borkh.). Nature Genetics 42.10, 833. https://doi.org/10.1038/ng.654

Viggiano, L., de Pinto, M. C., 2017. Dynamic DNA methylation patterns in stress response. Plant Epigenetics, Springer, Cham, pp. 281-302. https://doi.org/10.1007/978-3-319-55520-1_15

Wang, L., Xie, J., Hu, J., Lan, B., You, C., Li, F., Wang, Z., Wang, H., 2018. Comparative epigenomics reveals evolution of duplicated genes in potato and tomato. The Plant Journal 93.3, 460–471. https://doi.org/10.1111/tpj.13790

Wang, Q. M., Wang, Y. Z., Sun, L. L., Gao, F. Z., Sun, W., He, J., Gao ,X., Wang, L., 2012. Direct and indirect organogenesis of *Clivia miniata* and assessment of DNA methylation changes in various regenerated plantlets. Plant Cell Reports 31, 1283–1296. https://doi.org/10.1007/s00299-012-1248-6

West, P. T., Li, Q., Ji, L., Eichten, S. R., Song, J., Vaughn, M. W., Schmitz, R. J., Springer, N.M., 2014. Genomic distribution of H3K9me2 and DNA methylation in a maize genome. PLoS One 9.8, e105267. https://doi.org/10.1371/journal.pone.0105267

Xu, P., Chen, H., Jin, J., Cai, W., 2018. Single-base resolution methylome analysis shows epigenetic changes in *Arabidopsis* seedlings exposed to microgravity spaceflight conditions on board the SJ-10 recoverable satellite. Microgravity 4.1, 12. https://doi.org/10.1038/s41526-018-0046-z

Zemach, A., McDaniel, I. E., Silva, P., Zilberman, D., 2010. Genome-wide evolutionary analysis of eukaryotic DNA methylation. Science 328.5980, 916-919. https://doi.org/10.1126/science.1186366

Zemach, A., Kim, M. Y., Hsieh, P. H., Coleman-Derr, D., Eshed-Williams, L., Thao, K., Harmer, S.L., Zilberman, D., 2013. The *Arabidopsis* nucleosome remodeler DDM1 allows DNA methyltransferases to access H1-containing heterochromatin. Cell 153.1, 193-205. https://doi.org/10.1016/j.cell.2013.02.033

Zhong, S., Fei, Z., Chen, Y. R., Zheng, Y., Huang, M., Vrebalov, J., McQuinn, R., Gapper, N., Liu, B., Xiang, J., Ying Shao, Y., Giovannoni, J. J., 2013. Single-base resolution methylomes of tomato fruit development reveal epigenome modifications associated with ripening. Nature Biotechnology 31.2, 154. https://doi.org/10.1038/nbt.2462

Zhou, M., Sng, N. J., LeFrois, C. E., Paul, A. L., Ferl, R. J., 2019. Epigenomics in an extraterrestrial environment: organ-specific alteration of DNA methylation and gene expression elicited by spaceflight in *Arabidopsis thaliana*. BMC Genomics 20.1, 205. https://doi.org/10.1186/s12864-019-5554-z

Legends to figures

Fig. 1. Global DNA methylation levels in CpG, CHG and CHH contexts (A) and sequence distribution of significant differences in DNA methylation in genes based on the CpG, CHG and CHH contexts (B), based on biological processes (C), molecular functions (D) and cellular components (E).

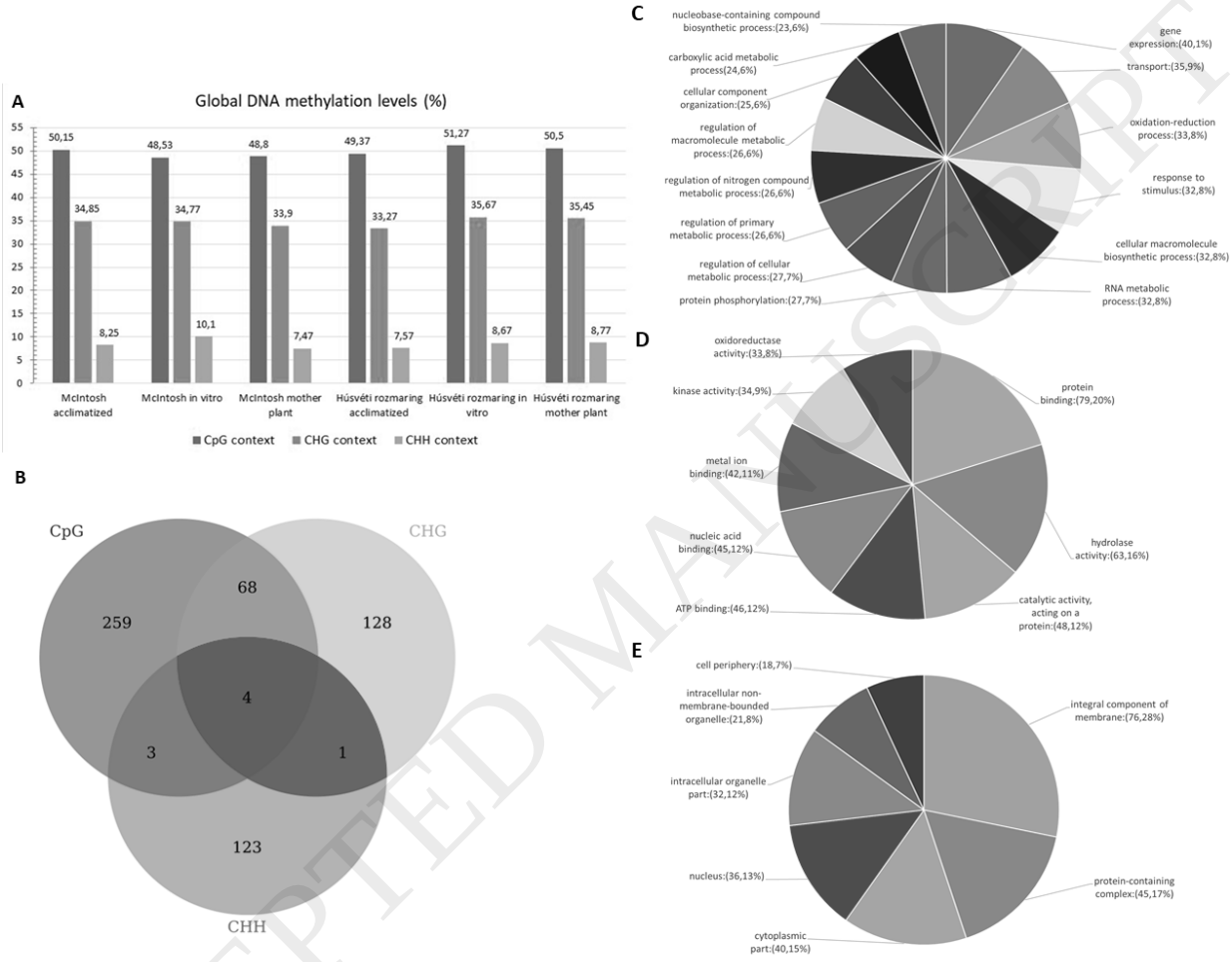

Fig. 2. Differences in the levels of DNA methylation of CpG (A, B, C), CHG (D, E, F) and CHH (G, H, I) contexts between acclimatized and *in vitro* plants (A, D, G), acclimatized and mother plants (B, E, H), and *in vitro* and mother plants (C, F, I) of scion cultivar 'McIntosh'.

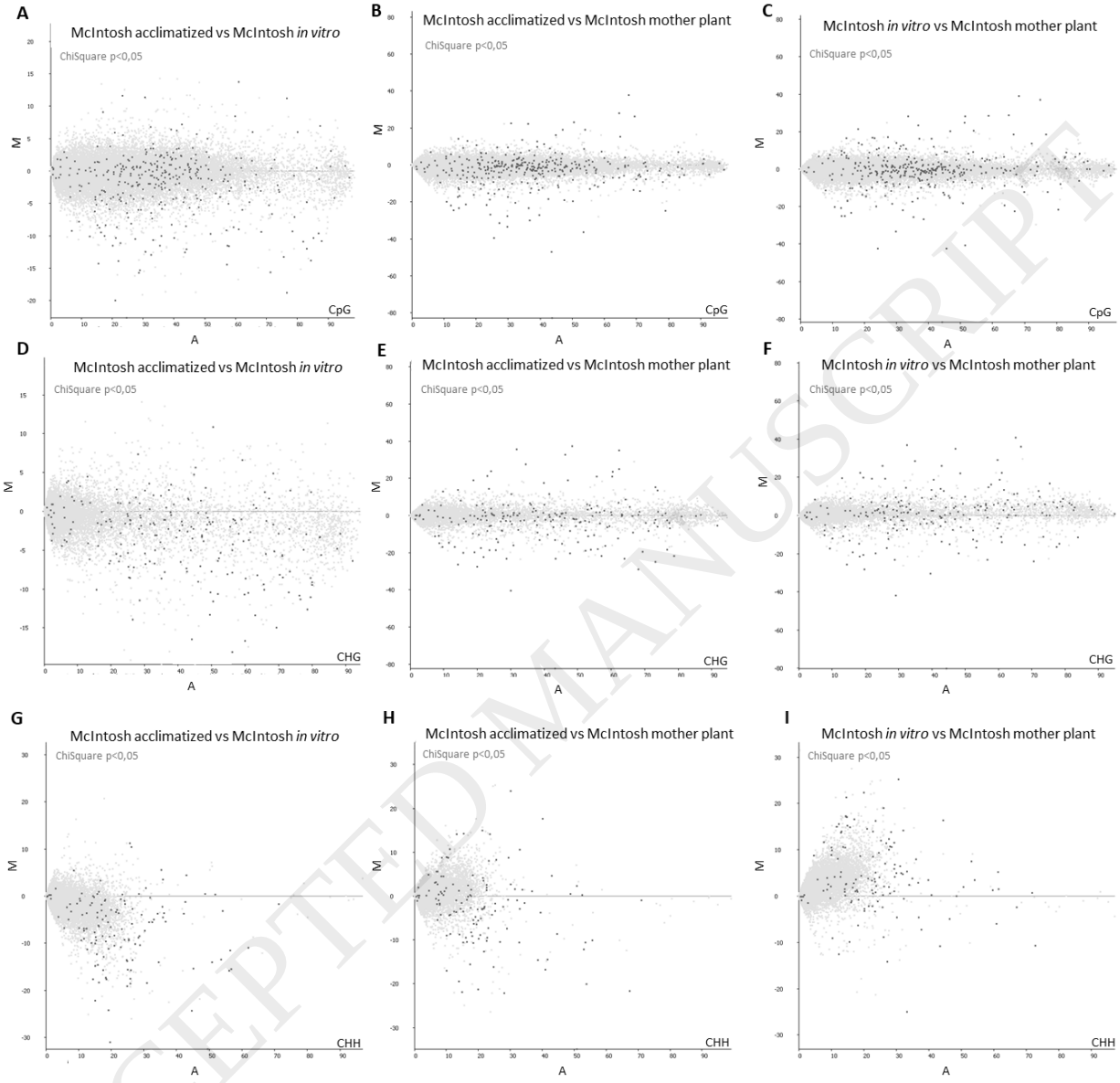

Fig. 3. Differences in the levels of DNA methylation of CpG (A, B, C), CHG (D, E, F) and CHH (G, H, I) contexts between acclimatized and *in vitro* plants (A, D, G), acclimatized and mother plants (B, E, H), and *in vitro* and mother plants (C, F, I) of scion cultivar 'Húsvéti rozmaring'.

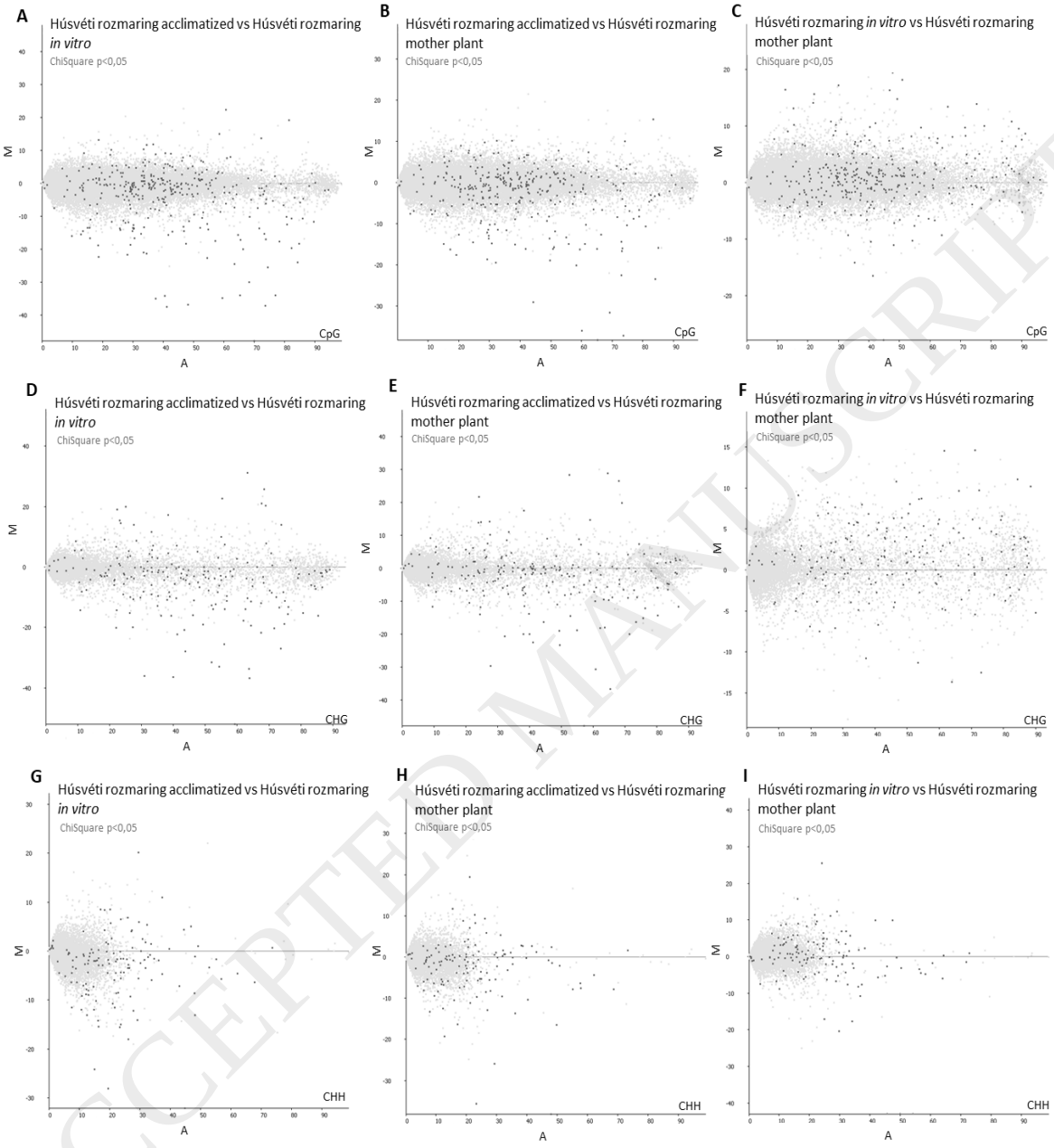

Fig. 4. Differences in the levels of DNA methylation of CpG (A, B, C), CHG (D, E, F) and CHH (G, H, I) contexts between apple scion cultivars 'McIntosh' and 'Húsvéti rozmaring' in acclimatized (A, D, G), *in vitro* (B, E, H), and mother plants (C, F, I).

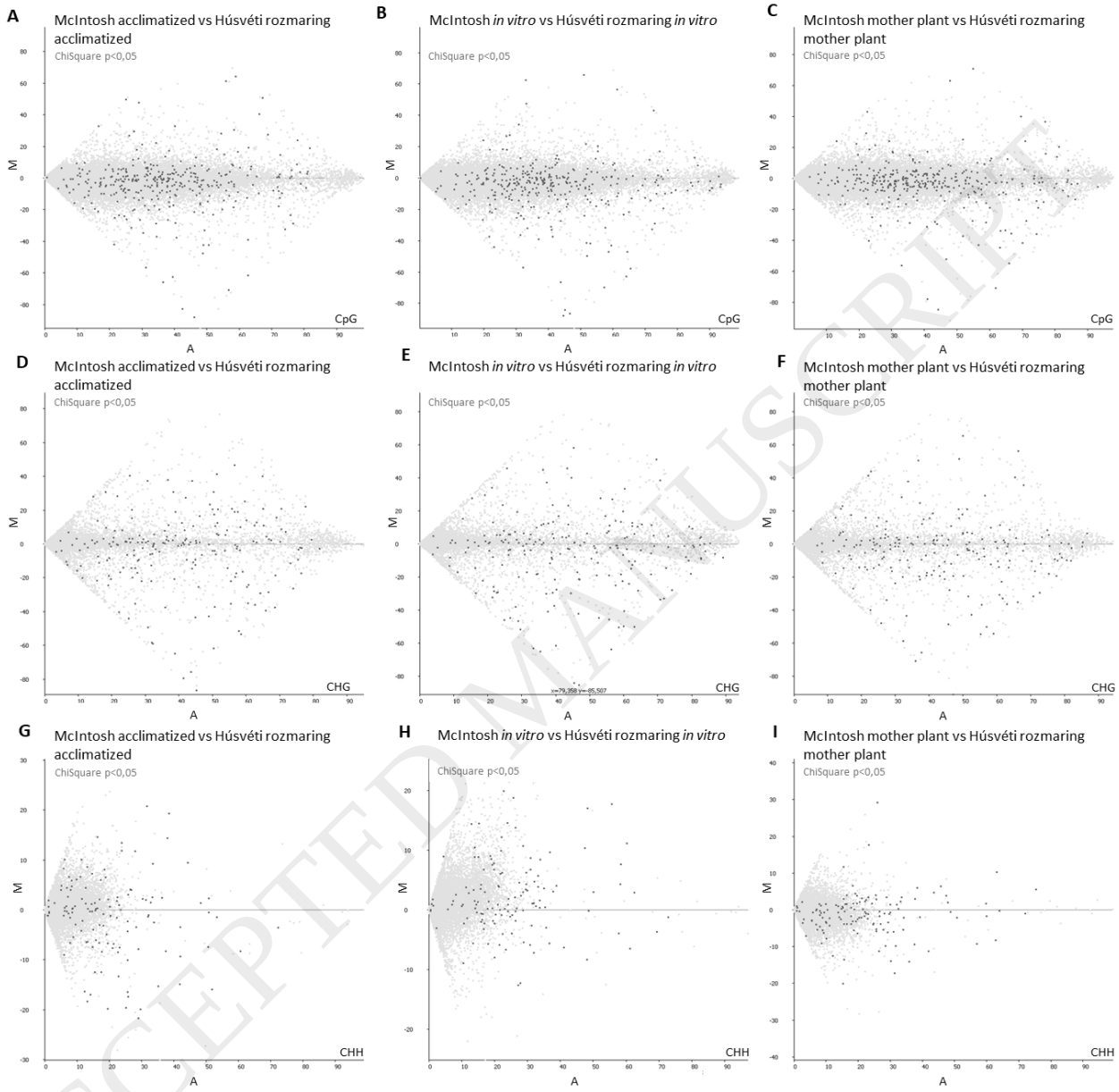

Fig. 5. Differences in the levels of DNA methylation of CpG (A, B, C), CHG (D, E, F) and CHH (G, H, I) contexts between acclimatized and *in vitro* plants (A, D, G), acclimatized and mother plants (B, E, H), and *in vitro* and mother plants (C, F, I) based on the average of both scion cultivars.