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Genetic stability and epigenetic changes of *in vitro* cultivated apple (*Malus* × *domestica* Borkh.)

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Background of the work and its aims

Apple (*Malus* × *domestica* Borkh.) is one of the most popular and widely grown fruits around the world. The maturity of cultivated varieties, their shelf life, and the ability to transport their fruit makes it possible for apple to be consumed every month of the year. Apple production is estimated at 87.2 million tonnes (t) worldwide, with Asia (64.6 %), Americas (11.2 %) and Europe (19.6 %) being the biggest contributors (FAOSTAT 2019), output that is 1.6 % more than in 2018. Although there is an extremely high number (more than 7,500) of apple varieties (CORTÉS *et al*. 2019), only a small proportion of them is grown in commercial apple production. Apple has been grown for a long time, starting 4,000 years ago.

In Hungary, apple occupies a dominant position in the fruit production sector with 30,974 hectares (ha) in 2019, where the total harvested yield was 498,330 tonnes which are lower than the previous year (31,838 ha and 678,775 t) (KSH 2019). The most popular apple varieties in Hungary are Idared, Florina (Querina), Jonagold, Remo, Golden Delicious, Golden Delicious Reinders, Golden B, Jonathan M 40, Gala and Relinda. Plant biotechnology and micropropagation also provides an opportunity for breeders to produce new breeds or breeding lines (ALDWINCKLE and MALNOY 2009, MOYO *et al*. 2011). The *in vitro* micropropagation helps the production of uniform propagating material regardless of the season, place or climate and the rapid propagation of new varieties, breeding lines and variants gained from breeding.

Epigenetics involves hereditary changes in gene regulation which is not related to the changes of DNA sequence itself. The popularity of epigenetic research has been developing in the last decades and it has been found to play a key role in many genetic processes. However, the question may arise as whether, and if so, what changes in the genetic stock are caused

by environmental effects, whether we should expect changes in gene expression due to epigenetic regulation (QUADRANA and COLOT 2016). Epigenetic changes, including methylation of DNA, histone proteins and microRNA may affect DNA function, RNA transcription. Epigenetic changes also play an important role in plant microproduction. Various methylation states can also occur during somatic embryogenesis and regeneration processes, which can result in gene expression changes during plant tissue culture but can also occur during *in vitro* micropropagation and in shoot cultures maintained for gene bank purposes (DUDITS and HESZKY 2000). Understanding methylation changes at the molecular level may contribute to the development of a better plant growth strategy for *in vitro* cultures (KARIM *et al*. 2016).

Our aim was to investigate:

1. if there is an epigenetic change in *in vitro* tissue culture compared to the mother plant. Does *in vitro* micropropagation cause change in DNA sequence?
2. if there is any DNA-based change during *in vitro* micropropagation that can cause problems in the genetic stability of off-springs?
3. if there is any difference between the epigenomes of ‘McIntosh’ and ‘Húsvéti rozmaring’ apple scion cultivars as a function of environmental change?
4. if the epigenetic change is a reversible or irreversible process?

Materials and methods

Experiments were carried out on two apple (*Malus* × *domestica* Borkh.) scion cultivars, 'McIntosh' and 'Húsvéti rozmaring'. Mother plants were sourced from University of Debrecen, Institutes for Agricultural Research and Educational Farm, Research Institute of Újfehértó. *In vitro* apple shoots maintained in the Plant Biotechnology Laboratory of the Centre for Agricultural Genomics and Biotechnology of the University of Debrecen have been sourced from the mother plants at the Research Institute of Újfehértó were used for the experiments. Plants were acclimatized from *in vitro* cultures. We call as mother plants apple samples that come from trees that have been exposed to the weather more than 20 years, grown on infield. The mother samples derived from the Research Institute of Újfehértó of Institutes for Agricultural Research and Educational Farm of University of Debrecen, where the apple trees used in the experiment can be found. 16-year-old *in vitro* shoot cultures sourced from these trees.

The shoot samples ('McIntosh' and 'Húsvéti rozmaring') were taken from the collection of the University of Debrecen, Institutes for Agricultural Research and Educational Farm, Research Institute of Újfehértó. Leaves were removed from the shoot pieces and surface disinfected, as follows: initial washing in tap water containing 0.05% Tween-20 (Sigma-Aldrich, St. Louis, MO, USA). After removal of shoot tips, disinfection was made with 70% ethanol (Sigma-Aldrich, Saint Louis, MO, USA) for 2 minutes, single wash in sterilized distilled water (SDW), a dip in 0.1% $HgCl_2$ (Sigma-Aldrich, St. Louis, MO, USA) solution for 3 minutes and three rinses in SDW. Surface-disinfected shoot tips were then placed on Murashige and Skoog (MS) basal medium (MURASHIGE and SKOOG 1962), which, in addition to MS macro- and microelements, vitamins, contained 3% sucrose (Sigma-Aldrich, St. Louis, MO, USA) and 0.7% agar (plant cell culture tested, SIGMA), 0.3 mg/L

IBA (Sigma-Aldrich, St. Louis, MO, USA) and 0.2 mg/L GA_3 (Sigma-Aldrich, St. Louis, MO, USA) growth regulators. The citokinin-content of the medium was 1.0 mg/L BA (Sigma-Aldrich, St. Louis, MO, USA) for 'McIntosh' and 0.5 mg/L BAR for 'Húsvéti Rozmaring'. The pH of the medium was adjusted to 5.8 before autoclaving. To maintain the shoots, 400 ml glass jars (OROSházaGLAS, Orosháza, Hungary) (4 shoots/jar; 50-ml MS medium/jar) were used, the shoots were grown in a culture room with a 16-h photoperiod (105 µmol m^{-2} s^{-1}) and 22±2 ºC. Shoots were transferred to fresh medium every 4 weeks. For the experiments *in vitro* shoot cultures maintained in *in vitro* culture for 16 years were used.

In vitro shoots with 45–50 mm long were placed vertically into root induction medium (R-72; DOBRÁNSZKI *et al*. 2000) and put in a Memmert Din 40050-IP-20 incubator (Memmert, Germany) where cultures were incubated at 26±2ºC in darkness for one week. After then plants were transferred to root induction medium (R6; DOBRÁNSZKI *et al.* 2000) and put back then to the culture room for two weeks. Acclimatization was performed according to the method described by BOLAR *et al*. (1998). Plantlets were placed in Jiffy-7® pellets (Jiffy International, Norway), irrigated with 1.5 ml/L Previcur 607 SL (Bayer, Leverkusen, Germany) 1/10 MS, placed in a VEG-box (Sat-Elit Ltd, Budapest, Hungary) and completely sealed with plastic foil (folpack, Borsod Chemical Combinate, Hungary) to ensure humidity. Plants were grown in an acclimatization room with a 16-h photoperiod and 22±2 ºC at 70–80% relative humidity. After 3 weeks, plants were transferred to a plastic pot (8 cm in diameter) which contained a mixture of perlite and peat (1:3, v/v) and covered with plastic bags. When new leaves appeared, small holes were made in each bag. Acclimatized plants remained in the acclimatization room until they became one year old (Figure 1) (TEIXEIRA DA SILVA *et al*. 2019).

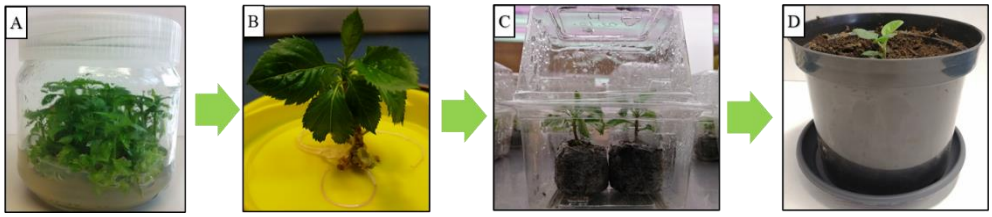

Figure 1. Acclimatization process (A: *in vitro* shoots, B: *in vitro* with roots, C: plantlet, D: acclimatized plant) (photographer: Andrea Gulyás)

Genomic DNA was isolated with the NucleoSpin Plant II kit (Macherey-Nagel, Germany) using the manufacturer's instructions. Three biological replicates and three technical replicates for the *in vitro* shoots, *in vivo* mother plants and one-year acclimatized plants were pooled (three replicates) for DNA extraction. The quality of the isolated DNA was checked by gel electrophoresis (Cleaver Scientific Ltd., Warwickshire, UK). Samples were also checked with a NanoDrop ND-1000 microcapillary spectrophotometer (NanoDrop Technologies/Thermo Scientific, Wilmington, DE, USA).

Polymerase chain reactions with the extracted DNA were performed in an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). Before the reaction, the DNA stock solutions were diluted to a concentration of 20 ng/µl in each case. The volume of the PCR mixture was 25 µl, which contained the following components: 2.5 µl of 10X DreamTaq Buffer (Fermentas, Thermo Fisher Scientific, CA, US) (containing 20 mM magnesium chloride ($MgCl_2$)), 0.5 µl of dNTP (10 mM) (Thermo Fisher Scientific, CA, US), 0.5 µl (10 µM) forward and reverse primer (Integrated DNA Technologies, Coralville, IA), 0.2 µl 5 U/µl DreamTaq DNA Polymerase (Fermentas, Thermo Fisher Scientific, CA, US), 2 µl template DNA (15 ng/µl) and 18.8 µl MQ water (high purity water Milli-Q water system, UK). The reaction mixture was prepared on ice. The 17 primers used in the SSR (LIEBHARD *et al.* 2002) analysis.

To determine the length of microsatellite alleles, an ALF-Express II (Amersham Pharmacia Biotech, Uppsala, Sweden) instrument was used, which automatically detects fluorescently labelled DNA fragments during polyacrylamide gel electrophoresis. To determine the exact size of the alleles, forward primers were labelled with Cy5 fluorescent dye. During the electrophoresis, the laser beam passes through the gel and excites the fluorescently labelled DNA fragments. The emitted light emitted is detected by a photodetector. Detector signals are collected and transmitted to the computer for processing. The signals in the form of vertices can be followed continuously during the run. By using the ALFwin Fragment Analyzer 1.00 software, the results can be evaluated, and the size of the fragments quantified.

Whole-genome Bisulfite Sequencing (WGBS) method was applied by using 100 ng of genomic DNA (*in vitro*, acclimatized and mother plant), to determine the status of cytosine methylation with the Pico Methyl-Seq Library Prep Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. The libraries were quality checked (QC) on Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) to determine exact size and concentration. WGBS was performed on Illumina HiSeq 2500 (Illumina, San Diego, CA, USA). WGBS sequencing provides a single base resolution of 5-mC in the genome, allowing profiling of the whole genome's DNA methylation. Library preparation, bisulfite conversion, paired-end sequencing on Illumina platforms, and primary data processing are all part of the study. The following bioinformatics programs were used to evaluate the whole genome sequencing of apples (Figure 2).

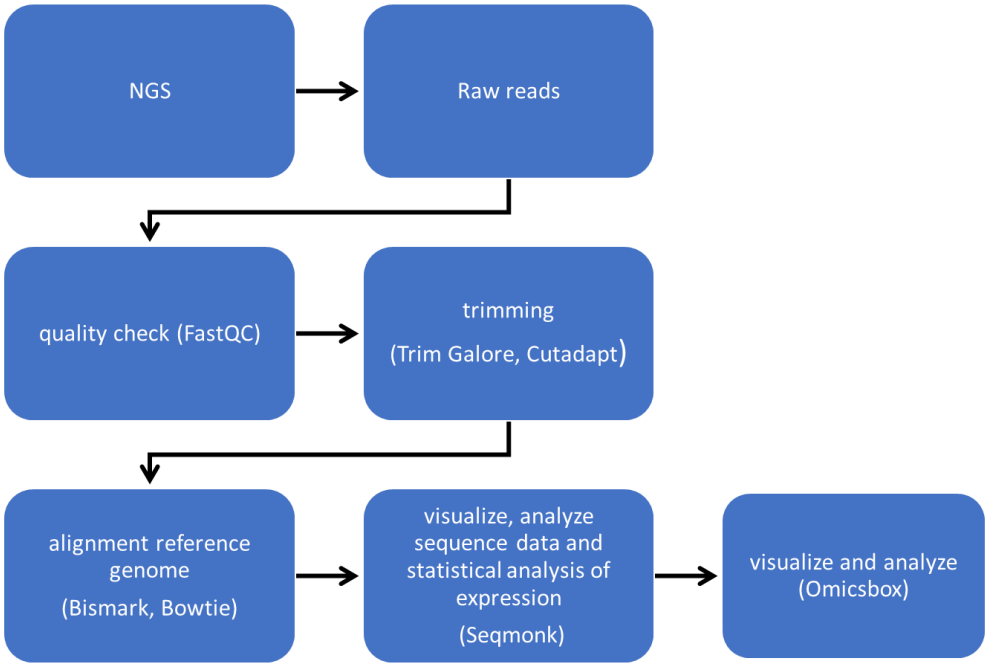

Figure 2. Bioinformatic pipeline of Whole-genome Bisulfite Sequencing (illustrated by Andrea Gulyás)

Results and discussion

The allele sizes determined by Alf-Express II device at 17 microsatellite loci are compiled in Table 1. No length polymorphism could be observed between the mother plant, *in vitro*, and acclimatized apple cultivars (GULYÁS *et al*. 2019a).

Table 1. Comparison of apple microsatellite allele length (bp) (GULYÁS *et al*. 2019a)

SSR marker name	'Húsvéti rozmaring' mother plant	'Húsvéti rozmaring' *in vitro*	'Húsvéti rozmaring' acclimatized	'McIntosh' mother plant	'McIntosh' *in vitro*	'McIntosh' acclimatized
CH01f02	170:184	170:184	170:184	174:206	174:206	174:206
CH01h01	112:112	112:112	112:112	114:116	114:116	114:116
CH01h02	203:206	203:206	203:206	249:249	249:249	249:249
CH02c02	171:185:191	171:185:191	171:185:191	179:183	179:183	179:183
CH02c06	216:220:252	216:220:252	216:220:252	230:254	230:254	230:254
CH02c09	241:247	241:247	241:247	231:255	231:255	231:255
CH02c11	222:232	222:232	222:232	226:226	226:226	226:226
CH02d08	211:217	211:217	211:217	211:229	211:229	211:229
CH03a02	135:145	135:145	135:145	119:157	119:157	119:157
CH03g07	119:129	119:129	119:129	126:166	126:166	126:166
CH04e03	193:197:203	193:197:203	193:197:203	185:199	185:199	185:199
CH04e05	174:182	174:182	174:182	184:204	184:204	184:204
CH04g10	135:135	135:135	135:135	139:143	139:143	139:143
CH05c02	168:172	168:172	168:172	168:168	168:168	168:168
CH05c04	185:207	185:207	185:207	207:207	207:207	207:207
CH05d11	187:195:205	187:195:205	187:195:205	173:175	173:175	173:175
CH05e03	162:172:190	162:172:190	162:172:190	162:162	162:162	162:162

In some loci three alleles appeared in 'Húsvéti rozmaring', which is not expected if the tested genotype is diploid. However already GUILFORD and colleagues (1997) observed that from the 14 primers they had developed 4 resulted in complex pattern, proving that microsatellite regions can multiply from several loci. Similar results were obtained by WICHMANN *et al*. (2010),

when they analysed Hungarian apple cultivars with a European standard set of microsatellite markers. KHAI and LANG (2005) distinguished between phenotypic and genotypic mutants of the indica rice plant *in vivo*. The phenotypic mutants differed externally, however, they could not show a significant difference with SSR markers. However, the genotypic mutants did not differ phenotypically, but showed significant differences with SSR markers. MODGIL *et al.* (2005) identified the apple plant as genetically stable *in vitro* by RAPD. JIN *et al.* (2008) studied a cotton (*Gossypium arboreum* L.) plant *in vitro* under the influence of 2,4-D and kinetin hormone. Significant difference with SSR and RAPD primers was revealed due to the combination effects of hormones. PRADO *et al.* (2010) studied grape (*Vitis vinifera* L.) plants *in vitro* regenerated by somatic embryogenesis. SSR markers could identify a mutant allele (231 bp) compared to the normal one (237 bp) in the 'Torrontés' variety only in one case. Their research could not prove that this had anything to do with micropropagation, the medium used, or possible hormone combinations.

GREHL *et al.* (2020) research suggests that although there are pipelines that are already optimized, unfortunately many important plants are still missing this process. Here, eight read mappers were compared: Bismark, BismarkBwt2, BSMAP, BS-Seeker2, Bwameth, GEM3, Segemehl, and GSNAP to assess the effect of read mapping results on DMR (differentially methylated regions) prediction. The user need the most accuracy, and Bismark, which requires the least memory and results in accuracy and a large number of individually mapped readings. That is why we used the Bismark program. Using Bismark, we were able to determine the total number of C's analysed, the amount of total methylated CpG, CHG, and CHH, and the amount of total unmenthylated CpG, CHG and CHH. The highest value in the total number of C's analysed was obtained with the 'Húsvéti rozmaring' mother plant R1+R2 (1,208,834,689). This value was highly distinguished

from the other samples examined. On average, the total number of C's analysed 1,139,052,600 was 6,12 % lower than this value for 'Húsvéti rozmaring' mother plant R1+R2. 'Húsvéti rozmaring' mother plant R1+R2 also outperformed all samples in the total number of CpG. For CpG, the average was 83370364.2. In the case of total CHG, although 'Húsvéti rozmaring' mother plant R1+R2 stands out. In this context the average of methylated cytosines were 68,549,708.3. It can be observed that of the 6 plants samples we examined, 'McIntosh' mother plant R1 + R2 'Húsvéti rozmaring' acclimatized R1 + R2 had a much lower methylated cytosine number. Even for unmethylated contexts, 'Húsvéti rozmaring' mother plant R1 + R2 shows the highest values. The same was observed for CHG as for CpG. For CHH, nearly identical results were obtained except for 'McIntosh' *in vitro* R1 + R2 (678,283,313) where the number of unmethylated cytosines was much lower. The methylated cytosin for CpG can be observed that the values are almost the same, only the values of 'Húsvéti rozmaring' *in vitro* R1+R2 and 'Húsvéti rozmaring' mother plant R1+R2 are higher than the others. In the CHH context, however, the highest value was found 'McIntosh' *in vitro* for R1 + R2.

After Bismark, the resulting CpG, CHG, and CHH methylation levels were visualized and analysed using SeqMonk. We used SeqMonk to compare our samples based on the GDDH13 Version 1.1 reference genom (https://iris.angers.inra.fr/gddh13/). Each context, CpG, CHG, and CHH had to be imported into the program to get a complete picture of the 'McIntosh' and 'Húsvéti rozmaring' apple scions, what we studied. After multiple-test correction, methylation dynamics is determined by analyzing data sets using the SeqMonk chi-square(χ^2) filter with a p value requirement of < 0.05. The SeqMonk help to visualize our results, which we present through the scatter plots, based on the generated statistical analysis in log fold change (LFC) values. Differentially methylated genes (DMG) were described as genes with

significant differences in DNA methylation in either their promoter or coding regions, as determined by the χ^2 test. The χ^2 test was visualized using a SeqMonk-generated MA plot (Bland-Altman plot), in which the differences in measurements between two of the three samples in all permutations were measured by transforming the data into M (log ratio) and A (mean average) scales using SeqMonk, and then plotting these values (BLAND and ALTMAN 1999).

In ‘McIntosh’and ‘Húsvéti rozmaring’, paired similarities 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, and by considering the average of all cultivars (GULYÁS *et al*. 2019b). *In vitro,* acclimatized, and mother plants of ‘McIntosh’ and ‘Húsvéti rozmaring’ were also compared for DNA methylation patterns between cultivars.

DMGs were downregulated in *in vitro* plants relative to acclimatized plants when DNA methylation patterns of ‘McIntosh’ were compared. The level of methylation in *in vitro* plants was higher than in mother plants, indicating that DMGs were upregulated, resulting in lower methylation in the mother plant. When comparing the MA plots of acclimatized and mother plants, no significant directional differences were seen, suggesting that after acclimatization, the DNA methylation pattern became identical to that of the mother plant.

DMG was reduced in *in vitro* plants compared to acclimatized plants in all contexts. In the mother plant, DMGs were upregulated, thus methylation levels were lower compared to *in vitro*. No significant differences were found between acclimatized and mother plants here either. The same was true for ‘Húsvéti rozmaring’ as it was for ‘McIntosh’.

Both up- and downregulation of DMGs were found in all three contexts when the two scion cultivars, ‘McIntosh’ and ‘Húsvéti rozmaring’,

were compared. The two scion cultivars had different methylation patterns, showing that the epigenome was regulated differently and, in a cultivar-specific method during apple adaptation to various environments. Our results show that cultivar-specific DNA methylation patterns may play a regulatory role in sensing and respon by regulating stress-responsive gene expression independently of the environment.

In both cultivars and all three contexts, significant differences in DNA methylation patterns were found. When apples were examined in general, we compared the *in vitro* to the acclimatized, the mother plant to the acclimatized, and the *in vitro* to the mother plant. We found that acclimatized plants were much less methylated than *in vitro*. When compared to the acclimatized with the mother plant, the methylation pattern tended to be similar.

Using SeqMonk, we were able to illustrate the differences between the different apple samples in all three contexts. ROTHKEGEL *et al.* (2020) studied different cultivars of cherries (*Prunus avium* (L). L.). For low-cool "Royal Dawn" and high-temperature "Kordia" cultivars, flower bud methylome was examined using MethylC-seq. Their results were visualized using SeqMonk, among others, as described by BUTLER *et al.* (2013) also when *Pseudomonas syringae* pv. *actinidiae* were studied. A total of 45,116 genes, including their promoters and coding regions, were examined using the program. Significant differences were identified in the differentially methylated genes in a total of 586 genes (334 in CpG, 201 in CHG, and 131 in CHH), of which only 446 were found in the DNA methylation Blas2GO database. This shows a 1.3 % difference in gene DNA methylation pattern in response to a change in environment, such as *in vivo* versus *in vitro*, or when the *in vitro* environment is replaced with an *in vivo* environment. In all three contexts, 72 DMGs were similar in CHG and CpG, five DMGs were identical in CHG and CHH, and seven DMGs were identical in CpG and CHH. Furthermore, four DMGs were found to be similar in all three cases, while

259, 128 and 123 DMGs were found to be unique to the CpG, CHG, and CHH contexts, respectively (Figure 3).

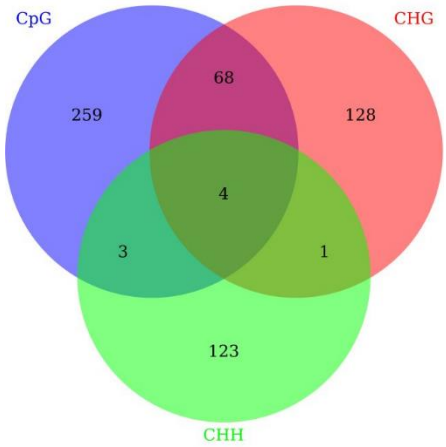

Figure 3. The CpG, CHG, and CHH contexts, the sequence distribution of significant differences in DNA methylation in genes (GULYÁS *et al.* 2019b)

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. The highest level of methylated DMGs was found in *in vitro* tissue culture. Some DMGs that participate in oxidation-reduction processes, metabolism, and biosynthesis (KAWAKATSU *et al.* 2017, BOUYER *et al.* 2017) but are not needed during *in vitro* culture were primarily methylated. In 'McIntosh' the amount of DNA methylation of DMGs was lower than in 'Húsvéti rozmaring'. After acclimatization, the amount of methylation in DMGs decreased in both cultivars.

KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment analysis was performed to identify differentially regulated biological pathways by apple cultivars from different environments. In the analysis, we

found a total of 60 pathways that can be affected due to different methylation in the various environments regarding the two apple cultivars examined. This means a total of 166 DMGs and 43 enzymes.

Conclusion and recommendation

We started our experiments by using microsatellite markers because several studies have been conducted on their ability to detect genotypes within the genus *Malus* (Liebhard *et al*. 2002). Based on our results, we found no difference with the 17 SSR markers we selected on the mother plants, *in vitro*, and between the acclimatized plants, either the 'McIntosh' or in 'Húsvéti rozmaring'. JIN *et al*. studied the genetic fidelity of regenerated plants and spontaneous shoot regeneration from the leaves of the apple rootstock 'Pingyitiancha' (*Malus hupehensis* var. *pinyiensis*) in 2014. MSAP profiling was performed on the leaf tissues of six plants (*Salvadora persica* L. (arak)), *Commiphora wightii* (Arn.) Bhandari (Indian bdellium-tree), male and female *Simmondsia chinensis* (Link) C.K.Schneid. (Jojoba), *Jatropha curcas* L. (physic nut), and female genotype of *Withania coagulans* (Stocks) Dunal (Indian cheese maker) developing *in vivo* and *in vitro*. *In vivo*-grown tissues of *S. persica*, *S. chinensis* (male), and *W. coagulans* and *in vitro*-grown tissues of *C. wightii*, *S. chinensis* (female), and *J. curcas* had higher percentage methylation in genome. The plasticity of the genomes of plants developing in two distinct habitats is reflected in the differences in DNA methylation and polymorphism in genomes. Under *in vitro* and *in vivo* conditions, different patterns of DNA methylation of homologous nucleotide sequences and polymorphism in the methylated DNA in tissues indicate that these fragments could be involved in complex processes controlling plant growth and development (RATHORE *et al*. 2015). When comparing the DMG patterns of 'McIntosh', DMGs were downregulated in *in vitro* plants compared to acclimatized plants. The DNA methylation level was higher in *in vitro* plants than in mother plants. There was no considerable directional changes in MA plots when acclimatized and mother plants were compared, indicating that after acclimatization, the DNA methylation pattern became similar to mother

plant. According to GO annotation function of the DMGs were present in: 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. The DNA methylation patterns of 'Húsvéti rozmaring' showed a similar tendency in all three contexts (CpG, CHG and CHH), as for 'McIntosh'. DMGs were present in i) metabolic, 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 and cell parts among their cellular components. We observed several DMGs in the three different environmental conditions, which confirm the findings of a series of earlier studies that have also implicated DNA methylation in the regulation of genes controlling pathways in plant development or tissue differentiation, during embryogenesis under *in vitro* propagation (US-CAMAS *et al*. 2014). DNA methylation is a key regulatory system in gene expression and plant development under stress conditions (CRISP *et al*. 2016, LÄMKE and BÄURLE 2017). We identified DMGs between 'McIntosh' and 'Húsvéti rozmaring' scion cultivars, which were both up- and down-regulated in all three contexts, independent of the environment. DMGs were present in i) metabolic cellular, biosynthetic and transfer processes, and 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. Our results suggest that the potential role of cultivar-specific DNA methylation constitute an important regulatory mechanism for sensing and responding to stress, such as drought and salinity (RAJKUMAR *et al*. 2019), as well as adaptation to other biotic and abiotic

stresses (VIGGIANO and DE PINTO 2017). In general, DMGs were detected in both cultivars and in all three contexts: i) *in vitro* plants were highly methylated compared to acclimatized plants (*in vitro* plants were downregulated); ii) *in vitro* plants were downregulated relative to mother plants; iii) DNA methylation patterns became similar to mother plants after acclimatization in both scion cultivars. The genotype of the donor plant influences the level of DNA methylation and pattern of regenerants and the ability of the genotype how to adopt to the *in vitro* environment (MACHCZYŃSKA *et al*. 2014).

New scientific results

1. As firstly in the international scientific community, we published the DNA methylome of diploid apple and identified DMGs in the whole genome of apple with whole genome bisulfite sequencing technique between the mother, acclimatized and *in vitro* plants.
2. We observed similar global DNA methylation levels for both 'McIntosh' and 'Húsvéti rozmaring' under different environmental conditions.
3. We found DMGs in the *in vitro* plants at all of the CpG, CHG and CHH contexts, which were caused by the micropropagation process in laboratory environmental conditions.
4. We could justify that there are very low but significant differences between acclimatized and mother plants, that means the DNA methylation pattern of acclimatized plant became very similar to that of the mother plant.
5. We published first, by investigating the full genome, that the methylome of apple that has been sustained *in vitro* for more than 16 years began to regress during one year of acclimatization and the 1-year-old acclimatized plant showed a very close DNA methylation pattern like the mother plant. This demonstrates that *in vitro* environment as a stressor can be quenched by adaptation and thereby can be restored by acclimatization.
6. We observed DMGs between the two scion cultivars ('McIntosh' and 'Húsvéti rozmaring'). The different scion cultivars were acclimatized to the three environmental conditions (mother plant, acclimatized plant and *in vitro* plant) in different ways. There are more easily adaptable scion cultivars to micropropagation, such as 'Húsvéti rozmaring', which regulate only few genes to adapt to stress.

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