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Analysis of Cytosine Methylation in Genomic DNA of *Solanum* × *michoacanum* (+) *S. tuberosum* Somatic Hybrids

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Abstract: Interspecific somatic hybridization is a noteworthy breeding strategy that allows the production of novel genetic variability when crossing barriers exist between two parental species. Although the genetic consequences of somatic hybridization have been well documented, little is known on its impact at the epigenetic level. The objective of our research was to investigate the epigenetic changes, in particular DNA methylation, occurring in a population of potato somatic hybrids. The analysis of 96 *Solanum* × *michoacanum* (+) *S. tuberosum* somatic hybrids from five fusion combinations and their parents was carried out by methylation-sensitive amplified polymorphism (MSAP) and high-performance liquid chromatography (HPLC) methods. Six MSAP primer combinations generated 622 unique bands, of which 295 were fully methylated. HPLC analysis showed from 15.5% to 16.9% total cytosine methylation within the parental forms. Overall, the MSAP and HPLC methods indicated an increase in DNA methylation in the somatic hybrids in comparison to their parents. Among the latter, a lower degree of DNA methylation in the wild *S.* × *michoacanum* species than *S. tuberosum* was found. Our findings indicated that somatic hybridization changed the level of cytosine methylation in the studied potato somatic hybrids.

Keywords: epigenetic; potato; somatic fusion; MSAP; HPLC

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1. Introduction

Somatic fusion is a useful tool in plant breeding to overcome crossing barriers and enable the transfer of important traits. The obtained hybrids, named somatic hybrids, are artificially produced polyploids (allopolyploids) containing a proper mixture of parental nuclear and organellar genomes able to determine their survival, growth and development. Relationships between nuclear genomes as well as between nuclear and organellar genomes are re-established after somatic fusion. Somatic hybridization results in changes in the structure and sequence of DNA and also affects the epigenetic regulation of gene expression [1,2]. The DNA methylation, histone modifications and RNA interference are epigenetic mechanisms that regulate the gene expression with no changes in the DNA sequence [3–5]. The most studied epigenetic mechanism in plants is the alteration of DNA methylation, which involves the addition of a methyl group to the 5′-carbon of the pyrimidine ring of the cytosine nucleotides [6] by DNA methyltransferases [7]. The methyl group added by covalent bonds provides the basis to which various protein structures are attached and modifies gene expression [8]. DNA methylation affects plant development and has an important role in plant responses to abiotic and biotic stresses [5], e.g.,

drought and salinity [3,9,10], low temperature [11–13], drugs [14], maintenance of plants in long-term storage [15,16] and pathogens [17–19]. In plants, genomic DNA methylation is not accidental, and it covers cytosines located at the coding and noncoding regions in three DNA sequence motifs: CG, CHG and CHH, where H means T, A or C [7,20]. The levels of methylation are diverse among various plant species and depend on the type of plant tissue [21]. The highest methylation level in plants is always in the CG motif [22]. Changes in the epigenome may be reversible or stable and are transmitted during meiosis and mitosis [16,23,24]. The methylation pattern can also change during the growth and development of plants [25]. This aspect is particularly important in polyploid crops, such as coffee, cotton, potato, peanut, sugarcane and wheat [21]. Indeed, compared to diploids, there is a higher copy number of repetitive sequences in the genomes of polyploids; because of this, a higher methylation level is required to silence such elements and ensure the normal development of polyploid plants [20,26]. Little is known about such changes in methylation patterns in somatic hybrids.

Methods of methylation analysis are dedicated to whole genome methylation profiling (global methods) or to searching for differentially methylated regions of the genome or specific genes (specific methods) [27–29]. In recent years, various methodologies have been developed and applied in different species [30]. Among them, the methylation-sensitive amplified polymorphism (MSAP) analysis is the most popular one and has been successfully used in various plant species [17,31–38], including potato [13,39,40]. This method is based on the use of methylation-sensitive restriction enzymes, which recognize and cleave 5′-CCGG-3′ sites based on differentially located methylated cytosine residues [41,42]. Another global technique frequently used for cytosine methylation analysis in plants is HPLC [14,43]. In this technique, the level of methylated and unmethylated cytosine is determined and the proportion of methylated cytosine is calculated with the appropriate formula [37]. MSAP and HPLC techniques provide a general picture of the methylation status and are relatively inexpensive and simple to perform. For these reasons, both techniques have been selected in the present study for investigation of methylation changes occurring in *S.* × *michoacanum* (+) *S. tuberosum* (*mch* (+) *tbr*) somatic hybrids. *Mch* (+) *tbr* somatic hybrids were obtained in our laboratory to transfer resistance to *Phytophthora infestans* from *mch* into the *tbr* genome [44]. Two somatic hybrids from the 97 tested were resistant to *P. infestans* [45]. Previous analysis of the genetic composition of *mch* (+) *tbr* somatic hybrids, evaluated by diversity arrays technology (DArT) markers, indicated that 13.9 to 29.6% of the markers that were present in the fusion parents were lost in the hybrids [45]. Analysis showed losses of single DArT markers spread over the whole length of every chromosome of each somatic hybrid. The explanation of this phenomenon may be the change in the methylation pattern in genomes of the *mch* (+) *tbr* hybrids due to the somatic hybridization process. Digestion of genomic DNA in DArT technology is carried out with a methylation-sensitive PstI restriction enzyme. Changes in methylation pattern in somatic hybrids could change PstI enzyme digestion, resulting in different DArT patterns than in their parental forms.

Our hypothesis is that the somatic fusion process generates changes in the level of methylation in the genome of somatic hybrids compared to their parents. Moreover, epigenetic changes have a role in plant resistance responses by regulating the expression of the genes related to resistance to pathogens [19], which may also result in a lack of expression of resistance to *P. infestans* and may explain the low frequency of obtained resistant hybrids [44,45]. The aim of this research was to determine the methylation levels of *mch* (+) *tbr* somatic hybrids and their parental forms based on MSAP and HPLC methods.

2. Materials and Methods

2.1. Plant Material

We used 96 interspecific *mch* (+) *tbr* somatic hybrids (MS1–MS31, MS33–MS95 and MS97–MS98) from five fusion combinations and their five parents: diploid ($2n = 2x = 24$) clones of *S.* × *michoacanum mch*/8 and *mch*/39, an interspecific potato hybrid; DG 81-68, a

dihaploid clone dHBard ($2n = 2x = 24$) derived from cv. Bard; and a tetraploid ($2n = 4x = 48$) cultivar Rywal. Somatic hybrids from combinations *mch*/8 (+) dHBard, *mch*/39 (+) DG 81-68 and *mch*/39 (+) dHBard are all tetraploid, and plants from *mch*/8 (+) cv. Rywal and *mch*/39 (+) cv. Rywal combinations have a ploidy higher than $4x$. Further details on the somatic hybrids and their parents are described in [44,45].

2.2. DNA Isolation

Genomic DNA was extracted from 200 mg fresh, young leaves of greenhouse-grown plants with the Qiagen DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The DNA concentration and quality were determined with a NanoDrop Lite Spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA). The DNA quality was also checked on 1% agarose gels, stained with ethidium bromide, separated during electrophoresis in 1 × TBE (Tris-Borate-EDTA) buffer followed by visualization under UV light.

2.3. Conversion of DArT Markers and PCR Analysis

In total, 18 DArT markers were chosen to develop the PCR markers. The names of the selected DArT markers, converted to PCR markers, their chromosomal localization and primer sequences are given in Table S1. The selection of markers was carried out based on previous research by [45]. Ten of the selected markers were present in *mch* parental forms but absent in *tbr* parents. Another eight were absent in *mch* and present in *tbr*. All 18 markers were absent in somatic hybrids. PCR primers were designed based on the DNA sequence of the DM 1-3 reference potato genome in regions that included sequences of the selected DArT markers. PCR primers were designed using Primer3 software [46]. PCR was performed in a T3000 thermocycler (Biometra GmbH, Göttingen, Germany) in a 20 µL volume reaction mixture containing 2 µL 10 × buffer including 20 mM $MgCl_2$ (Fermentas Life Sciences, Thermo Fischer Scientific, Waltham, MA, USA), 0.5 mM of each dNTP, 0.4 µM each primer, 1 U/µL DreamTaq polymerase (Fermentas Life Sciences, Thermo Fischer Scientific, Waltham, MA, USA) and 30 ng DNA template. The PCR conditions were as follows: one cycle at 95 °C for 10 min followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 60 s and one final extension at 72 °C for 5 min. The PCR products showing no polymorphism at the parental level were digested with 12 different restriction enzymes (MseI; TaqI; RsaI; AluI; DraI; XapI; BsuRI; HindIII; EcoRI; MvaI; BamHI; MwoI) (Fermentas Life Sciences, Thermo Fischer Scientific, Waltham, MA, USA) to obtain differences between the *mch* and *tbr* parents. Digestion of the amplicons with selected restriction endonucleases was performed according to the manufacturer's protocol at 65 °C for 3 h (MseI and TaqI) or 37 °C for 3 h (RsaI; AluI; DraI; XapI; BsuRI; HindIII; EcoRI; MvaI; BamHI; MwoI). The PCR products were separated in 1.5% agarose gels (EURx, Gdańsk, Poland), stained with ethidium bromide and assessed under UV light after electrophoresis in 1 × TBE buffer (Tris-Borate-EDTA). A 100-bp DNA marker (Fermentas Life Sciences, Thermo Fischer Scientific, Waltham, MA, USA) was used to determine the product size.

2.4. Methylation-Sensitive Amplified Polymorphism Analysis

MSAP reactions and analyses were performed as described by [47], with modifications. Sequences of the adapters and pre- and selective amplification primers are shown in Table S2. Restriction and ligation were performed in the same step. For each genotype, two separate restriction/ligation reactions with different combinations of restriction enzymes were performed. The restriction/ligation mix with 350 ng of genomic DNA was incubated overnight at 37 °C in a 50 µL reaction volume. Preamplification PCR was prepared in a 50 µL reaction volume with 0.5 µM of both EcoRI- and MspI/HpaII + 1 primer, 0.2 mM dNTPs, 1 × PCR buffer, 1.5 mM $MgCl_2$ and Taq DNA polymerase at a concentration of 1 U μL^{-1}. The reaction was carried out as follows: one cycle at 72 °C for 1 min, one cycle of 94 °C for 45 s, 65 °C for 30 s, 72 °C for 1 min, 94 °C for 30 s and a reduction of 0.7 °C per cycle from 64.3 °C to 56.6 °C for 30 s and 72 °C for 1 min, followed by 20 cycles of 94 °C for 30 s, 55.9 °C for 30 s, 72 °C for 1 min and one cycle of elongation at 72 °C for 30 s. PCR

products were checked on 1% agarose gel. Selective amplification was performed in a 50 µL reaction volume with 0.5 µM EcoRI primer and 0.5 µM MspI/HpaII primer, 0.2 mM dNTPs, 1 × PCR buffer, 2 mM $MgCl_2$ and Taq DNA polymerase at a concentration of 0.4 U μL^{-1}. PCR was performed under the same conditions described for preamplification, except for an initial step at 72 °C for 1 min. Analysis of PCR fragments was performed in a mixture of 1 µL of PCR product, 9 µL Hi-DiTM Formamide (Life Technologies Polska Ltd. Warszawa, Poland) and 0.5 µL GeneScanTM 600 LIZ® Size Standard v2.0 (Life Technologies Polska Ltd. Warszawa, Poland) on an ABI3500 DNA Analyser with 50-cm capillaries and Polymer POP-7 (Life Technologies Polska Ltd. Warszawa, Poland) using GeneMapper v.4.0 software (Life Technologies Polska Ltd. Warszawa, Poland). All PCRs were repeated twice, and reproducible and good quality peaks were scored for analysis. The presence of the DNA fragment was marked as (1), and the absence was (0). The profile of bands of genomic DNA digested with a combination of both MspI and EcoRI was compared to DNA digested with HpaII and EcoRI. By comparing the methylation pattern of every single band of a given size, four MSAP pattern types (MspI/HpaII = 1/1; 1/0; 0/1; 0/0) were expected. The obtained results for somatic hybrids and their parents were transformed into two groups: A (MspI/HpaII = 1/1—unmethylated) and B (MspI/HpaII = 1/0; 0/1; 0/0—methylated). Somatic hybrids possessed eight different methylation patterns in comparison to their parents. The methylation changes observed in the genomes of somatic hybrids are presented below:

***mch* parent**	***tbr* parent**	**somatic hybrid**	**methylation changes in somatic hybrid**
A	A	A	No change
A	A	B	Hyper-methylation of the somatic hybrid
A	B	A	Methylation pattern of the *mch* parent
B	A	A	Methylation pattern of the *tbr* parent
B	B	B	No change
B	B	A	Hypo-methylation of the somatic hybrid
B	A	B	Methylation pattern of the *mch* parent
A	B	B	Methylation pattern of the *tbr* parent

The analysis of the DNA methylation level of the somatic hybrids and their parental forms consisted of two parts: (1) quantitative analysis, in which the percentage of unmethylated and methylated CCGG sites was determined, and (2) qualitative analysis, in which the percentage of bands in various methylation patterns was determined.

2.5. High-Performance Liquid Chromatography Analysis

DNA samples (4 µg) were dried, dissolved in deionized water (100 µL), denatured at 100 °C for 2 min, and transferred to ice for 5 min. To each sample, 5 µL of 10 mM $ZnSO_4$ and 10 µL of 1.0 U mL^{-1} nuclease P1 in 30 mM NaOAc (pH 5.4) were added and incubated at 37 °C for 17 h. In the next step, 10 µL of 0.5 M Tris at pH 8.3 and 10 µL of 10.0 U mL^{-1} alkaline phosphatase in 2.5 M $(NH_4)_2SO_4$ were added, mixed carefully, and incubated at 37 °C for 2 h. After incubation, prepared samples were centrifuged for 5 min at 12 × 103 rpm. HPLC analysis was performed using a Waters 625 LC System Synergy Max-RP C12 (250 × 4.6 mm, 4u, Phenomenex, Torrance, CA, USA) column combined with a Synergy Max-RP C12 precolumn according to [48,49]. Buffer 'A', consisting of 0.5% v/v methanol in 10 mM KH2PO4 at pH 3.7, and buffer 'B', consisting of 10% v/v methanol in 10 mM KH_2PO_4 at pH 3.7, were used for the separation of nucleosides. The gradient used for separation consisted of 100% of buffer 'A' to 100% of buffer 'B' for 10 min, then 10–25 min of 100% of buffer 'B' and 5 min of 100% of buffer 'A' with the flow rate 1 mL per min at 30 °C and UV detection at a wavelength of 280 nm. The external standards were (1) DNA (0.5–50 µM), (2) RNA nucleosides (1.5–150 µM) and (3) 5-methyl-2′-deoxycytidine (5 mdC) dissolved in deionized water. Peaks of 2′-deoxycytidine (dC) and 5 mdC had retention times of 6.5 min and 9.3 min, respectively. The 5 mdC component was defined based on Millennium 32 v. 4.0 software (Waters Corporation, Milford, MA, USA). The estimate of nucleosides was determined on the automatically integrated surface areas (µV s^{-1}) of the

chromatograph peaks. The amount of cytidine (dC) and 5-methyl-2′-deoxycytidine (5 mdC) in relation to all nucleosides was evaluated using the following formula: dC = dC/(dC + 5 mdC + dG + dT + dA) × 100 and 5 mdC = 5 mdC/(dC + 5 mdC + dG + dT + dA) × 100. The level of global DNA methylation was calculated as the concentration of 5-methyl-2′-deoxycytidine (5 mdC) in relation to the whole amount of cytidine according to the following formula: 5 mdC/(5 mdC + dC) × 100.

3. Results

PCR analysis revealed the presence of all 18 markers converted from the missing DArT markers in the genomes of all 30 selected somatic hybrids from the five fusion combinations. The selected DArT markers were located on various potato chromosomes (I, II, III, IV, V, VI, VII, VIII and X) (Table S1). The known chromosomal assignment allowed the checking of different points in the genome of the somatic hybrids. For example, DArT marker 656,549 (assigned to chromosome I) was present in *mch*, absent in the *tbr* parent, and missing within somatic hybrids from all fusion combinations in the DArT analyses, as a PCR marker was detected in all the selected somatic hybrids (Figure 1).

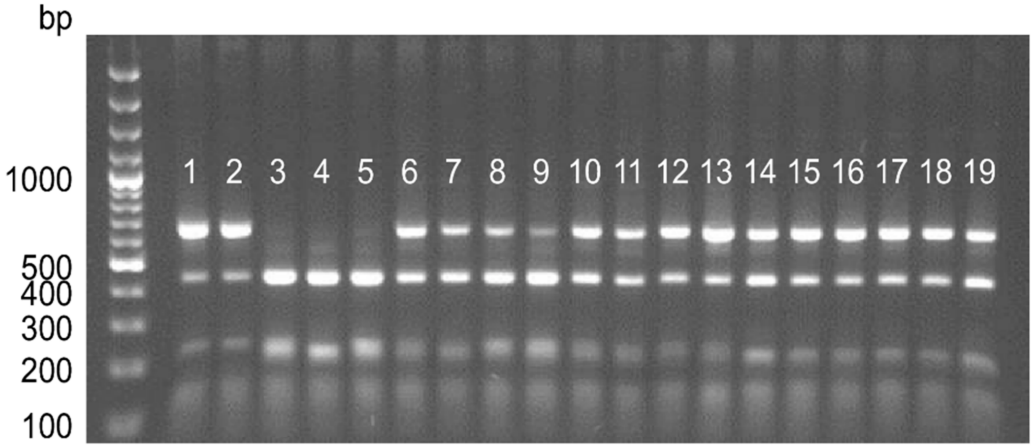

Figure 1. PCR marker converted from DArT marker 656549, digested by XapI. 1—*mch*/8; 2—*mch*/39; 3—DG 81-68; 4—dHBard; 5—cv. Rywal; 6–19—somatic hybrids from combinations: 6–7—*mch*/8 (+) dHBard, 8–9—*mch*/8 (+) cv. Rywal, 10–13—*mch*/39 (+) DG 81-68 and 14–19—*mch*/39 (+) dHBard.

MSAP analysis was applied to check the methylation changes induced by somatic hybridization in random CCGG sites. Overall, out of 14 primer combinations tested (Table S3), six (EHM_2; EHM_3; EHM_8; EHM_9; EHM_12; EHM_13) were polymorphic between the parental forms of the somatic hybrids and generated a total of 622 fragments, ranging from 75 (EHM_3) to 137 (EHM_2) bands per combination (Table 1). The sizes of the analyzed bands were between 51 bp and 630 bp. Although the total number of methylated CCGG sites was 295, the frequency changed by combination, with EHM_12 being the least polymorphic (38.3%) and EHM_9 the most (56.4%) (Table 1). Cytosine methylation levels differed among the parental forms (Table 2). On average, 182.8 bands were analyzed among them, with *mch*/39 and cv. Rywal showing the lowest and the highest number (148 and 213, respectively). Most of the CCGG sites were unmethylated in all parental forms, except for dHBard, for which 54.3% of the polymorphic sites were otherwise methylated. The CCGG methylation levels of the wild parents *mch*/8 and *mch*/39 were lower than those of the *tbr* (DG 81-68; dHBard and cv. Rywal) parents based on MSAP analysis.

Table 1. Sum of the scored MSAP bands amplified by six selected primer combinations in five parental forms.

Name of Combination	Primer Combination	No. of Bands Scored	Band Size Range (bp)	No. of Methylated CCGG Sites *	% of Methylated CCGG Sites
EHM_2	fam_EcoRI+3_CCA (+) HpaII/MspI+3_AAC	137	52–592	58	42.3
EHM_3	fam_EcoRI+3_CCA (+) HpaII/MspI+3_ACA	75	54–584	36	48
EHM_8	fam_EcoRI+3_CCA (+) HpaII/MspI+3_ACT	86	54–567	44	51.1
EHM_9	fam_EcoRI+3_CCA (+) HpaII/MspI+3_AGC	85	51–588	48	56.4
EHM_12	fam_EcoRI+3_CAA (+) HpaII/MspI+3_AGC	120	51–600	46	38.3
EHM_13	fam_EcoRI+3_CAA (+) HpaII/MspI+3_AGG	119	51–630	63	52.9
Total		622		295	

* In at least one parental form.

Table 2. Differences in the methylation of the CCGG sites detected by six MSAP primers and in the total cytosine methylation based on the HPLC technique in five parental forms of somatic hybrids.

		MSAP		HPLC	
Genotype	**Total no. of Bands**	**Unmethylated CCGG Sites (%)**	**Methylated CCGG Sites (%)**	**% of Cytosine DNA Methylation**	**Range of % of Single Readings**
mch/8	190	123 (64.7)	67 (35.3)	15.8	15.7–15.9
mch/39	148	105 (70.9)	43 (29.1)	15.5	15.4–15.6
DG 81-68	177	90 (50.8)	87 (49.2)	15.9	15.9–16.0
dHBard	186	85 (45.7)	101 (54.3)	15.7	15.6–15.9
cv. Rywal	213	121 (56.8)	92 (43.2)	16.9	16.7–17.0
Mean	182.8	104.8	78		

Between 236 (*mch*/39 (+) DG 81-68) and 272 (*mch*/8 (+) cv. Rywal) bands per fusion combination were analyzed (Table 3). The number of methylated CCGG sites differed between the fusion populations. Regarding the single fusion combinations, the highest mean percentage of methylated CCGG sites per individual occurred in the *mch*/39 (+) cv. Rywal combination (53.2%) and the lowest within *mch*/8 (+) dHBard (45.1%). No new bands appeared among the somatic hybrids, which were not observed in the parents. However, we observed a higher percentage of CCGG methylated motifs relative to the methylation level of the parental forms for fusion combinations *mch*/8 (+) cv. Rywal, *mch*/39 (+) DG 81-68 and *mch*/39 (+) cv. Rywal based on MSAP analysis. The methylation levels of the combinations *mch*/8 (+) dHBard and *mch*/39 (+) dHBard were lower than those of their parental forms.

Besides the quantitative analysis of the methylation changes triggered by somatic hybridization, a qualitative analysis was also performed (Table 4). Three major groups of banding patterns were identified (Table 4). The first one comprised those patterns that were unchanged across the parents and their somatic hybrids. Most of them (20%) belonged to the "B-pattern", which is linked to the methylated status of the CCGG sites. In the second group, the methylation changes occurring in the somatic hybrids were determined. On average, 6.1% of the analyzed MSAP bands were hypermethylated (AAB pattern), and the *mch*/39 (+) cv. Rywal combinations were the most affected (7.1%). By contrast, 14.8% of the analyzed bands were hypomethylated (BBA, ABA and BAA patterns), with means ranging from 7.2% (*mch*/8 (+) cv. Rywal) to 19.5% (*mch*/39 (+) dHBard). Lastly, many of the analyzed somatic hybrids resembled the methylation patterns of one of their parents. Thus, most of the somatic hybrids (*mch*/8 (+) cv. Rywal, *mch*/39 (+) DG 81-68, *mch*/39 (+) cv.

Rywal) inherited the banding pattern from the *tbr* parents, whereas the *mch*/8 (+) dHBard and *mch*/39 (+) dHBard banding patterns resembled those of the wild parent. Among the two *P. infestans*-resistant somatic hybrids, 7.2% (MS21) and 7.5% (MS52) of the analyzed MSAP bands showed hypermethylation (AAB), while the hypomethylated (BBA, ABA and BAA) sites accounted for 45% and 48% of the total sites in MS21 and MS52, respectively.

Table 3. Differences in the methylation of the CCGG sites detected by six MSAP primers and in the total cytosine methylation based on the HPLC technique in five populations of somatic hybrids.

	MSAP				HPLC	
Fusion Combination	**Number of Individuals**	**Total No. of Analysed Bands**	**Mean Unmethylated CCGG Sites Per Individual (%)**	**Mean Methylated CCGG Sites Per Individual (%)**	**Mean % of Cytosine DNA Methylation Per Individual**	**Mean Range of % of Cytosine Methylation Per Individual**
mch/8 (+) dHBard	4	261	140.5 (54.9)	118 (45.1)	18.8	16.1–21.5
mch/8 (+) cv. Rywal	2	272	134.5 (52)	130.5 (48)	18.3	17.5–19.0
mch/39 (+) DG 81-68	38	236	117.7 (50.6)	116.6 (49.4)	19.4	3.9–22.5
mch/39 (+) dHBard	44	240	127.3 (53)	112.7 (47)	17.3	13.1–19.7
mch/39 (+) cv. Rywal	8	251	117.4 (46.8)	133.6 (53.2)	15.9	14.0–19.2
Total	96					

Table 4. Mean percentage of the analyzed MSAP bands in eight different methylation patterns in five populations of somatic hybrids.

			Mean Percentage of the Analyzed Bands in the Fusion Combinations					
Parent *mch*	**Parent *tbr***	**Somatic hybrids**	***mch*/8 (+) dHBard**	***mch*/8 (+) cv. Rywal**	**mch/39 (+) DG 81-68**	***mch*/39 (+) dHBard**	***mch*/39 (+) cv. Rywal**	**Mean**
No changes								
A	A	A	10.4	15.5	7.0	6.1	8.4	9.5
B	B	B	20.5	24.5	18.5	17.5	18.9	20.0
								14.7
Changes in somatic hybrids								
A	A	B	5.7	5.9	5.7	5.6	7.6	6.1
B	B	A	17.0	7.2	17.3	19.5	12.9	14.8
								10.4
Parent-like banding patterns								
A	B	A	19.4	11.8	12.2	15.1	10.8	13.9
B	A	B	7.9	4.2	12.4	11.1	15.0	10.1
B	A	A	8.2	18.3	12.8	12.2	14.8	13.3
A	B	B	10.8	12.5	14.2	12.8	11.6	12.4

Regarding the HPLC analysis, the data highlighted variation in the total cytosine methylation, ranging from 15.5% (*mch*/39) to 16.9% (cv. Rywal) (Table 2). The total cytosine methylation levels of the wild parents, *mch*/8 and *mch*/39, were lower compared to the DG 81-68 and cv. Rywal parents, which was in line with the results obtained in the MSAP analysis. There was a one exception, dHBard, which had slightly lower total cytosine methylation level than wild *mch*/8. Based on the HPLC system, the highest mean percentage of methylated cytosine per individual was 19.4% for the *mch*/39 (+) DG 81-68 combination, and the lowest 15.9% for the *mch*/39 (+) cv. Rywal combination. The HPLC results indicated that the levels of cytosine methylation for the somatic hybrids from four combinations were higher than the level of methylation of the parental forms. In combination *mch*/39 (+) cv. Rywal, a lower level was noticed compared to the cv. Rywal

parent (Tables 2 and 3). The mean percentage of total cytosine methylation for the two late blight-resistant somatic hybrids was 20% and 19.04% for MS21 and MS52, respectively.

4. Discussion

The somatic fusion process is an 'extreme' procedure that is complicated and stressful for cells [24]. Somatic hybrids are obtained from electrofused protoplasts; they develop through calluses and are maintained under in vitro conditions. They consist of two different genomes, with increased ploidy levels and new nuclear–cytoplasmic interactions. In the present study, most of the *mch* (+) *tbr* somatic hybrids had higher mean CCGG and total cytosine methylation levels than their parents, as shown by the MSAP and HPLC results. It should be pointed out that MSAP detects changes in cytosine methylation in the CCGG motifs, while HPLC detects all the methylated cytosines in the whole genome. In the case of somatic hybrids, the methylation pattern can be changed to a greater extent than in sexual hybrids. It is known that during the plant tissue culture process, epigenetic changes are noted [50], and the DNA methylation level can either increase or decrease [24]. For example, the long-term tissue culture of potato generated 12.56 to 26.13% cytosine methylation changes, as evaluated by MSAP analysis [15]. Changes in the level of cytosine methylation determined by MSAP were also observed in other plant species, such as *Cymbidium hybridum* [51], *Freesia hybrida* [52] and *Hordeum brevisubulatum* [53]. Epigenetic changes can indirectly lead to changes in the DNA sequence by inducing point mutations or activating transposable elements [54].

A few examples of epigenetic changes induced by somatic hybridization have been described in potato. In *S. tuberosum* (+) *S. etuberosum* somatic hybrids, 2–6% of the total methylation patterns changed compared to those of their mother plants maintained in tissue culture, as evaluated by MSAP analysis [55]. Changes ranging from 3.2 to 8.5% were reported for *S. tuberosum* 'C-13' (+) *S. pinnatisectum* [16]. Besides potato, increased methylation levels also have been observed in somatic hybrids of cabbage [56] and citrus [57], as well as in asymmetric somatic hybrids between tall fescue and wheat [58]. Analysis of the methylation of the somatic hybrid *Ipomoea batatas* (+) *I. triloba* indicated a reduction in methylation levels compared to the parents [59]. Another factor that may have contributed to the methylation changes observed in our study is the increase in the ploidy level of our hybrids compared to the parental diploid genotypes. A previous study by [60] demonstrated that the allotetraploid form of cotton has a higher methylation level than its ancestral diploid genome and diploid hybrid. In *S. commersonii*, a wild potato species, the authors of [40,61] compared the level of methylation of the synthesized autotetraploids vs. their diploid parent. The authors noticed an increase in methylation in the synthetic polyploids. In our previous work [45], the genetic composition of the *mch* (+) *tbr* hybrids was determined using DArT markers. The analysis provided evidence that significant numbers of DArT markers present in the parental genotypes were missing in the hybrids. The PstI enzyme used in DArT technology is a methylation-sensitive rare cutter that recognizes the CTGCAG sites, and their digestion is blocked by methylation of cytosine at position ^{5m}CTGCAG [62,63]. This makes differential methylation a likely explanation for the loss of DArT markers in our somatic hybrids. We confirmed the presence of DArT DNA sequences missing in the genomes of hybrid forms according to the DArT analysis for 18 selected markers converted into methylation-insensitive PCR markers. These results are consistent with our hypothesis that the methylation pattern in somatic hybrids changes in comparison to their parents. Thus, DArT markers were not detected in the hybrids not because of deletion, chromosome loss or genome rearrangement, but most likely due to methylated restriction of the PstI sites. The authors of [62] noted that 1.2% of the DArT markers could not be grouped into a genetic linkage map of the barley lines because of their non-Mendelian behavior. This phenomenon has been explained by the formation of new, unstable cytosine methylations. DArT technology offers a high-throughput whole-genome genotyping method that can be used for the analysis of genetic diversity, population structure and gene mapping in different organisms. DArT markers have been applied

with success to crop plants, including rice [64], barley [62,65], wheat [66], potato [67,68] and many others. These markers can also be used to study the genetic composition of hybrids obtained through somatic hybridization and to dissect recombination patterns, but it should be taken into account that the analysis of the *mch* (+) *tbr* somatic hybrids with changed methylation levels induced by the somatic hybridization process may slightly affect the obtained DArT results.

Epigenetic modifications have an impact on the expression of genes. They may cause silencing or activation of some genes, leading to changes in the phenotype [59]. Qualitative analysis of the *mch* (+) *tbr* hybrids indicated not only an increase in the methylation level in their genomes but also the hypomethylation of some CCGG sites. A decrease in methylation may cause the expression of silenced genes, chromosome recombination and the activation of transposable elements [21,59]. The purpose of creating somatic hybrids is to transfer important traits, including resistance to biotic and abiotic stresses, into cultivated forms. An important problem associated with somatic hybridization is obtaining a low number of somatic hybrids expressing the desired trait. In our previous research, only two somatic hybrids were resistant to *P. infestans*, whereas the majority of the tested hybrids were classified as susceptible to late blight. Among the resistant somatic hybrids, a higher percentage of hyper- and hypomethylated CCGG sites was observed compared with the average percentage of the hyper- and hypomethylated CCGG sites for their fusion combinations. HPLC also indicated their higher mean percentage of methylated cytosines compared to the means of their fusion combinations. Both MSAP and HPLC methods did not allow us to accurately determine in which chromosome or chromosome region methylation changed, which could affect the resistance to *P. infestans*. Therefore, further research is needed. A reduced or a lack of expression of resistance to *P. infestans* in somatic hybrids has also been reported in *S. pinnatisectum* (+) *S. tuberosum* hybrids [69] and in *S.*× *michoacanum* (+) *S. tuberosum* hybrids [70]. One plausible hypothesis to explain these results is the change in the methylation status in the genome of somatic hybrids.

5. Conclusions

Our results confirmed changes in DNA methylation in the genomes of the *mch* (+) *tbr* hybrids. The impact of the DNA methylation level of the somatic hybrids on the expression of the desired trait, such as resistance to *P. infestans*, requires further detailed studies. Comprehensive characteristics of the somatic hybrids of various plant species, including potato, will widen the knowledge of the process of somatic hybridization and will allow for effective use of the obtained somatic hybrids in plant breeding.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11050845/s1, Table S1: Selected DArT markers converted to PCR markers. DArT markers were present in the *mch*/*tbr* genome but absent in the genome of somatic hybrids; Table S2: Sequences of adapters and primers used in methylation-sensitive amplified polymorphism (MSAP) analyses; Table S3: Combinations of selective amplification primers. The primer combinations used for analysis of the level of cytosine methylation in somatic hybrids are shown in bold.

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