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Phenotypic and epigenetic variation induced in newly synthesized allopolyploids and autopolyploids of potato

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ABSTRACT

Polyploidy is one of the most important events in plant evolution: the evidence suggests that more than 70% of the angiosperms underwent some event of genomic duplication during their diversification. In potato, the third more important crop worldwide, the effects of autopolyploidization at morphological, physiological and molecular levels have been explored and no evident phenotypic superiority of polyploid lines respect to the parental diploid lines has been found. On the other hand, the consequences of allopolyploidization have not been analysed so far in potato from a molecular point of view. In this work we study the variability induced by whole genome duplication in potato auto and allotetraploids obtained by chromosomal duplication of a *S. kurtzianum* genotype (autopolyploid model) and by chromosomal duplication of a diploid interspecific hybrid between *S. tuberosum* and *S. kurtzianum* (allopolyploid model). Polyploid lines in both models showed a tendency to increase the vigor in phenotypic characters compared with diploid parent they derived from. AFLP analysis showed no polymorphism between parental diploid and derived polyploids lines. However, there were DNA methylation changes between diploids and polyploids, indicating that polyploidization alters the epigenetic patterns in potato. It is possible that the superiority of the tetraploid can be explained as a heterotic response dependent on the presence of heterozygous loci and epistatic interactions inducing alterations in the gene regulation of the polyploid genomes.

1. Introduction

Polyploidy is one of the most important events in plant evolution and several authors refer that more than 70% of angiosperms have experience genome duplication during their evolution (Soltis et al., 2009). Whole-genome duplication of the same or very similar genomes results in autopolyploidy (polysomic polyploidy) and the duplication of two or more divergent genomes in allopolyploidy (disomic polyploidy). Polyploidization can be achieved mainly in two ways, by somatic doubling of the chromosomes (asexual polyploidization) or by 2n gametes with the sporophytic chromosome number (sexual polyploidization) (Iwanaga and Peloquin, 1982; De Storme and Geelen et al., 2013; Spoelhof et al., 2017).

The advantages of polyploids over diploids, such as higher yields, enhanced vigour and novel variation were thoroughly states by several authors (Leitch and Bennett, 1997; Wendel, 2000; Comai, 2005). Genome duplication entails the reorganization of two divergent subgenomes in the allopolyploid or the similar duplicated genome in the autopolyploid inducing gene expression modifications. Investigations on synthetic autopolyploids showed that modifications are not as intense as in allopolyploids, and it has been proposed that differences could be originated by the presence of diverged regulatory hierarchies in allopolyploids (Comai, 2005; Yu et al., 2010). Tan et al. (2015) found that tetraploidization of citrus (*Citrus junos* Sieb. ex Tanaka) had a limited effect on transcriptome changes when diploid parent and tetraploid derived lines were compared. Results obtained in *Arabidopsis* suggested that doubling the same genome in autopolyploids has much smaller effects on gene regulation than combining divergent genomes in allopolyploids (Wang et al., 2006). In cabbage, leaf and stem proteomes remained globally unchanged between diploid and autopolyploid lines (Albertin et al., 2005). Additional examples of changes in gene expression induced by allopolyploidization in vegetable crops have been reported in *Brassica* (Xu et al., 2009) and in wheat (Kashkush et al., 2002; He et al., 2003).

The cultivated potato (*Solanum tuberosum* L., tbr, 2n = 4x = 48), the third most important food crop after rice and wheat, is considered an autotetraploid with a chromosome base number of 12 (Iwanaga and Peloquin, 1982). However, Cribb and Hawkes (1986) provide evidences

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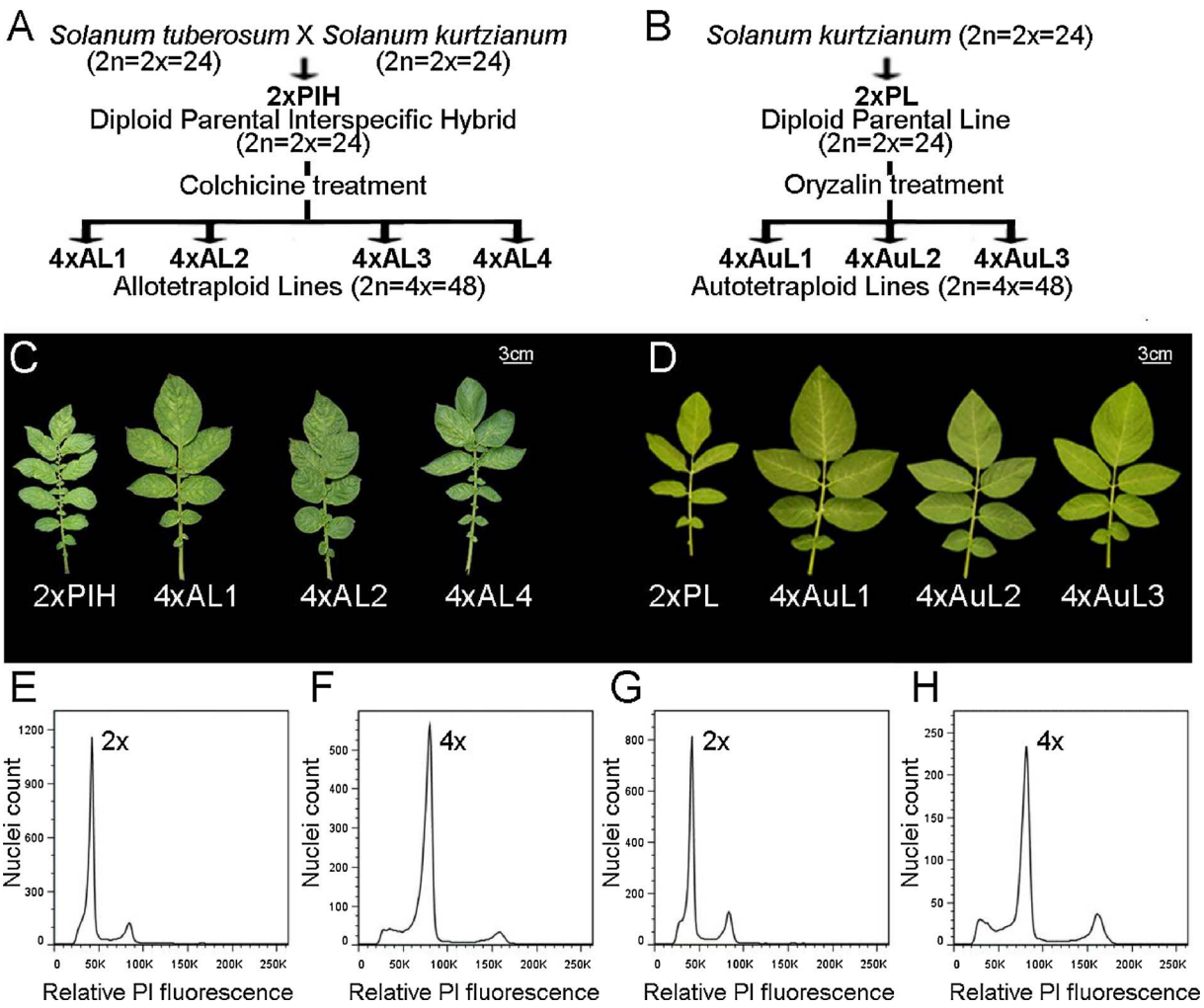


Fig. 1. Outline of the assayed experimental models, leaves and flow cytometry analyses of diploid and tetraploid lines. (A), allopolyploid model: a diploid parental interspecific hybrid was compared with four tetraploid lines derived from it after colchicine treatment. (B), autopolyploid model: a diploid *Solanum kurtzianum* genotype was compared with three tetraploid lines derived from it after oryzalin treatment. (C) and (D), photography of the sixth leaf of the diploid and three derived tetraploid lines in the allopolyploid and autopolyploid models, respectively. (E–H), representative analysis by flow cytometry of nuclei stained with propidium iodide (PI) of the diploid parental interspecific hybrid 2xPlH (E), the allotetraploid line 4xAL2 (F), the diploid parental line 2xPL (G) and the autotetraploid line 4xAuL3 (H).

that support the allotetraploid origin of tbr. Matsubayashi (1991), based on cytological analysis, propose that tbr is a segmental polyploid with two subgenomes (AAA^tA^t). Martinez-Zapater and Oliver (1984), analysing isoenzyme segregation suggested that autopolyploidy or amphidiploidy with lack of chromosome differentiation of the parental species may explain the tetrasomic segregation in potato. Therefore, it is possibly that divergent genomes participated in the origin of the tetraploid potatoes through the spontaneous doubling of a diploid hybrid (Ugent, 1970) or by functioning of 2n gametes (Iwanaga and Peloquin, 1982).

Previous studies of phenotypic, genetic, epigenetic and transcriptomic changes in three potato species (*S. bulbocastanum* Dun., *S. commersonii* Dun. and *S. phureja* Juz. et Buk.) were focused on newly synthesized autotetraploids, in which subtle transcriptomic changes were observed between the autotetraploids and the diploid parent they derived from; and no clear superiority of tetraploids was observed in terms of leaf thickness and area, vessel number, lumen size and vessel wall thickness, stomata pore length and width, guard cell width, and stomatal density compared with their diploid progenitors (Stupar et al., 2007; Caruso et al., 2011; Aversano et al., 2013, 2015b). However, hundreds of cultivated and wild potato species have been described (Spooner et al., 2014); therefore, the explored autopolyploid model in the three species above mentioned could be not representative of the induced variability nor the evolutionary relevance of genome duplication in potato. In addition, there are no studies focused on the effect of allopolyploidization in tuber-bearing *Solanum* species.

Extensive genetic and epigenetic modifications have been described in newly synthesized allopolyploids. Genetic changes include chromosomal rearrangements, transpositions and deletions (Shaked et al., 2001; Pontes et al., 2004). Epigenetic changes include alteration in DNA methylation, histone modifications and differential sRNAs accumulation (Comai, 2005). Rapid sequence rearrangements derived from autopolyploidization also has been reported (Martelotto et al., 2007). Scarce experiments have addressed direct comparisons between closely related allo and autopolyploid models. Most findings reported in the literature, seems to corroborate the hypothesis that, due to the absence of hybridization, autopolyploids experience less dramatic genetic and genomic alterations than those induced by allopolyploidization (Spoelhof et al., 2017).

Our aim was to study the phenotypic, genetic and epigenetic changes induced by allopolyploidization and autopolyploidization in potato and to compare the magnitude of the variability generated between these two models. The hypotheses of the research were that allopolyploids would have higher rates of genetic and epigenetic changes than autopolyploids and that the polyploidization in potato would not generate a phenotypic superiority respect to the diploid counterpart. To

test these hypotheses, allopolyploids and closely related autopolyploids were chemically resynthesizing and compared with their respective diploid parental lines and with each other.

2. Material and methods

2.1. Plant material

2.1.1. Allopolyploid lines

Synthetic allopolyploids was generated from a previously described diploid interspecific hybrid obtained from a cross between a tbr dihaploid (2n = 2x = 24) and one genotype of the wild potato species *Solanum kurtzianum* (ktz, 2n = 2x = 24) (Marfil et al., 2006). This hybrid was considered the diploid parental interspecific hybrid and labelled as 2xPIH. 2xPIH was introduced and multiplied in vitro in MS medium. Uninodal stem segments, each including an axillary bud, were cut from in vitro grown plants and placed back into fresh MS medium to allow the bud to grow to 10–15 mm in length. After about ten days, the bud was excised with a needle and discarded. Stem segments, with axillary buds excised, were immersed in 0.25% w/v sterile aqueous colchicine solution and placed in a shaker for 24 h in darkness (flasks with 20 ml of colchicine solution each containing 20 stem segments were used). After the colchicine treatment, the shoots were rinsed two times in 50 ml sterile distilled water and transferred to solidified MS medium in a growth room under the same conditions used for growing the original plants. After seven weeks, the surviving colchicine plantlets were transferred into pots with sterile substrate and cultivated in greenhouse. One hundred regenerants were subjected to flow cytometry for ploidy estimation (see below in *Flow cytometric and cytological analyses* section). Four allotetraploids lines (4xAL1, 4xAL2, 4xAL3 and 4xAL4) were selected for further analysis (Fig. 1). The five studied lines were cultivated in greenhouse during three months until tubers produced were collected and conserved for four months at 4 °C. This cultivation cycle was repeated four times and all comparisons were performed on plants of the fifth generation of clonal reproduction. For each analysed line, biological replicates were grown from tubers obtained in the previous season and bred at the same time under uniform conditions in an insect-proof screen house.

2.1.2. Autopolyploid lines

Synthetic autopolyploids were obtained from the diploid ktz genotype used as male parent of the interspecific hybrid described above (Marfil et al., 2006). This ktz genotype was considered the diploid parental line and labelled as 2xPL In order to obtain autotetraploid lines, a similar methodology to that previously described for allotetraploid lines was used, although instead of using colchicine, the in vitro treatment was performed with oryzalin on uninodal stakes obtained from 2xPL. Uninodal cuttings were introduced in 0.30% v/v sterile aqueous solution of oryzalin. The in vitro culture procedure was the same as described above for allotetraploid lines. After seven weeks, the surviving oryzalin seedlings were transferred into pots with sterile substrate and cultivated in greenhouse for their rustication. A total of 16 regenerants were subjected to flow cytometry analysis for the ploidy estimation (see below in *Flow cytometric and cytological analyses* section). Three autotetraploids lines (4xAuL1, 4xAuL2 and 4xAuL3) were selected for further analysis (Fig. 1). The four studied lines were cultivated in greenhouse during three months until tubers produced were collected and conserved for four months at 4 °C. This cultivation cycle was repeated four times and all comparisons were performed on plants of the fifth generation of clonal reproduction. For each analysed line, biological replicates were grown from tubers obtained in the previous season and bred at the same time under uniform conditions in an insect-proof screen house.

2.2. Flow cytometric and cytological analyses

The two-step protocol published by Doležel et al. (2007), which use Otto's buffers (Otto, 1990) for preparation of intact nuclei suspension and 50 mg ml^{-1} propidium iodide to stain nuclear DNA, was followed. Five expanding terminal leaflets of each analysed line were pooled for nuclei isolation; and tomato cv. Roma and tbr cv. Spunta nuclei from fully expanded leaflets were used as internal references. From the results obtained in the flow cytometric analyses, four allotetraploid and three autotetraploid lines were selected for further analyses. The ploidy in the selected lines was confirmed by direct chromosome counting on cytology samples. Chromosome counting was performed on samples obtained from root apices treated with 0.002 M 8-hidroxiquinoleine for 24 h, hydrolysed in HCL 1N for 10 min at 60 °C and staining in 2% acetorceine (0.2 g orceine, 45 mL 45% glacial acetic acid and 55 mL distilled water). Squash preparations were observed under a Nikon Eclipse E200 optical microscope (Tokyo, Japan).

2.3. Morphological analyses

Fifteen biological replicates of each analysed line were grown from tubers into 5 L pots filled with organic compost and cultivated in an insect-proof screen house under uniform conditions. Top dry weight (TDW), total tuber fresh weight (TTW), main tuber weight (MTW) and leaf area (LA) were measured. Total leaf area of 130-day-old well-watered plants was assessed by removing leaves from the whole plant and using a LI-COR LI-3100C Area Meter (Lincoln, Nebraska, USA). Vegetative tops (above-ground portion of the plant) were then dried in a forced-air oven at 60 °C to a constant weight and TDW per plant recorded. TTW and MTW produced per plant were determined five days after harvest.

2.4. Anatomical analysis

First, the exponential growth range of terminal leaflets was determined for each evaluated line according to Stupar et al. (2007). Then, only terminal leaflets in their predetermined exponential growth range were harvested (15–20 mm in length for diploid lines; 25–35 mm in length for tetraploid lines). For anatomical analysis, three terminal leaflets were selected from each line and fixed in FAA (40% formaldehyde: glacial acetic acid: 50% ethanol: 5:5:90 by volume) for 24 h. Terminal leaflet discs of 2 cm^2 containing the main vein were obtained, dehydrated in an ethanol series up to 100% and in a xylol series up to 100%, and then embedded in paraffin. Cross sections (5 µm thick) were cut with a microtome and collected on a glass slide. Samples were stained with fast green, mounted with Canadian Balsam and observed under a Nikon Eclipse E200 optical microscope (Tokyo, Japan). Samples were photographed with a Micrometrics 318 CU digital camera (Shangai, China) at various magnifications and analysed with the software ImageJ (Abramoff et al., 2004). The following parameters were measured: lamina thickness (LT), mesophyll/palisade ratio (M/P); vessels number (VN), vessel lumen area (VLA), and vessel wall thickness (VWT). VLA and VWT were measured for a minimum of ten vessels per section.

For scanning electron microscopy (SEM) analysis, two terminal leaflets for each individual plant were used. Discs of 2 cm^2 were obtained from the inter-nerval zone and fixed in FAA solution, dried and then gold coated with a sputter coater (DENTON VACUUM DESK IV, NJ, USA). Images were obtained using a Jeol SEM JSM- 6610LV at 5–10 kV. Photomicrographs were taken at ×300 magnification (standard area of 0.45 mm^2) and the following parameters were measured in almost 15 stomata per analysed plant: stomata pore length (SL), stomata pore width (SW) and guard cell width (CW). Stomata density was measured on a standard area of 1 mm^2.

2.5. DNA extraction and molecular analyses

Genomic DNA was extracted from meristem leaves and diluted according to Cara et al. (2013). The AFLP analysis was performed as described by Cara et al. (2014). For the allopolyploid model, fluorescence-labelled [*Eco*RI* + 3] and unlabelled [*Mse*I + 3] primers were used in selective amplification. Three primers combinations were used to amplify AFLP fragments. The primer combinations utilized were: E-ACG* + M-CAA, E-ACA* + M-CAA and E-AAG* + M-CAA. For the autopolyploid model, unlabelled amplification primers were used and three primers combination were assayed: E-ACA + M-CAA, E-ACA + MCTC, E-ACG + M-CTG. For allotetraploid lines, samples were then denatured at 90 °C for 3 min and run on an Applied Biosystems Genetic Analyzer 3130. Semi-automated scoring was performed on the resulting electronic profiles using GeneMapper version 3.7 (Applied Biosystems, California, USA). The fragments obtained from the amplification of lines from the autotetraploid model, were resolved in 6% polyacrylamide denaturing gels followed by silver staining. The preparation of the acrylamide gels and electrophoresis were carried out in a vertical Sequi-Gen GT vessel (BIO-RAD) following Cara et al. descriptions (2014). For both, allotetraploid and autotetraploid models, three biological replicates per line were analysed to validate the procedure, and only reproducible pattern on all replicates were used for analysis.

In order to demonstrate if epigenetic changes could be induced by polyploidization, DNA methylation was analysed with MSAP (methylation sensitive amplification polymorphism) technique using the protocol developed by Reyna-López et al. (1997) and adapted by Xiong et al. (1999). The methylation pattern at the 5′-CCGG sites was analysed using the isoschizomer methylation-sensitive enzymes *Hpa*II and *Msp*I. *Hpa*II is sensitive to full methylation (both strands methylated) of either cytosine but cleaves the hemimethylated external cytosine, whereas *Msp*I is sensitive only to methylation of the external cytosine (Roberts and Macelis, 1997). The isoschizomers *Hpa*II and *Msp*I were used as frequent cutters and *Eco*RI was used as rare cutter. For sample preparation, the protocol described by Cara et al. (2013) was followed. In the allotetraploid model, [*Eco*RI* + 3] labelled primer and [*Hpa*II/*Msp*I + 3] unlabelled primers were used. The following two primer combinations were assayed to amplify MSAP fragments: E-AAT* + H/M-ACA, and E-ATC* + H/M-AGC. Unlabelled primers were used for autotetraploid model, in which six primers combinations were assayed: E- ACC + H/M- AAT, E-ACC + H/M-ATC, E-ACG + H/M- ATC, E-AAA + H/M-ATC, E-AAA + H/M- AAT, and E-AGA + H/M-AAT Pre-amplification and selective amplification reactions and resolution and analysis of fluorescent amplified fragments in the allopolyploid model were performed as described previously in the AFLP protocol. The fragments obtained from the amplification in the autotetraploid model were resolved in 6% polyacrylamide denaturing gels in the same manner as detailed in the AFLP technique. For both, allotetraploid and autotetraploid models, three biological replicates per line were analysed to validate the procedure, and only reproducible pattern on all replicates were used for analysis.

Fifteen nuclear microsatellite (SSR) primer pairs described by Feingold et al. (2005) were assayed in the 2xPIH and 2xPL (Supplementary S1). PCR reactions were performed in a final volume of 15 μL containing 1X reaction buffer with 1.5 Mm $MgCl_2$, 0.04 mM of each dNTPs, 0.125 μM of forward and reverse SSR primers, 0.5 U of Taq DNA polymerase (Invitrogen, USA), and 50 ng of genomic DNA. The cycling profiles for each SSR marker were set as described by Feingold et al. (2005). PCR products were separated by electrophoresis on a 6% denaturing polyacrylamide gel, silver stained following the protocol described by Cara et al. (2014) and scanned for manual scoring.

2.6. Data analysis

Results obtained from morphological, physiological and anatomical parameters were subjected to analysis of variance ANOVA using Duncan's test for normally distributed variables. The allopolyploid and autopolyploid models were analysed separately, comparing in each the parental diploid line with its tetraploid derived lines. The differences were determinate with a significance level of $P \leq 0.05$ using InfoStat (InfoStat version 2009 software; Grupo InfoStat, Córdoba, Argentina).

In order to compare the magnitude of the variability induced by the polyploidization between allotetraploids and autotetraploids, ratios of relative changes were compared between models. For each model and parameter studied, the mean value of replicates from the diploid parental line were set as the reference value and used to calculate the relative change in each replicate from tetraploid lines. Once the values of the ratios were obtained, the distribution of the data for each variable was plotted. The variables were compared using ANOVA and generalized linear mixed-model (GLMM) for data with normal and non-normal distributions, respectively. For ANOVA analysis, the Duncan test was used. For the GLMM analysis, the Gamma family and the Bonferroni statistic were selected The proportion of changes between models were determined with a significance level of $P \leq 0.05$ using InfoStat (InfoStat version 2009 software; Grupo InfoStat, Córdoba, Argentina).

In the AFLP analysis, a binary matrix was constructed scoring fragments as absent (0) or present (1) and then monomorphic and polymorphic fragments among the five analysed lines for the allotetraploid model and among the four analysed lines for the autotetraploid model were computed. For the MSAP analysis, the different patterns of presence and absence of fragments in *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I profiles for each evaluated line were converted into four different methylation patterns as indicted in Fig. 3, and then, this codification was converted into a binary matrix for presence (1) or absence (0) of the particular pattern. Using the MSAP data set, similarity matrices were generated based on the Dice coefficient, and the UPGMA linkage method was used for dendrogram construction using NTSYS-pc 2.11 software (Rohlf, 1992).

3. Results

3.1. Agronomical and physiological analysis

Allotetraploid lines showed greater vigor in morphological traits compared with 2xPIH, including increased TDW and higher LA (Fig. 2A and B). Although only 4xAL3 developed greater tubers than the parental 2xPIH (Fig. 2C), the four allotetraploid lines increased TTW respect to the diploid parental interspecific hybrid 2xPIH (Fig. 2D). In the autopolyploid model, two out of three derived autotetraploid lines (4xAuL2 and 4xAuL3) increased TDW and LA respect to the diploid parental line 2xPL (Fig. 2A and B), the three autotetraploid lines significantly increased the greater tuber size (Fig. 2C), although only 4xAuL2 has bigger total tuber production than the diploid parental line 2xPL (Fig. 2D).

3.2. Morpho-anatomical analyses

Data on SL, SW, GCW and SD obtained by SEM analysis are shown in Table 1. After genome doubling SL and GCW were significantly increased in the four allotetraploid lines respect to the diploid parental intespecific hybrid 2xPIH (except GCW for the 4xAL3 line). Autotetraploid lines showed higher SL and GCW respect to the diploid parental line 2xPL, while only 4xAuL1and 4xAuL3 significantly increased SW respect to 2xPL (Table 1). The SD significantly decreased in allo and autotetraploid lines respect to their parental diploid lines (Table 1). Histological analysis of leaf cross sections showed that allotetraploid lines increased LT, VLA, VN and VWT respect to the diploid parental interspecific hybrid 2xPIH, except for 4xAL2 which not showed more VN than 2xPIH (Table 2). In the autopolyploid model, also tetraploid lines developed thicker leaves and vessel with higher lumen area and thicker wall than the diploid parental line 2xPL,

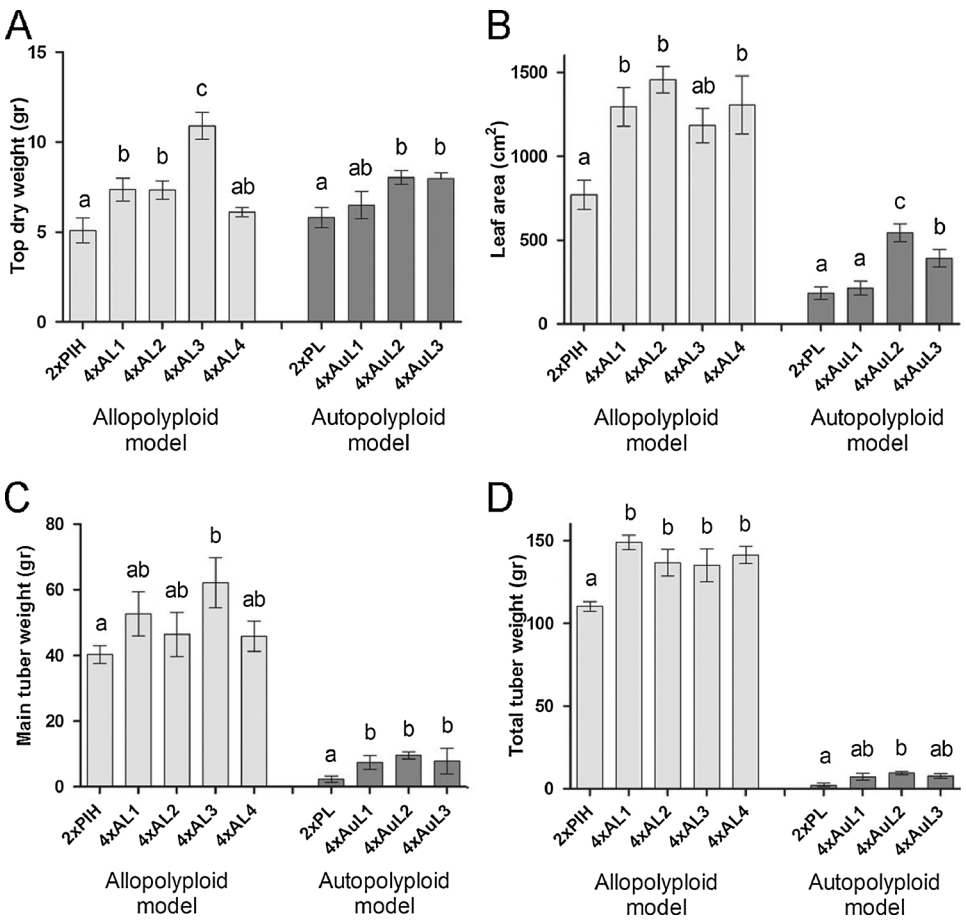


Fig. 2. Biomass production in diploid and tetraploid potato lines. In the allopolyploid model, a diploid parental interspecific hybrid *Solanum tuberosum* x *Solanum kurtzianum* (2xPIH) was compared with four tetraploid lines derived from it after colchicine treatment (from 4xAL1 to 4xAL4). In the autopolyploid model, a diploid *S. kurtzianum* genotype (diploid parental line, 2xPL) was compared with three tetraploid lines derived from it after oryzalin treatment (from 4xAuL1 to 4xAuL3). Results of top dry weight (A), leaf area (B), main tuber weight (C) and total tuber weight (D) of diploid and tetraploid studied lines.

without differences in VN among diploid and tetraploid lines (Table 2; Supplementary S2).

3.3. Ratios of relative changes between allotetraploid and autotetraploid lines

The trend towards a superiority in traits related with productivity and cell size of tetraploid lines respect to their respective diploid parental line was confirmed (Table 3). From 12 traits in which the ratios of relative changes were compared, eight showed differences between models. The increases respect to the parental diploid lines in total tuber weight, in the greater tuber weight, in the vessel wall thickness and in the palisade thickness were significantly higher in autopolyploids than in allopolyploids (Table 3). On the other hand, the increases respect to the parental diploid lines in stomata pore width, stomata pore length, lamina thickness and vessel lumen area were significantly higher in allopolyploids than in autopolyploids (Table 3).

3.4. Genetic and epigenetic analyses

In order to assess if structural changes were induced by polyploidization, diploid lines were compared with its respective tetraploid derived lines using AFLP markers. In the allopolyploid model, a total of 335 analysed fragments showed no polymorphism among the five evaluated lines. For autopolyploid model, 206 AFLP fragments were obtained, all of which were monomorphic among the four compared lines.

Epigenetic variation was studied by MSAP markers (Fig. 3). From the comparison of *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I amplification patterns, four types of CCGG methylation status were identified: i) unmethylated,

Table 1
Results of stomata pore length (SL), stomata pore width (SW), guard cell width (GCW) and stomata density (SD) of parental diploid (2x) and derived tetraploid (4x) potato lines.

Model[a]	Lines	SL (μm)[b,c]	SW (μm)	GCW (μm)	SD (N° of stomata/mm^2)
Allopolyploid	2xPIH	7.2 ± 1.8 A	1.8 ± 0.1 A	5.7 ± 0.1 A	440.8 ± 12.4 C
	4xAL1	14.8 ± 2.5 C	2.0 ± 0.2 A	7.9 ± 0.1 B	245.5 ± 7.4 AB
	4xAL2	15.0 ± 0.8 C	2.0 ± 0.2 A	8.5 ± 0.3 B	161.2 ± 22.7 A
	4xAL3	15.4 ± 2.9 C	2.2 ± 0.1 A	7.7 ± 0.5 AB	151.3 ± 151.3 A
	4xAL4	11.5 ± 1.3 B	1.9 ± 0.1 A	8.2 ± 0.9 B	349.9 ± 11.3 B
Autopolyploid	2xPL	8.5 ± 2.0 A	1.1 ± 0.3 A	5.0 ± 0.2 A	429.0 ± 23.2 C
	4xAuL1	14.3 ± 2.4 C	2.9 ± 0.6 C	7.3 ± 0.8 B	167.0 ± 37.0 A
	4xAuL2	13.8 ± 2.6 C	2.3 ± 0.5 C	7.2 ± 0.8 B	204.6 ± 26.6 A
	4xAuL3	10.4 ± 1.3 B	1.4 ± 0.5 A	7.5 ± 0.8 B	341.8 ± 20.1 B

[a] Allopolyploid and autopolyploid lines were analysed separately.
[b] Mean ± standard error from 15 replicates for each measured parameters.
[c] In each column means with the same letter did not differ significantly at P ≤ 0.05 according to Duncan's multiple range test.

Table 2
Results of leaf lamina thickness (LT), vessel lumen area (VLA), number of vessels per main vein (VN), vessel wall thickness (VWT) and mesophyll/palisade (M/P) ratio of parental diploid (2x) and derived tetraploid (4x) potato lines.

Model[a]	Lines	LT (μm)[b,*]	VLA (μm^2)	VN (*n*)	VWT (μm)	M/P ratio
Allopolyploid	2xPIH	72.7 ± 15.8 a	32.6 ± 14.1 a	21.9 ± 4.8 ab	0.9 ± 0.2 a	3.0 ± 0.1 b
	4xAL1	133.5 ± 41.6 c	438.5 ± 99.5 d	29.6 ± 3.8 c	2.1 ± 1.3 d	2.4 ± 0.2 a
	4xAL2	229.3 ± 59.1 e	258.8 ± 105.7 c	20.7 ± 5.3 a	2.1 ± 0.6 cd	2.4 ± 0.3 a
	4xAL3	109.8 ± 12.5 b	102.7 ± 49.2 b	30.6 ± 3.1 c	1.9 ± 0.4 c	2.4 ± 0.2 a
	4xAL4	165.5 ± 21.0 d	112.1 ± 37.3 b	27.9 ± 3.5 c	1.5 ± 0.3 b	2.8 ± 0.2 b
Autopolyploid	2xPL	80.5 ± 0.9 a	48.3 ± 14.5 a	18.5 ± 1.5 a	1.0 ± 0.1 a	5.8 ± 2.1 b
	4xAuL1	102.5 ± 2.6 b	103.5 ± 42.3 b	22.7 ± 1.4 b	1.6 ± 0.3 b	1.3 ± 0.4 a
	4xAuL2	100.1 ± 3.6 b	92.9 ± 25.8 b	21.6 ± 1.8 b	2.1 ± 0.1 b	1.2 ± 0.1 a
	4xAuL3	100.1 ± 5.8 b	136.9 ± 47.3 c	22.3 ± 1.7 b	1.4 ± 0.1 c	1.4 ± 0.5 a

[a] Allopolyploid and autopolyploid lines were analysed separately.
[b] Mean ± standard error from 15 replicates for each measured parameters.
* In each column means with the same letter did not differ significantly at P ≤ 0.05 according to Duncan´s multiple range test.

Table 3
Ratios of relative changes of polyploidy potato plants respect to their diploid parental lines and comparison of the variability induced by genome doubling between allopolyploid and autopolyploid models.

Traits	Mean ratio		p-value
	Allotetraploid model	Autotetraploid model	
Total tuber weight	1.08 ± 0.21	1.82 ± 0.51	*0.0002*
Greater tuber weight	1.19 ± 0.53	3.96 ± 1.32	*0.0001*
Top dry weight	1.29 ± 0.39	1.33 ± 0.28	0.6540
Leaf area	1.46 ± 0.45	1.37 ± 0.74	0.5880
Stomata pore width	1.77 ± 0.41	1.35 ± 0.49	*0.0001*
Stomata pore length	2.01 ± 0.62	1.57 ± 0.32	*0.0001*
Stomatal density	0.50 ± 0.10	0.54 ± 0.10	0.5770
Lamina thickness	2.11 ± 0.80	1.25 ± 0.07	*0.0001*
Mesophyll/palisade ratio	0.85 ± 0.09	0.22 ± 0.05	0.0001
Vessel number/main vein	1.18 ± 0.24	1.20 ± 0.09	0.5610
Vessel wall thickness	2.59 ± 1.03	2.93 ± 0.76	*0.0290*
Vessel lumen area	7.90 ± 2.58	2.41 ± 0.98	*0.0001*

ii) hemymethylated, iii) methylated, and iv) methylated ambiguous. In the MSAP technique, the absence of fragments in both profiles (*Eco*RI/*Hpa*II and *Eco*RI/*Msp*I) could be due to complete site methylation or may also represent a mutated site when genetically distinct samples are compared. Because of in AFLP analysis no polymorphism was detected among polyploid lines and their respective diploid parental lines, it was inferred that the polymorphisms detected in MSAP analysis was originated from methylation changes. The methylation status "methylated ambiguous" was taken because of full methylation (both strands methylated) of external cytosines could be not differentiated from full methylation of inner and external cytosines (Fig. 3A).

For the allopolyploid model 224 fragments were analysed using two primer combinations. An extensive remodelling in methylation patterns were observed in the four evaluated lines respect to the parental line 2xPIH: from the total of analysed fragments, only 49 (21.9%) were monomorphic (Fig. 3B). The total percentage of epigenetic changes comparing 2xPIH with the allotetraploid lines ranged from 33.9 to 40.6 (Fig. 3C). In 75 epiloci (33.5%), methylation changes respects to 2xPIH were identified in only one out of four allotetraploid lines. Of the polymorphic epiloci, 14 (6.3%) showed novel methylation patterns across the four derived lines respect to the diploid parental 2xPIH, and 29 (13.0%) and 57 (25.4%) exhibited novel methylation patterns in almost three or two of the allotetraploid lines, respectively (Fig. 3C).

For the autopolyploid model, six primer combinations were tested and 205 MSAP fragments were analysed. A higher percentage of monomorphic epiloci were observed in this model compared with the

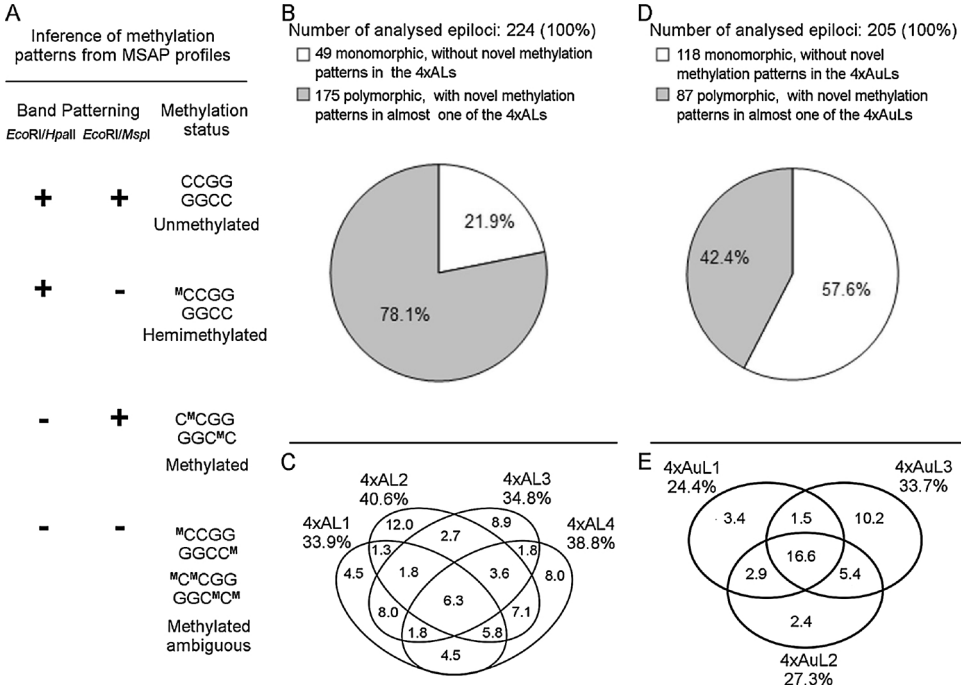
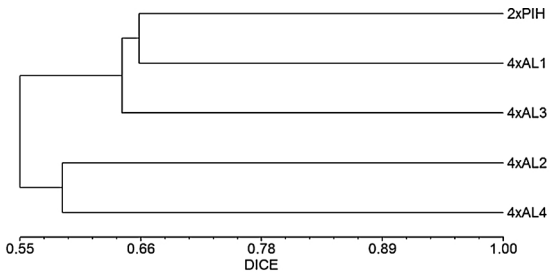


Fig. 3. Methylation-sensitive amplified polymorphism (MSAP) analysis in diploid and tetraploid potato lines. (A) Inference of methylation status of CCGG sites from the band patterning determined by *Hpa*II/*Msp*I isoschizomers. (B–C), allopolyploid model: a diploid parental interspecific hybrid *Solanum tuberosum* x *Solanum kurtzianum* (2xPIH) was compared with four tetraploid lines derived from it after colchicine treatment (from 4xAL1 to 4xAL4). (B) Total number of analysed epiloci and percentage of monomorphic and polymorphic patterns observed among the compared lines. (C) Venn diagrams showing the percentage of epiloci with novel DNA methylation patterns in each of the 4xALs respect to 2xPIH and distribution of changes among the allotetraploid lines. (D–E), autopolyploid model: a diploid *S. kurtzianum* genotype (diploid parental line, 2xPL) was compared with three tetraploid lines derived from it after oryzalin treatment (from 4xAuL1 to 4xAuL3). (D) Total number of analysed epiloci and percentage of monomorphic and polymorphic patterns observed among the compared lines. (E) Venn diagrams showing the percentage of epiloci with novel DNA methylation patterns in each of the 4xAuLs respect to 2xPL and distribution of changes among the autotetraploid lines.

A

	2xPIH	4xAL1	4xAL2	4xAL3	4xAL4
2xPIH	1.00				
4xAL1	0.66	1.00			
4xAL2	0.59	0.51	1.00		
4xAL3	0.65	0.64	0.50	1.00	
4xAL4	0.61	0.59	0.59	0.50	1.00



B

	2xPL	4xAuL1	4xAuL2	4xAuL3
2xPL	1.00			
4xAuL1	0.76	1.00		
4xAuL2	0.73	0.85	1.00	
4xAuL3	0.66	0.74	0.78	1.00

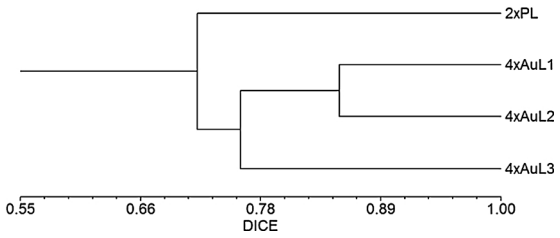


Fig. 4. Epigenetic variability generated by the polyploidization in potato. Dice similarity matrices (left) and dendrograms (right) obtained by cluster analysis based on presence/absence of MSAP profiles in a diploid parental interspecific hybrid*Solanum tuberosum* x *Solanum kurtzianum* (2xPIH) and its four derived allotetraploid lines (from 4xAL1 to 4xAL4) (A); and in a diploid *S. kurtzianum* genotype (diploid parental line, 2xPL) and its three derived autotetraploid lines (from 4xAuL1 to 4xAuL3) (B).

allopolyploid model: from the total of analysed fragments, 118 (57.6%) were monomorphic among the four compared lines (Fig.3D). On the other hand, the total percentage of epigenetic changes comparing 2xPL with the autotetraploid derived lines ranges from 24.4 to 33.7 (Fig.3E). Analysing the polymorphic epiloci, 34 of them (16.7%) showed novel methylation patterns in the three analysed lines with respect to 2xPL, 20 epiloci (9.7%) were modified in almost two out of three evaluated lines and 33 (16.1%) of the analysed epiloci showed methylation changes respect to 2xPL in only one of the autotetraploid lines (Fig.3E).

In order to obtain more evidences about the epigenetic variability induced by the allopolyploidization and autopolyploidization, epigenetic distance among lines within each model was calculated based on Dice similarity coefficient. For the allopolyploid model, the variability in the studied methylation patterns of the CCGG sites among the compared lines varied between 34% and 50% (Fig. 4A). On average, the variability among the parental line 2xPIH and its derived tetraploid lines was 37%. In the cluster analysis, obtained from the presence/absence of the analysed methylation patterns, lines were arranged into two main clusters, one grouping diploid parental hybrid and tetraploid lines 4xAL1 and 4xAL3, and the other grouping the tetraploid lines 4xAL2 and 4xAL4 (Fig.4A). For the autopolyploid model, the variability in the methylation patterns among the compared lines varied between 15% and 34% (Fig. 4B). On average, the variability among the diploid parental line 2xPL and its derived tetraploid lines was 28%. In the cluster analysis, the three derived autotetraploid lines were arranged together and separated from the diploid parental line (Fig. 4B).

3.5. Microsatellite analysis

Fifteen SSR loci were analysed to estimate the individual heterozygosity values of the diploid lines. In the diploid interspecific hybrid 2xPIH, 13 SSR (86%) amplified two alleles and were considered heterozygous; while in the *S. kurtzianum* genotype (diploid parental line, 2xPL), nine out of 15 (60%) were heterozygous (Supplementary S1).

4. Discussion

The evaluated allopolyploid and autopolyploid lines responded uniformly to polyploidization, showing a general increase on phenotypic characters compared with their respective diploid parental lines. Polyploidy has been associated with increases in mesophyll cell volume, leaf thickness and number of chloroplast per guard cell (Warner and Edwards, 1993; Romero-Aranda, 1997). The higher above- and below-ground biomass production in tetraploid lines respect their diploid parental lines may be associated to an increment in the lamina area and thickness. An increment in these characters is related with larger mesophyll cells with more chloroplast that support an increment in the photosynthetic activity (Fujimoto et al., 2012). The higher carbon gain of the potato tetraploids evaluated in the present study could be based on larger and thicker leaves with increases in cell size palisade mesophyll cells and more chloroplast per cell. In addition, a stronger xylem system with larger vessels in tetraploids compared with their respective diploid parental lines could transport more efficiently water and nutrients.

Variation in dosage-dependent gene regulation, altered regulatory interactions, and/or rapid genetic and epigenetic changes could be altering gene expression and phenotype in polyploids (Osborn et al., 2003). In AFLP analysis, the four allotetraploid and three autotetraploid evaluated lines did not showed genetic polymorphisms in comparison with 2xPIH and 2xPL, respectively. Due to the lack of detection of genetic changes, the wide phenotypic variation observed in the tetraploids unlikely could be attributed to rapid genetic rearrangement. These results are consistent with previous evidence obtained with potato autopolyploids, in which no polymorphisms were observed among diploid and synthetic derived autotetraploids using RAPD, AFLP and SSR markers (Stupar et al., 2007; Aversano et al., 2013). The results obtained in the present work, which in addition to the autopolyploid model explores for the first time an allopolyploid model, could confirm that the whole genome duplication in potato is unlikely a source of rapid genetic changes.

On the other hand, large methylation changes were observed comparing the tetraploids *versus* 2xPIH and 2xPL. In the allopolyploid model, most of these changes (33% of the analysed epiloci) showed a stochastic trend, because of the epigenetic modification was observed in only one out of four allotetraploid lines. However, in 19.3% of the analysed epiloci, the methylation patterns were simultaneously modified on at least the 75% (three out of four) of the allotetraploid lines, indicating that these genomic regions could be a hotspot of epigenetic modifications in response to whole genome duplication in potato. Additional evidence supporting the presence of hotspot region was obtained from the autopolyploid model, in which a more uniform response was observed: the 16.6% of the analysed epiloci showed novel methylation patterns in the three autotetraploid assayed lines, exceeding even the total epiloci that were modified in only one of the

4xAuLs (16.4%); and the 26.4% of the epiloci were modified in almost the 66.6% of the autotetraploid derived lines (two out of three). It has been highlighted that the alteration of the DNA methylation pattern of only one transcription factor could be sufficient to alter the expression of other genes (del Pozo and Ramirez-Parra, 2015) and that the genome doubling also significantly effects gene expression, resulting in epigenetically induced gene silencing (Osborn et al., 2003). Whether the large phenotypic differences observed in potato polyploids may be due to epigenetic modifications and altered expression of genes acting in processes such as energy metabolism and transport has to be further investigated. The epigenetics remodelling of the polyploidy genomes could leads to both the activation and suppression of gene expression. Accordingly to Comai (2005), many of these changes may be advantageous and others could be deleterious causing instabilities of the neopolyploids.

The allopolyploids and autopolyploids compared in this study demonstrated that synthetic allotetraploids experience on average 8.5% more of DNA methylation changes than do synthetic autotetraploids respect to the parental diploid lines (37.0% vs. 28.5%, respectively). Almost two explanations are possible. First, the autotetraploids were obtained with colchicine and the autotetraploids with oryzalin. These antimitotic agents could have a differential toxicity on potato uninodal segments, which in turn could have caused epigenetic changes of different magnitude between allo and autotetraploids. The comparative toxicity of these chemical agents is highly influenced by the used doses and the species assayed (Pickens et al., 2006; Ascough et al., 2008; Yu et al., 2009; Gallone et al., 2014;) and we lack compelling data to address the possible differences induced by the chemical treatment. In order to minimize the possible effects on genome instability induced by the antimitotic agents, we used in the comparison plants obtained after five cycle of vegetative reproduction. Other possibility is that the higher percentage of epigenetic changes observed in the potato allopolyploids were originated from the hybridization event, an additional genomic shock that experience the allopolyploids compared with autopolyploids (Parisod et al., 2010). In a previous study working with MSAP markers, it was demonstrated that F_1 diploid potato interspecific hybrids *S. tuberosum* x *S. kurtzianum* presented on average 8.85% of novel methylation patterns compared with the parental genotypes (Marfil et al., 2006). This difference is in the same order of magnitude as the observed between the allopoplyploid and autoplolyploid models assayed in the present work and could reflect the genomic instability inherent to the hybridization event (Parisod et al., 2010). When the average increase over the diploid parental value was compared for biomass parameters (*i.e.* total tuber weight, main tuber weight and top dry weight), the autotetraploids showed increases almost an order of magnitude greater than those observed in allotetraploids, indicating that the greater epigenetic variability observed in allotetraploids could be operating in response to additional instability generated by hybridization at the expense of yield. On the other hand, the comparison of the average percentage of methylation changes observed in the synthetic poliploids evaluated in the present study with those reported in interspecific hybrids (Marfil et al., 2006), show that genome doubling triggered broader epigenetic restructuration than hybridization in the evaluated potato lines.

The phenotypic superiority of polyploids evaluated in this study respect to the diploid parental lines contrast with the subtle phenotypic changes observed in potato autotetraploids by Stupar et al. (2007) and Aversano et al. (2013; 2015b). Previous works in potato had demonstrated that non-additive genetic effects (*i.e.* intra and interlocus interactions) were the major components of the tuber yield (*i.e.* plant biomass) and that to increase yields had to maximizing diversity and heterozygosity at the tetraploid level (Mendiburu, 1971; Mok and Peloquin, 1975). In these previous observations probably lies the response about the differences among the different polyploid potato models. In the present work, lines with 0.86 and 0.6 individual heterozigosity values (calculated from the analysis of fifteen randomly chosen neutral loci) were analysed in the allopolyploid and autopolyploid model, respectively. In the study performed by Stuppar et al. (2007), isogenic lines derived from a *S. phureja* monoploid (1x) clone were compared, therefore the heterozygosity of these lines would be zero. Aversano et al. (2013, 2015b) compared autotetraploid lines derived from a diploid *S. commersonii* clone with an individual heterozygosity value of 0.36 (estimated from 11 SSR loci) (Aversano et al., 2013). Subsequently, a 1.5% of heterozygosity was calculated for this *S. commersonii* clone from a whole-genome shotgun sequence and assembly approach (Aversano et al., 2015a). The heterozygosity differences outlined above would be the major argument to explain the discrepancies on phenotypic responses among potato polyploids. The polyploid phenotypic superiority reported in the present work may be considered as an heterotic response, in which the increased levels of biomass displayed by tetraploids relative to their parental diploids is dependent on the presence of heterozygous loci and epistatic interactions (Mok and Peloquin, 1975). Recently, it has been demonstrated that non-additive phenotypic effects are mediated by small RNAs conducting to wide epigenetic and gene expression changes in tomato hybrids; and part of the novel DNA methylation patterns reported here in potato polyploids could be secondary to the initiating events associated with novel intra and interlocus interactions in polyploids (Shivaprasad et al., 2011). However, when autotetraploid lines of *S. bulbocastanum* derived from a diploid clone with an individual heterozigosity value of 0.64 (estimated from 11 SSR loci) were compared, no general tendency towards a polyploidy superiority was observed (Aversano et al., 2013; 2015b), suggesting that part of the polyploidization induced phenotypic variation would be species and/or genotype dependent (Mok and Peloquin, 1975; Aversano et al., 2015b).

In conclusion, an overall harmonization among the tetraploid biomass increases and the evaluated morpho-anatomical parameters was observed in the closely related allopolyploid and autopolyploid models assayed in the present study. This polyploid phenotypic superiority unlikely could be explained by changes in gene expression induced by genetic rearrangements. On the other hand, novel methylation patterns were induced by the polyploidization, with a greater epigenetic variability in allotetraploid than in autotetraploid, presumably originated by the additional genomic instability induced by hybridization in the former. The higher biomass production in potato polyploids could be interpreted as a heterotic response dependent on the presence of heterozygous loci and epistatic interactions. Future research can be directed to identify specific genes with altered expression among different ploidy levels and to explore additional epigenetic mechanisms as small RNAs, which could be acting as buffer against the genomic shock induced by poliploidization in potato.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.scienta.2018.02.022.

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