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Variation of DNA methylation and phenotypic traits following unilateral sexual polyploidization in *Medicago*

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Abstract Sexual hybridization is an important generator of biodiversity and a powerful breeding tool. Hybridization can also overcome ploidy barriers when it involves 2*n* gametes, as in the case of unilateral sexual polyploidization (USP) that has been utilized in several crops, among which alfalfa. This research was aimed at gaining insights into the effects of USP on genome methylation and on phenotypic traits in alfalfa, an important forage species. The Methylation-Sensitive Amplified Polymorphism technique was used to estimate the cytosine methylation changes occurring in a tetraploid ($2n = 4x = 32$) USP progeny from crosses between a diploid *Medicago sativa* subsp. *falcata* genotype that produces 2*n* eggs and a cultivated tetraploid *Medicago sativa* subsp. *sativa* variety. De novo methylation or demethylation in the USP progeny were observed for 13% of the detected genomic sites, indicating that methylation changes can be relevant. USP plants showed larger surface area of the leaf epidermis cells than both parents, but this did not result in larger leaf size or higher plant biomass. They displayed significant higher ovule sterility than the tetraploid parent, but normal fertility was observed in crosses with unrelated male testers. We conclude that hybridization and sexual polyploidization resulted in novel variation in terms of remodeling of the methylation landscape as well as changes in phenotypic traits in alfalfa.

Keywords Alfalfa · *Medicago falcata* · MSAP · 2*n* Eggs · Hybridization

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Introduction

Merging genomes through hybridization is a ubiquitous feature of the plant kingdom, particularly among angiosperms. The significance of hybridization has been reevaluated in the last decades, following the evidence that bringing different genomes together can promote genetic changes and, consequently, leads to adaptive variations and functional novelties (Rieseberg and Carney 1998). Thus, sexual hybridization is an important generator of biodiversity and its creative signature in evolution is clear. It also represents a powerful breeding tool, in that it allows breeders to broaden the genetic diversity by introducing new genes for improvement of economic traits and by increasing allelic diversity to maximize intra/interlocus interactions. The success of hybridization mainly

depends upon the genetic relationships between parents and the possibility of chromosome pairing and genetic recombination. Equally important is the absence of sexual barriers, like those causing hybrid sterility or endosperm degeneration (Stoskopf et al. 1993). Hybridization may sometimes exploit 2*n* gametes (Bretagnolle and Thompson 1995). The most popular methods of using 2*n* gametes are the so-called sexual polyploidization crossing schemes. Bilateral sexual polyploidization requires functioning of both 2*n* pollen and 2*n* eggs. Unilateral sexual polyploidization (USP) requires functioning of either 2*n* pollen or 2*n* eggs from one parent and n gametes from the other parent with higher ploidy. The usefulness of sexual polyploidization has been reported in several crops, such as potato (Peloquin et al. 1989) banana (Ortiz and Vuylsteke 1995), blueberry (Lyrene et al. 2003), alfalfa (Veronesi et al. 1986, and references therein; Sledge et al. 2002).

The effects of sexual interspecific/intergeneric hybridization have been studied by several authors (reviewed by Soltis and Soltis 2009) and progress towards understanding the molecular mechanisms underlying phenotypic variation in hybrids has been made (Paun et al. 2007). An important role during plant hybridization is played by epigenetic changes, namely heritable modification of gene expression that are not due to changes in DNA sequence. One of the key epigenetic regulation mechanisms is DNA methylation (the addition of a methyl group to a cytosine base), which is associated with gene silencing (Grant-Downton and Dickinson 2006). DNA methylation is often a source of heritable phenotypic variation and many examples exist of epialleles that cause segregating phenotypes (Stokes et al. 2002; Johannes et al. 2009; Marfil et al. 2009; Reinders et al. 2009). The use of restriction enzymes sharing the same recognition site (isoschizomeres) but having differential sensitivity to DNA methylation is an efficient and reliable method to detect cytosine methylation changes in DNA (Reyna-Lòpez et al. 1997). In particular, the Methylation-Sensitive Amplified Polymorphism technique (MSAP) is based on the use of the isoschizomers *Hpa*II and *Msp*I, both recognizing the 5′CCGG sequence, but affected by the methylation state of the outer or inner cytosine residues. *Msp*I is inhibited by methylation of the outer cytosine (^{m}CCGG), but cleaves when the inner cytosine (C^{m}CGG) is hemi-(only one strand) or fully (both strands) methylated. By contrast, *Hpa*II cleaves when the outer cytosine is hemi methylated but it is inhibited by methylation (both hemi or full) of inner cytosine (C^{m}CGG), as well as by full methylation of outer cytosine. When the genomic DNA of two individuals is digested with either enzyme, in conjunction with a common esacutter enzyme, different banding patterns demonstrate different methylation status of the CCGG sequences.

In alfalfa (*Medicago sativa* L.), an agriculturally important autotetraploid ($2n = 4x = 32$) forage species, USP has been used to introgress new traits from wild diploid germplasm into cultivated tetraploid accessions via either $4x - 2x$ or $2x - 4x$ crosses. However, no information exists on epigenetic changes associate to sexual polyploidization crossing schemes. The objective of this work was to gain insights into the effects of unilateral sexual polyploidization on genome methylation and on the phenotype in alfalfa. The practical implications of epigenetic changes are discussed.

Materials and methods

Plant material

Seeds of a tetraploid F1-USP progeny were already available at Department of Applied Biology—University of Perugia. They were obtained from $2x \times 4x$ controlled pollinations between a diploid *M. sativa* subsp. *falcata* genotype (PG-F9, hereafter named P1-2*x*) that produces 55–70% 2*n* eggs (Tavoletti 1994; Barcaccia et al. 1997), and the Italian *M. sativa* variety Adriana ($2n = 4x = 32$, hereafter named P2-4*x*). *M. sativa* subsp. *falcata* carries useful traits such as cold and frost tolerance. One hundred F1 hybrid seeds were sown in jiffy 7 arranged in flats as well as those of the P2-4*x* parent variety. All the plants were reared in the greenhouse of the Department of Applied Biology under continuous illumination until flowering. Flower color was used as a phenotypic marker of hybridity. Hybrid plants between *M. sativa* subsp. *falcata* (yellow flowers) and *M. sativa* subsp. *sativa* (purple flowers) display a greenish flower color termed variegated. Root tips of 20 1-month-old plants with variegated flowers were excised and treated with an alphabromonaphthalene saturated solution.

The Feulgen technique was used for root tip chromosome counts using a Leica DMLP optical microscope. All the plants with variegated flowers had 32 chromosomes, and six of them were retained for further studies (4*x* USP progeny, hereafter named F1-4*x*). The F1-4*x* progeny and the parental plants were cloned by cuttings. Eight rooted cuttings per genotype were reared in pots in the greenhouse with supplementary illumination (16/8 h day/night) at temperature ranging from 15 to 25°C during late winter-early spring with complete randomization.

MSAP analysis

Parental plants and the six F1-4*x* hybrids were used for molecular analysis. Two DNA pools were created by mixing equal amounts of genomic DNA from eight cuttings of three hybrid genotypes each. They were coded F1-4*x*A and F1-4*x*B. Fully developed young leaves were collected. DNA was isolated using the DNeasy Plant mini kit (Qiagen) following the manufacturer's instructions. DNA quality and integrity was checked by gel electrophoresis and fluorometric assay. The methylation pattern at the 5′-CCGG sites was analyzed using the methylation-sensitive amplification polymorphism (MSAP) technique, which employs the isoschizomer methylation-sensitive enzymes *Hpa*II and *Msp*I. The MSAP protocol developed by Reyna-Lòpez et al. (1997) and adapted by Xiong et al. (1999) for rice was followed. Primer and adapter sequences were as described by Liu et al. (2001). Two samples of 500 ng genomic DNA were prepared from each sample (P1-2*x*, P2-4*x*, F1-4*x*A, F1-4*x*B). Digestions with *Eco*RI + *Hpa*II and *Eco*RI + *Msp*I. were performed using 5U *Eco*RI and 5U *Hpa*II (or alternatively *Msp*I) (New England Biolabs) in a final volume of 40 µl of the appropriate buffer, for 3 h at 37°C. The digested fragments were ligated to the adapters using 20U T4 DNA ligase (New England Biolabs) in the appropriate buffer at 37°C for 3 h. Preamplifications and selective amplifications were performed according to the protocol of AFLP™ (Vos et al. 1995) plant mapping kit of Applied Biosystems. For selective amplifications, one FAM-labeled *Eco*RI primer (*Eco*RI-TCCA) was combined with six *Hpa*II-*Msp*I primers (*Hpa*II-*Msp*I-AAC, *Hpa*II-*Msp*I-AAG, *Hpa*II-*Msp*I-AGG, *Hpa*II-*Msp*I-ACG, *Hpa*II-*Msp*I-ACA, *Hpa*II-*Msp*I-ACC), for a total of six primer combinations. MSAP fragments were electrophoretically separated on 6% denaturing polyacrylamide gels and fluorescence detected with a Typhoon 9210 scanner (Amersham). MSAP images were analyzed with ImageQuant TE, v2002.01 (Amersham), and by visual inspection. To have reproducible and clear banding patterns, each amplification was repeated at least three times, and only bands showing consistent amplification were considered. For each genotype, polymorphic fragments were recorded as 1/0 binary matrices, where 1 indicates the presence and 0 the absence of a given fragment. GeneScan 500 ROX (Applied Biosystems) was used as the size standard.

Because of the different sensitivity exhibited by the isoschizomers (McClelland et al. 1994) the methylation of the internal cytosine ($C^{5m}CGG$) or the hemi- (single strand) methylation of the external cytosine ($^{5m}CCGG$) can be unequivocally distinguished (Online resource 1). In particular, when *EcoR*I/*Hpa*II and *EcoR*I/*Msp*I restrictions are compared, four different patterns are possible, depending on the ability of either enzyme to cut at their recognition sequence: 1/1, corresponding to unmethylated sites (hereafter coded as U) in which a fragment (represented by an amplicon) is detected in both the *Hpa*II- and the *Msp*I-digested samples (*Hpa*II = 1/*Msp*I = 1); 1/0, corresponding to hemi- (only one strand) methylation of the external cytosine sites (coded as E), in which the amplicon is present only in the *Hpa*II, but not in the *Msp*I sample (*Hpa*II = 1/*Msp*I = 0); 0/1, reflecting hemi- or fully (both strands) methylation of the internal cytosine (coded as I), where the band is present in the *Msp*I, but absent in *Hpa*II sample (*Hpa*II = 0/*Msp*I = 1); 0/0, corresponding to either site mutation or full methylation at CCGG sites (coded as F). The F banding pattern was excluded from the analysis to avoid the noise produced by confounding the effects of mutation and methylation (Ochogàvia et al. 2009).

Genetic similarity (GS) between the samples was calculated by the simple matching coefficient SM = (a + d)/(a + b+c + d), where a and d are the cases where the values of both variables agree (i.e. the same band is either present o absent in two genotypes, respectively), and b and c those in which they disagree. The genetic similarities were graphically represented by a dendrogram constructed using the UPGMA (unweighted pair group method with arithmetic mean) clustering algorithm (NTSYS-pc package, Rohlf 1998).

Phenotypic analyses

Fresh and dry matter yield (g per plant) was assessed after clipping the plants when about three stems had open flowers. Dry matter yield was determined after desiccating the fresh material for 48 h at 100°C. Pollen fertility and diameter were assessed by mixing pollen from four random florets per genotype, staining it on a microscope slide with a drop of acetocarmine (acetic acid 45 ml, water 55 ml, carmine 1 g, glycerin 100 ml). Digital pictures were taken using a Leica DMLP optical microscope equipped with a Leica ICCA digital camera. The percentage of stained grains was evaluated on three random microscope fields counting at least 50 pollen grains each. Pollen diameter was measured using the Leica IM1000 software. Female fertility was assessed in the greenhouse during winter and spring of 2008 using continuous illumination (sodium halide lamps). Four racemes (replicates) per plant were hand-crossed and selfed without emasculation. An unrelated, male fertile, cultivated *M. sativa* subsp. *sativa* pollen donor was hand-crossed with all the genotypes. P1-2*x* was also crossed with an unrelated 2*x M. coerulea* plant (the diploid form of *M. sativa*). Self-fertility was estimated by tripping florets of 2–4 racemes per plant. Ovule fertility was estimated by assessing callose deposition in ovules at flower maturity, as described by Rosellini et al. (1998). Seed set was estimated by calculating the number of seeds per floret. Yield and fertility data were subjected to the analysis of variance using the GLM procedure of SAS (Statistical Analysis System, Inc. Cary, NC, USA, 2009). Digital pictures of leaves, flowers and mature pods were taken for each plant. Leaf shape was assessed as the ratio of width to length of the central leaflet of 5 random fully expanded leaves per genotype. Pieces of leaf lower epidermis were peeled off using fine forceps and placed onto a microscope slide in a drop of water. Digital pictures of the epidermal cells were taken and the surface area of cells surrounding the stomata were measured with the equipment described above.

Results

MSAP analysis yielded roughly 1280 bands, representing 273 distinguishable genetic loci (data not shown). The two parents, P1-2*x* and P2-4*x* exhibited a different percentage of methylated sites, namely 53% for P1-2*x* and 45% for P1-4*x*. By contrast, the F1-4*x*A and F1-4*x*B hybrid pools showed the same percentage of cytosine methylation (51%). Among the 273 loci, 104 showed the F pattern and consequently were not taken into account. The remaining 169 markers were used to compare the methylation patterns of the two parents and their F1 hybrids. The null hypothesis was that the F1 hybrids had the combined methylation patterns of both parents. Any deviation from this additivity expectation was considered a change in methylation caused by USP (Online Resource 2). In particular, the methylation pattern of the heterozygous 4*x* male parent may be reproduced or not depending on which two chromosomes of the four are transmitted to the F1-4*x*. For instance, a single chromosome with the U methylation status would be dominant with respect to the E or I status of the other three chromosomes. Bulking three F1-4*x* plants in each of the two analyzed samples likely reduced, but did not eliminate, variation due to heterozygosity of the 4*x* male parent.

Three major classes of banding patterns, including 21 subclasses, were identified among the MSAP fragments (Table 1). The first class (named N) comprised 147 unchanged sites (87% of all methylated sites), whereof 108 (N1, N6 and N11) were monomorphic among parents and F1-4*x* hybrids. In the second class (H), the patterns associated with de novo methylation were reckoned (specifically, differential cytosine methylation patterns between parents and USP hybrids). They accounted for 9.5% (16/169) of all methylated sites, and reflected both events of outer cytosine methylation (CC → ^{m}CC or U → E) and internal cytosine methylation (CC → C^{m}C or U → I). Five bands (classes H1 and H2) indicated de novo methylation of a 2*x* parent chromosomal site (Table 1). It should be noticed that de novo methylation of P2-4*x* chromosomal sites can go undetected due to its heterozygosity, when the newly methylated chromosome(s) are not transmitted to the progeny. Lastly, in the third class (D), six markers (3.5% of all methylated sites) deriving from demethylation events were grouped. Demethylation occurred either at external (^{m}CC → CC or I → U) or internal cytosine (C^{m}C → CC or E → U).

A dendrogram was constructed (Fig. 1) using data obtained from the MSAP experiments (Cervera et al. 2002). In particular, for each genotype 0 was assigned to U and F patterns (undetectable methylation change),

Table 1 Results from MSAP fingerprinting profiles of *Medicago sativa* subsp. *falcata* (P1-2*x*), *M. sativa* subsp. *sativa* (P2-4*x*) and their tetraploid progeny (F1-4*x*)

Methylation status[a]	Methylation profile[b]			Number of amplicons
	P1-2*x*	P2-4*x*	F1-4*x*	
No-changes [N]				
N1	U	U	U	52
N2	U	E	U	0
N3	U	I	U	3
N4	E	E	E	36
N5	I	I	I	20
N6	E	U	U	12
N7	I	U	U	10
N8	E	I	U	2
N9	I	E	U	1
N10*	E	U	E	4
N11*	I	U	I	7
Total				147 (86.9%)
De novo *methylation [H]*				
H1	U	E	E	1
H2	U	I	I	4
H3	U	U	E	6
H4	U	U	I	5
Total				16 (9.5%)
Demethylation [D]				
D1	E	I	I	0
D2	E	I	E	2
D3	I	E	I	0
D4	I	E	E	0
D5	E	E	U	1
D6	I	I	U	3
Total				6 (3.5%)

[a] The methylation patterns of the P1-2*x* and P2-4*x* were compared with their F1-4*x* hybrids. The hybrids were expected to have the combined methylation pattern of both parents (No-changes group). De novo methylation was observed when the parents showed unmethylated (U = 1/1) profiles and the hybrids either internal (I = 0/1) or external (E = 1/0) profiles. By contrast, demethylation was assumed when the parents showed either internal (I = 0/1) or external (E = 1/0) methylation profiles, and the hybrids showed unmethylated (U = 1/1) profiles

[b] See Online resource 2

1 to E and I patterns (detectable methylation change). The P1-2*x* female parent and the F1-4*x* hybrids clustered together, revealing a higher similarity of the progeny with the 2*x* rather than with the 4*x* parent.

Biomass, leaf morphology and fertility traits

Biomass production was not statistically different among the genotypes tested (Table 2). The shape of the central leaflet of the F1-4*x* progeny resembled that of the diploid parent (Table 2). Interestingly, the surface area of the leaf epidermis cells was significantly higher in the F1-4*x* progenies with respect to both parents (almost twice that of P1-2*x*), showing high-parent heterosis in this cross. The flower color of the F1-4*x* progeny plants was variegated, as a result of crossing yellow-flowered subsp *falcata* with purple-flowered ssp *sativa.* Flower size was visibly larger in all 4*x* plants with respect to P1-2*x* (Fig. 2). Pollen viability was 81, 85, and 89% for P2-4*x*, F1-4*x*, and P1-2*x*, respectively, and not significantly different. The F1-4*x* progeny had a number of ovules per floret intermediate between the parents (Table 2). This was not the case for ovule sterility, which was similar in P1-2*x* and the F1-4*x*, but much lower in P2-4*x*. However, when crossed by hand with unrelated male testers, the F1-4*x* plants produced only slightly (and non significantly) fewer seeds than the 4× parent, but many more than P1-2*x* (Table 3).

Discussion

In this study, we observed that the cytosine methylation status at CCGG sites changed between parents and hybrids obtained through USP. In particular, approximately 13% of the detected sites were altered, both through de novo methylation and demethylation. The literature lacks reports on the epigenetic changes associated with interploidy hybridization involving 2*n* gametes. A few studies on changes in DNA methylation are available only for progenies derived from interspecific hybridization involving parents with the same ploidy. In rice, for example, Sakthivel et al. (2010) observed methylation changes for 25% of the loci in F1-4*x* hybrids. In wheat (Shaked et al. 2001) changes occurred in 13% of the loci and in potato they varied between 28 and 53% (Marfil et al. 2009). Lower rates of DNA methylation alterations are generally found in F1 from intraspecific crosses. In rice, Xiong et al. (1999) reported that methylation patterns were changed only for 4% loci in hybrids between cultivated genotypes. Recently, Banaei Mughaddam et al. (2010) found that in intraspecific hybrids of

Table 2 Green and dry matter yield, leaf size and shape, surface area of epidermal cells, mean number of ovules and percentage of sterile ovules per floret of *Medicago* genotypes used in this study

Genotypes	Green matter yield (g plant^{-1})	Dry matter yield (g plant^{-1})	Length/width ratio of central leaflet	Leaf area (mm^2)	Cell surface area (μm^2)	Number of ovules per floret	Sterile ovules (%)
P1-2*x*	69 (3)	17 (3)	1.47 B (35)	172.2 C (10)	809 C (10)	7.6 C (20)	24.5 AB (20)
P2-4*x*	54 (17)	14 (17)	1.73 A (103)	519.8 A (23)	1251 B (23)	10.5 A (82)	17.5 B (82)
F1-4*x*	49 (39)	13 (39)	1.41 B (128)	431.6 B (40)	1487 A (40)	8.8 B (114)	25.3 A (114)

The numbers of observations are in parentheses. Means followed by different letters are significantly different according to the LSD test ($P < 0.05$)

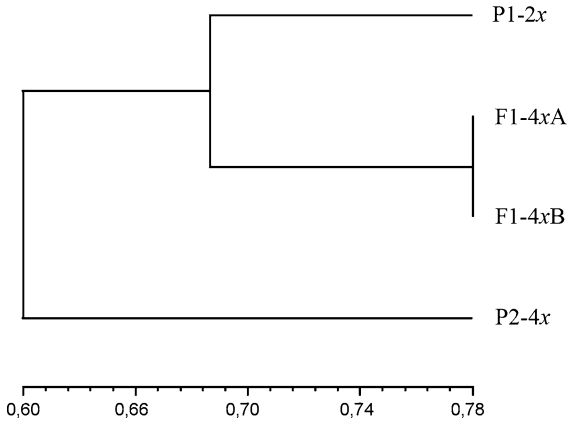

Fig. 1 UPGMA dendrogram depicting the relationship among 2*x* and 4*x* alfalfa genotypes based on MSAP analysis

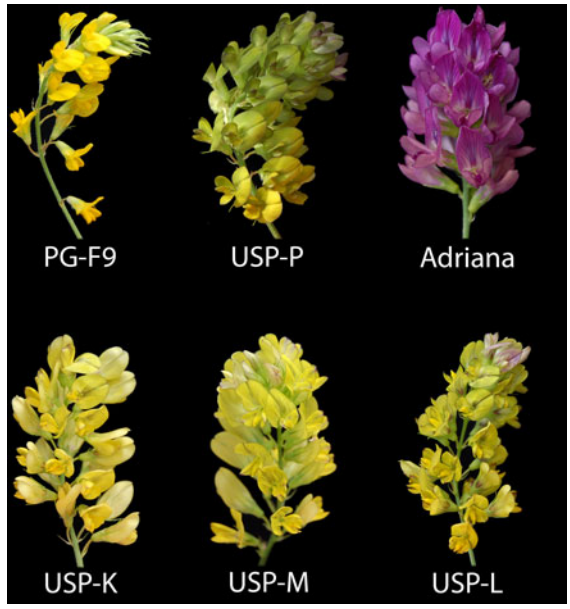

Fig. 2 Flowers of P1-2x (PG-F9), P2-4x (Adriana) and four F1-4x plants USP P, K, M, L). The variegated flower color typical of *Medicago sativa* ssp. *falcata* x ssp. *sativa* hybrids and the flower size difference between 2x and 4x plants are evident

Arabidopsis thaliana only 3% of the MSAP signals showed marks of altered methylation. Higher rates of DNA methylation changes were found in genotypes produced through other breeding strategies, such as somatic hybridization in *Festuca arudinacea* (+) *Triticum aestivum* (Cai et al. 2007), and in vitro differentiation in rose (Xu et al. 2004), hop (Peredo et al. 2006) and barley (Liu et al. 2007) genotypes. Our data for an intraspecific, interploid cross in alfalfa appear to be in the range reported for interspecific, homoploid crosses. This can be attributed to the fact that two subspecies were crossed in this study, so the genetic divergence between parents was high.

De novo methylation was observed at five maternal loci. As for the paternal genome, de novo methylation could not be fully elucidated due to heterozygosity. Indeed, the IUI (7 loci) and EUE (4 loci) profiles were assigned to the No-changes group, but are also compatible with de novo methylation of paternal sites, when at least one unmethylated site remained and was transmitted to the progeny. The methylation changes scored by MSAP are potentially underestimated also because *Hpa*II and *Msp*I cannot distinguish among unmethylated CCGG, fully methylated $^{m}C^{m}CGG$ or hemi-methylated $C^{m}CGG$ (Ashikawa 2001; Cervera et al. 2002; Dong et al. 2006). All this considered, the number of loci that experienced methylation changes as a consequence of USP appears to be very relevant and possibly capable to affect the phenotype. To explore whether the patterns of cytosine methylation were consistent with genetic relationship expected from USP, a dendrogram was constructed with MSAP data. It showed that, as expected, the female parent P1-2*x* and the 4*x* hybrids clustered together. In fact, a whole set of 16 chromosomes of P1-2*x* was

Table 3 Female fertility upon hand cross-pollinations with unrelated fertile 2*x* or 4*x* testers, and upon self-pollinations

Crosses	N[a]	Seeds per floret
P1-2*x* × 2*x*	4	1.2 BC
P1-2*x* × 4*x*	4	0.8 C
P2-4*x* × 4*x*	16	3.2 A
F1-4*x* × 4*x*	24	2.4 AB
Self-pollinations		
P1-2*x* self	4	0.0
P2-4*x* self	16	0.5
F1-4*x* self	24	0.2

Means followed by the same letter are not significantly different according to the LSD test ($P < 0.05$)

[a] Number of racemes crossed or selfed per plant; about 10–20 florets were present in each raceme

transmitted to the progeny through 2*n* gametes, whereas the reduced gametes of the heterozygous 4*x* male parent transmitted half of the parental genome and were genetically different. This result is likely affected by the inherent underestimation of de novo methylation of the male parent genomic loci.

Epigenetic variation is often overlooked as a source of phenotypic variation. Indeed, DNA methylation has been proved to be a powerful regulatory mechanism of gene expression and consequently its alteration could generate epigenetic variation of key genes involved in plant development, evolution and heterosis. In spite of the methylation changes observed, our F1-4*x* hybrids did not show altered morphologies. They possessed significantly larger leaf epidermis cells than both parents. However, this did not result in larger leaf size or higher plant biomass. In the literature, tetraploid progenies from *M. sativa* subsp. *falcata* x *M. sativa* 2*x* × 4*x* crosses generally produced more biomass than the diploid parent (reviewed in Barcaccia et al. 2003). Several hypotheses can be proposed to explain our results. First, fewer cells may be present in the tissues of F1-4*x* plants. This was hypothesized for maize, in which cell size increased with ploidy (Rhoades and Dempsey 1966), but plant height did not (Riddle et al. 2010). Second, it is also possible that differences in growth cycle may have conditioned plant biomass. Indeed, we observed that flowering dates differed among genotypes, with the P2-4*x* parental plants flowering first, the F1-4*x* after 4–5 days, and P1-2*x* 12 days later. The earlier flowering of the tetraploid plants resulted in a shorter time for biomass accumulation with respect to the diploid parent. Finally, it is possible that DNA methylation changes occurring in the F1 progeny have affected genes involved in biomass production. However, a correlation between DNA methylation and phenotypic variation in our hybrids remains to be proved. Sequencing the loci that change in methylation status may provide hints as to the functional significance of this phenomenon.

In this research we also analyzed some important fertility traits. The F1-4*x* progeny displayed significant higher ovule sterility than the tetraploid parent, possibly because the meiotic abnormalities of the P1-2*x* parent were transmitted to its 4*x* progenies. However, seed set of the F1-4*x* plants was similar to that of cultivated alfalfa when they were crossed with an unrelated 4*x* male tester, and higher than seed set of P1-2*x*. This suggests that fertility of a diploid can be immediately improved by sexual polyploidization. It should be pointed out that ovule sterility up to 50% may not be limiting for seed set, because usually no more than five seeds per pod are found in alfalfa, even though 10 ovules are available. This is probably why ovule sterility and seed set were not correlated in USP alfalfa plants (Barcaccia et al. 1998). Self-fertility was very low in all genotypes, as consistently observed in USP plants (Barcaccia et al. 2003).

We have shown that hybridization and sexual polyploidization resulted in novel variation in terms of remodeling of methylation landscape as well as changes in phenotypic traits in alfalfa. Since DNA methylation can be considered a general mechanism of transcriptional regulation, studying the effect of methylation on gene expression may shed light on the interplay between epigenetic and phenotypic variation in the context of plant breeding.

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