Sexual Polyploidization in Medicago sativa L.: Impact on the Phenotype, Gene Transcription, and Genome Methylation

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ABSTRACT Polyploidization as the consequence of 2n gamete formation is a prominent mechanism in plant evolution. Studying its effects on the genome, and on genome expression, has both basic and applied interest. We crossed two diploid (2n = 2x = 16) Medicago sativa plants, a subsp. falcata seed parent, and coerulea · falcata pollen parent that form a mixture of n and 2n eggs and pollen, respectively. Such a cross produced full-sib diploid and tetraploid (2n = 4x = 32) hybrids, the latter being the result of bilateral sexual polyploidization (BSP). These unique materials allowed us to investigate the effects of BSP, and to separate the effect of intraspecific hybridization from those of polyploidization by comparing 2x with 4x full sib progeny plants. Simple sequence repeat marker segregation demonstrated tetrasomic inheritance for all chromosomes but one, demonstrating that these neotetraploids are true autotetraploids. BSP brought about increased biomass, earlier flowering, higher seed set and weight, and larger leaves with larger cells. Microarray analyses with M. truncatula gene chips showed that several hundred genes, related to diverse metabolic functions, changed their expression level as a consequence of polyploidization. In addition, cytosine methylation increased in 2x, but not in 4x, hybrids. Our results indicate that sexual polyploidization induces significant transcriptional novelty, possibly mediated in part by DNA methylation, and phenotypic novelty that could underpin improved adaptation and reproductive success of tetraploid M. sativa with respect to its diploid progenitor. These polyploidy-induced changes may have promoted the adoption of tetraploid alfalfa in agriculture.

KEYWORDS alfalfa DNA methylation polysomic polyploids tetrasomic inheritance transcriptome

Polyploidization is an increase in the number of genomes per cell, and occurs in nature as the consequence of 2n gamete formation (sexual polyploidization, the most frequent mechanism; Brownfield and Köhler 2010), or as the consequence of somatic genome duplications (somatic polyploidization). A duplication of a species’ chromosomes results in the formation of an autoployploid having completely homologous duplicated chromosomes. The merging of the genomes of two species, concomitant with genome doubling, results in the formation of an allopolyploid. Polyploidy is widespread in plants, to the point that at least 70% of species have experienced polyploidization at some point of their evolution, and about 50% of economically important species are polyploid (Wendel 2000; Bowers et al. 2003; Wood et al. 2009).

The effects of polyploidization have been studied widely at the genetic, cytogenetic, and phenotypic level (New Phytologist 2010; Osborn et al. 2003; Chen 2007; Doyle et al. 2008; Hegarty and Hiscock 2008;
Aversano et al. 2012; Tayalé and Parisod 2013; Cheng et al. 2015). These studies have shown that gene expression changes can be brought about by polyploidization in many ways: gene dosage modification (copy number increase), alteration of the interactions among transcription factors, histone and chromatin state modifications, and DNA cytosine methylation. All these phenomena can translate into silencing or activation of genes and transposable elements, which, in turn, may result in novel traits such as increased cell size, changes in growth habit, or flowering time.

It is reasonable to expect that autopolyplody, a duplication of existing genes, does not result in modifications as deep as those caused by allopolyploidy, which involves the merging of genomes of different species (Guo et al. 1996; Wang et al. 2004, 2006; Madlung et al. 2005; Parisod et al. 2010; Pignatta et al. 2010). It is becoming accepted that hybridization, both intraspecific and interspecific, is a more powerful trigger of genomic and gene expression novelties than polyploidization per se (Albertin et al. 2006; Hegarty et al. 2006; Wang et al. 2006; Miller et al. 2012).

Research on polyploidy has been devoted mainly to allopolyploids, with comparatively little work in autopolyploids. In addition, in the vast majority of published research, polyploids were produced through somatic doubling, whereas, in nature, sexual events involving 2n gametes represent the main route to polyploid formation. In light of this, more data on the effect of autopolyplodontization would be useful (Stupar et al. 2007; Allario et al. 2011; Aversano et al. 2015).

Alfalfa (Medicago sativa L., 2n = 4x = 32) is a widely cultivated autotetraploid forage species with tetrasomic inheritance (Quirós 1982; Julier et al. 2003). The cultivated form is mostly Medicago x varia, originated by the hybridization of M. sativa subsp. sativa with M. sativa subsp. falcata (Small 2011). These subspecies have distinctive traits: subsp. sativa has purple flowers and coiled pods, and is adapted to warm and dry climates, whereas subsp. falcata has yellow flowers and sickle-shaped pods, and is adapted to cool and humid environments. The natural distribution of the two subspecies has overlapping areas, including Transcaucasia, Turkey, Iran and Southern Turkistan, where alfalfa is thought to have been initially cultivated about 8000–9000 yr ago. Both subspecies exist at the diploid (2x) and tetraploid (4x) level, but the diploids are not cultivated (reviewed by Small 2011). Sexual polyploidization is thought to be the mechanism by which tetraploid alfalfa originated (Barcaccia et al. 2003; Veronesi et al. 1986).

The objective of this work was to investigate the consequences of sexual polyploidization in alfalfa. We crossed two previously selected diploid (2x) plants, a M. sativa subsp. falcata seed parent, and a M. sativa subsp. coerulea x falcata pollen parent. Both are spontaneous meiotic mutants, producing a mixture of n and 2n eggs and pollen, respectively. Such a cross produced full-sib 2x and 4x hybrids, the latter being the result of bilateral sexual polyploidization (BSP). These unique materials allow us to investigate the effects of BSP, and separate the effects of intraspecific hybridization from those of polyploidization by comparing 2x vs. 4x full sibs. To make sure that they were true autopolyploids, we first characterized chromosome pairing behavior (random vs. preferential pairing) of the neopolyploids, by assessing segregation of simple sequence repeat (SSR) markers. Then, polyploidization-induced changes in leaf and leaf cell morphology, biomass production, and fertility traits were described. Finally, gene expression and epigenetic changes were studied from a genome-wide perspective by microarray and methylation-sensitive amplified polymorphism (MSAP) markers, respectively. Our findings contribute to understanding the success of polyploid M. sativa in agriculture, and could have practical implications in breeding of alfalfa and other polyploids.

MATERIALS AND METHODS

Plant material and ploidy determination

Two M. sativa meiotic mutants made this study possible. The M. sativa subsp. falcata genotype PG-F9 produces 55–70% 2n eggs (Tavoletti 1994; Barcaccia et al. 1997). The M. sativa genotype 12P was obtained by two cycles of recurrent selection for 2n pollen production from a cross of M. sativa subsp. coerulea and M. sativa subsp. falcata (Tavoletti et al. 1991). PG-F9 and 12-P were cloned by cuttings, reared in pots in a greenhouse under natural light, and crossed without emasculation using PG-F9 as the female parent. At maturity, seeds were harvested, and 200 of them were sown in jiffy pots. The plants were reared in flats in a greenhouse at the Department of Agricultural, Food and Environmental Sciences, University of Perugia under continuous illumination. Among the PG-F9 × 12-P hybrids, about 5% 4x and very few 3x hybrids are expected (Barcaccia et al. 1998). For the screening of ploidy level, a quick test based on chloroplast counts of guard cells was employed (Bingham 2006) on 10 plants displaying a “2x phenotype” (small, narrow leaflets), and 10 displaying a “4x phenotype” (large, wide leaflets). To confirm
chromosome number, root tips were used to count mitotic chromosomes of six putative 2x and 4x plants as previously described (Baracca et al. 1995). Three randomly taken plants per ploidy level were used in this study. Greenish flower color of these plants (Figure 1) confirmed that they derived from crossing, and not from selfing of the PG-F9 female parent.

**Chromosome pairing behavior of BSP neopolyploids**

The three 4x BSP plants (S29, S48, S60) were crossed with a pollen donor from the Italian variety Classe (4x). Sixty plants per progeny were reared in a greenhouse until genomic DNA was extracted (SIGMA Genelute plant kit). Twenty-eight published SSR markers (Diwan et al. 2000; Julier et al. 2003; Sledge et al. 2005; Mun et al. 2006) were tested for their ability to provide parent-specific alleles, that is, alleles present in only one of the diploid parents and not shared with the tetraploid tester (Classe). Primers were selected based on chromosome location. Amplifications were performed as follows: buffer 1X, MgCl2 1.5 mM, dNTP 0.2 mM, primer FOR/REV 0.5 μM, Taq polymerase (Sigma) 1 U, genomic DNA 30 ng, in 20 μl final volume. PCR cycling was 94° C 3 min, 40 cycles at 94° C 30 sec, Ta 72° C 30 sec, where Ta is the marker-specific annealing temperature (Supplemental Material, Table S1). After screening in agarose, fluorescein isothiocyanate (FITC)-labeled primers for nine selected primer pairs (Table S1) were used for amplification of the three parental plants and the three hybrids (60 plants each), and capillary electrophoresis was performed for three of the three BSP plants. The expected segregations of the markers were determined, under the assumption of no double reduction, for the hypotheses of complete preferential pairing, or complete random pairing (Figure S1), and the significance of the marker segregation data in single progenies of the three BSP plants. See Table S3, Table S4, and Table S5 for marker segregation data in single progenies of the three BSP plants.

**Significance of Chi Square Values**

| Chromosome | S29 |     | S48 |     | S60 |
|------------|-----|-----|-----|-----|-----|
|            | Disomic | Tetrasomic | Disomic | Tetrasomic | Disomic | Tetrasomic |
| I          | **   | NS   | **  | NS   | **   | NS   |
| II         | *    | **   | NT  | NS   | **   | NS   |
| III        | **   | NS   | **  | NS   | NS   | NT   |
| IV         | NT   | NS   | NT  | NS   | NT   | **   |
| V          | *    | **   | NS  | **   | NS   | **   |
| VI         | **   | NS   | NT  | NS   | **   | NS   |
| VII        | **   | NS   | NS  | NS   | **   | —    |
| VIII       | **   | NS   | NS  | NS   | NT   | NS   |

* Significant at P < 0.05; **significant at P < 0.01; NS, not significant; NT, non-testable (because one or more of the expected numbers is 0).

Data from two SSR loci of chromosome V are available for plants S29 and S60.

**Leaf morphology, biomass and fertility assessment**

The parents and the selected 2x and 4x progeny plants were cloned from cuttings. Eight rooted cuttings per genotype were reared in pots containing a soil:sand:neutral peat moss (3:1:1) mix in the greenhouse with natural light during late winter to early spring with complete randomization. Fresh and dry matter yield (g per plant) was assessed after clipping the plants, when about 10 stems had open flowers; due to differences in flowering times among genotypes, harvesting was not performed at the same time. Dry matter yield was determined after desiccating the fresh material for 48 hr at 100° C. Biomass yield was evaluated again in two subsequent regrowth cycles in a screen house with drip irrigation during the summer under natural sunlight. Flowering time was evaluated as days from March 1 (first summer assessment), and as days from the previous cut (second summer assessment). Pollen production per floret, estimated visually by tripping 10 random florets per plant was scored from 0 (no visible pollen), to 3 (abundant pollen). Pollen fertility and diameter were assessed by mixing pollen from four random florets per genotype, staining it on a microscope slide with acetocarmine. Digital pictures were taken using a Leica DMPLP optical microscope equipped with a Leica ICCA digital camera. The percentage of stained grains was calculated on three random microscope fields. Pollen diameter was measured using the Leica IM1000 software. Seed set was assessed in the greenhouse during the winter and spring using continuous illumination (sodium halide lamps) and, on the regrowth of the same plants, during the summer in a screen house with natural light. Ovule fertility was estimated by assessing callose accumulation within the nucellus in ovules at flower maturity, as described by Rosellini et al. (1998). Four racemes (replicates) per plant were crossed without emasculation using three unrelated *M. sativa* subsp. *coerulea* (2x), and one unrelated cultivated *M. sativa* subsp. *sativa* (variety Classe) pollen donors were used for crosses. All plants were fertilized with pollen from both the 2x and the 4x pollen donors, thus performing intraploidy (2x-2x, 4x-4x), and interploidy (2x-4x, 4x-2x), crosses. Hand-crosses in all combinations were also made between 2x hybrids, and between 4x hybrids (full sib crosses). Self-fertility was estimated by hand-tripping florets of two to four racemes per plant. Seed set was estimated by calculating the number of seeds per floret.

**Microarray analyses**

Six cloned plants of PG-F9, 12P, and their 2x and 4x hybrids, were reared in a screen house under natural conditions, with complete
samples were immediately frozen in liquid nitrogen and stored at 
plants, thus obtaining three biological replicates per genotype. The 
shoots of each plant at the vegetative stage, bulking the leaves of two 
randomization. Young, fully expanded leaves were harvested from

shoots of each plant at the vegetative stage, bulking the leaves of two plants, thus obtaining three biological replicates per genotype. The samples were immediately frozen in liquid nitrogen and stored at –80°C. About three trifoliate leaves per sample were finely ground in liquid nitrogen, and total RNA was further purified with the Qiagen RNeasy minikit. RNA quantified on a NanoDrop ND-1000 spectrophotometer, and quality checked using a 2100 Bioanalyzer (Agilent Technologies). Twenty four RNA samples were used for microarray hybridizations. Nimblegen Microarrays were designed at the Centro di Genomica Funzionale, University of Verona, Italy. Probes for 41755 M. truncatula genes (TC or singleton ET) were designed based on the Medicago Gene Index, release 11.0. For 41,340 genes, it was possible to design three different probes per gene, whereas for the remaining 184 genes, one or two probes were designed.

Labeling and hybridization was performed according to Nimblegen gene expression user guide version 3.2. Scanning was performed with Axon GenePix 4400A scanner. Scanner settings were set according to Nimblegen gene expression user guide version 3.2. Raw data were quantile normalized and summarized with the RMA algorithm (Irizarry et al. 2003), as implemented in Nimblenscan 2.5 software using default parameters (Nimblegen).

Differential expression analysis was performed using Linear Models Microarray Analysis (LIMMA; Smyth 2005) as follows. To identify genes affected by hybridization, two groups of samples corresponding to parents (PG-F9, three replicates; 12P, three replicates), and to 2x hybrids (s8-2x, three replicates; s16-2x, three replicates; s24-2x, three replicates) were contrasted using a two-class unpaired design. To identify genes affected by hybridization and polyplidization, two groups of samples corresponding to parents (as above), and to 4x hybrids (s29-4x, three replicates; s48-4x, three replicates; s60-4x, three replicates) were contrasted using a two-class unpaired design. To identify genes affected by polyploidization, two groups including 2x hybrids (as above) and 4x hybrids (as above) were contrasted using a two-class unpaired design. To identify genes affected by hybridization and polyploidization, two groups of samples corresponded to parents (as above), and to 4x hybrids (s29-4x, three replicates; s60-4x, three replicates) were contrasted using a two-class unpaired design.

To estimate genotype-specific transcriptional differences, the following pairwise contrasts were performed using a two-class unpaired design: PG-F9 vs. 12P; S8-2x vs. S16-2x, S8-2x vs. S24-2x, S16-2x vs. S24-2x, S29-4x vs. S48-4x, S29-4x vs. S60-4x, and S48-4x vs. S60-4x. Genes were considered as differentially expressed among the groups compared if |log2FC| > 1 and adjusted P-value < 0.05 (Benjamini and Hochberg 1995).

The Blast2GO software (Conesa and Götz 2008; https://www.blast2go.com/) was used to perform a semi-automatic Gene Ontology (GO) annotation, and data mining of probe sequence sets of the PS genes, in order to enhance knowledge on the unannotated sequences. The Biological Networks Gene Ontology tool (BINGO, Maere et al. 2005) — a plugin for Cytoscape (Shannon et al. 2003) — was adopted to identify enrichment of GO terms in PS genes with respect to all genes in the microarray. The BinGO analysis tests the probability that the frequency of a GO term in a set of genes (test set) taken from a larger set of genes (reference set) is different from the frequency in the reference set.

To confirm the results of microarray analyses, six genes from those evidenced by the Bingo analysis were tested by qRT-PCR performed in a Mx3000P Stratagene system (Agilent Technologies Inc., Santa Clara, CA) using FastStart SYBR Green Master Mix (Roche Life Science, Italy) with the following settings: 2 min at 95°C, followed by 40 cycles at 95°C for 30 s, 58°C for 1 min, and 72°C for 20 s. For each genotype, three reactions were run from a cDNA synthesis, and the mean values calculated. Primers were designed using the Primer3Plus Software (Table S15). The specificity of amplicons (Table S16) was confirmed by dissociation curve analysis, generated after the last PCR cycle, and by sequencing. Data analysis and calculations, to compare transcript accumulation data, were performed through the 2-ΔΔCt (threshold cycle) method, with β-actin as the endogenous reference.

DNA from leaves of plants used for microarray analysis was purified using the DNeasy Plant Maxi Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. DNA purity was evaluated using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE), and nucleic acids concentrations were determined using a Qubit fluorometer (Life Technologies, Carlsbad, CA). The methylation pattern at 5′-CCGG sites was analyzed using the MSAP technique, which is based on the use of the isoschizomeric restriction

Figure 2 Determination of the ploidy state of 2x and 4x progeny plants (one plant per ploidy state is shown as an example). In 4x plants, leaf size is larger, and leaflets shape are less elongated, seeds are bigger, and the number of chloroplasts in stomata guard cells is higher (for example, S16-2x, 8 chloroplasts; S29-4x, 13 chloroplasts) than in 2x plants. Root tip cell chromosomes counts (for example, S16-2x, 16 chromosomes; S29-4x, 32 chromosomes) confirmed the ploidy estimation based on morphological traits (see text for details).
enzymes HpaII and MspI, which recognize the same restriction site (5'-CCGG-3'), but have different sensitivities to methylation of the cytosine residues. In particular, HpaII digests only nonmethylated CCGG sequences and hemi- (single strand) methylated mCCGG sequences of all possible methylated CCGG variants. MspI can cleave nonmethylated CCGG sequences, and hemi- or fully (both strands) methylated CmCGG sequences, but not hemi- and fully methylated mCCGG and mCmCGG sequences (Reyna-López et al. 1997; Mann and Smith 1977). MSAP analysis was performed as reported previously (Aversano et al. 2012). For selective amplifications, one FAM-labeled EcoRI primer (EcoRI-TCCA) was combined with six HpaII-MspI primers (HpaII-MspI-AAC, HpaII-MspI-ACA, HpaII-MspI-ACT, HpaII-MspI-AGA, HpaII-MspI-AGC, HpaII-MspI-AGG), for a total of six primer combinations. 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 position in the gel, the following HpaII-Mspl fragment pattern variants, referring to fragment presence (1) or absence (0), were observed: a 1-1 pattern (a fragment of definite length visualized in both the HpaII and Mspl lanes) was attributed to digestion by both enzymes at a nonmethylated CCGG site, and, therefore, associated to unmethylated sites; a 1-0 pattern, representing a fragment of definite length visualized in the HpaII, but not in the Mspl, lane may be interpreted as two different situations: 1) the cutting of hemi-methylated mCCGG sites with HpaII but not Mspl, and 2) the presence of internal hemi-methylated CmCGG site(s) between the cleaved distal CCGG and the EcoRI site; in both cases the 1-0 pattern was attributed to digestion by both enzymes at a nonmethylated CCGG site, and, therefore, associated to unmethylated sites. A 0-1 pattern corresponds to digestion with Mspl but not HpaII, and refers to the presence of a fully methylated CmCGG site. Finally, a (0-0) pattern could be caused either by restriction target absence due to a mutated site when genetically distinct samples are compared, or inhibition of digestion with both enzymes at a fully methylated mCmCGG site when another sample shows the presence of a fragment at that position (Schulz et al. 2013; Fulneček and Kováč 2014). Therefore, the 0-0 profile is not informative and was excluded from the analysis to avoid the noise produced by confounding the effects of mutation and methylation. To decipher hyper- and hypomethylation changes, we adopted the MSAP scoring method of Fulneček and Kováč (2014). One-way ANOVA was applied to analyze the cytosine methylation level differences in the diploid and tetraploid hybrids using XLSTAT-PRO 7.5.3 software (Addinsoft, http://www.xlstat.com). The Duncan test was performed to compare mean values. To compare de novo methylation and demethylation frequencies, the methylation ratio (MR) was calculated as the percent ratio of the number of markers revealing de novo methylation over the number of markers revealing demethylation.

Data availability
The M. sativa meiotic mutants, and their 2x and 4x hybrids, are available upon request for research purposes. The Supplemental Material files contain 17 tables and 11 figures with detailed information on SSR markers, chromosome segregation data, phenotypic traits, the results of GO, and homology searches of nonadditively expressed genes, and RT-PCR data. Raw phenotypic data are available on request. Gene expression data are available at GEO under the accession number: ID: GSE71559, and are available at this link: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=gxkdcaabvgltxs8ac&gse=71559.

RESULTS

Chromosome segregation in neotetraploids
Informative SSR markers were found for all eight chromosomes of the three BSP plants, with the exception of chromosome VII in S60-4x (Table S2). In particular, in S29-4x, tetrasomic inheritance was demonstrated for six of the eight chromosomes (Table 1 and Table S3), while segregation of markers of chromosomes II and V fit with both disomic and tetrasomic transmission. In S48-4x (Table 1 and Table S4), chromosomes V and VII showed disomic inheritance. Chromosome VIII showed an intermediate behavior. In S60-4x, chromosome IV deviated significantly from the expectations of the tetrasomic model (Table 1 and Table S5) due to segregation distortion rather than to preferential pairing. Overall, in these three BSP plants, preferential pairing appears to be limited, leading to disomic or intermediate inheritance for one to three of the eight chromosomes. Chromosome V showed disomic inheritance, as it consistently tended to pair preferentially in these three BSP plants.

The number of alleles found in 4x BSP plants was less than the sum of the alleles of the parental plants for 17 out of 27 SSR loci (Table S6).
Although this may be explained by segregation in parental plants (restitutional meiotic divisions in both parents allow for allele segregation), DNA loss in neopolyploids could also be hypothesized (Song et al. 1995; Feldman et al. 1997; Shaked et al. 2001). In particular, the presence in S29 of only one allele of marker MTIC48 of chromosome V (Table S2), may be explained by loss of the PG-F9 allele.

Phenotypic effect of hybridization and polyploidization

The effects of hybridization per se was estimated by comparing 2x hybrids with the parental mean. Several traits exhibited clear midparent heterosis: leaf area and stomata size were larger, and biomass production was moderately higher in 2x hybrids (Figure 3, A, D, E, H, and J, Table S7, Table S8, Table S9, Table S10, Table S11, and Table S12). Stomata density decreased in 2x hybrids (Figure 3, F and G). As for the reproductive traits, 2x hybrids flowered later, had fewer ovules per ovary, and higher seed set than their parents (Figure 4, A, C, and F, and Table S12).

We estimated the added effect of polyploidization to hybridization by comparing full-sib 4x with 2x hybrids. Leaf size, epidermal cell surface and stomata size were increased by tetraploidization (Figure 3, A, and C–E), and stomata density was concomitantly strongly reduced (Figure 3, D–E and Table S7). Leaflet length/width ratio differed between the 4x and the 2x hybrids due to a less elongated shape (Figure 2 and Figure 3C). Green and dry biomass of 4x hybrids was higher than that of 2x hybrids (second cut, Figure 3, H–J, Table S8, Table S9, and Table S10). Delayed flowering observed in 2x hybrids was not present in 4x hybrids. Flowers and pollen grains of 4x hybrids were larger, and ovule sterility lower (Figure 1, Figure 4, D and E, and Table S12). Surprisingly, in intraploidy crosses, seed set of 4x hybrids (1.97 seeds per floret) was 2.7-fold higher than that of 2x hybrids, and seeds were 37% heavier (Figure 2 and Figure 4G). In particular, 4x plants S48 and S60 produced more seeds per floret than both parents (Table S12), consistent with a "high-parent polyploid heterosis" behavior. When the hybrids were pair-crossed in all combinations within ploidy level (full-sib crosses, Table S10), the picture was reversed: 4x hybrids had much lower seed set than 2x hybrids (Table S12).

Effect of hybridization and polyploidization on gene transcription

In 2x hybrids, nonadditive gene transcription was recorded for only 118 genes (Figure 5). We defined them as “hybridization-sensitive” (HS), because their expression deviated from the parental mean as a consequence of hybridization. When the parents were compared with their 4x hybrids, 605 genes were nonadditively expressed; since their expression was affected by the combined effects of hybridization and polyploidization, we defined them as “hybridization- and polyploidization-sensitive” (HPS). Comparison of gene expression of 2x and 4x hybrids revealed that 566 genes were differentially expressed, 341 of which only between 2x and 4x hybrids, and not between parents and 4x hybrids. Therefore, they are candidates for being affected only by the ploidy change, and not by concurrent hybridization. We defined them as “polyploidization-sensitive” (PS), and will examine them in more detail below.

The small number of HS genes is probably due to the large transcriptional difference between the parents (Figure 6): for many genes, the parental mean was affected by a large SE, and this restricted the number of statistically significant instances in parent–progeny comparisons. However, the number of HPS genes was about five times that of HS genes, clearly indicating that polyploidization, combined with hybridization, has a significant effect on gene expression.

The comparison of single progeny genotypes with the parental mean (Table 2) showed that there were more overexpressed than underexpressed genes in all genotypes but one (S60-4x). The ratio between the numbers of over and underexpressed genes was 2.13 for 2x and 1.20 for 4x hybrids, indicating that hybridization mostly increased gene transcription, but hybridization combined with polyploidization resulted in a modest increase of the transcription level.

Due to large genetic distance, wide transcriptional differences between the parental genotypes were expected; in fact, 4839/41,538 = 11.6% of the genes differed significantly (Figure 6 and Table S13). The differences between single genotypes within the 2x and 4x hybrids were comparatively small, involving 357–1010 genes. This is consistent with the high genetic similarity between the full-sib plants of each progeny.
Gene ontology analysis and term enrichment of PS genes

Of 341 PS genes, 240 were annotated (Figure S2). The vast majority of Blast hits were *M. truncatula* sequences, followed by *Glycine max*, *Cicer arietinum* and *Vitis vinifera* (Figure S3). Blast2GO analysis showed that in the "biological process" vocabulary, the more frequent GO term was "oxidation–reduction process" (Figure S4). In the "cellular component" vocabulary, "protein complex" and "integral to membrane" were the most represented terms (Figure S5). In the "molecular function" vocabulary, the most frequent term was "ATP binding", and related terms were also present ("nucleoside-triphosphatase activity", "hydrolyase activity, acting on ester bonds"). The term "oxidoreductase activity" was well represented, and "tetrapyrrole binding" was present (see below) (Figure S6). The GOslim procedure was also adopted to restrict the ontology search to the plant kingdom. In the "biological process" vocabulary, "response to stress" and "biosynthetic processes" were the most frequent terms, followed by "cellular protein modification process" and "signal transduction" (Figure S7). In the "cellular component" vocabulary, the plastid compartment clearly predominated, with the "plastid" and "tuflocoid" terms (Figure S8). In the "molecular function" vocabulary, nucleic acid binding was the most frequent function with several terms related to it. "Hydrolase activity" and "protein binding" were also frequent (Figure S9).

We used BinGO to determine whether a GO term was more or less frequent than expected in PS genes with respect to the whole microarray. Six "Molecular function", 18 "Biological process", and 16 "Cellular component" GO terms were significantly enriched in the PS genes (Table S14). These 40 terms originate from 25 genes (Table 3); 15 of them had higher expression in 4x than in 2x hybrids, and showed homology with chlorophyll-binding proteins (six genes), lipoxigenases (three genes), heat shock proteins (two genes), ribulose 1,5-bisphosphate carboxylase small subunit, UDP-glucosyltransferase, and photosystem I subunit *PsAD* (one gene each). Therefore, most of the genes in this group are related to photosynthesis. Ten genes were less expressed in 4x than in 2x hybrids, and showed homology with a diverse set of proteins: a protein kinase, a chaperonin, an alpha-dioxygenase, a peroxidase precursor, a glutathione S-transferase, a Myb transcription factor, a replication licensing factor, a disease resistance response protein, GDSL esterase/lipase, and thaumatin-like protein 1a (one gene each) (Table 3). No general pattern of variation with respect to the parents was observed in this sample of genes, and stochastic variation within the progeny group was observed (not shown).

Six genes among those pinpointed by the Bingo enrichment analysis (three from the 4x > 2x group, three from the 4x < 2x group) were tested by qRT-PCR (Figure S9). Sequencing of the amplicons confirmed the identity of the genes (Table S16). The 2x–4x expression differences from the microarray experiment (Figure S10) were confirmed for all genes, and the trends were validated.

**DISCUSSION**

The polyploidization system used in this study exploits spontaneous mutations of gametogenesis leading to 2n gamete formation, and, as such, simulates natural polyploidization events. Since the natural distribution range of subsp. *coerulea* and *falcata* overlap, and hybrids occur in nature at both 2x and 4x levels (Small 2011), a cross similar to PG-F9 × 12P could have occurred spontaneously. From this point of view, our experimental materials differ from those used in previous studies on plant neopolyploids, obtained by artificial chromosome doubling involving tissue culture or spindle-inhibiting substances. Therefore, this work offers an original perspective with which to investigate the adaptive advantages of polyploidy (Hilu 1993, Mayrose et al. 2011; Madlung and Wendel 2013).
Neopolyploids show tetrasomic inheritance for most chromosomes

In alfalfa, quadrivalent pairing is infrequent (Stanford et al. 1972), so cytogenetic investigations are not useful to demonstrate tetrasomic inheritance; molecular marker segregation, on the contrary, can give clear evidence of pairing behavior. Cytogenetic studies have shown differences in C-banding patterns between *M. sativa* subsp. *falcata* and *M. sativa* subsp. *coerulea* (Bauchan and Hossain 1997), revealing differences in constitutive heterochromatin content of chromosomes. Therefore, preferential pairing may be expected in the BSP plants because they have two *falcata* chromosome sets from PG-F9, and two mixed *falcata* x *sativa* chromosome sets from 12P, deriving from two meiotic recombination rounds after the *falcata* x *sativa* cross. Preferential pairing implies disomic inheritance and allopolyploidy, whereas random pairing leads to tetrasomic inheritance, which is characteristic of autotetraploids (reviewed by Parisod et al. 2010). Therefore, the first question is whether or not our BSP plants are true autopolyploids. SSR marker segregation allowed us to answer this question: tetrasomic inheritance was the rule, with only a few exceptions, indicating that chromosome homology between *falcata* and *sativa* is high, in spite of the large morphological difference between the subspecies. Chromosome V was the only one showing a consistent tendency toward disomic segregation. Preferential pairing of this chromosome could depend on the amount and pattern of recombination between *sativa* and *falcata* chromosomes occurred in 12P (Figure S11).

Polyploid alfalfa hybrids show phenotypic superiority and novel variation for adaptive traits

For several traits our autopolyploids performed better than both their parents and 2x hybrids, suggesting that sexual polyploidization between heterozygous, diverse genotypes resulted in increased heterozygosity of 4x BSP plants, a possible cause of “polyploid heterosis”. By contrast, in maize, tobacco, and potato autopolyploids obtained through somatic doubling, 4x plants did not display a polyploid superiority (Riddle et al. 2010; Stuper et al. 2007; Assourn et al. 2009; Aversano et al. 2015). This probably reflects the fact that somatic polyploids have the same alleles as the diploid parents, and thus do not have increased allele interactions that can enhance heterosis. Higher seed set in crosses was the most striking effect of polyploidization. It is likely that sterility factors present in parents were partly offset by complementation in the 2x hybrids, resulting in heterosis for seed set, and this complementation was much higher at the 4x level. Masking of unfavorable parental alleles as a consequence of sexual polyploidization form the basis of the high fitness of first-generation polyploids, and contribute to reproductive success. Such advantage of neopolyploids may not be uncommon (Gross and Schiesl 2015). A positive effect of chromosome doubling *per se* on fertility in alfalfa was demonstrated by Obajimi and Bingham (1973). Higher seed set would allow more abundant seed production before summer drought in the southern part of the species distribution area in which cultivation of 4x alfalfa was established. Higher seed weight has been associated with adaptation advantages of polyploids (Hahn et al. 2013). Such a combination of traits might also form the basis of the adoption of tetraploid *M. sativa* in agriculture. In this view, sexual polyploidization can be regarded as a key factor underpinning the agricultural adoption of alfalfa, as was proposed for wheat (Dubcovsky and Dvorak 2007). Flowering time is an important adaptive trait, and has been shown to be affected by chromosome doubling in *Arabidopsis thaliana*, with autotetraploids flowering later than diploids (Chen 2010). In our study, 4x hybrids tended to flower earlier than 2x hybrids, indicating that polyploidization may result in a shorter growth cycle. These different responses to autopolyploidization are reminiscent of the responses to hybridization: early flowering is heterotic in maize, whereas late flowering is heterotic in *Brassica napus* (Chen 2010). It can be hypothesized that alfalfa 4x hybrids experience adaptive advantages in the wild. Indeed, faster development leading to earlier flowering can be an advantage in the wild to outcompete other species. In cultivated fields, flowering time directly reflects biomass production—the most important agronomic trait of forage crops. In alfalfa, fast development is desirable to gain more harvests per year and to better compete against weeds.

Polyploidization increases nonadditive gene transcription

In this work, gene transcription levels were compared using equal amounts of total RNA per sample. We assumed that the total amount of RNA per genome was constant or, in other words, that the total RNA per cell (transcriptome) doubled with chromosome number. Therefore, relative transcription levels per genome were compared in this work (Guo et al. 1996; Stuper et al. 2007). Since cell size increased with ploidy (Beaulieu et al. 2008; documented here for leaf epidermal and stomata cells), when a gene showed equal transcription levels in the 2x and 4x plants, it could be assumed that there was an increase of transcription level per cell proportional to the increase in cell size (Guo et al. 1996; Riddle et al. 2010). On the contrary, different expression between ploidy levels implies that the change in transcription was not proportional to the change in cell size.

When two individuals are crossed, some genes can show nonadditive gene expression in the hybrids, that is, the transcription level of some genes can differ significantly from the parental mean. Such nonadditivity may be at the base of heterosis (reviewed in Chen 2013). This was tested in alfalfa, by comparing the numbers of nonadditively expressed genes in heterotic vs. nonheterotic population hybrids (Li et al. 2009). The heterotic hybrids showed substantially more (4.4–7.7%) nonadditively expressed genes than the nonheterotic hybrid (0.5%). Polyploidization can affect gene expression in hybrids: in fact, nonadditivity of parental gene expression is a common feature of allopolyploids (reviewed in Jackson and Chen 2010; Chen 2013). In neo-autotetraploids, subtle gene expression changes have been found. For example, in maize, about 6% of the genes were transcriptionally affected due to transition from the 2x to the 4x state (Riddle et al. 2010). In potato, about 12% of 9029

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**Table 2 Numbers of genes whose transcription levels differed significantly between single progeny plants and the parental mean**

|                      | 2x Hybrids | 4x Hybrids |
|----------------------|------------|------------|
|                      | S8-2x      | S16-2x     | S24-2x     | Mean | S29-4x     | S48-4x     | S60-4x     | Mean |
| Overexpressed (O)    | 236        | 240        | 562        | 346  | 432        | 818        | 393        | 547  |
| Underexpressed (U)   | 74         | 117        | 490        | 227  | 299        | 680        | 404        | 461  |
| Ratio O/U            | 3.19       | 2.05       | 1.15       | 2.13 | 1.44       | 1.20       | 0.97       | 1.20 |
| Differentially       | 310        | 357        | 1052       | 573  | 731        | 1498       | 797        | 1009 |
| expressed (O+U)      | 0.75       | 0.86       | 2.53       | 1.38 | 1.76       | 3.61       | 1.92       | 2.43 |
| Percentage           |            |            |            |      |            |            |            |      |
| differentially       |            |            |            |      |            |            |            |      |
| expressed (%)        |            |            |            |      |            |            |            |      |
Table 3 Transcripts differentially expressed between 2x and 4x hybrids whose GO IDs are significantly enriched in the polyploidization-sensitive group of genes with respect to the genes present on the microarray, according to BiNGO analysis.

| Gene            | Log₂ 2x/4x Expression | Best Blast Hit* | E Value |
|-----------------|-----------------------|-----------------|---------|
| TC172620        | -4.419                | XP_003622001:1: Heat shock protein Hsp70, Medicago truncatula | 0.0     |
| TC198142        | -2.17                 | XP_00363970:1: Ribulose bisphosphate carboxylase small chain 3A, M. truncatula | 3e-118  |
| TC183902        | -1.723                | AACC25775:1: Chlorophyll a/b binding protein, M. sativa | 1e-90   |
| TC177786        | -1.697                | AACC25775:1: Chlorophyll a/b binding protein, M. sativa | 2e-167  |
| TC201583        | -1.59                 | XP_00362718:1: Lipoxygenase, M. truncatula | 0.0     |
| TC173777        | -1.571                | XP_00362718:1: Lipoxygenase, M. truncatula | 0.0     |
| TC195187        | -1.537                | AACC25775:1: Chlorophyll a/b binding protein, M. sativa | 2e-167  |
| TC178665        | -1.305                | XP_00450250:1: UDP-glucose flavonoid 3-0-glucosyltransferase 7-like, Cicer arietinum | 0.0     |
| TC174046        | -1.293                | XP_003610706:1: Photosystem I reaction center subunit II, M. truncatula | 9e-138  |
| TC178639        | -1.226                | XP_00362718:1: Lipoxygenase, M. truncatula | 0.0     |
| TC180095        | -1.168                | XP_003618482:1: Chlorophyll a/b binding protein, M. truncatula | 4e-131  |
| TC188202        | -1.158                | AACC25775:1: Chlorophyll a/b binding protein, M. sativa | 7e-101  |
| TC182218        | -1.154                | XP_003592196:1: Heat shock protein, M. truncatula | 1e-180  |
| TC179482        | -1.058                | XP_003618482:1: Chlorophyll a/b binding protein, M. truncatula | 2e-174  |
| TC172508        | -1.001                | XP_00362196:1: Lipoxygenase, M. truncatula | 0.0     |

The genes are listed according to decreasing Log₂ 2x/4x expression ratio: negative values mean that the gene has higher expression in 4x that in 2x plants, and vice-versa. The best blast hit of each transcript and its E value are presented.

* Best hit from Blastx as of March 15, 2014. When the best hit was an ‘unknown protein’ the best annotated hit was taken instead.

Genes were differentially expressed in leaves between isogenic 2x and 4x potato plants (Stupar et al. 2007). Here, nonadditivity was observed for a small number of genes in 2x hybrids (1.38%, in the range observed by Li et al. 2009), but polyploidization determined a five-fold increase of nonadditively expressed genes in 4x hybrids. Cheng et al. (2015) found a similar response. The fact that, in the comparison of pooled 4x hybrids with parents, underexpressed genes prevailed, whereas in comparisons between single 4x hybrids with parents, overexpressed genes prevailed, suggests that polyploidization exerted a stochastic effects on gene transcription. A few hundred differentially expressed genes were evidenced in the comparison between 2x and 4x hybrids. Since the 2x and 4x plants are full sibs, we concentrated our attention on those genes that we consider to be “polyploidization-sensitive”. Polyploidization preferentially affected the expression of genes related to energy metabolism, response to stress, and the plastid compartment. GO terms significantly enriched in the PS group of genes derived from only 25 differentially expressed genes, 15 of them upregulated, and 10 downregulated in 4x vs. 2x plants.

Eight photosynthesis-related genes were upregulated, six of them encode chlorophyll binding proteins. These proteins bind to chlorophyll.
Progenies (Wasternack et al. 2013). Increased expression of LOX in 4x conditions has been shown that, despite the significant effect of interspecific hybridization, polyploidy led to a secondary effect on methylation, with rever-
sion to additivity at some loci, and novel methylation at others. Furthermore, 33 HSP genes, 31 were underexpressed, and three overexpressed, compared with average expression in tetraploid parents (Wang et al. 2006), but the comparison with diploid parents was not made.

Only 10 PS, downregulated genes contributed to the GO terms enrichment, with no obvious link between them. Among them, a minichromosome maintenance (MCM) protein gene got our attention. The eukaryotic replicative helicase is composed of six distinct, but related, subunits (Benn et al. 2001). The MCM complex binds to chromatin at the site of replication initiation, and is required for chromosome replication in eukaryotes (Bell and Botchan 2013). Its release from the replication initiation site and degradation prevents reinitiation of replication in the same cell cycle. In humans, high expression of MCM protein is observed in some cancerous cells (see, for example, Ishimi et al. 2003). Given that, it can be speculated that reduced expression of a MCM subunit in polyploid alfalfa on a per genome basis might correlate with a decrease in the number of cell divisions in leaf tissue, contributing to the observed lower cell number, and larger cell size. Further studies are needed to test this hypothesis.

Polyploidization does not induce DNA methylation
On average, hybridization promotes decreases in cell size, but not when combined with polyploidization. Indeed, less fully methylated sites were found in 4x than in the 2x hybrids. It is possible that, when the balanced parental genomes merge in the 4x hybrids, the resulting 4x genome is more balanced than that of the 2x hybrids, in which de novo methylation might be necessary to balance gene expression. Whether cytosine methylation variation is induced by hybridization or genome duplication in polyploid plants is still an open question. Madlung et al. (2002) and Salmon et al. (2005) argued that changes in cytosine methylation arise most likely as an effect of hybridization rather than genome doubling per se. However, Hegarty et al. (2011) investigated the non-additive changes of cytosine methylation in allopolyploid Senecio and showed that, despite the significant effect of interspecific hybridization, polyploidization led to a secondary effect on methylation, with reversion to additivity at some loci, and novel methylation at others. Changes in cytosine methylation levels and patterns during the

Figure 8 Results of principal coordinates analysis (PCA) of MSAP patterns. The first two coordinates (PCO1 and PCO2) are displayed with the indication of the percentage of variance explained in brackets. Labels indicate the centroids of each group.

Table 4 Results of MSAP analysis

| Genotypes | Total | Monomorphic | PG-F9 like | 12-P like | Demethylation | De novo Methylation | Ambiguous | MR |
|-----------|-------|-------------|-----------|-----------|---------------|---------------------|-----------|----|
| 2x progenies |       |             |           |           |               |                     |           |    |
| S8        | 221   | 100a (45.25)b | 41 (18.55) | 28 (12.67) | 24 (10.86)    | 22 (9.95)           | 6 (2.71)  | 0.9 |
| S16       | 235   | 101 (42.98)  | 54 (22.98) | 21 (8.94)  | 26 (11.06)    | 27 (11.49)          | 6 (2.55)  | 1.0 |
| S24       | 234   | 87 (37.18)   | 43 (18.38) | 39 (16.67) | 16 (6.84)     | 46 (19.66)          | 3 (1.28)  | 2.9 |
| Total     | 690   | 288 (41.74)  | 138 (20.00)| 88 (12.75) | 66 (9.57)     | 95 (13.77)          | 15 (2.17) | 1.4 |
| 4x progenies |       |             |           |           |               |                     |           |    |
| S29       | 250   | 90 (36.00)   | 63 (25.20) | 30 (12.00) | 30 (12.00)    | 33 (13.20)          | 4 (1.60)  | 1.1 |
| S48       | 242   | 79 (32.64)   | 50 (20.66) | 42 (17.36) | 42 (17.36)    | 28 (11.57)          | 1 (0.41)  | 0.7 |
| S60       | 250   | 87 (34.80)   | 41 (16.40) | 43 (17.20) | 43 (17.20)    | 32 (12.80)          | 4 (1.60)  | 0.7 |
| Total     | 742   | 256 (34.50)  | 154 (20.75)| 115 (15.50)| 115 (15.50)   | 93 (12.53)          | 9 (1.21)  | 0.8 |

Number and percentage of methylation markers scored in each 2x or 4x hybrid in comparison with their parents. MR, methylation ratio.

a Methylation changes difficult to interpret according to Fulneček and Kovalík (2014).
b MR: methylation ratio is the percent ratio of the number of markers revealing de novo methylation over the number of markers revealing demethylation.
c The number of corresponding sites.
d The frequency of the corresponding sites.

These two figures differ significantly at P ≤ 0.05.

* a and b to form the light-harvesting complexes (LHC) I and II, that capture and deliver light excitation energy to photosystems I and II. The photosystem I subunit PsAD, and the Rubisco small subunit, were also overexpressed as a consequence of ploidy change. It can be speculated that the observed per genome increases in the expression of photosynthetic genes contributed to the increased biomass of tetraploids.

Four overexpressed lipoxygenase genes were enriched in the PS group. One of the main pathways of lipid alteration is the formation of polyunsaturated fatty acids (PUFAs) (Feussner and Wasternack 2002). Their oxidation to hydroperoxides can be catalyzed by lipoxygenases (LOX). Among plastid LOX, 13-LOX is the first enzyme of the jasmonate pathway. Jasmonates are ubiquitous lipid-derived signaling compounds active in plant development, and in plant responses to biotic and abiotic stresses (Wasternack et al. 2013). Increased expression of LOX in 4x plants may influence many cellular processes related to biotic and abiotic stress responses. Two heat shock protein (HSP) genes, homologous to Hsp70 (the gene with the highest fold change) and to Hsp90, respectively, were nonadditively overexpressed in 4x hybrids. These chaperone proteins are involved in the response to heat, and to many other stress factors, and their increased expression may provide advantages to the 4x condition. In A. thaliana allopolyploids, of 33 HSP genes, 31 were underexpressed, and three overexpressed, compared with average expression in tetraploid parents (Wang et al. 2006), but the comparison with diploid parents was not made.

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process of hybridization and allopolyploidization have been well documented, and our data are consistent with the findings in polyploids of *Cucumis* (Chen and Chen 2008), watermelon (Wang et al. 2009), sage (Li et al. 2012), and poplar (Suo et al. 2015). We found that methylation patterns differed among genotypes within 2x and 4x hybrids. Similar differences in methylation levels among individuals were reported in polyploids of *Taraxacum officinale* (Verhoeven et al. 2010), *Solanum* spp. (Aversano et al. 2013) and *Populus* (Suo et al. 2015). Due to instability of cytosine methylation at different genomic loci (Zhao et al. 2007), it may be speculated that a number of metastable loci show random alterations as a consequence of hybridization, resulting in individual differences.

**Conclusion**

In a recent interesting review, Parisod et al. (2010), stated that: “the successful range expansion and radiation demonstrated in various natural autopolyploids suggest that genome duplication *per se* may represent an evolutionary advantage”. In this work, we showed that sexual polyploidization conferred on alfalfa traits that can be advantageous both in the wild, and in cultivation. Several hundred genes, related to diverse metabolic functions, changed their expression level as a consequence of polyploidization. The meaning of these transcriptional changes will be investigated in further research. In addition, we found that DNA cytosine methylation was affected by both hybridization and polyploidization, suggesting that it acts as a regulatory mechanism in that DNA cytosine methylation was affected by both hybridization and polyploidization, suggesting that it acts as a regulatory mechanism in the success of polyploid wheat under domestication. Science 316: 1862–1866.

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