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ORIGINAL PAPER

Intraspecific hybrids of *Arabidopsis thaliana* revealed no gross alterations in endopolyploidy, DNA methylation, histone modifications and transcript levels

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Abstract Arabidopsis accessions Col-0 and C24 and their reciprocal hybrids were employed as a model system to investigate the potential relationship between changes in DNA methylation, chromatin structure, endopolyploidization and gene expression in heterotic genotypes. Nucleolus size, endopolyploidization level and distribution of DNA and histone H3 methylation at the microscopic level does not differ between parents and their hybrids. Methylation sensitive amplified polymorphism revealed a largely constant pattern of DNA methylation (97% of signals analyzed) after intraspecific crosses. The parental expression profile of selected genes was maintained in hybrid offspring. No correlation was found between expression pattern and DNA methylation levels at restriction sites within 5′ regulatory regions. Thus, the results revealed only minor changes of chromatin properties and other nuclear features in response to intraspecific hybridization in *Arabidopsis thaliana*.

Introduction

Heterosis refers to superior performance of progeny obtained from crossing two inbred lines in comparison to both parental lines. Although the application of heterosis to breeding and development of crop hybrids has made an enormous contribution to twentieth-century agriculture, the genetic basis for this phenomenon remains conjectural. It is becoming apparent that heterosis cannot adequately be explained in terms of simple mutual complementation of multiple unfavorable alleles. Rather, complex phenomena, including the interplay of alleles to cause gene expression in the hybrid that deviate from the additive mid-parent prediction, are likely to be involved (Birchler et al. 2003; Hochholdinger and Hoecker 2007). Accordingly, studies using high-throughput transcript profiling identified heterosis-related gene expression changes for a large number of genes in all possible modes of action (Guo et al. 2006; Hochholdinger and Hoecker 2007; Swanson-Wagner et al. 2006; Uzarowska et al. 2007).

Beyond variation at the DNA sequence level, epigenetic variation, that is, heritable variation in DNA methylation or covalent histone modifications at particular genomic regions, might also play a role in heterosis. In general, chromatin can be divided into euchromatin which is transcriptionally active and shows a low DNA methylation level, and condensed heterochromatin which is thought to be transcriptionally inert and is associated with a high DNA methylation level. In contrast to DNA, where methylation is the only covalent mark, the N-terminal tails of histones are subjected to acetylation, methylation and a number of other post-translational modifications (Fuchs et al. 2006). The acetylation and methylation of selected lysine (K) residues in the N-terminal tails of histones H3 and H4 in particular seem to have a crucial role in heterochromatin versus euchromatin formation in many organisms. In mammals and in plants, the accumulation of H3 methylated at K9 and K27 as well as strong DNA methylation have been attributed to heterochromatin,

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whereas sparse DNA methylation and the presence of H3 methylated at K4 are considered to be characteristic of euchromatin (Kouzarides 2007; Tariq and Paszkowski 2004).

Inbred accessions of *Arabidopsis thaliana* display a high level of epigenetic variation (Vaughn et al. 2007). Thus, intercrosses between inbred lines could potentially lead to modification of chromatin marks. In fact, there is accumulated evidence for a role of chromatin modification in the expression of heterosis. Early studies on chromatin composition of animals and plants have demonstrated differences in the protein-to-DNA ratio of the chromatin (Naso 1976; Shannon and McDaniel 1979; Tallman et al. 1977) and in histone composition (Martinez and McDaniel 1981) between inbred parental lines and their respective heterotic hybrids. These authors proposed that variations in chromatin characteristics must be partly responsible for patterns of heterotic development. More recently, a possible role of DNA methylation in the expression of heterosis has been put forward (Tsaftaris et al. 1997). Differential methylation in CG or CNG sequence context was observed in F_1 hybrids and their respective parental lines in maize (Tsaftaris et al. 1997) and rice (Xiong et al. 1999).

Interestingly, modification of chromatin marks is also frequently observed after combining distinct but related genomes during allopolyploid formation. In interspecies hybrids, one parental set of genes was silenced within a few generations but could be reactivated by blocking both, DNA methylation and histone deacetylation, suggesting a role for chromatin modification in the regulation of expression of orthologous genes (Lawrence et al. 2004; Lee and Chen 2001). Changes of DNA methylation induced by wide hybridization were reported for synthetic *Cucumis* alloploylpoids (Chen and Chen 2008). Gene expression studies in synthetic allopolyploid *Arabidopsis* (Comai 2000) and in cotton (Brubaker et al. 1999) revealed that gene silencing occurs during the first or second generation after hybridization. Comparative gene expression studies in synthetic allpolyploid lines of *Arabidopsis* demonstrated that around 5% of genes had a nonadditive expression level (Wang et al. 2006). Contrary to these findings, studies in allopolyploid *Spartina anglica, Brassica juncea* and cotton showed that the activity of parental genomes remained unchanged (Baumel et al. 2002; Axelsson et al. 2000; Liu et al. 2001). These contrasting observations among different species may suggest that genome responses to allopolyploidization are variable and species dependent (Ma and Gustafson 2005).

Similar to heterotic progeny from crosses of inbred lines, *Arabidopsis* allopolyploids often also show superior vigor (Comai 2005). It is, therefore, tempting to speculate that similar mechanisms of regulation of gene expression and/or chromatin modification are involved. Nevertheless, heterotic effects induced by intra- and interspecific hybridization are not mutually exclusive, as crosses of inbred lines of allohexaploid wheat can produce heterotic progeny (Wu et al. 2003).

There is evidence that the growth rate of organisms is correlated with cellular ribosomal RNA (rRNA) content, with higher levels enabling faster protein synthesis and growth (Elser et al. 2000). Therefore, in cells with high growth rate most of the transcripts belong to rRNA (Grummt and Pikaard 2003). Increased cellular rRNA levels may be based on expansion of rDNA copy number, or increase in the transcription rate per rDNA unit (Elser et al. 2000). Endoreduplication may be another way by that plant cells can achieve fast growth (Rogers and Bendich 1987). So far no differences in endopolyploidy between hybrid plants and their parents have been reported.

Intraspecific hybrids between *A. thaliana* accessions Col-0 and C24 provide a useful model system for analysis of heterosis (Meyer et al. 2004). In order to test whether the increased biomass production of such hybrids (Meyer et al. 2004) correlates with an increase of rDNA transcription activity and/or with the level of endopolyploidization, we compared the nucleolus size and endopolyploidization level between the parental accessions and their reciprocal hybrids. To improve our understanding of the potential relationship between intraspecific hybridization as a basis for heterosis and histone and DNA modifications, the distribution of DNA and of histone H3 methylation was compared between parental and hybrid plants at the microscopic level and for selected genes.

Materials and methods

Plant material

Seeds of *A. thaliana* Col-0 and C24 were sown in soil and stratified for 3 days at 4°C. After 15 days under short-day conditions (8 h light, 16 h dark, 21°C), plant culture was continued under long-day conditions (16 h light, 8 h dark, 21°C). For intraspecific crossing, five flower buds on the primary shoot were emasculated and manually cross pollinated, while all other flower buds were removed. The same procedure was applied to the plants selected to produce parental seeds (Col-0 and C24) except that the remaining five flower buds were permitted to self-pollinate. The seeds of each genotype were pooled. Plantlets to be harvested at 4 and 6 days after sowing (DAS) were cultivated on nylon mesh put on soil; plantlets to be harvested at 10, 15 and 21 DAS were grown on soil only. Details are provided in Supplementary Material and Methods.

Determination of the endopolyploidization and flow sorting of nuclei

The endopolyploidization levels of nuclei isolated from seedlings, cotyledons or leaves at 4, 6, 10 and 15 DAS, respectively, were measured using a FACStarPLUS (BD Biosciences) according to Barow and Meister (2003). The cycle values, indicating the mean number of endoreduplication cycles per nucleus in a cell population, were calculated based on the formula:

$$\text{cycle value} = (0 \cdot n2\text{C} + 1 \cdot n4\text{C} + 2 \cdot n8\text{C} + 3 \cdot n16\text{C} \cdots)/(n2\text{C} + n4\text{C} + n8\text{C} + n16\text{C} \cdots)$$

where n2C, n4C, n8C… are the numbers of nuclei with the corresponding C-value (2C, 4C, 8C,…) and coefficient numbers (0, 1, 2,…) are the number of endoreduplication cycles necessary to reach the corresponding ploidy level.

For silver staining and immunostaining experiments 2C and 4C nuclei from 6 DAS seedlings were flow sorted into tubes using a FACSAria (BD Biosciences) and dropped onto slides as described in Pecinka et al. (2004).

Silver staining and indirect immunostaining of *A. thaliana* nuclei

Nucleoli of 2C and 4C nuclei from 6 DAS seedlings were silver-stained according to Hizume et al. (1980) and Rufas et al. (1982). Polyclonal rabbit antibodies against histone H3K27me3, H3K4me2 and H3K9me2 (Millipore, cat. nos. 07-449, 07-030 and 07-441, respectively) were used to detect the distribution of these marks in isolated 4C nuclei from 6 DAS seedlings as described by Houben et al. (2003). 5-methylcytosine (Eurogentec, cat. no. MMS-900P-A) immunodetection was performed according to Ruffini Castiglione et al. (2002). Microscopic images were recorded using an Olympus BX61 microscope equipped with an ORCA-ER CCD camera. Images were analyzed using the SIS software (Olympus).

Methylation sensitive amplified polymorphism assay

DNA methylation patterns in pooled samples of Col-0, Col-0xC24, C24xCol-0 and C24 plants harvested 6 and 21 DAS were compared by MSAP analysis according to Cervera et al. (2002) in four biological replicates. Briefly, genomic DNA of each genotype was cleaved with restriction endonucleases *Eco*RI (recognition sequence GAATTC) and either *Hpa*II (recognition sequence CCGG, but inhibited by methylation of the inner C, but not inhibited by hemi-methylation of the outer C) or *Msp*I (recognition sequence CCGG, cleavage not inhibited by methylation of the inner C, cleavage inhibited by methylation of the outer C) (Supplementary Material and Methods). After restriction cleavage, *Eco*RI and *Hpa*II/*Msp*I adapters (Supplementary Table S1) were ligated using T4 DNA ligase. DNA fragments with ligated adapters served as templates for primary PCR amplifications using primers complementary to the *Eco*RI and *Hpa*II/*Msp*I adapters with one additional selective nucleotide at the 3′ end (Supplementary Table S2). PCR products were diluted and used as templates for secondary selective amplification with combinations of primers complementary to the *Eco*RI and one *Hpa*II/*Msp*I adaptors, but this time with two or three selective nucleotides, respectively, at the 3′ end (Supplementary Table S3). Secondary primers complementary to *Eco*RI adapters were 5′ end-labeled with IRD700 fluorescent dye. PCR products were mixed with the size marker microSTEP 15a which was differentially labeled (Microzone, Lewes, UK) and size separated by capillary electrophoresis in a BECKMAN COULTER CEQ 8000 Genetic Analysis System. Obtained data were analyzed using CEQ 8000 software.

Cloning and sequencing of C24 alleles of selected genes

For seven genes that were identified as being specifically regulated in Col-0xC24 and reciprocal hybrid seedlings (Maria von Korff Schmiesing and Thomas Altmann, personal communication), sequence differences between accessions Col-0 and C24 were determined by PCR amplification, cloning and sequencing of promoter and transcribed regions of the C24 alleles. Sequences were submitted to the DDBJ/EMBL/GenBank DNA sequence database under accession numbers FJ899135 (C24-At1g03420), FJ899136 (C24-At1g29270), FJ899137 (C24-At2g33220), FJ899138 (C24-At3g19520), FJ899139 (C24-At3g25905), FJ899140 (C24-At4g29200), and FJ899141 (C24-At5g15360).

Allele-specific 5′ regulatory region DNA methylation assay

Genomic DNA extracted from seedlings 6 DAS was incubated with cytosine methylation-sensitive restriction enzymes specific for sites common to the 5′ regulatory regions of Col-0 and C24 alleles (Supplementary Table S4). As control, the same amount of DNA was incubated under the same conditions, but without restriction enzyme. Restriction enzymes were inactivated, and DNA samples were used as templates for PCR with primers (Supplementary Table S5) designed in a way that the amplified region included not only the common methylation-sensitive site, but also an additional restriction site(s) unique either to the Col-0 or C24 allele (Supplementary Table S4). After PCR product purification,

aliquots were cleaved with the allele-specific restriction enzymes and size fractionated side-by-side with un-cleaved controls by agarose gel electrophoresis. Allele-specific methylation levels were estimated from the intensity of the corresponding bands after ethidium bromide staining.

Quantitative transcript analysis

Based on the Col-0 and C24 sequence information, primers were designed (Supplementary Table S6; Supplementary material and methods) to equally amplify transcripts from Col-0 and C24 alleles of the seven candidate genes (Supplementary Table S7). Quantitative RT-PCR was performed in a BioRad iQ5 Real-Time PCR system using a BioRad iQ SYBR Green Supermix kit. Data were analyzed with BioRad iQ5 software (Supplementary material and methods). *ACTIN2* (At3g18780) (Supplementary Table S8) was used as a constitutively expressed control gene for calibration in quantitative comparisons according to Livak and Schmittgen (2001).

Allele-specific transcript assay

Allele-selective transcript analysis was developed based on Col-0- or C24-specific restriction sites in cDNA sequences. PCR was performed on cDNA with the same gene-specific primers used for quantitative RT-PCR (Supplementary Table S6) for numbers of cycles that would avoid reaction saturation. After PCR product purification, aliquots were cleaved with allele-specific restriction enzymes (Supplementary Table S7). Cleaved PCR products were size fractionated adjacent to un-cleaved controls by agarose gel electrophoresis. Allele-specific transcript levels were estimated from the intensity of the corresponding bands after ethidium bromide staining.

Results

The level of endoreduplication and nucleolus size does not differ between inbred parents and their intraspecific hybrids

Somatic plant cells can undergo increasing ploidy levels by successive rounds of chromosomal DNA replication without intervening mitosis in a process called endoreduplication. In *A. thaliana*, endoploidy levels ranging from 4C to 32C are commonly found (Sugimoto-Shirasu and Roberts 2003). To test whether the level of endopolyploidization differs between inbred parents and their hybrid progeny, flow cytometric analysis was conducted on three biological replicates of each genotype. The ploidy levels were determined for nuclei from leaves (15 DAS), cotyledons (15 and 10 DAS) or seedlings (6 and 4 DAS) and used to calculate cycle values according to Barow and Meister (2003) as an indicator of endoreduplication (Fig. 1). No differences in endoreduplication were obvious between Col-0, C24 and their reciprocal hybrids.

The nucleolus area correlates with the activity of rRNA genes (Hubbell 1985) and was used to compare the relative rRNA gene activity of inbred parental accessions and their hybrids (Fig. 2). To ensure that the nucleolus areas were determined in nuclei of the same ploidy level, flow-sorted 2C and 4C nuclei from seedlings 6 DAS were used. The average nucleolus area showed a high degree of variability within all samples tested, and the nucleolus area of intraspecific hybrids was similar to that of their parent nuclei. Hence, the nucleolus area does not indicate an increased rRNA gene activity in hybrid offspring.

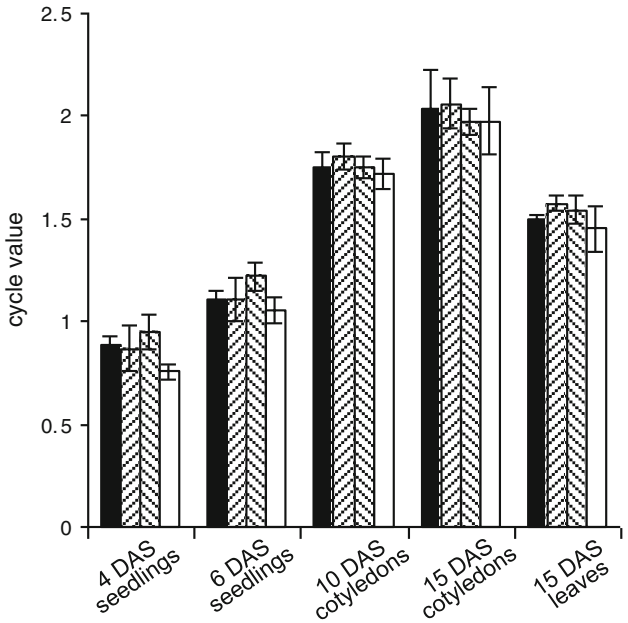


Fig. 1 Endopolyploidisation levels in inbred parents and their hybrid offspring. Cycle values were determined in accessions Col-0 (*black bars*) and C24 (*white bars*) and their reciprocal hybrid offspring (*hatched bars*) at different growth stages. Seedlings were analyzed 4 DAS (days after sowing) and 6 DAS, cotyledons 10 DAS and 15 DAS, and leaves 15 DAS. The cycle values provide a measure for the average frequency of endoreduplication in a cell population and are calculated according to the numbers of nuclei with particular ploidy levels in relation to the total number of nuclei analyzed. The error bars indicate standard deviations among three biological replicates. Cycle values differ among time points and different organs, but in no case differences between inbred parents and their hybrid offspring were detected. Approximately 10,000 nuclei per sample were analyzed

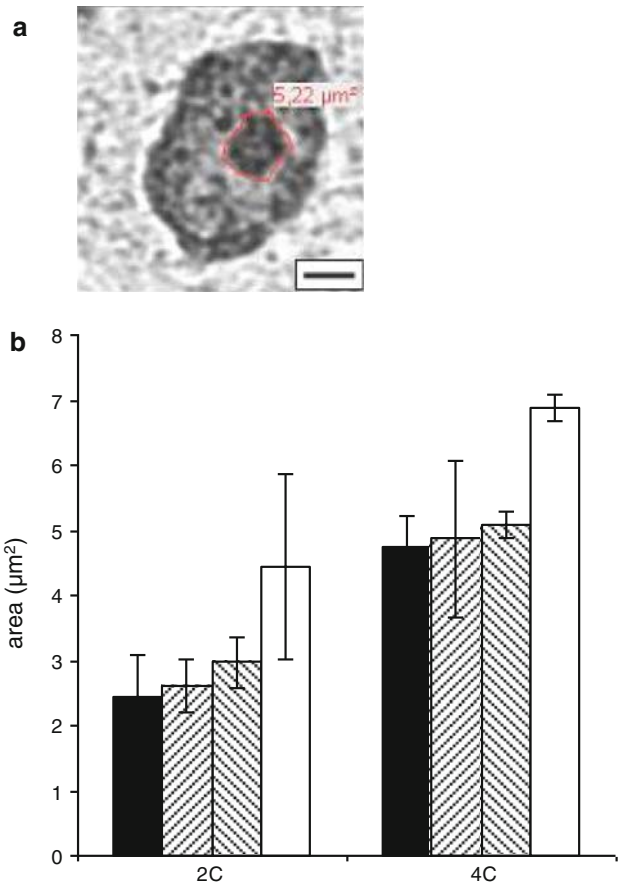


Fig. 2 Nucleolus size in inbred parents and their hybrid offspring. Flow-sorted 2C and 4C nuclei isolated from seedlings 6 DAS were silver-stained. **a** Nucleoli (surrounded by a *red line*) appeared darker than the surrounding nucleoplasm. The nucleolar area was manually encircled and measured using SIS software (Olympus). The scale bar indicates 2 µm. **b** Nucleolus area measurements were performed on 2C and 4C nuclei of accessions Col-0 (*black bars*) and C24 (*white bars*) and their reciprocal hybrid offspring (*hatched bars*) in two biological replicates. On average, 130 nuclei were measured for each sample per replicate. The nucleolus area (μm^2) of nuclei with a defined ploidy level varied among individuals (bars show standard deviation), with nucleolus areas of 4C nuclei being larger than the ones of 2C nuclei

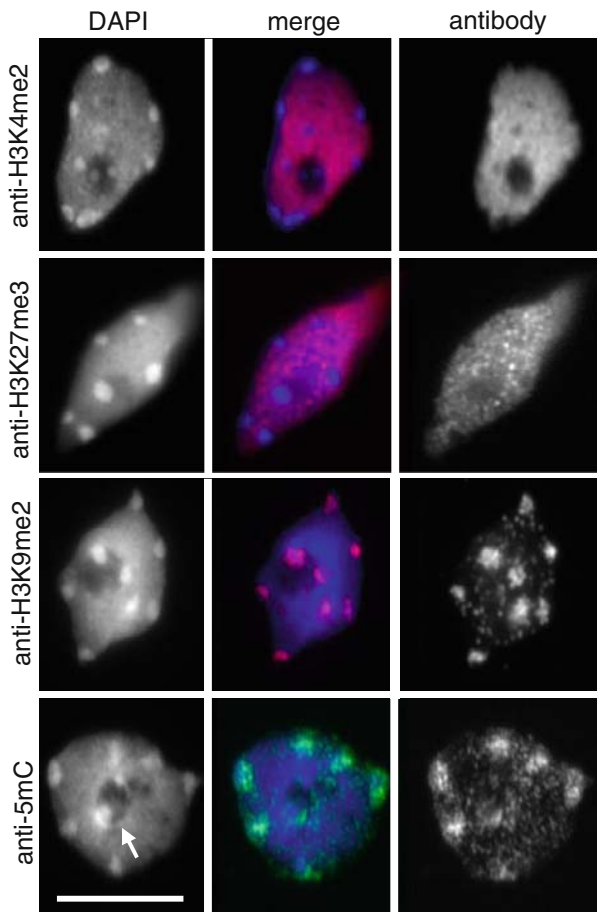


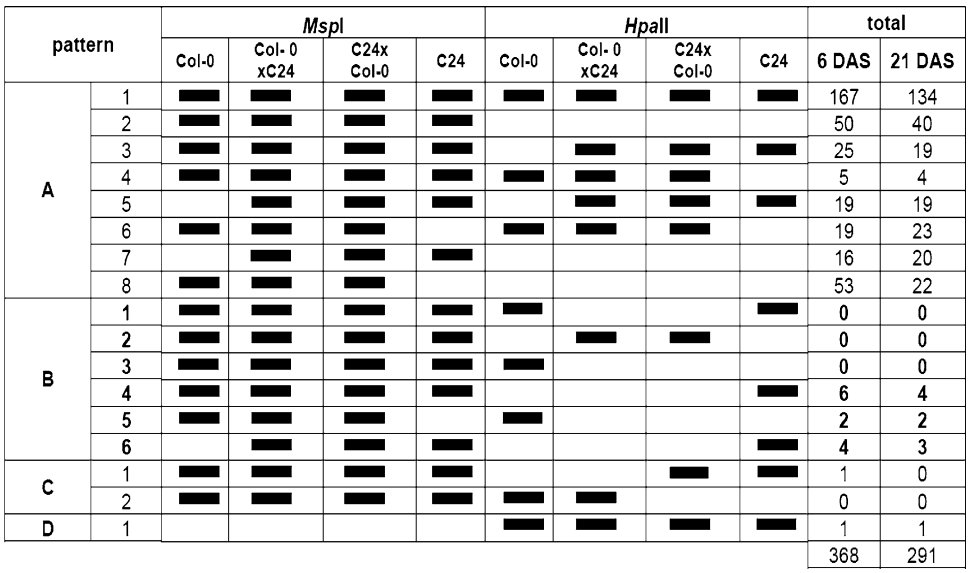
Fig. 3 Nuclear distribution of histone H3-methylation at positions K4, K9 and K27 and DNA methylation. Indirect immunolabeling of histone H3K4me2, H3K9me2, H3K27me3 and 5mC was done on 4C nuclei isolated from seedlings 6 DAS. Images from nuclei of Col-0xC24 hybrid progeny are shown as representatives. Images from Col-0 and C24 inbred parents and C24xCol-0 hybrid progeny showed identical signal patterns. DAPI-bright spots in nuclei represent heterochromatic chromocenters. The dark round area indicated with a *white arrow* represents a nucleolus. The scale bar indicates 2.5 µm

The nuclear distribution of DNA methylation and histone H3-methylation at positions K4, K9 and K27 in inbred parents and intraspecific hybrids is similar

DNA methylation (5-methylcytosine, 5mC), histone H3 dimethylation of lysines at position K4 (H3K4me2) or K9 (H3K9me2) and H3 trimethylation of lysine at position K27 (H3K27me3) were selected as representative chromatin marks for analysis at the microscopic level. Indirect immunolabeling was performed on isolated 4C nuclei and fluorescence signal distribution recorded (Fig. 3). As previously reported by Fuchs et al. (2006) co-localization of immunofluorescence signals with DAPI-stained heterochromatic chromocenters was observed for H3K9me2 and 5mC, while signals for H3K4me2 and H3K27me3 were dispersed in euchromatin of interphase nuclei and were excluded from chromocenters. Regardless of which chromatin modification was analyzed, there were no obvious differences in the distribution and intensity of immunofluorescence signals between Col-0, C24 and their reciprocal hybrids.

A limited gain of DNA methylation appeared in hybrid offspring compared to inbred parents

Methylation sensitive amplified polymorphism (MSAP) analysis was used to investigate possible changes in the overall distribution of DNA methylation in intraspecific hybrid offspring compared with inbred parents. In Fig. 4, MSAP patterns are grouped according to their information



pattern		*Msp*I				*Hpa*II				total	
		Col-0	Col-0 xC24	C24x Col-0	C24	Col-0	Col-0 xC24	C24x Col-0	C24	6 DAS	21 DAS
A	1	■	■	■	■	■	■	■	■	167	134
	2	■	■	■	■					50	40
	3	■	■	■	■		■	■	■	25	19
	4	■	■	■	■	■	■	■		5	4
	5		■	■	■		■	■	■	19	19
	6	■	■	■		■	■	■		19	23
	7		■	■	■					16	20
	8	■	■	■						53	22
B	1	■	■	■	■	■			■	**0**	**0**
	2	■	■	■	■		■	■		**0**	**0**
	3	■	■	■	■	■				**0**	**0**
	4	■	■	■	■				■	**6**	**4**
	5	■	■	■		■				**2**	**2**
	6		■	■	■				■	**4**	**3**
C	1	■	■	■	■			■	■	1	0
	2	■	■	■	■	■	■			0	0
D	1					■	■	■	■	1	1
										368	291

Fig. 4 MSAP analysis of inbred parents and their hybrid offspring. MSAP analysis using differentially methylation-sensitive isoschizomeres (*Msp*I and *Hpa*II) was performed on DNA from pooled seedlings 6 DAS and 21 DAS with eight primer pairs. Results from four biological replicates are summarized according to the pattern of presence (*black bar*) or absence (*empty cell*) of the corresponding signals in samples derived from inbred parents and their hybrid offspring. The presence of a signal indicates cleavage at a particular *Msp*I/*Hpa*II site, whereas absence indicates inhibition of cleavage. Presence of a signal in *Msp*I- and *Hpa*II-treated DNA from the same sample indicates absence of methylation. The presence of a signal in *Msp*I-treated DNA in combination with the absence of a signal in *Hpa*II-treated DNA indicates methylation of the inner cytosine of the CCGG recognition site (pattern category A, B, C). The absence of a signal in *Msp*I-treated DNA in combination with the presence of a signal in *Hpa*II-treated DNA from the same sample indicates hemimethylation of the outer cytosine of the CCGG recognition site (pattern category D). Methylation at a particular site in only some DNA copies in a sample cannot be detected by this method as it will be masked by the positive signal from the unmethylated copies. The absence of a signal in both *Msp*I- and *Hpa*II-treated DNA is inconclusive, as this could be caused either by simultaneous methylation at the outer and inner cytosine of the CCGG recognition site, or by absence of the restriction site (Cervera et al. 2002). Patterns of category B and C indicate changes between parents and hybrids, while patterns of category A and D indicate absence of changes. The numbers in the columns 6 DAS and 21 DAS indicate the numbers of MSAP signals for the respective pattern that were determined at these time points

content in the context of methylation changes related to hybrid formation. Approximately 97% (355 of 368 at 6 DAS and 282 of 291 at 21 DAS) of all MSAP signals analyzed showed patterns which did not indicate any methylation changes in hybrid offspring compared with their inbred parents (Fig. 4, pattern category A and D). In more than half of these cases, no methylation polymorphism between Col-0 and C24, and the reciprocal hybrids was detected. The patterns indicated either an absence of methylation at either cytosine in all samples (Fig. 4, pattern A1), methylation at the inner cytosine of the CCGG recognition site (Fig. 4, pattern A2) or, for one single signal, methylation at the outer cytosine (Fig. 4, pattern category D). In approximately 10% of cases, the presence of a signal after *Msp*I treatment was observed in both parents and their reciprocal hybrids, in combination with the presence of a signal after *Hpa*II treatment in all samples, except one parent. This indicated that methylation was present at the inner C of the CCGG recognition site in the respective parent, but not in the other parent (Fig. 4, pattern A3 and A4). In the remaining cases, the absence of a signal for one parent after *Msp*I and *Hpa*II treatments made it impossible to determine whether the restriction site in this parent was either methylated at both the inner and outer cytosine of the recognition site, or whether the restriction site was absent (Fig. 4, pattern A5 to A8). Nevertheless, absence of methylation at the outer and inner cytosine indicated by cleavage of the site by *Msp*I and *Hpa*II (Fig. 4, pattern A5 and A6) of the other parent was always inherited in the hybrids. This was applied to the absence of methylation at the outer cytosine and presence of methylation at the inner cytosine, which was indicated by cleavage by *Msp*I, but not *Hpa*II (Fig. 4, pattern A7 and A8).

Only approximately 3% (13 of 368 at 6 DAS and 9 of 291 at 21 DAS) of the analyzed MSAP signals showed signs of altered methylation in hybrids (Fig. 4, pattern category B and C). No case could be identified in which the hybrids had a methylation status different from both parents, neither by gaining (Fig. 4, pattern B1) nor losing (Fig. 4, pattern B2) methylation. Rather, methylation of the inner cytosine of the CCGG recognition site always increased in both reciprocal hybrids, as indicated by the loss of *Hpa*II cleavage. In one of the parents, methylation at the same position was present (Fig. 4, pattern B3 and

B4), or at least likely to be present (Fig. 4, pattern B5 and B6). In the latter, lack of cleavage by *Msp*I and *Hap*II in one of the parents once again could not discriminate between the presence of methylation at the outer and the inner cytosine at these sites and the possible absence of the recognition site (compare also Fig. 4, pattern A5 to A8). Finally, in one case, one of the two reciprocal hybrids did lose the *Hpa*II cleavage site indicating an increase in methylation at the inner cytosine (Fig. 4, pattern C1). As above, in one of the parents, methylation was already present at the same position. Notably, the total number of scorable MSAP signals declined from 368 to 291 with progressing development of plantlets (Fig. 4, column 6 DAS and 21 DAS), although the same set of primer pairs (Supplementary Table S3) was used for amplification for both points of time.

No changes in allele-specific methylation of 5′ regulatory regions of preselected genes were detected in intraspecific hybrid offspring

Previous studies have shown that the DNA methylation status of specific genes can vary in different maize inbred lines (Makarevitch et al. 2007) and that DNA methylation in maize can show parent-of-origin effects in hybrids (Lauria et al. 2004). To supplement our overall DNA methylation data obtained by the MSAP technique with exemplary gene- and also allele-specific information, seven genes were selected for analysis by methylation-sensitive restriction cleavage followed by PCR. In microarray-based analysis, transcript levels of At1g03420, At1g29270, At2g33220, At3g19520, At3g25905, At4g29200 and At5g15360 had been found to differ significantly between the parental lines Col-0 and C24, and in addition to possibly deviate from mid-parent values in the respective reciprocal hybrids (Maria von Korff Schmiesing and Thomas Altmann, personal communication). Partial sequences of the 5′ regulatory regions and open reading frames of the C24 alleles of these genes were determined (Table S4). Based on alignments of the obtained sequences with sequence information for Col-0 from public databases, PCR primers and restriction enzymes for allele-specific methylation analysis of the 5′ regulatory regions of these genes were selected and analyses were performed (Fig. 5, Supplementary Tables S4 and S5). Amplification with primers specific for the 5′ regulatory region of At2g33220 resulted in PCR products of 360 bp for Col-0, 357 bp for C24, and additively, 360 bp and 357 bp, for reciprocal hybrids (Fig. 5, panel a). After cleavage of genomic DNA with *HpyCH*4IV, no PCR products were obtained, indicating that the restriction site is unmethylated in all tested samples (Fig. 5, panel b). Incubation with *Xba*I cleaved the C24-specific PCR product into two fragments of 143 and

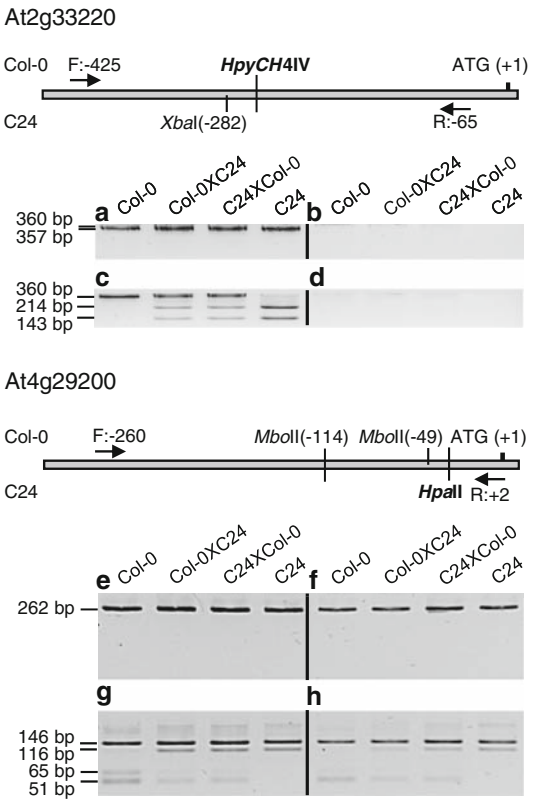


Fig. 5 Semi-quantitative allele-specific analysis of methylation in 5′ regulatory regions of selected genes in inbred parents and their hybrid offspring. DNA cytosine methylation in the 5′ regulatory region of genes in seedlings 6 DAS was probed by incubation of genomic DNA from with a methylation-sensitive restriction enzyme followed by PCR with primers flanking the respective restriction site (see gene models). In the presence of methylation at the recognition site, cleavage would be inhibited and a PCR product would be obtained, while in the absence of methylation, cleavage would occur and no PCR product would be obtained. In addition, primers were designed so that PCR products would include a restriction site polymorphic between Col-0 and C24 to discriminate PCR products according to their parental origin. Results are provided in detail for examples of the absence of methylation (At2g33220; **a**–**d**) and presence of methylation (At4g29200; **e**–**h**) at the methylation-sensitive restriction site. Inverse images of fluorescence signals after agarose gel electrophoresis and ethidium bromide staining are shown. Table 1 summarizes results for all genes that were examined

214 bp, but left the Col-0-specific PCR product un-cleaved. PCR products from reciprocal hybrids showed partial cleavage by *Xba*I, confirming equal amplification of both the Col-0- and the C24-derived allele (Fig. 5, panel c). As in Fig. 5, panel b, PCR products were absent after incubation of genomic DNA with *HpyCH*4IV (Fig. 5, panel d).

In conclusion, no methylation was detected at the *HpyCH*4IV site in the 5′ regulatory region of gene At2g33220. Amplification with primers specific for the 5′ regulatory region of At4g29200 resulted in PCR products of 262 bp for Col-0, C24 and their reciprocal hybrids (Fig. 5, panel e). After incubation of genomic DNA with

*Hpa*II, equal amounts of PCR products were obtained for all tested samples. Nevertheless, compared with Fig. 5e, the amounts of PCR products were lower and indicated the presence of partial, approximately equal methylation at the *Hpa*II site in genomic DNA of Col-0, C24 and their reciprocal hybrids (Fig. 5, panel f). Incubation with *Mbo*II cleaved the Col-0-specific PCR product into three fragments of 146, 65 and 51 bp and the C24-specific PCR into two fragments of 146 and 116 bp. PCR products from reciprocal hybrids showed partial cleavage by *Mbo*II with fragment sizes typical for both parents, confirming equal amplification of both the Col-0- and the C24-derived alleles (Fig. 5, panel g). *Mbo*II-cleavage of the PCR product from genomic DNA resistant to incubation with *Hpa*II resulted in band patterns specific for Col-0 and C-24. As in Fig. 5g, *Mbo*II-treatment resulted in partial cleavage for the reciprocal hybrids. In both hybrids, fragments of 65 and 51 bp specific for Col-0 and the fragment of 116 bp specific for C24 were present (Fig. 5, panel h). In conclusion, partial methylation at the *Hpa*II site is present in approximately equal levels in genomic DNA of Col-0, C24 and their reciprocal hybrids, and there is no shift in the relative methylation levels of Col-0- and C24-derived alleles in the hybrids compared with the inbred parental lines.

Methylation in the 5′ regulatory region was detected in genes At4g29200, At1g29270 and At3g19520 on both Col-0- and C24-derived alleles. In the other genes that were analyzed, methylation was absent (Table 1). No change of methylation status was observed in the reciprocal hybrids compared with the inbred parental lines for all genes.

No changes of allele-specific transcript levels of preselected genes were found in intraspecific hybrid offspring

Quantitative RT-PCR with primers equally efficient for amplifying Col-0 and C24 transcripts of the candidate gene set detected relative transcript levels in hybrid offspring equal to the intermediate levels for the inbred parental lines within the resolution of the method (Fig. 6). This observation is in agreement with the rather constant DNA methylation level between hybrid offspring and parents as analyzed by MSAP.

PCR primers and restriction enzymes for allele-specific transcript analysis were selected according to sequence alignments between Col-0 and C24 genomic sequences. Semi-quantitative RT-PCR in combination with restriction enzyme cleavage was performed (Fig. 7, Supplementary Table S6 and S7).

Amplification with primers specific for the transcript of gene At2g33220 resulted in PCR products of 176 bp for Col-0, C24, and their reciprocal hybrids. Band intensities were similar, indicating about equal transcript levels in all samples (Fig. 7, panel a). Incubation with *Hpa*II cleaved the Col-0-derived PCR product into two fragments of 132 bp and 44 bp, but left the C24-derived PCR product un-cleaved. PCR products from reciprocal hybrids showed partial cleavage by *Hpa*II, with relative band-intensities of the Col-0- and C24-specific fragments almost reflecting the relative band intensities of the respective fragments from the inbred parental lines (Fig. 7, panel b). Amplification with primers specific for transcripts of gene At4g29200 resulted in PCR products of 199 bp for C24 and the reciprocal hybrids, but no PCR products for Col-0. Band intensities were similar for C24 and the reciprocal hybrids, indicating about equal transcript levels in these samples (Fig. 7, panel c). After incubation with *Xho*I, only fragments of 113 and 86 bp typical for transcripts of the C24 allele were detected for C24 and the reciprocal hybrids. No un-cleaved 199 bp PCR product, which would indicate transcripts from the Col-0 allele, was detected (Fig. 7, panel d). In conclusion, transcription states observed in the parental inbred lines, i.e., bi-parental transcription from Col-0 and C24 alleles for gene At2g33220 or mono-parental transcription from the C24 allele, for gene At4g29200, remained unaltered in the reciprocal hybrids.

As summarized in Table 2, transcripts were detected from both parental alleles (At2g33220, At1g03420), or from only one parent (At4g29200, At5g15360, At3g19520). No case of a change of the allele-specific transcript status was observed in the reciprocal hybrids compared with the inbred parental lines.

Table 1 Summarized results from analysis of methylation in the 5′ regulatory regions of selected genes

Gene	Methylation in 5′ regulatory region
At1g03420	Not determined
At1g29270	Methylation at both alleles
At2g33220	No methylation
At3g19520	Methylation at both alleles
At3g25905	No methylation
At4g29200	Methylation at both alleles
At5g15360	No methylation

Discussion

Endopolyploidization level, nucleolus size and global distribution of chromatin marks do not differ between inbred parents and their intraspecific hybrids

Endopolyploidization levels have been reported to increase in *A. thaliana* with progressing developmental stage

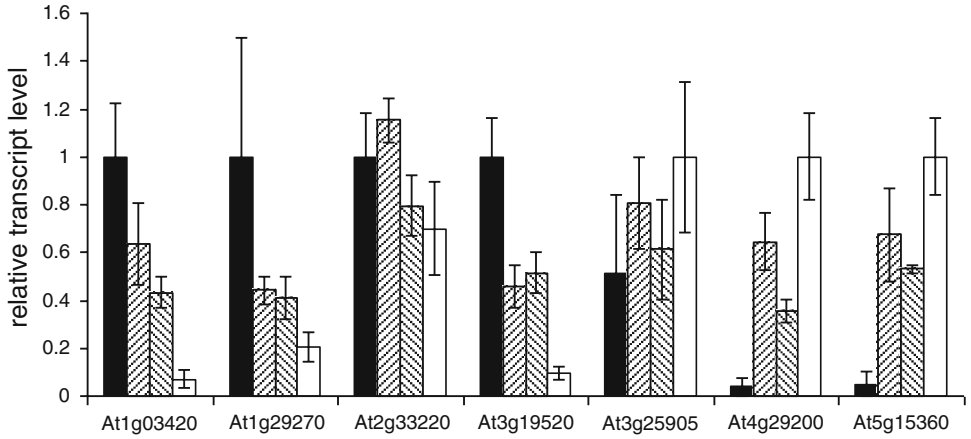


Fig. 6 Relative transcript levels of selected genes obtained by quantitative RT-PCR. The relative transcript levels are compared with those of *ACTIN2* (At3g18780) as a control gene according to the ΔΔCt method (Livak and Schmittgen 2001) using total RNA extracted from seedlings 6 DAS. Data for Col-0 (*black bars*) and C24 (*white bars*) and their reciprocal hybrid offspring (*hatched bars*) are shown. To facilitate comparison, the median relative transcript level of the parental line showing the higher RNA abundance was assigned the value 1.0 for each gene. The corresponding values for the relative transcript levels of the other parental line and the reciprocal hybrids were calculated accordingly. Error bars show standard deviation among three biological replicates

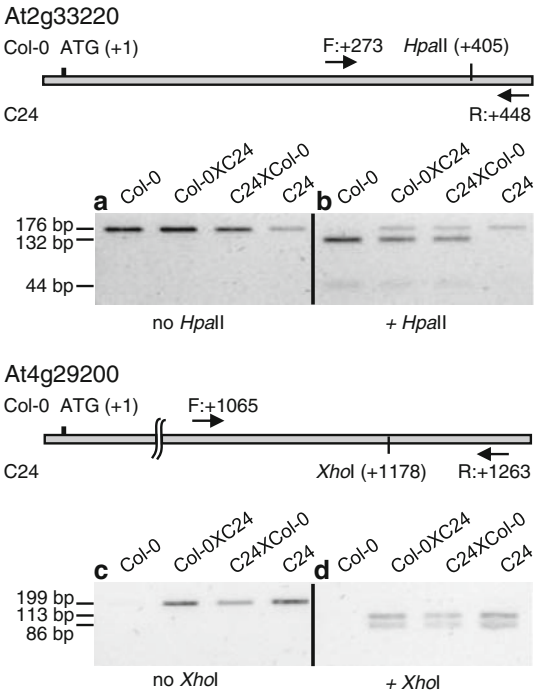


Fig. 7 Semi-quantitative allele-specific analysis of transcript levels of selected genes in inbred parents and their hybrid offspring. Semi-quantitative RT-PCR was done with total RNA from 6 DAS seedling. The primers were designed so that PCR products included a restriction site polymorphic between transcripts of Col-0 and C24 alleles to allow differentiating PCR products according to their parental origin (see cDNA models). Sample results present details for a case of presence of transcripts in both parental lines (At2g33220; **a** and **b**) and presence of transcripts in one parent, but not in the other (At4g29200; **c** and **d**). Inverse images of fluorescence signals after agarose gel electrophoresis and ethidium bromide staining are shown. Table 2 summarizes results for all genes that were examined

Table 2 Summarized results from allele expression assays of selected genes

Gene	Allele expression pattern
At1g03420	From both parents
At1g29270	Not determined
At2g33220	From both parents
At3g19520	From one parent, Col-0
At3g25905	Not determined
At4g29200	From one parent, C24
At5g15360	From one parent, C24

(Galbraith et al. 1991). Thus, increased vigor in hybrid offspring (Meyer et al. 2004) might be correlated with accelerated development, which could result in an increased level of endoreduplication. Nevertheless, no differences in endoreduplication were obvious between Col-0, C24 and their reciprocal hybrids in this study.

Increased rRNA gene activity in hybrid offspring was another assumption that could help to explain the increased growth vigor. Nucleolus area measurements did not detect a consistent increase in hybrids versus inbred parents. Rather, particularly for 4C nuclei, the nucleolus area of accession Col-0 and of hybrid offspring were more similar to each other than to those of C24. Variation of rRNA gene copy number and DNA methylation levels between *A. thaliana* accessions have been reported by Riddle and Richards (2002). Therefore, the subtle difference in nucleolus area measurements might reflect such differences between Col-0 and C24 and might result in slightly different rRNA synthesis. Similarly, no major reorganization

of DNA and histone methylation marks after intraspecific hybridization could be detected by microscopic analysis employing indirect immunolabeling. Nevertheless, it needs to be considered that already chromatin modifications at the single gene level could result in a different plant performance. This is exemplified by the *FWA* gene in *A. thaliana* and related species. Expression of *FWA* can vary dependent on the DNA methylation levels in the 5′ regulatory region of the gene, which has a profound influence on the timing of flowering (Soppe et al. 2000; Fujimoto et al. 2008). Therefore, it would be of interest to extend the study of dynamic histone modification marks to the resolution of DNA sequences by, for example, chromatin immunoprecipitation in combination with whole genome tiling array hybridization (Gendrel et al. 2005).

Intraspecific hybrid formation is accompanied by a slight increase of DNA methylation

Genome-wide DNA methylation analysis by MSAP detected in about 3% of the covered *Msp*I/*Hpa*II recognition sites a change of methylation in Col-0xC24 and C24xCol-0 hybrids in comparison with Col-0 and C24 inbred parental lines. These hybridization-related changes were always toward a gain of methylation at the inner cytosine of the CCGG restriction site. A similar level of differentially methylated sites (approximately 1% gain or loss) was found for intraspecific hybrids of two crop plants, rice (Xiong et al. 1999) and cotton (Zhao et al. 2008). In contrast, 6.8 and 8.3% of sequences of synthesized *Brassica napus* (Xu et al. 2009) and *Arabidopsis* allopolyploids (Madlung et al. 2002), respectively, showed DNA methylation changes in comparison to their diploid progenitors. Surprisingly, in independently formed hybrids of *Spartina* × *townsendii* and *Spartina* × *neyrautii*, 30% of the parental methylation pattern were altered in the hybrids (Salmon et al. 2005). Hence it is likely that intraspecific hybridization, in general, induces less DNA methylation changes than interspecific hybridization. However, in contrast to the crop plants, there was no instance of a loss of cytosine methylation in *A. thaliana* hybrids compared with their parents. These observations suggest that an intraspecific hybridization-driven loss of cytosine methylation is species dependent as also reported for rapid genome reorganization events after allopolyploidization (Ma and Gustafson 2005). A second conclusion can be drawn by comparing DNA methylation patterns in seedlings at 6 DAS and 21 DAS. The number of scorable MSAP signals declined during this time period from 368 to 291. As both *Msp*I and *Hpa*II are sensitive to DNA methylation, this indicates a progressive increase of DNA methylation over time and is consistent with the observations of Ruiz-Garcia et al. (2005) that over-all DNA methylation in *A. thaliana* increases with progressing developmental stages. This effect was independent of the genotype. Both inbred parents and their intraspecific hybrid offspring displayed increased methylation through development.

DNA methylation and transcript levels of single genes were largely unaffected by intraspecific hybrid formation

No change of methylation status was observed in the reciprocal hybrids compared with the inbred parental lines for all seven candidate genes that were analyzed. The same applies for the transcript levels, which were always close to calculated mid-parent-values in the intraspecific hybrids. In this context, it is important to note that the relative transcript levels of parental inbred lines and their reciprocal hybrids determined by quantitative RT-PCR (Fig. 6) and semi-quantitative RT-PCR (Fig. 7) were largely consistent. This confirms the results of allele-specific semi-quantitative transcript analysis. It is not uncommon that relative transcript levels determined by microarray analysis are only partially confirmed by subsequent quantitative RT-PCR. Similar observations were made in several studies on heterosis-related expression changes in maize (Uzarowska et al. 2007; Meyer et al. 2007; Hoecker et al. 2008). No clear correlation was detected between DNA methylation in the 5′ regulatory region (Fig. 5), and the allele-specific transcript levels in *A. thaliana* (Fig. 7). For example for gene At4g29200, DNA methylation was detected in the Col-0- and in the C24-allele at similar level, but transcripts were detected only from the C24 and not the Col-0 allele. This observation is in agreement with the finding that gene expression levels are not necessarily correlated with DNA methylation levels of 5′ regulatory regions of the respective genes (Zhang et al. 2006; Zhang 2008).

In summary, the results suggest that only minor changes of chromatin properties occur in response to intraspecific hybridization in *A. thaliana*. Nevertheless, it should be pointed out that the resolution of DNA and histone modification profiling needs to be drastically increased before any more definitive conclusions about the contribution of chromatin-based mechanisms to gene regulation in this system can be made. It will be interesting to be see whether this observation will hold true for crop species showing heterosis. However, a similarly low level of DNA MSAPs as detected in our system was also observed in rice (Xiong et al. 1999) and cotton (Zhao et al. 2008). It remains to be tested whether the phenomenon of heterosis could rather be explained by the more complex transcriptome composition of hybrids versus inbred lines.

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