

# Changes in genomic methylation patterns during the formation of triploid asexual dandelion lineages

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## Abstract

DNA methylation is an epigenetic mechanism that has the potential to affect plant phenotypes and that is responsive to environmental and genomic stresses such as hybridization and polyploidization. We explored *de novo* methylation variation that arises during the formation of triploid asexual dandelions from diploid sexual mother plants using methylation-sensitive amplified fragment length polymorphism (MS-AFLP) analysis. In dandelions, triploid apomictic asexuals are produced from diploid sexual mothers that are fertilized by polyploid pollen donors. We asked whether the ploidy level change that accompanies the formation of new asexual lineages triggers methylation changes that contribute to heritable epigenetic variation within novel asexual lineages. Comparison of MS-AFLP and AFLP fragment inheritance in a diploid × triploid cross revealed *de novo* methylation variation between triploid F<sub>1</sub> individuals. Genetically identical offspring of asexual F<sub>1</sub> plants showed modest levels of methylation variation, comparable to background levels as observed among sibs in a long-established asexual lineage. Thus, the cross between ploidy levels triggered *de novo* methylation variation between asexual lineages, whereas it did not seem to contribute directly to variation within new asexual lineages. The observed background level of methylation variation suggests that considerable autonomous methylation variation could build up within asexual lineages under natural conditions.

**Keywords:** apomixis, autopolyploidy, DNA methylation, epigenetics, MS-AFLP, within-clone variation

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## Introduction

In plants, DNA (cytosine) methylation plays a role in the suppression of transposon activity (Miura *et al.* 2001) and it influences gene expression (Zilberman *et al.* 2007). It is an important component of the epigenetic mechanisms that affects how genomes are translated into transcriptomes. DNA methylation is implicated in genomic imprinting (Grossniklaus *et al.* 2001) and can cause heritable phenotypes in morphological and life-history traits (Cubas *et al.* 1999; Soppe *et al.* 2000), for instance, when transposon silencing affects transcription of nearby genes (Lippman *et al.* 2004). Furthermore,

methylation patterns are responsive to environmental stimuli (Labra *et al.* 2002; Sherman & Talbert 2002; Aina *et al.* 2004; Chinnusamy & Zhu 2009). It has been proposed that stress-induced methylation repatterning can lead to increased genetic variation by facilitating somatic recombination (Bond & Finnegan 2007; Boyko *et al.* 2007), which could be adaptive during times of stress and in apomictic/clonal lineages.

Not only environmental stimuli but also genomic stresses such as hybridization and polyploidization can induce methylation changes (Adams & Wendel 2005; Dong *et al.* 2006; Grant-Downton & Dickinson 2006). After polyploidization events, sequence loss, methylation-dependent regulation of duplicated genes and re-activation of epigenetically silenced transposons are frequently observed (Comai *et al.* 2000; Lee & Chen

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2001; Shaked *et al.* 2001; Kashkush *et al.* 2002; Madlung *et al.* 2002, 2005; Wang *et al.* 2004a,b; Lukens *et al.* 2006; Chen 2007; Paun *et al.* 2007; Hegarty & Hiscock 2008). Such genomic instabilities may result from regulatory incompatibilities that are created by genome merging or genome doubling and that need to be reconciled (Chen 2007). Polyploidization can trigger immediate methylation alterations during the first or first few generations after the polyploidization event (Wang *et al.* 2004a; Paun *et al.* 2007). In addition, polyploidization can have an indirect effect on patterns of methylation variation that develop subsequently over evolutionary time possibly in response to environmental factors. This is because genome duplications create many opportunities for functional divergence between duplicated genes (Chen 2007; Otto 2007). The methylation effects of hybridization and allopolyploidization (in which two diverged genomes are brought together) are usually more pronounced than the effects of genome doubling per se (Salmon *et al.* 2005; Wang *et al.* 2006). But methylation repatterning is also observed during autopolyploidy (intraspecific genome doubling). For instance, a transgenic and active resistance gene in a diploid *Arabidopsis thaliana* strain experienced stochastic and methylation-associated silencing after autotetraploidization, and also in triploid offspring generated in intraspecific crosses with tetraploid strains, but not in diploid offspring (Scheid *et al.* 1996, 2003).

The methylation status of plant genomes is not reset each generation to the same degree as mammalian genomes are, and a considerable proportion of the methylation marks are stably transmitted across generations (Cervera *et al.* 2002; Riddle & Richards 2005; Vaughn *et al.* 2007). As DNA methylation patterns are dynamic and have the potential to affect phenotypes and to be inherited across generations, there has been speculation about the role of epigenetic inheritance in plant evolution (Kalisz & Purugganan 2004; Richards 2006; Bossdorf *et al.* 2008). Epigenetic mechanisms such as DNA methylation could allow, at least in principle, for transgenerational inheritance of the activity states of genes and not just the genes themselves. While it is currently an open question whether or not epigenetic inheritance plays a significant role in plant evolution, it has been suggested that epigenetic variation could be particularly important to the evolutionary potential of asexual species that harbour little genetic variation (e.g. Wilson *et al.* 2003; Vogt *et al.* 2008).

In the common dandelion (*Taraxacum officinale*), as in several other plant species, polyploid asexual individuals co-occur with diploid sexual conspecifics (van Dijk 2003). Asexual dandelions are mostly triploid apomicts, which derive from diploid sexual mother plants in hybridizations with triploid or tetraploid pollen donors

(van Dijk *et al.* 1999; Tas & Van Dijk 1999; van Dijk 2003; Verduijn *et al.* 2004). Thus, the formation of new apomictic (asexual) lineages is accompanied by a ploidy level change between diploid (sexual) mother and triploid (asexual) offspring. The question we address in this paper is whether the ploidy level change induces *de novo* methylation changes during the first two generations after the polyploidization event. Asexual dandelion lineages have limited opportunities for genetically based adaptation; however, epigenetic variation could be a relevant source of heritable phenotypic variation within lineages. It is therefore of interest to see if the polyploidization event that accompanies the generation of new asexual lineages generates instant epigenetic variation among genetically identical individuals within such new lineages.

Using methylation-sensitive AFLP® fingerprinting, we explored the methylation status of genomewide marker loci in the triploid asexual progeny of a diploid × triploid dandelion cross. Each triploid apomictic F<sub>1</sub> individual is the founder of a different asexual lineage, and *de novo* methylation changes that occur from the parents to the F<sub>1</sub> contribute to epigenetic variation between asexual lineages. Methylation changes that occur in the next generation, that is, in genetically identical triploid offspring of an F<sub>1</sub> apomict, contribute to epigenetic variation among individuals within apomictic lineages. In this study, we examined whether there is evidence for *de novo* methylation variation between and within newly formed asexual lineages.

## Methods

### Plant material

The study species, *Taraxacum officinale* section *Ruderalia* (Asteraceae), is an early-successional perennial herb that is common at disturbed sites and in meadows. In central and southern Europe, sexual and asexual plants occur sympatrically and are frequently found co-occurring within the same populations (Menken *et al.* 1995). Sexual plants are diploid ( $2x = 16$ ) and are usually self-incompatible. Asexual plants are polyploid (usually triploid,  $3x = 24$ ) obligate apomicts, producing clonal seeds in a process that involves unreduced egg cell formation (diplospory), parthenogenetic embryo development and autonomous endosperm formation (van Dijk *et al.* 1999). While apomicts produce unreduced egg cells, their pollen production involves meiosis with random chromosome pairing and segregation. Reduced pollen from triploid apomicts are mostly aneuploid and inviable, but at low frequency, viable haploid and diploid pollen are produced that can fertilize sexual mother plants. New triploids can therefore be generated

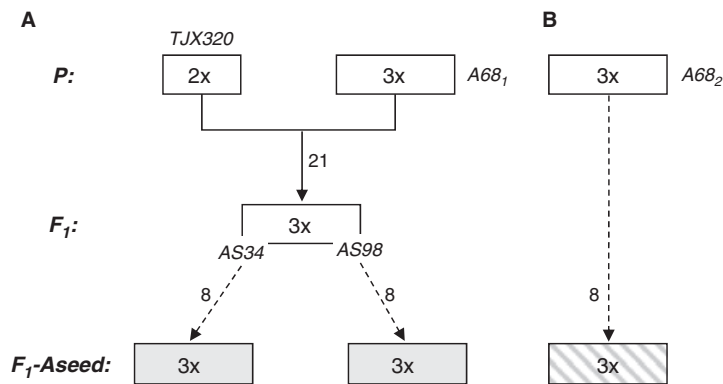
in experimental crosses when a diploid seed parent (with haploid egg cells) is fertilized by diploid pollen from a triploid apomict. Using codominant microsatellite markers, van Dijk & Bakx-Schotman (2004) showed that in triploid progeny of diploid  $\times$  triploid crosses, there is a random association of alleles from the triploid parent (that contributes diploid pollen), indicating that triploid individuals are conspecific to the diploid sexuals. As different components of apomixis segregate independently (van Dijk *et al.* 1999), only a proportion of triploid progeny are functionally apomictic. Via sexual  $\times$  apomict hybridizations, new apomictic genotypes are thought to arise frequently in mixed natural populations (Verduijn *et al.* 2004).

We studied a cross between a diploid sexual seed parent (TJX320) and a triploid apomict pollen donor (A68<sub>1</sub>) (Fig. 1). TJX320 is a male-sterile plant that was collected in east-central France (van der Hulst *et al.* 2004). Male sterility facilitates analysis of progeny because it prevents self-fertilization of the seed parent, which is often induced in diploid  $\times$  triploid matings in the normally self-incompatible plants by the presence of inviable pollen from the pollen donor (mentor effect; Tas & Van Dijk 1999). A68<sub>1</sub> is a plant from an apomictic lineage that was originally collected in an all-apomict population in a meadow near Heteren, The Netherlands. The A68 apomictic lineage arose via natural ploidy level change from a sexual ancestor at an unknown point in evolutionary history, presumably many generations prior to collection because sexual dandelions are absent at the collection site and very rare in this part of the species distribution (van Dijk 2003). This lineage was maintained in the lab for several generations prior to the experiment. F<sub>1</sub> individuals were

screened previously for ploidy level (by measuring nuclear DNA content in leaf samples by flow cytometry; Tas & Van Dijk 1999) and for functional apomixis (production of abundant viable seeds on isolated inflorescences of triploid plants). Diploid progeny, generated by fertilization with haploid pollen, were always sexual and were discarded. We generated 21 triploid progeny from the cross between TJX320 and A68<sub>1</sub>. Seventeen of these progeny were apomictic. In our genotyping study, we included both parents (TJX320 and A68<sub>1</sub>; throughout the paper we refer to these as the parental generation), all 21 triploid progeny (the F<sub>1</sub> generation) and offspring from two apomictic F<sub>1</sub> individuals AS34 and AS98 (eight offspring per individuals; hereafter called the apomictic seed (Aseed) generation F<sub>1</sub>-Aseed) (Fig. 1A). In addition, we included eight offspring from the apomictic plant A68<sub>2</sub> that is an individual of the same A68 apomictic lineage used as the paternal parent in the cross (Fig. 1B). We use the term lineage to denote individuals that belong to the same apomictic genotype and that are presumably genetically identical. Thus, AS34 and its F<sub>1</sub>-Aseed offspring represent one newly found apomictic lineage, AS98 and its F<sub>1</sub>-Aseed offspring represent another newly found apomictic lineage, and A68<sub>2</sub> and its offspring represent a long-established natural apomictic lineage.

#### Growing conditions

Parental and F<sub>1</sub> plants had been maintained for several years under common greenhouse conditions. They were repotted, leaves were clipped above the base of the rosette to promote growth of new leaves, and were subsequently grown in a climate chamber for 2 months (8H



**Fig. 1** Pedigree with genotyped individuals. Solid arrows indicate sexual reproduction and dashed arrows indicate apomictic reproduction. A. Triploid apomictic F<sub>1</sub> individuals in the diploid  $\times$  triploid cross differ genetically from one another and each is a founding individual of a different apomictic lineage. Shaded grey blocks are apomictic offspring from triploid F<sub>1</sub> plants AS34 and AS98; each block therefore consists of genetically identical sibling individuals within newly found apomictic lineages. B. The hatched grey block are apomictic offspring from a plant of the natural A68 apomictic lineage and consists of genetically identical individuals within a long-established apomictic lineage.

dark/16H light at 250 PAR; 20 °C/14 °C; 70% RH) after which leaf tissue was harvested and freeze-dried for subsequent DNA analysis. Previously collected seeds from AS34, AS98 and A68<sub>2</sub> (produced under greenhouse conditions) were germinated on water-saturated filter paper in petridishes for 10 days (14H light/10H dark; 18 °C/14 °C). Seedlings were grown under climate chamber conditions as described above for 5 weeks, after which leaf tissue was harvested and freeze-dried for subsequent DNA analysis. All sample collection took place at similar time of day (between 11:00 h and 13:00 h) to avoid any potential circadian rhythm-related variation. Plants were watered 1× per week with half-strength Hoagland nutrient solution and 2× per week with water.

#### *AFLP and MS-AFLP analysis*

Total DNA was isolated from 2 cm<sup>2</sup> leaf tissue using the hexadecyl-trimethyl-ammonium-bromide (CTAB) procedure (Doyle & Doyle 1990). Amplified fragment length polymorphism fingerprinting (AFLP<sup>®</sup>, patent and registered trademark owned by Keygene N.V.) was performed according to Vos *et al.* (1995). Five *EcoRI/MseI* primer combinations were used: *EcoRI*+TAA/*MseI*+CCA, *EcoRI*+TAA/*MseI*+CTA, *EcoRI*+TAC/*MseI*+CAA, *EcoRI*+TTC/*MseI*+CTA, and *EcoRI*+TTC/*MseI*+CTT. For restriction-ligation reactions per sample 250 ng of DNA was used. Fragments were separated on 4.5% denaturing polyacrylamide gels. Methylation-sensitive amplified fragment length polymorphism (MS-AFLP) fingerprinting followed the same general protocol as AFLP analysis described above, but the *EcoRI* restriction enzyme was replaced by the methylation-sensitive restriction enzyme *HpaII*. *HpaII* cleaves CCGG sequences, but cleaving is blocked when either or both cytosines are fully methylated and may be impaired or blocked when one or both of the cytosines are hemi-methylated (McClelland *et al.* 1994; Roberts *et al.* 2007). Thus, MS-AFLP polymorphisms can arise because of variation among individuals in methylation of the marker locus, also in the absence of genetic (sequence) variation at the locus. We used five *HpaII/MseI* primer combinations, each with 2 (*HpaII*) or 3 (*MseI*) selective nucleotides: *HpaII*+CT/*MseI*+CTA, *HpaII*+CC/*MseI*+CAC, *HpaII*+CA/*MseI*+CAG, *HpaII*+AT/*MseI*+CAG, and *HpaII*+AC/*MseI*+CT. Intensities of AFLP and MS-AFLP gel fragments were quantified using AFLP-Quantar<sup>®</sup> software (registered trademark of Keygene N.V., Wageningen, The Netherlands). In addition, gels were scored visually for presence/absence of fragments. All DNA extraction, AFLP and MS-AFLP analyses were carried out by the KeyGene laboratories, Wageningen, the Netherlands.

#### *Data analysis: segregation of AFLP and MS-AFLP fragments*

*EcoRI/MseI* (AFLP) polymorphisms are typically interpreted as genetic (sequence) variation between individuals. *HpaII/MseI* (MS-AFLP) polymorphisms result either from genetic variation or from methylation variation at the marker locus. To investigate methylation changes that occurred from the parents to the F<sub>1</sub>, we compared segregation of AFLP fragments with MS-AFLP fragments. *De novo* methylation changes are expected to affect MS-AFLP fragment inheritance, but not AFLP fragment inheritance. MS-AFLP markers capture the net effect of genetic plus methylation variation between individuals, where methylation variation may be attributable to segregation of pre-existing methylation variation that was present in the parents and that is stably transmitted to offspring, or *de novo* methylation changes. Genetic variation and pre-existing, stable methylation variation should segregate following Mendelian expectations. But *de novo* methylation variation adds an extra dimension of variability to fragment presence/absence scores that can cause deviations from Mendelian expectation. Thus, if large-scale methylation changes have occurred in the cross, MS-AFLP markers are expected to deviate from Mendelian expectation more often than AFLP markers. One caveat with this approach is that *EcoRI* is not completely methylation-insensitive, but has somewhat reduced cleavage when the cytosine within its restriction site is methylated (Nelson *et al.* 1993; Roberts *et al.* 2007). The difference in methylation-sensitivity between AFLPs and MS-AFLPs may therefore be quantitative and not absolute, but this will still lead to overall differences in segregation between MS-AFLP and AFLP markers if *de novo* methylation changes occur. The segregation analysis could also be affected by age- or developmental stage-dependent variation in methylation patterns within individuals (Lu *et al.* 2008). At the time of tissue collection, parents and F<sub>1</sub> were of different age (although sampled leaves were of similar developmental stage) and age-related methylation variation could therefore contribute to deviations from Mendelian segregation of MS-AFLP markers. However, comparing plants of different age from the A68 apomictic lineage (the pollen donor in the cross A68<sub>1</sub> plus eight apomictic offspring A68<sub>2</sub>), we observed only 1 out of 122 screened MS-AFLP markers that showed different methylation status between plants of different age (data not shown). Thus, age-dependent variation in methylation at the MS-AFLP loci seems to be rare and will therefore not be an important cause of deviations from Mendelian segregation of MS-AFLP markers.

Based on presence/absence scores, polymorphic markers were tested for deviation from Mendelian segregation

using exact chi square tests for goodness-of-fit. Mendelian expectations are worked out for different marker classes assuming that markers are scored dominantly and that triploid progeny randomly receive two chromosome sets from the pollen parent and one from the seed parent. The second assumption requires that chromosome pairing and segregation is random during male meiosis in the triploid pollen donor, and available observations are indeed consistent with this (van Dijk & Bakx-Schotman 2004). Fragments that are present in the diploid maternal parent, but absent in the triploid paternal parent (marker class 'M<sub>1</sub>P<sub>0</sub>''), either do not segregate (when the diploid parent is homozygous at the marker locus) or they segregate with an expectation of 1/2 (when the diploid parent is heterozygous, in which case, half of the progeny inherit the fragment). Fragments that are absent in the diploid parent, but present in the triploid parent ('M<sub>0</sub>P<sub>1</sub>''), either do not segregate (when the triploid parent has two or three copies of the fragment) or they segregate with an expectation of 2/3 (when the triploid parent has one copy of the fragment, which is transmitted to 2/3 of its diploid pollen). Note that because the markers are dominant, segregation goes unnoticed in diploid pollen when the triploid parent has two copies of the fragment. Some M<sub>0</sub>P<sub>1</sub> markers were absent in all 21 F<sub>1</sub> individuals and such markers with zero observations in one of the two categories cannot be analysed using chi square tests. We labelled these markers as significantly deviating from Mendelian expectation after observing that less extreme cases (such as 1 present vs. 20 absent) were significant in the exact chi square tests. Fragments that are present in both parents ('M<sub>1</sub>P<sub>1</sub>'') either do not segregate or they segregate with an expectation of 5/6 (when the diploid and triploid parents each have one copy of the fragment, in which case 1/2 × 1/3 of the offspring do not inherit the fragment from either parent).

#### *Data analysis: variation within apomictic lineages*

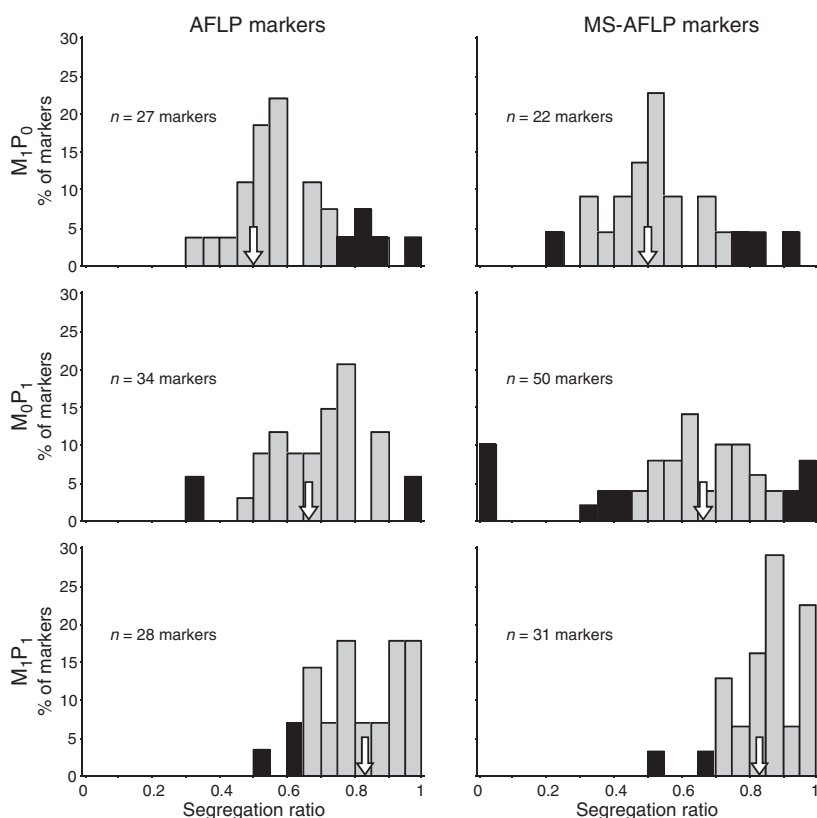
To study methylation changes that occurred from F<sub>1</sub> to their apomictic F<sub>1</sub>-Aseed offspring, we first used AFLP markers to check whether within-lineage apomictic sibs are genetically identical. Conditional on genetic similarity, we interpreted MS-AFLP variation within lineages as methylation variation. For reasons of dominant scoring, part of the variation between individuals is not captured by presence/absence data. In triploids, for instance, a methylation change is only exposed if it makes the difference between zero and ≥1 visible copies of the fragment, but any changes within the range of 1–3 visible copies are not detected. The F<sub>1</sub>-Aseed generation and the offspring from apomict A68<sub>2</sub> were therefore also analysed quantitatively using gel fragment intensity scores. While the relationship between fragment copy

number and intensity may not be linear (for instance, because of PCR steps in the AFLP protocol), intensity data are expected to contain at least some information on dosage variation that could be captured using quantitative analysis (see for instance, Castiglioni *et al.* 1999; Klahr *et al.* 2004). Another advantage of quantitative analysis is that it circumvents difficulties of labelling faint bands as either present or absent. Raw intensity scores were natural log-transformed and samples (gel lanes) were normalized by dividing each fragment score by the mean value of all fragments in the gel lane. This normalization accounts for overall differences in intensity scores between samples, for instance because of slight differences between samples in initial DNA concentrations. For each marker, we calculated the coefficient of variation (CV; standard deviation divided by the mean) of intensity scores among individuals from the same apomictic lineage. CV values were subjected to ANOVA (after natural log-transformation to improve normality of residuals) to reveal effects of apomictic lineage and marker type.

## Results

### *Methylation changes from parents to F<sub>1</sub>*

The fingerprinting yielded 89 AFLP and 103 MS-AFLP markers that were segregating among F<sub>1</sub> individuals or that were polymorphic between parents and F<sub>1</sub>. Observed segregation ratios are presented in Fig. 2. In one of the three marker classes (M<sub>0</sub>P<sub>1</sub>), MS-AFLP fragments deviated more often from Mendelian expectation than AFLP fragments (Table 1; significant deviation in 16 out of 50 MS-AFLP fragments vs. 4 out of 34 AFLP fragments; Fisher's Exact test for independence,  $P = 0.039$ ). In this marker class, extreme deviation from expectation was observed in five MS-AFLP markers, where none of the triploid progeny expressed the fragment that was present in the triploid parent. This is consistent with *de novo* methylation of the marker loci. For these markers, we checked whether fragment presence in the triploid parent could be a technical artefact (in which case fragment absence in the F<sub>1</sub> merely reflects the expected result of a 00 × 000 cross) by typing eight plants from the triploid parent's apomictic lineage A68 and at all five markers, the fragment was present in all eight apomictic individuals. This suggests that the fragments were present and were not affected by methylation changes during apomictic reproduction, but experienced methylation changes (e.g. from unmethylated to methylated restriction sites) in the diploid × triploid hybridization. In the M<sub>1</sub>P<sub>0</sub> and M<sub>1</sub>P<sub>1</sub> classes, no significant difference between MS-AFLP and AFLP fragment inheritance was observed.



**Fig. 2** Segregation ratios of all polymorphic AFLP and MS-AFLP markers in the triploid  $F_1$ , expressed as the proportion of  $F_1$  individuals showing the fragment on gel. Classification of markers by parental genotype:  $M_1P_0$  (top panels), fragment only present in diploid parent;  $M_0P_1$  (middle panels), fragment only present in triploid parent;  $M_1P_1$  (bottom panels), fragment present in both parents. Arrows indicate Mendelian segregation expectation. Black bars indicate markers that deviate significantly from Mendelian segregation expectation.

**Table 1** Proportion of AFLP and MS-AFLP markers that deviate significantly from Mendelian segregation in the  $F_1$ . Markers are classified by parental genotype:  $M_1P_0$ : fragment only present in diploid parent;  $M_0P_1$ : fragment only present in triploid parent;  $M_1P_1$ : fragment present in both parents

Type	Expected ratio	Number of markers	
		Total	Deviating*
<b>AFLP</b>			
$M_1P_0$	1/2	27	5 (18.5%)
$M_0P_1$	2/3	34	4 (11.8%)
$M_1P_1$	5/6	28	3 (10.7%)
<b>MS-AFLP</b>			
$M_1P_0$	1/2	22	4 (18.2%)
$M_0P_1$	2/3	50	16 (32.0%)
$M_1P_1$	5/6	31	2 (6.5%)

\*Exact chi square goodness-of-fit tests,  $\alpha = 0.05$ .

#### Methylation changes within apomictic lineages

In the  $F_1$ -Aseed generation, using 67 AFLP markers, we detected no polymorphisms among sibs from the same apomictic lineage and also apomictic offspring from A68<sub>2</sub> showed no AFLP polymorphisms (Table 2). This confirmed that the apomicts produced offspring that are genetic copies of their parent. Thus, MS-AFLP polymorphisms that are observed among sibs from the same

**Table 2** Within-lineage marker polymorphism in two new apomictic lineages (AS34 and AS98) and one established apomictic lineage (A68) based on eight sibling plants per lineage

Lineage	AFLP markers		MS-AFLP markers	
	Mono-morphic	Poly-morphic	Mono-morphic	Poly-morphic
<b>New</b>				
AS34	67	0	118	4
AS98	67	0	121	1
<b>Established</b>				
A68	67	0	121	1

apomictic lineage are unlikely to be confounded with genetic variation and we interpreted all MS-AFLP variation among sibs from the same apomictic lineage as methylation variation. In the  $F_1$ -Aseed generation and in the apomictic offspring from A68<sub>2</sub>, a vast majority of MS-AFLP markers were monomorphic within lineages and only 1–4 out of approximately 120 markers per lineage showed within-lineage polymorphism (Table 2). When within-lineage polymorphism was present, there were usually several of the eight individuals that showed the non-consensus genotype. In addition, some markers (three in AS34 and two in AS98) were mono-

morphic within lineages, but different from their apomictic  $F_1$  parent. None of these five markers showed evidence of *de novo* methylation change from parents to  $F_1$  (no significant deviation from Mendelian segregation). Thus, these inconsistencies between  $F_1$  apomicts and their  $F_1$ -Aseed offspring might reflect novel changes that occurred from  $F_1$  to the next generation, rather than reversions of methylation changes that had occurred from parents to  $F_1$ . Alternatively, as  $F_1$  apomicts and  $F_1$ -Aseed offspring were not of the same age at the moment of sampling, it cannot be ruled out that some of these differences reflect age-dependent methylation variation.

To explore within-lineage methylation variation in more detail, we analysed quantitative variation in fragment intensity scores among apomictic lineage members in the  $F_1$ -Aseed generation and in the apomictic offspring from A68<sub>2</sub> (Fig. 3). For individual markers, variation in fragment intensity between plants from the same lineage was typically higher for MS-AFLP than for AFLP markers (ANOVA, effect of Marker Type on CV values:  $F_{1,475} = 17.5$ ,  $P < 0.001$ ). This is consistent with within-lineage methylation variation. However,

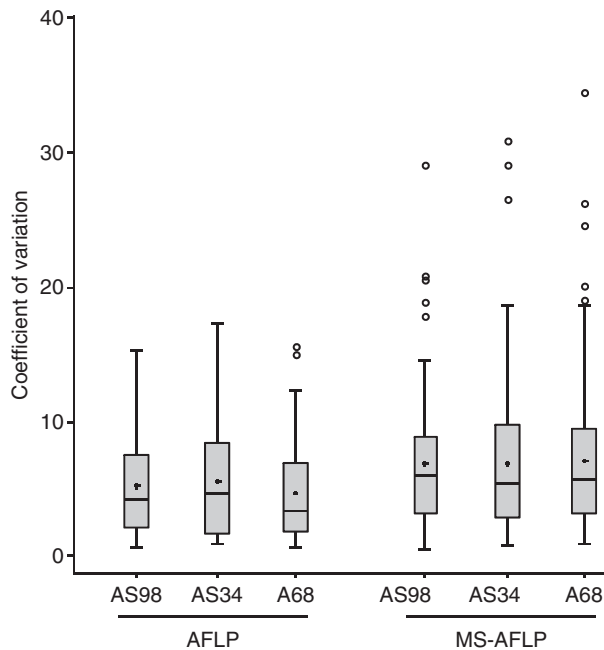


Fig. 3 Distribution of within-lineage coefficients of variation of fragment intensity scores, calculated for each marker among eight sibling individuals per lineage, for two new apomictic lineages (AS89 and AS34 of the  $F_1$ -Aseed generation) and one established apomictic lineage (A68). Left box plots are for AFLP markers ( $n = 50$ ) and right box plots are for MS-AFLP markers ( $n = 109$ ). Boxes cover the 25th–75th percentile and maximum whisker extension is to 1.5 interquartile range distance, with outliers denoted by circles. Lineage medians are indicated by a horizontal line and means by a + sign.

there was no significant difference in CV values between lineages (Lineage effect:  $F_{2,475} = 0.6$ , not significant; Lineage  $\times$  Marker Type interaction:  $F_{2,475} = 0.9$ , not significant). Thus, there is no evidence that methylation variation was generated at an increased level within the newly formed lineages (AS98 and AS34) compared to the established lineage (A68, see Fig. 3).

## Discussion

Using genome-wide marker data, we show that the formation of triploid asexual dandelion lineages in a diploid  $\times$  triploid cross is accompanied by *de novo* methylation changes that contribute to variation between newly formed asexual lineages. Some methylation variation was also observed within the newly formed asexual lineages. But, within-lineage levels of variation were not notably higher in the newly formed lineages than in a long-established natural asexual lineage. These changes may therefore reflect a background rate of methylation change rather than being triggered directly by the recent polyploidization event. Thus, the ploidy level change from diploid mother to triploid offspring triggered methylation repatterning in  $F_1$  plants, but our data do not provide clear evidence that an increased level of methylation or demethylation activity persisted also in the subsequent generation. The ploidy level change therefore does not seem to contribute directly to methylation variation within new asexual lineages. However, the background level of methylation changes that we observed, both in the newly generated and in the long-established apomicts, suggests that considerable methylation variation might build up within a lineage over short evolutionary time scales.

The observation that *de novo* methylation variation was generated among  $F_1$  plants adds to existing evidence that changes in ploidy level per se can initiate methylation repatterning. Methylation repatterning after the merging of two diverged genomes is well-established (Rapp & Wendel 2005), but relatively little is known about the effects of autopolyploidization. Methylation changes after autopolyploidization have been observed at a hygromycin-resistance transgene in *Arabidopsis* (and these changes were associated with modified resistance phenotypes; Scheid *et al.* 1996, 2003), but have not been detected at random marker loci in other systems (Martelotto *et al.* 2007). Furthermore, methylation effects of allopolyploidy are generally attributed to the merging of diverged genomes rather than genome doubling (Salmon *et al.* 2005; Wang *et al.* 2006). In our genomewide analysis of >100 methylation-sensitive markers, we obtained the strongest evidence for *de novo* methylation changes in five MS-AFLP markers that showed a fragment in the triploid pollen parent, but not in any of the  $F_1$  (marker

class  $M_0P_1$ ; see Fig. 2). We never observed the alternative situation: MS-AFLP fragments that were present in the diploid seed parent (marker class  $M_1P_0$ ) were always inherited by at least some of the  $F_1$ . This could indicate that most methylation changes in the cross involved methylation of paternally inherited marker alleles and not methylation of maternal alleles.

Our data indicate that methylation changes occurred from parents to  $F_1$ , but we provide no formal test that these changes exceed background levels of methylation changes occurring from one generation to the next even within apomictic lineages (see for instance Table 2 and Fig. 3). Background variation might be caused, for instance, by imperfect action of the enzymes that maintain cytosine methylation patterns across cell divisions (Vaughn *et al.* 2007). Out of 122 MS-AFLP markers, only one polymorphic marker was observed among sibs in the established apomictic lineage A68 (Table 2), which is consistent with previous studies in other plants that show overall faithful transmission of methylation patterns (e.g. Cervera *et al.* 2002; Riddle & Richards 2005). In contrast, *de novo* methylation in the cross was implicated for at least five MS-AFLP markers (out of 103) that showed extreme deviation from Mendelian segregation and presumably also for some other markers that showed significant, but less extreme deviations from expected segregation (see Fig. 2). Even though the results from MS-AFLP analysis in the cross and the apomictic lineage cannot be compared directly in a straightforward way (for instance, because significant deviation from Mendelian segregation can have other causes than methylation; see below), these results seem more consistent with a cross-induced increase in methylation activity than with a constant background-level rate of methylation changes.

We inferred methylation activity in the cross from the observation that MS-AFLP markers deviated more often than AFLP markers from Mendelian segregation. Notably, AFLP markers also deviated significantly from Mendelian segregation quite often (12 of 89 markers, where only 5% are expected by chance at the  $\alpha = 0.05$  significance level). The reasons for this remain unknown, but factors that can contribute to unexpected segregation include the presence of duplicated marker loci in the genome and systematic biases in scoring very faint bands as either present or absent. These factors are not expected to cause a systematic difference in segregation behaviour between methylation-sensitive and nonsensitive markers. Thus, under the assumption that methylation changes are the main cause of differential segregation, an overall AFLP vs. MS-AFLP comparison captures methylation effects also when AFLP polymorphisms do not segregate strictly following Mendelian rules.

Apart from polyploidization-induced methylation variation, a relevant question from an evolutionary perspective is whether background levels of methylation change, as observed in our study, can build up over time to generate important epigenetic variation within the apomictic lineages. In *Arabidopsis*, DNA methylation patterns are very similar among plants from the same accession that share a very recent common ancestor and very dissimilar among ecotypes (Cervera *et al.* 2002). In our experiment, we saw *de novo* variation arise at one or a few MS-AFLP markers within each lineage (of 122 markers studied) in one generation and under highly controlled environmental conditions. In the absence of genetic variation among apomictic sibs, this reflects autonomous epigenetic variation (Richards 2006) that is not under genetic control. The numbers indicate a rate of autonomous C-methylation change that is many orders of magnitude higher than genetic mutation rates (Baer *et al.* 2007). Environment-induced methylation changes may further contribute to methylation variation within apomictic lineages under natural conditions (Chinnusamy & Zhu 2009; Verhoeven *et al.* in press). Thus, even at very short evolutionary time scales of tens or hundreds of generations, appreciable levels of within-lineage methylation variation could build up, if methylation changes tend to be stably inherited. Whether or not this epigenetic variation rescues some of the evolutionary potential of asexuals (that lack evolutionary potential via genetic variation) is still an open question as phenotypic effects of the observed methylation changes are currently unknown. Striking levels of phenotypic variation are sometimes found in genetically identical clonal plants when grown in common garden experiments and it is hypothesized that methylation variation might underpin such phenotypic variation (e.g. Richards *et al.* 2008). Studies like ours suggest that there might be considerable opportunity for within-clone methylation variation to arise. But the relationship between methylation patterns as detected with MS-AFLP and phenotypic variation remains unclear and requires further study. For instance, while it is evident that some methylation changes can affect gene expression and phenotypes (e.g. Cubas *et al.* 1999; Zilberman *et al.* 2007), other methylation changes are unlikely to have such effects (Vaughn *et al.* 2007). Plants that have naturally low levels of genetic variation, such as apomictic plants, can provide a suitable tool to address the causes and phenotypic consequences of autonomous epigenetic variation.

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