Plant Cell Tiss Organ Cult (2014) 119:289–299
DOI 10.1007/s11240-014-0533-1

ORIGINAL PAPER

DNA methylation changes in triticale due to in vitro culture plant regeneration and consecutive reproduction

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Received: 25 February 2014 / Accepted: 9 June 2014 / Published online: 19 June 2014

Abstract Doubled haploids of triticale are of interest for plant breeders due to hybrid breeding programs based on cytoplasmic male sterility Tt phenomenon. However, (epi)mutations appearing during in vitro culture regeneration may lead to a phenotypic variation that makes the uniformity of plant materials questionable. Using RP-HPLC genomic DNA methylation of donor doubled haploid plants utilized as a source of tissues for the in vitro regeneration (via androgenesis and somatic embryogenesis) of triticale cv. Bogo and their consecutive generative progeny was evaluated. It was demonstrated that in vitro cultures induced a decrease of the DNA methylation of the regenerants independently of the approach used for plant regeneration. The decrease in DNA methylation of genomic DNA proceeded up to the first/second successive generations followed by the beginning of its reestablishment. Moreover, somatic embryogenesis resulted in a higher level of genomic DNA demethylation in regenerants than androgenesis and the process of methylation seems to be affected by donor plant. It is being speculated that long term changes in genomic DNA methylation may be a source of off-type individuals that may spontaneously arise during plant breeding.

Keywords Androgenesis · Doubled haploid · Epigenetics · RP-HPLC · Somatic embryogenesis · *Triticosecale*

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
DAPI	4′-6′ Diamidino-2-phenylindole
DH	Doubled haploid
IAA	Indole-3-acetic acid
ISSR	Inter-simple sequence repeat
MSAP	Methylation sensitive amplified polymorphism
NAA	α-Naphthaleneacetic acid

Introduction

Triticale is an artificial species that originated ca. 130 years ago from a cross between wheat and rye, with the first cultivars useful for breeders available in the 1960s (Mergoum et al. 2009). It exhibits high yield potential, grain quality, resistance to pathogens, favourable amino acid composition and adaptation to adverse conditions (Heger and Eggum 1991). Hybrid breeding based on cytoplasmic male sterility is a promising direction in its cultivation (Oettler et al. 2005). However, homozygous, genetically uniform and stable doubled haploids (DHs) are necessary to maximize heterosis. Such forms could be derived via

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androgenesis employing anther cultures or isolated microspores (Eudes and Amundsen 2005; Żur et al. 2008; Lantos et al. 2013; Würschum et al. 2013).

Tissue culture induced variation (TCIV) in regenerants (Li et al. 2007; González et al. 2013) and somaclonal variation (SV) in progeny of such regenerants (Linacero and Vazquez 1992; Ivanov et al. 1998) have been observed in crops. TCIV may be manifested at the morphological level of regenerants (Jaligot et al. 2000). Usually, off-type plants are eliminated from the breeding population. However, epigenetic changes without obvious morphological effect, are mostly not easily recognized (Bednarek et al. 2007) and may spontaneously come into being even after several generations (Brettell and Dennis 1991). Evidently, tissue culture may induce alterations in DNA methylation pattern (González et al. 2011; Shan et al. 2013) since it may impose stressful conditions (González et al. 2013).

Additionally, cell reprogramming may require resetting of the DNA methylation pattern (Baroux et al. 2011) causing epimutations. Relatively low levels of epigenetic change related to tissue culture were described in barley (Bednarek et al. 2007). Those changes occurred whether regeneration was through either androgenesis or somatic embryogenesis. However, it is not evident whether genome methylation of triticale regenerants derived via androgenesis and somatic embryogenesis and their progeny behaves similarly. Possibly, the complex genome of triticale may react differently as suggested by genomic studies (Bento et al. 2011) as well as molecular and cytological analyses (Brettell et al. 1986).

In triticale, off-type forms derived from tissue culture regeneration are not rare (Pauk et al. 2000). Cytogenetic analysis showed that triticale is genetically unstable during tissue culture manipulation (Lapitan et al. 1984). Chromosome analysis of the root tip cells of regenerated plants revealed that the rye genome was more frequently involved in chromosomal rearrangements than wheat genome (Charmet et al. 1986). Not only tissue culture regeneration, but also generative reproduction may cause genome instability (Suenaga and Nakajima 1993) of the progeny derived from regenerants.

To study the extent of changes induced by tissue culture and inherited by the progeny different methods have been used (Hossain et al. 2003; Peredo et al. 2009; Linacero et al. 2011; Díaz-Martínez et al. 2012). Most of them involved isoschizomers differing in their sensitivity towards DNA methylation sites (Ochogavía et al. 2009; Wang et al. 2013; Machczyńska et al. 2014). The limitation of the system to restriction sites recognized by the endonucleases is apparent (Schrey et al. 2013). Contrary to marker-based approaches, high performance liquid chromatography (HPLC) has been applied in studies of DNA methylation of plants regenerated via tissue culture (Renau-Morata et al. 2005; Rival et al. 2013). This technique supports general information on global DNA methylation and could be applied for the evaluation of differences among plant materials that could be related to spontaneously arising off-type plants (Johannes et al. 2009; Yi et al. 2010).

Tissue culture is a stressful environment that may lead to numerous epigenetic changes. These changes may be transmitted to the next generations. However, it is not clear how long such changes may persist in the progeny. The purpose of the study was to evaluate the level of global epigenetic changes related to genomic DNA methylation among donor triticale plants, their regenerants derived via different in vitro culture methods and consecutive generative progenies of the regenerants using RP-HPLC approach.

Materials and methods

Plant material

Four lines of DH donor plants (each line was represented by vegetatively multiplicated clones, Table 1) of winter triticale cv. Bogo (Oleszczuk et al. 2004) were used to generate regenerants via shed microspore culture (M), anther culture (A), and immature zygotic embryo culture (E). The regenerants were used to obtain the first, the second and the third generative progenies. Donor plants, their homozygous regenerants and their consecutive progenies formed a set. Each plant within the set had its complete pedigree to a specific donor from which it was derived. There were four of such sets encoded S^1, S^2, S^3 and S^4. The arrangement of the plant material is given in Table 1.

Androgenesis

Anther culture

Plants of winter triticale cv. Bogo have grown under controlled conditions in a chamber room at a photoperiod of 16 h day and 8 h night at 16 and 12 °C, respectively. The tillers were collected, when the microspores were at the mid to the late uninucleate stage and pretreated in a cool room for 4 weeks at 4 °C. Next, the spikes were sterilized (70 % ethanol then 10 % sodium hypochlorite) and rinsed three times for 1 min with sterile water. Subsequently, anthers from one half of the spikes were excised and cultured on solid induction medium 190-2 (Zhuang and Xu 1983) with Phytagel and supplemented with 90 g/l maltose,

Table 1 Number of plants encompassing four sets used for the RP-HPLC analyses

Plant material	Four sets derived from four lines of donor plants			
	S^1	S^2	S^3	S^4
	No. of regenerants and progenies comprising the sets			
D	9	8	12	10
R_M	13	8	6	12
P_M^1	23	14	14	18
P_M^2	20	19	16	16
P_M^3	18	12	18	15
R_A	25	20	22	14
P_A^1	20	13	14	22
P_A^2	15	17	18	24
P_A^3	15	15	18	15
R_E	26	15	15	17
P_E^1	22	14	14	15
P_E^2	15	12	18	18
P_E^3	17	9	18	9
Total	238	176	203	205

S^1, S^2, S^3, S^4 stands for the first, the second, the third and the fourth set, respectively. Each set consists of donors, regenerants and progeny. D, vagetatively multiplicated doubled haploid donor plants; R_M, regenerants derived from shed microspore; R_A, regenerants derived from anthers; R_E, regenerants derived from immature zygotic embryos; P_M^1, P_A^1 and P_E^1, the first progeny derived from R_M, R_A and R_E, respectively. P_M^2, P_A^2, P_E^2 and P_M^3, P_A^3, P_E^3 stands for the second and third generative progeny, respectively

400 mg/l glutamine, 2 mg/l 2,4-D and 0.5 mg/l kinetin at 26 °C in the dark, for a maximum of 8 weeks. Calli and embryo-like structures were transferred onto regeneration medium (190-2 with 0.5 mg/l NAA and 0.5 mg/l kinetin), when they were 2–5 cm in size. Afterwards, green plantlets were kept in an Erlenmeyer flasks, in rooting medium (190-2 with 2 mg/l IAA). After roots appeared, plants were vernalized for 6 weeks in 4 °C and transferred to soil in a greenhouse, then grew to maturity.

Shed microspore culture

The same protocol as for anther culture was used for shed microspore culture from anthers taken from the second half of the same spikes except with a liquid induction medium. Then, appearing callus pieces, as well as proembryos were transferred to solid regeneration medium.

Somatic embryogenesis

Immature zygotic embryo culture

Caryopses obtained after self-pollination of the DH mother plants were cut to remove the embryos at the coleoptile stage under aseptic conditions. Immature embryos were isolated and placed with the scutellum exposed onto MS medium (Murashige and Skoog 1962) with 30 g saccharose and 30 μM dicamba. Developing embryos were transferred onto medium supplemented with 0.9 μM 2,4-D for conversion of somatic embryos to plantlets. Cultures were maintained in controlled conditions at 26 °C under 16/8 h (day/night) photoperiod. Afterwards, the plantlets were transferred to the regeneration medium. The 5 cm long plantlets were transferred on a root formation medium (regeneration and rooting media are the same as for androgenesis). Next, small plants were kept in 4 °C for 6 weeks, then they were placed into soil in a greenhouse and grown to maturity.

Progeny

Individual DH regenerants derived via anther cultures and shed microspore cultures as well as regenerants derived from immature zygotic embryo cultures were selfed by bag isolation. Seeds of those regenerants were advanced to obtain progeny. Some plants were again bag isolated to obtain the second and third generations of progeny.

Flow cytometry

Ploidy status of regenerants after in vitro androgenesis was determined by flow cytometry (Partec; PAII; HBO lamp; Germany). Young regenerated plants were prepared according to Galbraith et al. (1983) with minor modification. Nuclei were isolated from leaves which were chopped with a razor blade in 2 ml of a lysis buffer containing DAPI, afterwards the suspension was filtered through a 30 μm pore diameter filter prior to analysis. Subsequently, histograms of the cell nuclei were generated. The ploidy level of regenerants was determined by comparing the G1 peaks of each sample to a reference (diploid plant).

DNA isolation

Total genomic DNA isolation was performed from young leaves (with flag leaf emerging) of donors, regenerants and their progeny following Plant DNeases MiniKit 250 (Qiagen) manufacturer protocol. DNA quantity was evaluated spectrophotometrically (NanoDrop 1000 Overview). The samples were tested for their integrity and purity on agarose gel with ethidium bromide (1.2 % gel, 1× TBE buffer, 160 V).

RP-HPLC analysis

The plant material used for the RP-HPLC is arranged in Table 1.

Sample preparation

DNA samples (6 μg) were dissolved in 100 μl deionised water and denatured by heating to 100 °C for 2 min following immediate placing on ice for 5 min. DNA hydrolysis was performed in a total volume of 135 μl consisting of 5 μl of 10 mM $ZnSO_4$ and 10 μl of 1.0 U ml^{-1} of P1 nuclease (in 30 mM NaOAc pH 5.4), the samples were gently stirred and incubated for 17 h at 37 °C. After nuclease digestion 10 μl 0.5 M Tris pH 8.3 and 10 μl of 10 U ml^{-1} of alkaline phosphatase (in 2.5 M $(NH_4)_2SO_4$) were added, followed by gentle mixing and incubating at 37 °C for 2 h. After digestion, the samples were centrifuged at 12,000 rpm for 5 min.

Chromatographic assessment of global DNA methylation

RP-HPLC quantification of overall DNA methylation was performed according to the procedure of Johnston et al. (2005) using Waters 625 LC System. Briefly, the 4u Max-RP C12 (250 × 4.6 mm, Phenomenex) column combined with 4u Max-RP C12 pre-column were applied using linear gradient of the eluent A comprising of 0.5 % methanol plus 10 mM KH_2PO_4 at a pH of 3.7 (v/v) and eluent B comprising of 10 % methanol in 10 mM KH_2PO_4, pH 3.7 (v/v) that changed from 100 % of A to 100 % of B within 10 min following 15 min of 100 % B eluent and ending with 100 % of the A eluent for 5 min at a flow rate equal to 1 ml min^{-1}. The absorbance was measured spectrophotometrically (UV detector, $\lambda = 280$ nm). Chromatograph was calibrated with external standards consisting of each DNA nucleosides (5–50 μM), 15–150 μM for RNA nucleosides and 1.5–15 μM of 5-methyldeoxycytidine dissolved in deionised water. On the basis of that data the calibration curves were evaluated and used for the quantification of the nucleosides in the samples with Millennium 32v 4.0 software. The percentage of deoxycytidine methylation in relation to the total content of cytidine was calculated according to the following equation: $5mdC\ \% = [5mdC/(5mdC + dC)] \times 100$, where 5mdC and dC states for 5-methyldeoxycytidine and deoxycytidine, respectively. Each RP-HPLC analysis was conducted in three repeats using DNA samples from plant material arranged in Table 1.

Statistics

One-way analysis of variance (ANOVA) with Tukey's contrasts analysis were performed in R CRAN software (*stats* and *multcomp* packages, respectively). The regression analysis was performed in R CRAN software using *stats* package and polynomial regression was evaluated. For all statistical analyses significance level $\alpha = 0.01$ was applied.

All RP-HPLC results (including three repeats for each sample) based on the whole plant material arranged in Table 1 were used for Tukey's contrasts and regression analysis.

Results

Donor plants

Each donor's genotype was represented by several vegetatively multiplicated individuals (Table 1). No morphological variation among multiplicated plants (representing the given genotype as well as all genotypes) was observed.

Plant regeneration

Embryos and embryo-like structures were observed after 4–8 weeks of culture in darkness in shed microspore culture (Fig. 1a). Microspore development resulted in complete embryo formation with visible cotyledons and embryo axis (Fig. 1b). Androgenesis in anther cultures resulted in lumpy embryo-like structures visible after ca. 6 weeks of culture (Fig. 1c). After transmission of such structures into the light some albino plants were detected (Fig. 1d). Somatic embryogenesis from cultured immature zygotic embryos is shown in Fig. 1e, f. Embryos appeared after 4 weeks of culture on MS medium with dicamba (Fig. 1e) and converted to plants on regeneration medium (Fig. 1f). Self-pollination of regenerants (Fig. 1g) resulted in formation of the first generative progeny (Fig. 1h).

With the exception of albinos, no obvious morphological changes were observed among fertile regenerants as well as among the first, second and third generations of the progeny. However, morphological differences were visible between haploids and diploids. The haploid plants were smaller, had thinner stems and narrower leaves compared to DH plants.

Analysis of ploidy level

There were 63.3 and 71.7 % haploids among regenerants derived via shed microspore and anther culture, respectively (Table 2) with spontaneous diploidization amounting to 27.1 and 27.7 % for the two approaches, severally. Aneuploids were observed for 0.6 and 0.62 % of cases, respectively (Table 2).

Changes in global DNA methylation

The RP-HPLC approach resulted in clearly distinguishable, well-separated peaks corresponding to dC and 5mdC, with retention time equal to 5.44 and 8.43 min, respectively.

The greatest degree (25.4 %) of average global DNA methylation was observed for donors. In case of all regenerants (R) the level of DNA methylation was equal to 24.1 %

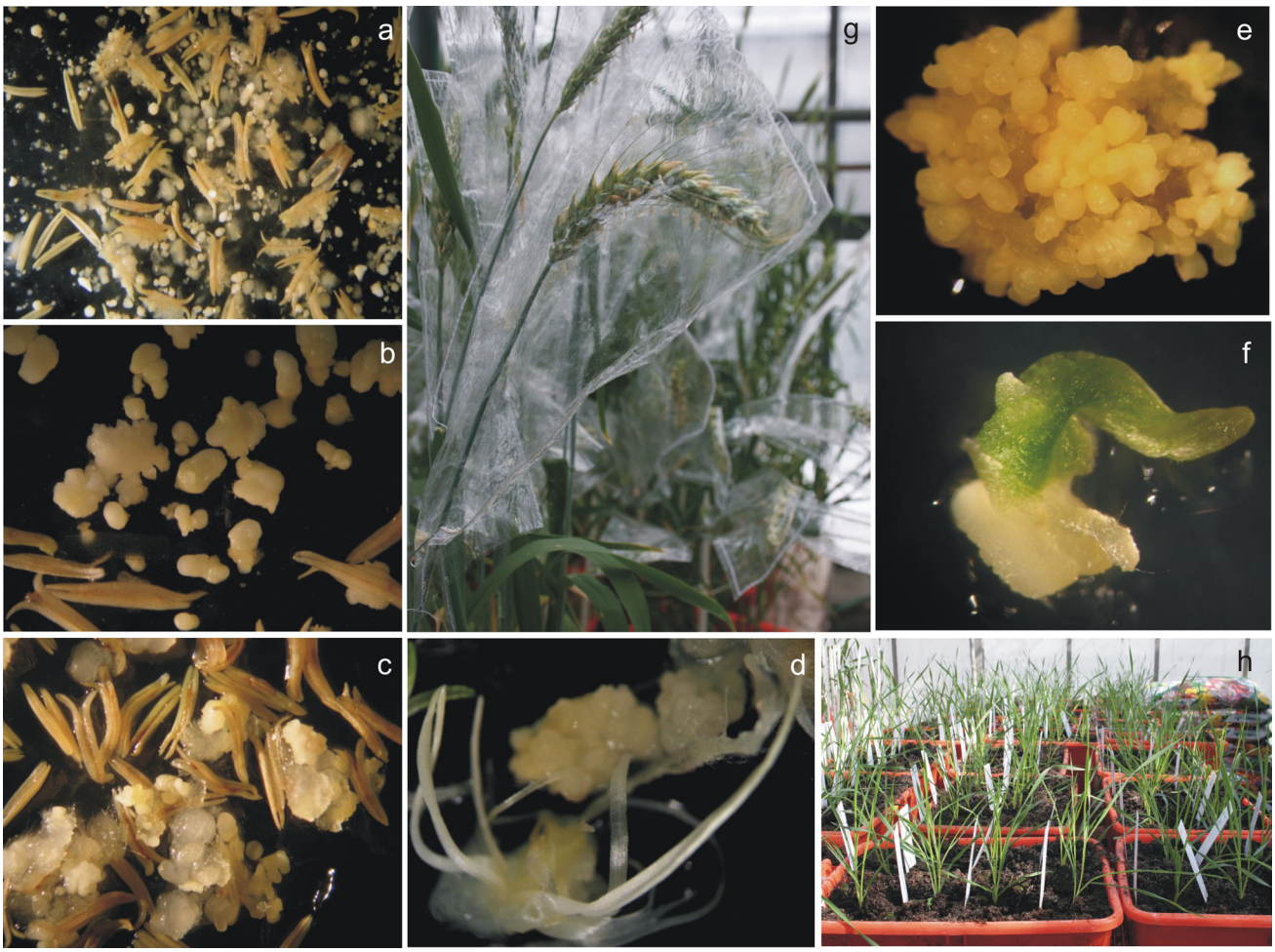

Fig. 1 *Triticosecale* Wittmack cv. Bogo regenerants derived via in vitro culture and their progeny; **a** androgenesis on liquid induction medium, **b** embryo formation from shed microspore culture, **c** androgenesis on solid induction medium, **d** albino plant derived from anther culture, **e** somatic embryogenesis from immature zygotic embryo, **f** germinating embryo, **g** fertile anther culture-derived plants, **h** progenies of regenerants

Table 2 Flow cytometry data. R_M and R_A states for the regenerants derived from shed microspore and anthe culture, respectively

Plant material	No. of plants analyzed	No. of plants with various ploidy levels		
		Haploid	Doubled haploid	Aneuploid
R_M	166	105	45	1
R_A	318	228	88	2

and was the lowest for the first generative progeny (P^1) (23.6 %). Starting from the second generative progeny, global DNA methylation was slightly greater than for P^1 (Table 3).

Comparison of global DNA methylation for D, R, P^1, P^2 and P^3 without considering sets and regeneration method

According to Tukey's grouping (Fig. 2) donor plants differed from regenerants due to genomic DNA methylation. No difference between R and P^1 was observed, however, R and P^1 differed from P^2 and P^3 that formed separate group (Table 4). Polynomial regression (Table 5) calculated for the abovementioned data demonstrated that global DNA methylation decreased from donors to the first generative progeny and then reversed (Fig. 3).

Comparison of global DNA methylation for regenerants and their three generations of progeny by regeneration method

Comparison (ANOVA) of the global DNA methylation of the regenerants derived via shed microspore (M), anther (A) and immature zygotic embryos (E) tissue culture approaches revealed (Fig. 4) that plants obtained via androgenesis differed from those regenerated via somatic embryogenesis (Table 4). The first generative progeny of shed microspore derived regenerants differed from those

Table 3 Arrangement of the mean values of the genomic cytosine methylation evaluated based on RP-HPLC analyses of donor plants, their regenerants divided according to the tissue culture approach and consecutive progeny of those regenerants

	Plant materials				
	D	R	P^1	P^2	P^3
Regeneration method	Mean value of total genomic cytidine methylation (±standard error)				
M	25.39 ± 0.14	24.68 ± 0.41	23.81 ± 0.41	23.96 ± 0.64	24.02 ± 0.22
A		24.43 ± 0.47	23.53 ± 0.47	23.88 ± 0.89	23.90 ± 0.39
E		23.19 ± 0.44	23.40 ± 0.44	23.52 ± 0.86	23.84 ± 0.35

D, Donor plants; R, regenerants; P^1, P^2, P^3, the first, the second, the third generations of regenerants; M, shed microspore culture; A, anther culture; E, immature zygotic embryo culture

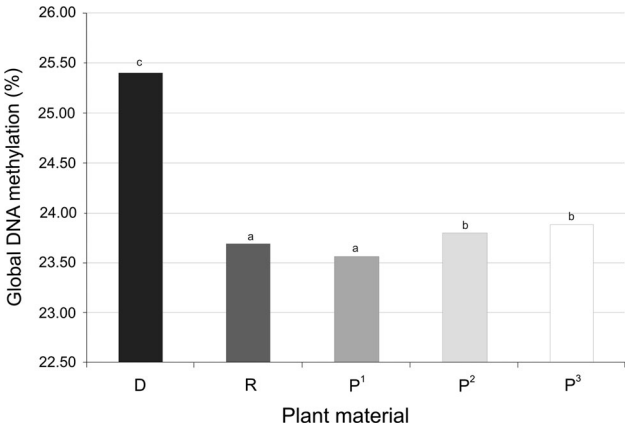

Fig. 2 Tukey's grouping of donors (*D*), regenerants (*R*), the first (P^1), the second (P^2) and the third (P^3) generative progeny of the regenerants without considering sets or tissue culture regeneration method, based on global DNA methylation evaluated via RP-HPLC. Global DNA methylation is expressed as the amount of methylated cytosine to the total amount of cytosine converted to percentages. Materials forming the same group are indicated by either '*a*', '*b*' or '*c*' letters

Table 4 Arrangement of the ANOVA for means comparisons

Plant material	ANOVA for means comparison
D–R–P^1–P^2–P^3	$F = 37.94$; $p < 0.01$
R_M–R_A–R_E	$F = 99.67$; $p < 0.01$
P^1_M–P^1_A–P^1_E	$F = 55.11$; $p < 0.01$
P^2_M–P^2_A–P^2_E	$F = 16.67$; $p < 0.01$
P^3_M–P^3_A–P^3_E	$F = 3.57$; $p < 0.01$
S^1	$F = 61.14$; $p < 0.01$
S^2	$F = 51.91$; $p < 0.01$
S^3	$F = 70.5$; $p < 0.01$
S^4	$F = 53.09$; $p < 0.01$

For the explanation of the abbreviations see Tables 1 and 3

obtained via anthers and the first progeny from somatic embryogenesis derived regenerants (Table 4). P^2_M and P^2_A did not differ from each other according to 5mdC content, although they were distinct from P^2_E (Table 4). In the case of the third generative offspring, overall DNA methylation in P^3_M differed from P^3_E (Table 4), whereas DNA methylation level in P^3_A was similar both to P^3_M and P^3_E.

Regression analysis (Fig. 5) performed for donors, R_A, R_M, R_E and their three generations demonstrated that global genomic DNA methylation decreased in regenerants compared to the donors. The decrease in DNA methylation was observed up to the first/second generative progeny following by reversion of the process. Independently of the tissue culture approach used for plant regeneration the observed trends were significant and followed the polynomial function (Table 5). A little bit higher level of DNA demethylation for R_E than for R_A and R_M was observed.

Comparison of global DNA methylation for sets

Tukey's test revealed significant differences in global DNA methylation of plant material encompassing each of the S^1, S^2, S^3 and S^4 sets (Fig. 6). In detail, in the first set D, R, P^2 differed from each other, whereas differences between P^1 and P^3 were insignificant (Table 4). In the S^2–D, P^1, P^2, P^3 differed from each other, whereas R were similar both to P^1 and P^3 (Table 4). In the third set D, R, P^1, P^2, P^3 differed from each other (Table 4) while in the fourth one D, R, P^2, P^3 differed from each other, whereas P^1 were similar both to R and P^2 (Table 4).

Global genomic DNA methylation changes followed polynomial regression in the case of all sets with local minimum at the level of the first/second generative progeny (Table 5; Fig. 7). All trends were significant. In the case of S^2 and S^4 local minimum was deeper compared with S^1 and S^3.

Discussion

There is ample evidence suggesting that tissue culture is a stressful environment that may be responsible for the induction of abiotic stress during plant regeneration

Table 5 Arrangement of the polynomial regression models

Plant material	Polynomial regression model
D–R–P^1–P^2–P^3	$F = 31.56$; $p < 0.01$; $Y = 24.81031 - 20.74299x + 0.11423x^2$
D–R_A–P^1_A–P^2_A–P^3_A	$F = 44.03$; $p < 0.01$; $Y = 26.45975 - 21.522122x + 0.20587x^2$
D–R_M–P^1_M–P^2_M–P^3_M	$F = 85.61$; $p < 0.01$; $Y = 26.40839 - 21.22876x + 0.14997x^2$
D–R_E–P^1_E–P^2_E–P^3_E	$F = 44.51$; $p < 0.01$; $Y = 25.04682 - 21.13266x + 0.18040x^2$
S^1	$F = 5.698$; $p < 0.01$; $Y = 24.65436 - 20.52956x + 0.07611x^2$
S^2	$F = 79.79$; $p < 0.01$; $Y = 26.91769 - 21.94924x + 0.27377x^2$
S^3	$F = 17.65$; $p < 0.01$; $Y = 25.4608 - 20.9356x - 20.1274x^2$
S^4	$F = 73.56$; $p < 0.01$; $Y = 26.72317 - 2.28005x + 0.35598x^2$

For the explanation of the abbreviations see Tables 1 and 3

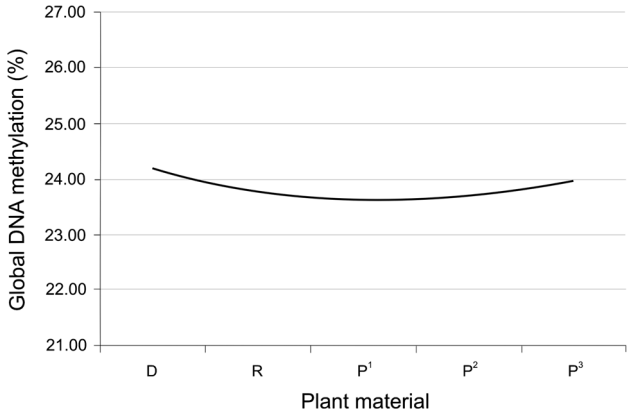

Fig. 3 Polynomial regression of donors (*D*), regenerants (*R*), the first (P^1), the second (P^2) and the third (P^3) progenies of the regenerants (without considering sets and regeneration method). Global DNA methylation is expressed as the amount of methylated cytosine to the total amount of cytosine converted to percentages

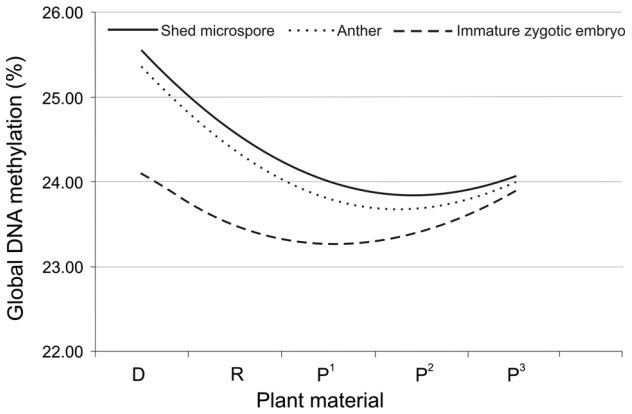

Fig. 5 Polynomial regression analysis of global DNA methylation changes exhibited between *D*, *R*, P^1, P^2 and P^3 materials according to the tissue culture regeneration method. The regression line for D–R_M–P^1_M–P^2_M–P^3_M, D–R_A–P^1_A–P^2_A–P^3_A and D–R_E–P^1_E–P^2_E–P^3_E are given in *black, dotted* and *dashed lines*, respectively

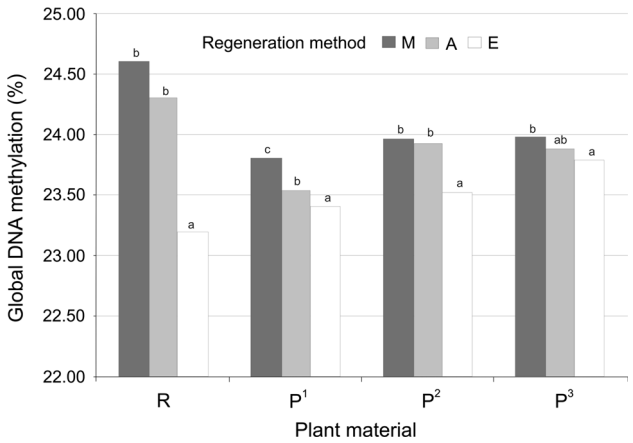

Fig. 4 Tukey's grouping of the regenerants (*R*), the first (P^1), the second (P^2) and the third (P^3) generative progeny of the regenerants based on tissue culture method used. Grouping is indicated by *small letters*. The materials with the *same letter* (e.g. '*a*') form the same group and are distinct from those classified as '*b*' within the group of *R*, P^1, P^2 and P^3, respectively. Global DNA methylation is expressed as the amount of methylated cytosine to the total amount of cytosine converted to percentages. The regenerants derived either via M (*dark grey*), A (*grey*) or E (*white*) approach and their progeny are indicated by *colours*

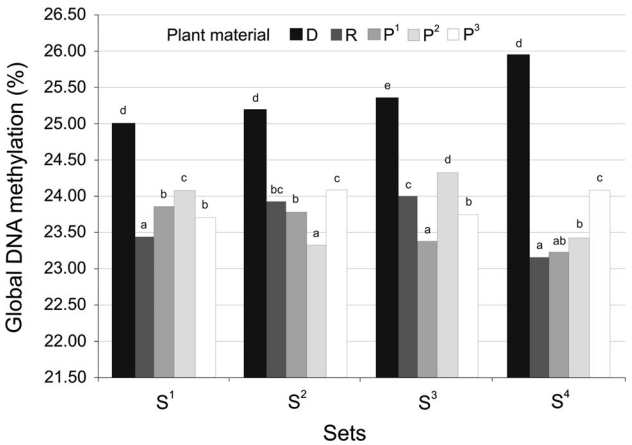

Fig. 6 Tukey's grouping of the donor plants, regenerants and consecutive generative progeny within sets without considering tissue culture approach used for plant regeneration. S^1, S^2, S^3 and S^4 state for sets comprising *D*, *R*, P^1, P^2 and P^3. For detailed description of the abbreviation see Fig 2. Materials with the same *small letter* (indicated on graph) of the given set belong to the same group

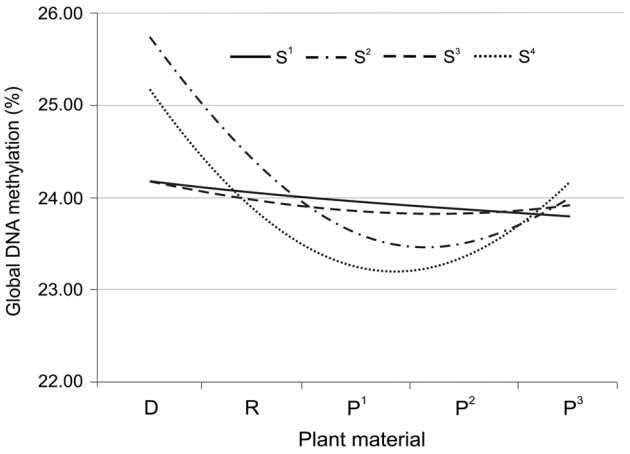

Fig. 7 Polynomial regression reflecting global DNA methylation changes among donors (*D*), regenerants (*R*), their successive generative progeny (P^1, P^2 and P^3) within each of the S^1, S^2, S^3 and S^4 sets

(Miguel and Marum 2011). Such stress may cause the regeneration of the off-type plants that could be detected at the morphological (Li et al. 2010), cytological (Linacero and Vazquez 1992), genetic or epigenetic level (Bednarek et al. 2007; Vining et al. 2013). Some of the changes may subsequently arise among progeny originated from tissue culture-derived regenerants even after many generative cycles (Bregitzer et al. 1998; Kirikovich et al. 2003).

Thus, it is essential to have several sets of plant material encompassing homozygous donor plants, the regenerants derived from them and the progenies obtained from the regenerants to be able to identify epigenetic differences among such materials. The plant material used in our study originated from DH plants that became the source of tissues for the regeneration. The donor plants represented distinct genotypes but did not exhibit any morphological variation. Moreover, generative progeny was obtained by self-pollination under controlled conditions. Thus, any variation among our materials within sets (donor plants, regenerants and their progenies) should be attributed to the changes induced in tissue cultures and transmitted to the next generations. However, possibly due to highly homozygous plant material, as well as the limited number of plants analyzed in our study, we failed to observe at the morphological level any off-type plants among regenerants and their progeny within as well as and among sets. Nevertheless, the differences were revealed for haploids and a few aneuploids detected via flow cytometry among regenerants. The level of spontaneously derived DH plants and aneuploids was similar to prior report (Oleszczuk et al. 2004).

The lack of morphological variation or even cytological uniformity does not, however, exclude changes at the DNA level and especially those related to DNA methylation, which may take place under tissue culture conditions, as showed in barley (Bednarek et al. 2007). Our RP-HPLC study demonstrated that the triticale genome was affected by global DNA demethylation which occurred during tissue culture regeneration. The same direction of DNA methylation changes was observed in our preliminary study on triticale cv. Bogo with metAFLP approach (Machczyńska et al. 2014), where demethylation prevailed over de novo methylation. Our data are congruent with the MSAP studies of genomic DNA methylation changes performed between rye donor plants and their regenerants where the level of demethylation was greater for the regenerants (González et al. 2013). Similar results were obtained also in rye using *Hpa*II and *Msp*I digested DNA amplified with ISSR markers (Linacero et al. 2011). It was demonstrated that the decrease in DNA methylation at the level of regenerants may be caused by the necessity of genes activation during cell reprogramming to facilitate plant regeneration (Kaeppler and Phillips 1993).

Our RP-HPLC results showed no difference in the genomic DNA methylation level between shed microspore and anther culture approaches while androgenic regenerants exhibited greater methylation than somatic embryogenesis derived ones. It is well documented that a callus phase induces variation during plant regeneration (Bouman and Klerk 2001). In our experiment immature zygotic embryo plant regeneration encompassed an obvious callus stage whereas androgenesis, especially in the case of shed microspore cultures, largely circumvents callus. Thus, the longer callus duration of somatic embryogenesis compared to androgenesis may have been responsible for the differences in the level of DNA methylation evaluated between regenerants derived via two paths (Xu et al. 2004). Alternatively, the tissue culture medium composition could be also responsible for the difference in global DNA methylation. Plant growth regulators may enhance in vitro induced variation resulting from epigenetic modifications (Nehra et al. 1992; Bairu et al. 2006). Study on *Malus xiaojinensis* revealed increased DNA methylation with increasing 2,4-D (Huang et al. 2012). To induce the androgenic response in our experiment 2,4-D was supplemented to the induction medium, which may have been responsible for the greater genome-wide methylation in androgenic than in somatic embryogenesis derived regenerants. Immature zygotic embryos were cultured on a medium containing dicamba reported to cause more negative changes than 2,4-D (Rakoczy-Trojanowska 2002). It might be suggested that higher demethylation in somatic embryogenesis derived regenerants in triticale was caused by the presence of dicamba. Alternatively, the differences in global DNA methylation observed between androgenesis and somatic embryogenesis derived regenerants could have been due to cold stress used to convert the gametophytic pathway to a sporophytic one. Studies on

stress-induced epigenetic changes confirm that cold treatment induces DNA demethylation (Fan et al. 2013; Shan et al. 2013). Moreover, the differences in DNA methylation observed between androgenesis and somatic embryogenesis derived regenerants could be also attributed to the ploidy level. The relationship between DNA methylation and haploids versus diploids was studied in rice (Zhang et al. 2006). The authors concluded that the higher methylation level in haploids might be a readjusting reaction to the decrease in ploidy because of survival. If DH plants inherit the increased DNA methylation e.g. via replication mechanisms then our data is congruent with the abovementioned results.

In our study significant methylation change induced in vitro and revealed by ANOVA affected not only regenerants but also their consecutive generative progeny. Reversion of the DNA demethylation in the first/second generation demonstrated by polynomial regression analysis may have reflected the very beginning of the genomic DNA methylation re-establishment. Apparently, plant regeneration via tissue cultures resulted in epigenetic changes that were not fully compensated even after several generative cycles. Based on our experiment, however, it was not possible to predict whether further generative cycles would result in DNA methylation comparable to that of donor plants or whether the process would behave differently. Although, in general, the trend of global DNA demethylation and its reversion was observed for all sets, the differences among them were obvious. Most probably this may reflect genotypic differences existing between donor plants. Obviously, revealed DNA methylation changes observed among sets suggest that triticale plant material derived via in vitro cultures and its progeny may be prone to epimutations dependent on DNA methylation that maybe exhibited among e.g. regenerants' progeny.

Based on our HPLC analysis, we demonstrated for the first time that the level of genomic DNA methylation alterations between donor triticale plants, their regenerants derived via different in vitro culture methods and consecutive generative progenies of the regenerants was affected by in vitro culture. Moreover, genomic DNA demethylation of the regenerants depended on both regeneration method and genotype of donor plants. Evidently, re-establishment of the triticale genomic DNA methylation level after tissue culture treatment may take time and special care needs to be taken to control epimutations in regenerants.

Acknowledgments The authors wish to thank Dr. Sylwia Oleszczuk for providing seeds of triticale cv. Bogo and for her substantial help in plant regeneration.

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