

Tissue culture-induced variation at simple sequence repeats in sorghum (*Sorghum bicolor* L.) is genotype-dependent and associated with down-regulated expression of a mismatch repair gene, MLH3

Meishan Zhang · Huan Wang · Zhenying Dong ·
Bao Qi · Kezhang Xu · Bao Liu

Received: 29 August 2009 / Revised: 19 October 2009 / Accepted: 25 October 2009 / Published online: 12 November 2009
© Springer-Verlag 2009

Abstract Somaclonal variation is a common phenomenon associated with plant tissue culture. Microsatellites or simple sequence repeats (SSRs) are ubiquitous components of eukaryotic genomes, and are intrinsically unstable under various stress conditions including tissue culture. Here, we assessed genetic stability of a set of 29 mapped SSR loci in calli and regenerated plants derived from a pair of reciprocal sorghum inter-strain F1 hybrids and their pure line parents. We further measured the steady-state transcripts of a set of nine mismatch repair (MMR)-encoding genes and a DEMETER (DME), a DNA glycosylase domain protein-encoding gene in these lines, and tested for a possible relationship between altered expression of a given MMR or DME gene and the SSR variations. We found that SSR variations occurred in calli and regenerated plants of both the studied pure lines though at sharply different frequencies (20.7 vs. 6.9%), but no variation was detected in calli and regenerated plants of the pair of F1 hybrids. Compared with the donor seed plants, markedly altered expression of all nine studied MMR genes and the DME gene was observed

in calli, and more conspicuously, in the regenerated plants. However, only one gene, i.e., MLH3, showed an altered expression pattern that is genotype specific and significantly correlated with the occurrence of SSR instability.

Keywords Simple sequence repeat (SSR) · Mismatch repair (MMR) genes · Somaclonal variation · Tissue culture · Sorghum · Pure line versus hybrid

Introduction

Plant tissue culture, being comprised of a dedifferentiation (formation of callus) and a redifferentiation (regeneration into plants) process, is known to induce an array of genetic and epigenetic instabilities, collectively termed somaclonal variation (Larkin and Scowcroft 1981). It has been repeatedly found that a typical feature of tissue culture-induced instability is its complexity, i.e., the variations are comprised of diverse types that were manifested at various levels, phenotypic, chromosomal and molecular (Kaeppeler et al. 2000; Mohan Jain 2001). Largely based on this feature, Phillips et al. (1994) proposed that plant tissue culture-induced variations are likely a “self-imposed” mutagenesis due to breakdown of normal cellular controls.

The difference between a hybrid genome and a pure line one remains a tantalizing enigma, as it underlies the basis of the fundamental biological phenomena such as hybrid vigor (heterosis) and inbreeding depression. We recently reported in sorghum (*Sorghum bicolor* L.) that there exists a sharp difference in the degree of both genetic and epigenetic instabilities at randomly sampled genomic loci under tissue culture conditions between F1 hybrids and their parental pure lines, with the former being highly stable while the later highly mutable (Zhang et al. 2009).

Communicated by P. Kumar.

Electronic supplementary material The online version of this article (doi:10.1007/s00299-009-0797-9) contains supplementary material, which is available to authorized users.

M. Zhang · H. Wang · Z. Dong · B. Qi · B. Liu (✉)
Key Laboratory of Molecular Epigenetics of MOE,
Institute of Genetics and Cytology,
Northeast Normal University,
130024 Changchun, People’s Republic of China
e-mail: baoliu@nenu.edu.cn

M. Zhang · K. Xu
Department of Agronomy, Jilin Agricultural University,
130118 Changchun, People’s Republic of China

More interestingly, this differential genomic instability in the F1 hybrids versus their parental pure lines appears highly correlated with the ability to tune expression of a set of genes responsible for maintenance and perpetuation of cytosine methylation states in a coordinated manner, in the former but not in the later, during the course of tissue culture (Zhang et al. 2009). This study suggests that the epigenetic difference (ability to coordinately tune the expression of a set of genes responsible for maintaining DNA methylation state) between plant genotypes (i.e., a pure line and a hybrid) plays an important role in protecting the genomic integrity under certain stress conditions. This is consistent with the findings in *Arabidopsis* cell suspension cultures, which have elegantly established that misregulated epigenetic mechanism under the cell culture conditions is largely responsible for the incurred somaclonal variations (Tanurdzic et al. 2008).

Microsatellites or simple sequence repeats (SSRs) are tandem repeats of short (1–6 bp) DNA sequences, which exist throughout the genome. Such genomic regions are intrinsically unstable, as the repeated sequences undergo frequent changes in their tract length, causing either contractions or expansions of the number of repeat units (size variation in the repeat-motif) (Nag et al. 2004). Because of this feature, SSRs have several advantages as molecular markers for monitoring genome instability, including their strong discriminatory power, co-dominant inheritance, and greater reproducibility (Varshney et al. 2005). In addition, recent studies have indicated that changes in the repeat-number may also alter the function and/or expression pattern of a cellular gene, depending on the position of the SSR tracts (Nag et al. 2004). Therefore, SSRs represent targeted genomic loci that are not only suitable for detecting tissue culture-induced somaclonal variations, but also for studying the mutagenic basis of the phenomenon. Although there are several studies reporting the use of SSRs to assess somaclonal variation in plants (Chowdari et al. 1998; Rahman and Rajora 2001; Rodríguez López et al. 2004; Ryu et al. 2007; Schellenbaum et al. 2008; Gao et al. 2009; Marum et al. 2009), none has gone a step further as to explore the possible causes for the SSR variations under the tissue culture conditions. In this respect, it is notable that several studies have indicated that alteration of the dinucleotides' repeat-tract length is characteristic of certain types of human carcinogenic cells, which is associated with mismatch repair (MMR) defects (Yu et al. 2006; Haugen et al. 2008; Vilar et al. 2008; Bertagnolli et al. 2009). These studies suggest that mutations in SSRs might be a consequence of, or at least related to, disproportional titration and/or malfunctioning of the MMR proteins under certain developmentally and/or environmentally perturbed conditions such as in vitro cell and tissue culture.

As a first step to address the mechanistic aspect of tissue culture-induced SSR instability, we assessed genetic stability of a set of SSR loci in the same set of calli and regenerated plants of a pair of sorghum inter-strain F1 hybrids and their parental pure lines that we used previously to assess genetic and epigenetic variations at random genomic loci (Zhang et al. 2009). We further measured the steady-state transcripts of a set of MMR-encoding genes and a DEMETER (DME), a DNA glycosylase domain protein-encoding gene (which is known to be involved in base-excision repair, and hence might be related to maintaining SSR stability) in these lines, and tested for the possible correlations between altered expression of a given repair gene and the SSR variations. Here, we report that genetic instability of the set of studied SSR loci occurred at sharply different frequencies in calli and regenerated plants among the sorghum genotypes including two pure lines and their resultant pair of reciprocal F1 hybrids, and that the occurrence of SSR instability is negatively correlated with the extent to which a particular MMR gene (MLH3) being up-regulated in calli and regenerated plants of a given sorghum genotype.

Materials and methods

Plant materials

A pair of sorghum inter-strain F1 hybrids, designated as AD and DA (the first and second letters denoting maternal and paternal parents, respectively), and their corresponding parental pure lines, YN336 (A) and YN267 (D), were kindly provided by the Institute of Crops, Jilin Academy of Agricultural Sciences, Changchun, China. The pure lines had been maintained in our hands by strict self-pollination for many generations, while the hybrids were made by careful manual pollination.

Tissue culture

Two weeks after pollination, immature seeds were collected from the plants of sorghum pure lines and hybrids. The immature seeds were immersed in 70% ethanol for 30 s, and then immersed in 0.1% (w/v) mercuric chloride solution for 3 min and finally rinsed three times with sterile-distilled water. Twenty immature embryos were aseptically removed and inoculated onto MS basal medium supplemented with 2 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 mg l^{-1} kinetin (KT) and 3% sucrose, which was solidified by 0.68% agar. The pH of the medium was adjusted to 5.8 before autoclaving. After 3 weeks of culture at $26 \pm 1^\circ\text{C}$ in darkness, calli were subcultured every 2–3 weeks on MS basal medium supplemented with

0.5–1.0 mg l⁻¹ 2,4-D, 0.5 mg l⁻¹ KT, 3% sucrose, and 0.68% agar at 26 ± 1°C in darkness for 6 months. Then the embryogenic calli were selected out and transferred to MS basal medium supplemented with 1,000 mg l⁻¹ proline and 1,000 mg l⁻¹ polyvinylpyrrolidone (PVP), 3% sucrose, and 0.72% agar at 26 ± 1°C under a 14 h photoperiod for plant regeneration. Regenerated shoots over 5 cm long were transferred onto a rooting medium (growth regulator-free half strength MS medium containing 1,000 mg l⁻¹ PVP, 2% sucrose, and 0.68% agar) for root development and shoot strengthening. When grown to 10 cm in height, the plantlets with healthy roots were removed from medium, rinsed in tap water and transplanted into a mixture of sterilized soil, and grown under humid conditions in a growth room.

DNA isolation and polyacrylamide gel electrophoresis

Genomic DNA was isolated from expanded leaves of the donor seed plants and four independent regenerated plants, and from pooled calli, of a given genotype using the high salt CTAB method. Twenty-nine SSR primers (Wu and Huang 2007) were used (supplementary Table 1). The amplification products were resolved in a 5% denaturing polyacrylamide gel electrophoresis and visualized by silver staining. Only clear and completely reproducible bands in two experiments using independent DNA extractions were scored.

Recovery and sequencing of SSR bands

The fragment showing SSR sequence alteration in calli or/and regenerated plants relative to the donor plants was excised from polyacrylamide gels, eluted and re-amplified and placed in 1 × TE, heated at 70°C for 90 min to elute the DNA. The recovered DNA was re-amplified with the original SSR primer pairs. Sizes of the PCR products were verified by agarose gel electrophoresis, and then cloned into the pMD18-T vector (Takara Biotech. Inc., Dalian). The cloned DNA segments were sequenced with vector primers by automated sequencing.

Real-time reverse transcriptase (RT)-PCR analysis

Total RNA was extracted by Trizol Reagent (Sangon, Shanghai, China) following the manufacturer's protocol. The RNA was treated with DNaseI (Invitrogen) and the RT reaction was performed using a RT system (Invitrogen) following the manufacturer's protocol. Real-time PCR was performed using a Roche Light Cycler 480 apparatus (Roche Inc.) according to the manufacturer's instruction and SYBR Green Real-time PCR Master Mix (A3477L) as a DNA-specific fluorescent dye. Expression of a sorghum actin gene (accession no. X79378) was used as the internal

control. The primers for the sorghum actin gene, a set of nine MMR genes and a DNA glycosylase (DME) gene, were designed by the Primer 3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and are given in supplementary Table 2. Thermal cycling conditions consisted of an initial denaturation step at 95°C for 30 s, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C. Three batches of independently prepared total RNAs were used as technical replications. The relative amounts of the gene transcripts were determined using the Ct (threshold cycle) method. The Ct (threshold cycle) was defined as the number of cycles required for the fluorescence signal to exceed the detection threshold. The relative amounts of the gene transcripts were determined using the software provided by the Roche Company, and calculated by the 2^{-ΔΔCt} method [$\Delta\Delta C_t = (C_t \text{ gene} - C_t \text{ actin}) \text{ calli or regenerant} - (C_t \text{ gene} - C_t \text{ actin}) \text{ seed plant}$]. Quantitative results were given as mean expression (means ± SD), by assuming expression level of the seed plants as “1” and those of the calli and regenerants as percentages of 1 (Wang et al. 2009).

Statistics

Statistical significance was determined using SPSS 11.5 for Windows (<http://www.spss.com/statistics/>) and analyzed by independent samples Student's *t* test. Correlations between the SSR variation frequencies and expression state (relative steady-state transcript abundance) of the MMR- and DNA glycosylase-encoding genes were calculated using the Pearson correlation analysis.

Results

Variation occurred at both the repeat-motifs and their flanking regions of certain SSR loci in calli and regenerated plants of sorghum in a genotype-dependent manner

Twenty-nine mapped SSR primer pairs (supplementary Table 1) were used to investigate possible microsatellite DNA instability in calli and regenerated plants from two sorghum pure lines and their reciprocal F1 hybrids. No variation was observed at 22 SSR loci in any of the studied plant lines; however, six (*SBKAFGK1*, *Xtxp57*, *Xtxp60*, *Xtxp197*, *Xcup15* and *Xcup11*) and two (*Xcup15* and *Xtxp50*) SSR loci showed size variation at the repeat-motif in calli and all four independently regenerated plants in pure lines A and D, respectively. Repeat-motif size variation of the *Xtxp15* locus occurred in both A and D (Table 1; Figs. 1, 2). Taken together, SSR repeat-motif size variation occurred in tissue culture of sorghum pure lines A and D at frequencies of 20.7% (6/29) and 6.9% (2/29), respectively,

Table 1 Variation at SSR loci in sorghum tissue culture determined by pairwise sequence comparison between the donor seed plant and the calli/regenerants

SSR locus	Genotype	Changes in calli and/or regenerants
<i>SBKAFGK1</i>	A	S ACAGCGC(AGC) ₃ ~ ~ ~ ~ ~ (AAC) ₅ AGTTCTTGTTCAGCACTCAGTCCAC
		C/R ACAGCGC(AGC) ₃ <u>AGCAACAACAACAAC</u> (AAC) ₅ AGTTCTTGTCA <u>T</u> CACTCAGT <u>G</u> CAC
<i>Xtxp50</i>	D	S ATGCAGAAAGG(TC) ₁₂ <u>TC</u> (AC) ₁₂ ~ ~ ~ ~ ~ GCACGC
		C/R ATGCAGAAAGG(TC) ₁₂ ~(AC) ₁₂ <u>ACACACACACACACCCCAAC</u> GCACGC
<i>Xtxp57</i>	A	S GGCA(GT) ₁₂ <u>GT</u> (GT) ₃ <u>GT</u> GATTGCTTCGCGCCTTCGCTTCAACTCTTCAAGAGGGTT
		C/R GGCA(GT) ₁₂ <u>AT</u> (GT) ₃ ~GATTGCTTCGCGCCTTCGCTTCAACTCTTCAAGAGGGTT
		S GGCA(GT) ₁₆ <u>GTGTGT</u> GATTGCTTCGCGCCTTCGCTTCAACTCTTCAAGAGGGTT
		C/R GGCA(GT) ₁₆ ~GATTGCTTCGCGCCTTCGCTTCAACTCTTCAAGAGGGTT
<i>Xtxp60</i>	A	S ACTA(GT) ₄ GC(GT) ₆ ~ ~ GGAAAGAATCT
		C/R ACTA(GT) ₄ GC(GT) ₆ <u>GT</u> GGAAAGAATCT
<i>Xtxp197</i>	A	S TCAGGT(CA) ₂ A(CA) ₉ ~ ~ ~ ~ ~ (A) ₁₀ CTTGGCC
		C/R TCAGGT(CA) ₂ A(CA) ₉ <u>CACAAAAAA</u> (A) ₁₀ CTTGGCC
		S TCAGGT(CA) ₂ A(CA) ₉ ~ ~ ~ ~ ~ (A) ₁₂ CTTGGCC
		C/R TCAGGT(CA) ₂ A(CA) ₉ <u>CACAA</u> (A) ₁₂ CTTGGCC
<i>Xtxp15</i>	A	S AT(TC) ₈ <u>TCTCTCTCTCTCTCT</u> TCTCATTCACTTCTTCTCTCTCTTTCCCATCCA
		C/R AT(TC) ₈ ~TTCTCATTCACTTCTTCTCTCTCTTT <u>T</u> CATCCA
	D	S ATT <u>C</u> (TC) ₇ ~ ~ ~ ~ ~ TTCTCATTCACTTCTTCTCTCTCTTTCTCATCCA
		C/R ATT <u>T</u> (TC) ₇ <u>TCTCTCTCTCT</u> TCTCATTCACTTCTTCTCTCTCTTTCTCATCCA
<i>Xcup11</i>	A	S GCCAGCTCTA(GCTA) ₄ T ~ ~ ~ ~ ~ (AGTACTC)AGTAGGAGCTAGCGAG
		C/R GCCAGCTCTA(GCTA) ₄ <u>TAGTACTC</u> (AGTAG <u>T</u> C)AGTAGGAGCTAGCGAG

Repeat-number variations (additions or deletions) and point mutations are highlighted in bolded and underlined, respectively

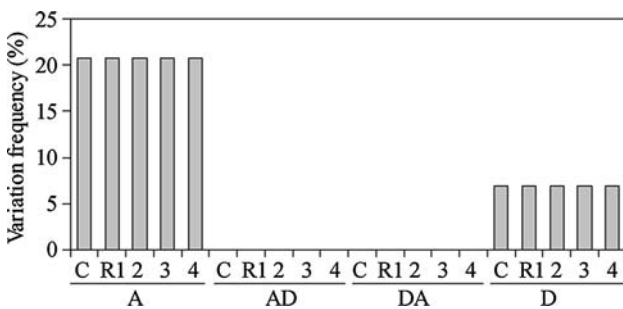


Fig. 1 SSR variation frequencies in calli and regenerated plants from two sorghum pure lines (A and D) and their reciprocal F1 hybrids (AD and DA)

indicating substantial genotypic difference between the two pure lines (Fig. 1). In contrast to the pure lines, no repeat-motif size variation was detected by any of the studied SSR loci in calli and four regenerated plants of the reciprocal F1 hybrids AD and DA. This suggests that, relative to a sorghum pure line genome, an inter-line F1 hybrid genome has an enhanced ability to resist to tissue culture-induced genetic instability at the microsatellite loci, thus further pointing to genotypic difference as a major determinant for genomic instability.

Sequencing of all the seven SSR loci that exhibited genetic instabilities in calli and regenerated plants (relative to the donor seed plant) of either or both of the sorghum

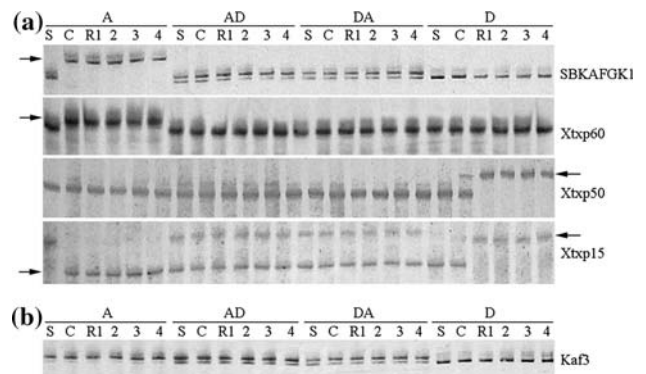
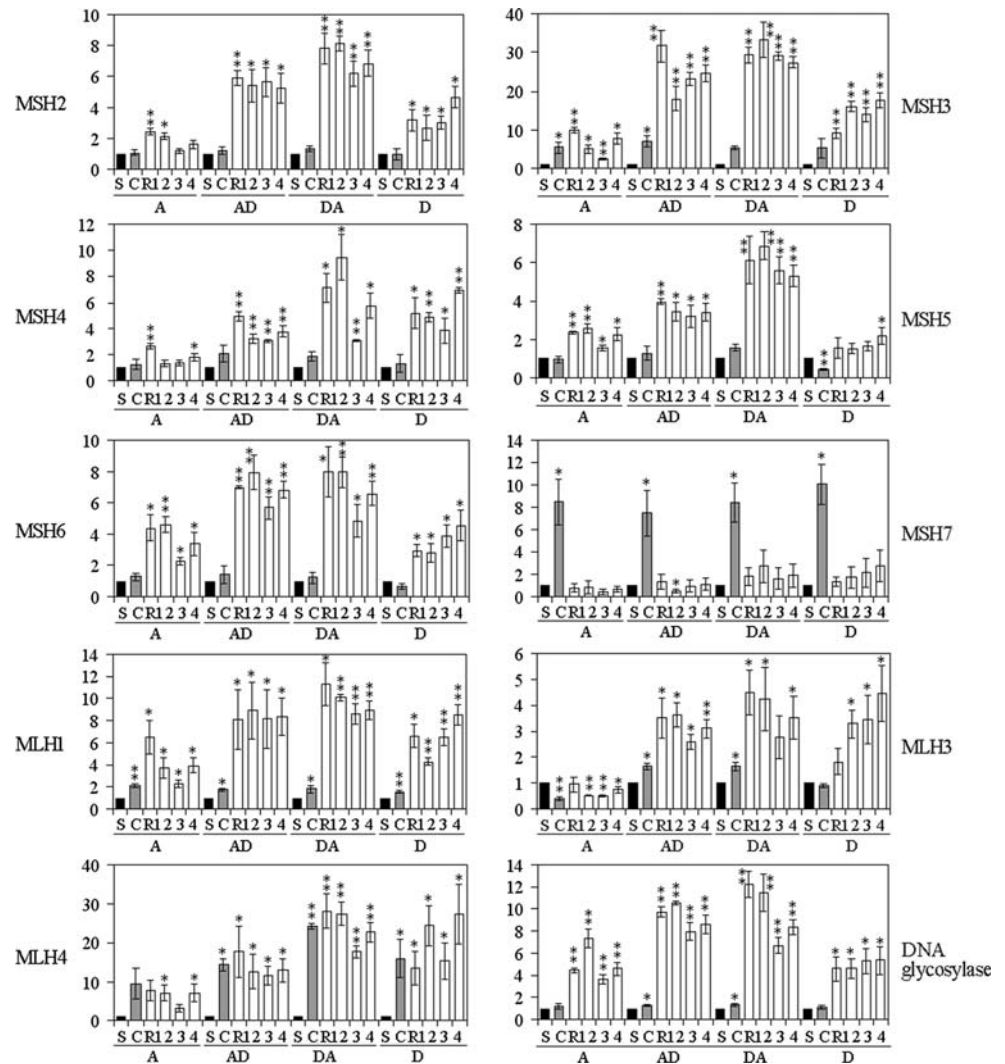


Fig. 2 Examples of SSR fingerprints in the donor seed plants, calli (C) and four independent regenerated plants (R1 to R4) in each of the pair of reciprocal sorghum inter-strain F1 hybrids (AD and DA) and its parental pure lines (A and D). **a** Four SSR loci (labeled on the right margin) showing SSR variations that occurred in calli and regenerated plants of one or both the pure lines. *Arrows* are denoting the SSR variants. **b** A polymorphic SSR locus (*Kaf3*) between the two sorghum parental lines which shows genetic stability in tissue culture of both lines, and hence authenticating identity of the pair of F1 hybrids

pure lines (A and D) verified that they indeed contained alteration in size of the repeat-motifs (Table 1). Four SSR loci (*SBKAFGK1*, *Xtxp60*, *Xtxp19* and *Xcup11*) showed only increase in the repeat-unit number, one (*Xtxp57*) showed only decrease in the repeat-unit number, one

Fig. 3 Alteration in transcript abundance of nine MMR genes and a DNA glycosylase (DME) gene in calli and regenerated plant of a pair of sorghum reciprocal F1 hybrids (*AD* and *DA*) and their parental pure lines (*A* and *D*). Real-time quantitative RT-PCR analysis on transcript abundance of the ten genes was performed on three batches of independent RNA-derived cDNAs with gene-specific primers (supplementary Table 2). The relative amounts of the gene transcripts were determined using the Ct (threshold cycle) method, which was defined as the number of cycles required for the fluorescence signal to exceed the detection threshold. Data were analyzed using the software provided by the Roche Company and calculated by the $2^{-\Delta\Delta Ct}$ method, where, $\Delta\Delta Ct = (\text{Ct gene} - \text{Ct actin})$ calli or regenerant - $(\text{Ct gene} - \text{Ct actin})$ seed plant. Quantitative results were given as mean expression (means \pm SD). The gene names are labeled. Asterisk and double asterisk denote statistical significance at the 0.05 and 0.01 levels, respectively



(*Xtp50*) showed concomitant increase in one repeat-motif (AC) and decrease in another repeat-motif (TC) units, and one (*Xtp15*) showed an increase in number of the same repeat-unit in pure line A but a decrease in pure line D (Table 1).

The sequencing analysis further revealed that apart from alteration in size of the repeat-motifs, three of the SSR loci harbored point mutations within the repeat-motifs. Specifically, a single base transition from G to A occurred within the “GT” repeat-motif at locus *Xtp57* in line A, a single base (T) insertion occurred within the repeat-motif “TC” at locus *Xtp15* in line D, and a single base transversion from C to G occurred within the repeat-motif “AGTACTC” at locus *Xcup11* in line A (Table 1). In addition, two SSR loci showed point mutations in the repeat-motif flanking regions: two single base transversions (G to T and C to G) at locus *SBKAFGK1* in line A, and the same single base transition (C to T) but in different positions at locus *Xtp15* in both line A and line D (Table 1).

Alteration in expression of genes encoding for a set of DNA MMR enzymes and a DNA glycosylase (DME) in calli and regenerated plants relative to the donor seed plants

Quantification of the steady-state transcript abundance of genes encoding for nine DNA MMR enzymes and a DNA glycosylase (DME) by real-time quantitative PCR was summarized in Fig. 3. Analyzing this set of data enabled generalization of the following results: (1) for seven of the nine MMR-encoding genes (except MSH7 and MLH3) and the DME gene, the four independently regenerated plants of each line showed conspicuous up-regulation relative to their donor seed plant, and even relative to the calli from which they were regenerated; (2) regenerated plants of the pair of F1 hybrids showed clear heterosis largely consistent with the over-dominance model (Luo et al. 2009) in the extent of up-regulation of the above eight genes (seven MMR genes and a DME gene) relative to the parental pure

lines; (3) in calli, most of the genes were also up-regulated relative to the donor seed plants, but only four (MLH1, MLH4, MSH3 and MSH7) reached statistical significance, with the most marked one being MSH7; (4) one MMR gene (MLH3) showed the most clear genotype-dependent expression patterns across the plant lines: relative to the corresponding donor seed plants, it was significantly down-regulated in both calli and all four regenerated plants in pure line A, it showed no change in calli but was significantly up-regulated in three of the four regenerated plants of pure line D, and it was significantly up-regulated in both calli and all regenerated plants in each of the pair of reciprocal F1 hybrids (Fig. 3). Taken together, it is clear that tissue culture in sorghum is associated with substantial alteration (predominantly up-regulation) in expression of the MMR genes, which are not only being largely perpetuated (except MSH7) through the regeneration process but in most cases being further augmented in the regenerated plants. The consistent up-regulation of the MSH7 gene in calli of all four sorghum genotypes, which is otherwise meiosis-specific during normal plant development (Wu et al. 2003), is interesting, and strongly implicating a functional role of this gene in the rapidly dividing callus cells, conceivably to sustain a default level of genomic stability. Finally, the extent of alteration in the expression of most of the MMR genes manifests apparent heterosis in reciprocal F1 hybrids, which is largely consistent with the over-dominance model.

Correlation of SSR variation with altered expression patterns of a particular MMR gene, MLH3

Given the concomitant occurrence of SSR variation and alteration in expression of the MMR genes, an interesting question to ask is whether the two phenomena are intrinsically correlated or occurred independently. Because except in one case (Fig. 2, locus *Xtxp50*), all the

independently regenerated plants showed exactly the same variations with their corresponding calli (Fig. 2), it can be deduced that almost all the variations should have occurred during the callus culture stage, namely, the regeneration process had rarely been accompanied by further variations. Therefore, if altered expression of the MMR genes had played a role in genesis of the variations, then only those occurred at the callus stage are relevant. As described above, although most of the MMR genes showed up-regulation in callus, only four (MLH1, MLH4, MSH3 and MSH7) are significantly altered relative to the donor seed plants (Fig. 3). Nonetheless, three of these four genes did not show a distinct difference among the genotypes which showed fundamental difference in the occurrence of SSR variations (Fig. 3); in contrast, one of these genes, i.e., MLH3, manifested clear genotypic difference in both the tuning direction (up-regulation) and transcript quantity, which were well-mirrored by both the occurrence and frequencies of the SSR variations among the genotypes (Fig. 3). Specifically, MLH3 showed significant down-regulation in callus of pure line A which exhibited the highest frequency (20.7%) of SSR variations (Fig. 1); the gene was also down-regulated (but the extent did not reach a statistically significant level) in callus of pure line D, which also showed SSR variations but at a markedly lower frequency (6.9%) than line A (Fig. 1); the gene was significantly up-regulated in callus of both F1 hybrids, which did not show any SSR variation of the studied loci (Figs. 1, 2). To further investigate the seemingly correlation between tuned expression of the MMR genes and SSR variations, we calculated the correlation coefficients of all possible pairs by plotting the quantified steady-state mRNA levels of each of the MMR genes and the DNA glycosylase gene against the SSR variation frequencies by the Pearson's test of SPSS 11.5 for Windows. We found that MLH3 is indeed the only gene for which the expression level in calli is negatively correlated with frequencies of SSR

Table 2 Pearson correlation between the frequencies of simple sequence repeat (SSR) variation and the steady-state transcript abundance of a set of nine DNA mismatch repair (MMR)-encoding genes and a DNA glycosylase

MMR genes	Calli		Regenerants	
	Pearson correlation	Significance (two-tailed)	Pearson correlation	Significance (two-tailed)
MSH2	-0.554	0.446	-0.866**	0
MSH3	-0.405	0.595	-0.875**	0
MSH4	-0.828	0.172	-0.614*	0.011
MSH5	-0.470	0.530	-0.613*	0.012
MSH6	-0.077	0.923	-0.691**	0.003
MSH7	0.229	0.771	-0.462	0.072
MLH1	0.736	0.264	-0.831**	0
MLH3	-0.957*	0.043	-0.848**	0
MLH4	-0.750	0.250	-0.660**	0.005
DNA glycosylase	-0.572	0.428	-0.684**	0.003

*, ** statistical significance at the 0.05 and 0.01 levels (two-tailed), respectively

variation (Table 2). We also tested the possible correlations between expression of the MMR genes and the DME gene with SSR variation frequencies in the regenerated plants, and we found that expression of eight of the nine MMR genes and the DME gene showed significant, negative correlation with SSR variation (Table 2). This may suggest that enhanced expression of these genes is required to ensure genomic stability at the SSR loci during the plant regeneration process, which is conceivably being accompanied by further epigenetic reprogramming, and hence entails more tight control.

Discussion

From an evolutionary point of view, an organism's genomic stability and mutability need to be intricately balanced for the sake of survival and adaptation (Joyce et al. 2003). Thus, it is not surprising that a sizable number of cellular genes are evolved to exert a function for the tight control of genomic integrity under normal, favorable conditions, and in relaxing the control to allow mutations to be generated in times of severe stress (Boyko and Kovalchuk 2008). Plant tissue culture represents a traumatic stress for the plant cells (McClintock 1984), and as such, simultaneous induction of various kinds of genomic instability has been found as a rule rather than an exception with refined resolution in the detecting methods, which are consistent with the proposal that tissue culture-induced somaclonal variation is self-imposed as a consequence of disrupted normal cellular controls (Phillips et al. 1994; Kaeppeler et al. 2000; Joyce et al. 2003; Madlung and Comai 2004; Krizova et al. 2009). Although the exact controlling mechanisms, which are apparently prone to disruption by the tissue culture process, remain to be fully understood, a thorough investigation in *Arabidopsis* suspension cell cultures by Tanurdzic et al. (2008) has provided compelling evidence pointing to epigenetic regulation as the underlying instigator for the genesis of somaclonal variations. In that study, it was found that expression of a large number of genes responsible for chromatin regulation was altered (predominantly up-regulated) in cell suspension cultures relative to the seedling plants, which was concomitant with shift in profiles of the two kinds of siRNAs (21 nt vs. 24 nt), locus-specific alteration in cytosine methylation, as well as transcriptional activation of transposable elements (Tanurdzic et al. 2008). Consequently, the authors have proposed that it is the misregulation of chromatin structure/state-related genes and hence loss of epigenetic targeting (by siRNA) under the cell culture conditions that is largely responsible for the occurrence of genomic instabilities in the cell suspension cultures (Tanurdzic et al. 2008). We have reported recently in sorghum that the various cytosine

methyltransferases and DNA glycosylases are extremely sensitive to perturbation by tissue culture, and their differential ability to be tuned toward up-regulation in a coordinately fashion between inter-strain F1 hybrids and their parental pure lines is intimately correlated with the frequencies of both genetic and epigenetic variations (Zhang et al. 2009). These results are in line with the findings by Tanurdzic et al. in *Arabidopsis* cell suspension cultures, and together they reiterate that epigenetic regulation functions as a common cellular mechanism underlying both genetic and epigenetic instabilities in plant tissue cultures, and probably also in some other stress conditions.

SSRs, being ubiquitous components of eukaryotic genomes, are intrinsically unstable either stochastically due to developmental/metabolic cues or predictably under perturbed environmental conditions, and hence, are ideal markers for monitoring genome instability under various circumstances (Varshney et al. 2005). As expected, nearly all investigations employing this marker have detected instability in tissue cultures of diverse plants (Chowdari et al. 1998; Rahman and Rajora 2001; Ryu et al. 2007; Schellenbaum et al. 2008; Gao et al. 2009; Marum et al. 2009). However, the mechanism for tissue culture-induced SSR variations remains uninvestigated. Nonetheless, it is likely that it shares similar mechanistic basis with other situations, like in certain types of cancer cells, wherein SSR variations occur abundantly (Yu et al. 2006; Haugen et al. 2008; Vilar et al. 2008; Bertagnolli et al. 2009). Indeed, several studies in human cancer cells have documented that disproportional titration and/or malfunctioning of the MMR proteins play a major role in generation of SSR mutations (Yu et al. 2006; Haugen et al. 2008; Vilar et al. 2008; Bertagnolli et al. 2009).

Given that dramatic dysregulation of a large number of genes related to chromatin structure or state occurred under the tissue culture conditions (Tanurdzic et al. 2008; Zhang et al. 2009), it is not difficult to imagine that other cellular genes responsible for retaining genomic integrity including those encoding for MMRs are also likely being dysregulated, and hence causing escape of SSR mutations from being corrected in a timely manner. Indeed, we showed in this study that expression of all nine studied MMR genes was altered in callus and/or regenerated plants (conspicuously more in the later), and the great majority are up-regulations. Although the altered expression patterns of most of the MMR genes are genotype-independent and hence appeared uncoupled with the detected SSR variations that are strictly genotype-dependent, that of one gene, i.e., MLH3, was in perfect accordance with the SSR variations, which was further bolstered by correlation analysis. Therefore, whereas tuning on expression of the majority of the MMR genes by the tissue culture conditions is probably

merely passive responses and hence inconsequential to SSR instability, the genotype-specific ability to tune the expression of a critical MMR gene (like MLH3) toward up-regulation likely plays a critical role in maintaining SSR stability.

The observation that calli and regenerated plants derived from the pair of reciprocal F1 hybrids showed no variation in the studied SSRs whereas those derived from the pure line parents showed high levels of variation is striking. Not surprisingly, this SSR stability in the F1 hybrids is concomitant with the over-dominant model of expression in seven of the nine studied MMR-encoding genes and the demeter gene, thus strongly implicating an intrinsic connection between the two phenomena. This is consistent with our previous observation on both the genetic and epigenetic stability at randomly analyzed genomic loci of these hybrid lines (Zhang et al. 2009). Together, these data suggest that inter-strain sorghum F1 hybrids possess a higher capability in canalizing genomic stability in times of certain stress conditions.

Further studies are needed to establish the possible casual relationships between tuning on expression of the MMR genes and SSR mutations in plants, and for which plant tissue culture will continue to represent a tractable experimental system.

Acknowledgments This study was supported by the National Natural Science Foundation of China (30870178, 30870198), and the Program for Introducing Talents to University (111 project #B07017). We are grateful to Professor Diter von Wettstein of the Washington State University for his interest in this study and constructive suggestions to improve the manuscript.

References

- Bertagnoli MM, Niedzwiecki D, Compton CC, Hahn HP, Hall M, Damas B, Jewell SD, Mayer RJ, Goldberg RM, Saltz LB, Warren RS, Redston M (2009) Microsatellite instability predicts improved response to adjuvant therapy with irinotecan, fluorouracil, and leucovorin in stage III colon cancer: cancer and leukemia group B protocol 89803. *J Clin Oncol* 27:1814–1821
- Boyko A, Kovalchuk I (2008) Epigenetic control of plant stress response. *Environ Mol Mutagen* 49:61–72
- Chowdari KV, Ramakrishna W, Tamhankar SA, Hendre RR, Gupta VS, Sahasrabudhe NA, Ranjekar PK (1998) Identification of minor DNA variations in rice somaclonal variants. *Plant Cell Rep* 18:55–58
- Gao DY, Vallejo VA, He B, Gai YC, Sun LH (2009) Detection of DNA changes in somaclonal mutants of rice using SSR markers and transposon display. *Plant Cell Tissue Org Cult* 98:187–196
- Haugen AC, Goel A, Yamada K, Marra G, Nguyen TP, Nagasaka T, Kanazawa S, Koike J, Kikuchi Y, Zhong X, Arita M, Shibuya K, Oshimura M, Hemmi H, Boland CR, Koi M (2008) Genetic instability caused by loss of MutS homologue 3 in human colorectal cancer. *Cancer Res* 68:8465–8472
- Joyce SM, Cassells AC, Jain SM (2003) Stress and aberrant phenotypes in in vitro culture. *Plant Cell Tissue Org* 74:103–121
- Kaeppler SM, Kaeppler HF, Rhee Y (2000) Epigenetic aspects of somaclonal variation in plants. *Plant Mol Biol* 43:179–188
- Krizova K, Fojtova M, Depicker A, Kovarik A (2009) Cell culture-induced gradual and frequent epigenetic reprogramming of invertedly repeated tobacco transgene epialleles. *Plant Physiol* 149:1493–1504
- Larkin PJ, Scowcroft WR (1981) Somaclonal variation—a novel source of variability from cell cultures for plant improvement. *Theor Appl Genet* 60:197–214
- Luo X, Fu Y, Zhang P, Wu S, Tian F, Liu J, Zhu Z, Yang J, Sun C (2009) Additive and over-dominant effects resulting from epistatic loci are the primary genetic basis of heterosis in rice. *J Integr Plant Biol* 51:393–408
- Madlung A, Comai L (2004) The effect of stress on genome regulation and structure. *Ann Bot* 94:481–495
- Marum L, Rocheta M, Maroco J, Oliveira MM, Miguel C (2009) Analysis of genetic stability at SSR loci during somatic embryogenesis in maritime pine (*Pinus pinaster*). *Plant Cell Rep* 28:673–682
- McClintock B (1984) The significance of responses of the genome to challenge. *Science* 226:792–801
- Mohan Jain S (2001) Tissue culture-derived variation in crop improvement. *Euphytica* 118:153–166
- Nag DK, Suri M, Stenson EK (2004) Both CAG repeats and inverted DNA repeats stimulate spontaneous unequal sister-chromatid exchange in *Saccharomyces cerevisiae*. *Nucleic Acids Res* 32:5677–5684
- Phillips RL, Kaeppler SM, Olhoft P (1994) Genetic instability of plant tissue cultures: breakdown of normal controls. *Proc Natl Acad Sci USA* 91:5222–5226
- Rahman MH, Rajora OP (2001) Microsatellite DNA somaclonal variation in micropropagated trembling aspen (*Populus tremuloides*). *Plant Cell Rep* 20:531–536
- Rodríguez López CM, Wetten AC, Wilkinson MJ (2004) Detection and quantification of in vitro-culture induced chimerism using simple sequence repeat (SSR) analysis in *Theobroma cacao* (L.). *Theor Appl Genet* 110:157–166
- Ryu TH, Yi SI, Kwon YS, Kim BD (2007) Microsatellite DNA somaclonal variation of regenerated plants via cotyledon culture of hot pepper (*Capsicum annuum* L.). *Kor J Genet* 29:459–464
- Schellenbaum P, Mohler V, Wenzel G, Walter B (2008) Variation in DNA methylation patterns of grapevine somaclones (*Vitis vinifera* L.). *BMC Plant Biol* 8:78. doi:10.1186/1471-2229-8-78
- Tanurdzic M, Vaughn MW, Jiang H, Lee TJ, Slotkin RK, Sosinski B, Thompson WF, Doerge RW, Martienssen RA (2008) Epigenomic consequences of immortalized plant cell suspension culture. *PLoS Biol* 6:2880–2895
- Varshney RK, Graner A, Sorrells ME (2005) Genic microsatellite markers in plants: features and applications. *Trend Biotechnol* 23:48–55
- Vilar E, Scaltriti M, Balmã J, Saura C, Guzman M, Arribas J, Baselga J, Tabernero J (2008) Microsatellite instability due to hMLH1 deficiency is associated with increased cytotoxicity to irinotecan in human colorectal cancer cell lines. *Br J Cancer* 99:1607–1612
- Wang HY, Chai Y, Chu XC, Zhao YY, Wu Y, Zhao JH, Ngezahayo F, Xu CM, Liu B (2009) Molecular characterization of a rice mutator-phenotype derived from an incompatible cross-pollination reveals transgenerational mobilization of multiple transposable elements and extensive epigenetic instability. *BMC Plant Biol* 9. doi:10.1186/1471-2229-9-63
- Wu YQ, Huang Y (2007) An SSR genetic map of *Sorghum bicolor* (L.) Moench and its comparison to a published genetic map. *Genome* 50:84–89
- Wu SY, Culligan K, Lamers M, Hays J (2003) Dissimilar mismatch-recognition spectra of *Arabidopsis* DNA-mismatch-repair

- proteins MSH2-MSH6 (Mus α) and MSH2-MSH7 (Mus γ). *Nucleic Acids Res* 31:6027–6034
- Yu J, Mallon MA, Zhang W, Freimuth RR, Marsh S, Watson MA, Goodfellow PJ, McLeod HL (2006) DNA repair pathway profiling and microsatellite instability in colorectal cancer. *Clin Cancer Res* 12:5104–5111
- Zhang MS, Xu CM, Yan HY, Zhao N, Von Wettstein D, Liu B (2009) Limited tissue culture-induced mutations and linked epigenetic modifications in F1 hybrids of sorghum pure lines are accompanied by increased transcription of DNA methyltransferases and 5-methylcytosine glycosylases. *Plant J* 57:666–679