Molecular characterization of genetic variation in somaclones of durum wheat (*Triticum durum* Desf) using SSR markers

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Abstract

This study is a continuation of a previous work; the main objective is to characterize the induced somaclonal variation in the first generation of durum wheat plants. In a previous experiment, *in vitro* plants of three genotypes of durum wheat were obtained following treatment with PEG6000 (0%, 10% and 20%). First generation offspring was produced. Five microsatellite markers were used for genotyping 26 durum wheat somaclones obtained under stressful and non-stressful conditions from 3 durum wheat cultivars. Amplifiable and reproducible alleles were obtained in three of the tested loci. These loci provided in total 78 monomorphic alleles of which 69 were detected in somaclones while the remaining 9 in the mother plants. High values of among-population genetic diversity were found, which accounted for 71 % of the total genetic variation. The number of alleles per locus varied from three to six. Estimated genetic distances varied from 0.83 to 1.67 between populations. The somaclonal variation was identified with 2 SSR markers. Five new alleles were identified in somaclonal variants DKR_1-S1, DKR_1-S2, OZR_1-S2, WR_1-C3 and WR_1-S1at loci *gmw131* and *gwm427*. Genetic variation rate was 21.74%. Eighty % of the genetic variation was identified in plants obtained from callus undergoing high osmotic pressure. The presence of selective agent in the medium could explain the observed genetic variations. Somaclonal variation cannot always be detected at the gross morphological level. The selected SSR markers

could be used to study the uniformity of plants obtained from tissue culture and varietal identification.

Keywords: *Triticum durum* Desf, somaclones, somaclonal variation, SSR markers.

Introduction

Wheat is encountering different environmental challenges, such water stress, that affects growth and development of wheat. The development of new varieties is a practical strategy for improving yield, especially under abiotic stress and future climate changing conditions. Diversity in plant genetic resources provides opportunity for plant breeders to develop new and improved cultivars with desirable characteristics (Bellatreche et Gaouar 2012, Govindaraj et al., 2015). Somaclonal variation resulted from tissue or cell culture (Larkin and Scowcroft, 1981) is an alternative source of new genetic diversity that is very useful for plant breeding programs including generating a novel character like drought tolerance. Several works with wheat have revealed that the somaclonal variation might result from point mutation, chromosomal translocation, chromosomal aberration, alterations in gene expression and mtDNA rearrangement (Larkin et al., 1981; Hartmann et al., 1989; Sato et al. 2011), although little is known about the mechanism regulating this processes. A selection pressure applied by stress agent could induce genetic variation, which confer broad resistance to multiple biotic or abiotic stresses (Zair et al., 2003). Several strategies are available for detecting genetic variation including morphological traits, cytogenetic analysis, molecular and biochemical markers (Heinze and Mee 1971; Hsissou and Bouharmont 1994; Mehta et al. 2000), most of them have limitations. Nowadays, DNA marker techniques based on PCR amplification have become increasingly important to study the genetic relationships among plants. Simple sequence repeat (SSR) markers are the most popular type of co-dominant molecular markers in phylogenetic studies, evaluation of genetic diversity, cultivar identification and genotypic variations. There are limited numbers of research reports on somaclonal variation detection in durum wheat using microsatellites. In this paper, we used SSR analysis of first generation (R_1) durum wheat somaclones.

Material and methods

Plant material

In a previous study, three durum wheat varieties (Djenah Khetifa-DK, Oued Zenati-OZ and Waha-W) were screened for drought tolerance at cellular level. Several *in vitro* plants (R_0) with different level of tolerance were developed using polyethylene glycol (PEG-6000) as a drought

simulation agent (Kacem et al., 2016). R_0 plants were self-pollinated to produce R_1 seed. The obtained R_1 plants were subjected to analysis of the induced genetic variation (Table 1). From a morphological point of view, plants obtained from non-stressed callus are identical to their mother plants with the exception of one somaclone obtained from stressed callus of the Oued Zenati variety (Figure 1).

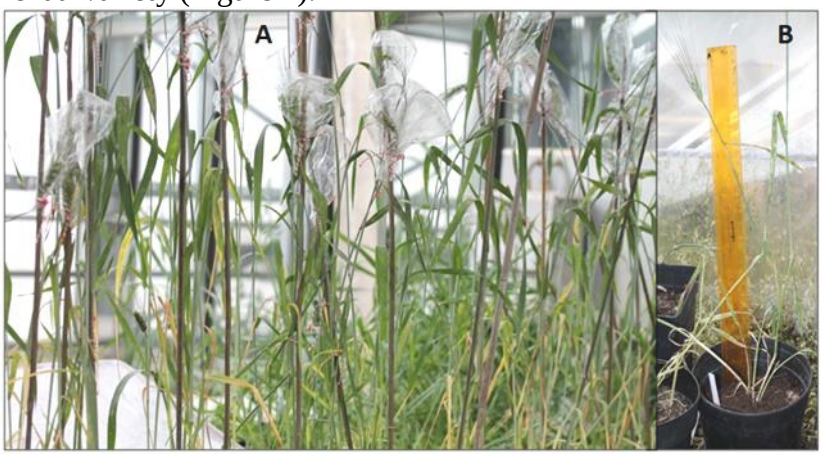

Figure 1. R_0 plants at the heading stage. **A**. Plants obtained from non-stressed callus. **B**. Plants obtained from stressed callus (20% PEG).

DNA extraction

Genomic DNA of somaclones (R_1) was extracted from 50 mg leaf tissue. For mother plant, genomic DNA was extracted from 25 mature embryos per each genotype. The total genomic DNA was extracted using the CTAB method by Doyle and Doyle (1990). The quantity and quality of the DNA was checked on nano-spectrophotometer (IMPLEN, Germany) and by 0.8% agarose gel electrophoresis, respectively. Absorbance ratio between 260 and 280 nm was computed and the quality of the genomic DNA was confirmed.

Table 1. Number and origin of R_1 somaclones used.

Genotypes	**Origin of R_1 somaclones**	
	Control callus (0% PEG)	Stressed callus (20% PEG)
Djenah Khetifa	6	5
Oued Zenati	6	2
Waha	3	1

PCR Amplification and SSR Analysis

A total of 5 SSR markers were used in this study (Table 2). These markers were developed in *Triticum aestivum* (Röder et al., 1998). PCR reactions were performed in a total reaction volume of 20 µl containing about 20 ng of genomic DNA, 1× PCR buffer, 0.2mMof each dNTP, 1.5mMMgCl2, 1 µg/µl BSA, 0.25 µM of each primer, and 0.5 U Taq DNA polymerase (Fermentas). Reactions were performed in iCycler (Bio-Rad)

thermal cycler with the following conditions: 94 °C for 4 min, followed by 30 cycles at 94 °C for 60 s, 55–60 °C depending on the primer pair for 60 s (Table 2), and 72 °C for 2 min. The last step was at 72 °C for 10 min. The electrophoresis was performed in 6.5 % denaturing polyacrylamide gel using a Li-Cor global edition IR2 DNA sequencer (Westburg). Allele scoring was done using Gene ImagIR software, v 4.03 (Westburg).

Table 2. Sequences of SSR primers used in PCR-reactions. All primer sequences are given in the 5 to 3 direction

Locus	Primer sequences (forward)	Primer sequences (reverse)	Core Motifs	Location	Tm (C°)
gwm131	*AAT CCC CAC CGA TTC TTC TC*	*AGT TCG TGG GTC TCT GAT GG*	(CT)22	1B-3B	60°C
gwm135	*TGT CAA CAT CGT TTT GAA AAG G*	*ACA CTG TCA ACC TGG CAA TG*	(GA)20	1A	60°C
gwm427	*AAA CTT AGA ACT GTA ATT TCA GA*	*AGT GTG TTC ATT TGA CAG TT*	(CA)31 (CA)22	6A	50°C
gwm088	*CACTACAACTATGCGCTCGC*	*TCCATTGGCTTCTCTCTCAA*	(GT)18TT (GA)4	6B	60°C
gwm186	*CACTACAACTATGCGCTCGC*	*TCCATTGGCTTCTCTCTCAA*	(GT)18TT (GA)4	6B	60°C

Statistical Analysis

The distance and genetic identity matrix were estimated according to Nei's coefficient (Nei, 1978) using GENETIX software version 4.05 (Belkhir et al., 2004).

Results

Detection of somaclonale variation

After size determination of the alleles for each locus in the tested individuals, we decided to exclude data related to markers *gwm088* and *gwm186* due to bad separation and difficulty of reading the size of alleles. Three amplifiable and reproducible SSR markers: *gwm131*, *gwm135* and *gwm427* were selected. We started our study by verifying the obtained profiles using microsatellites *gwm131, gwm135* and *gwm427* on mother plants (Figure 2). A total of 9 monomorphic and reproducible fragments were produced, with the studied genotypes of durum wheat. The amplification products were between 139 bp to 225 bp (Table 3).

With the 23 analyzed somaclones, we obtained a total of 69 reproductible and monomorphic fragments. The amplification products were between 136 bp to 225 bp. With the marker *gwm131* two deletions of 4 and 16 base pairs were identified in the somaclones DKR_1-S1 and DKR_1-S2, respectively. With the same marker, we recorded another case of deletion of one base pair in the somaclone WR_1-S1 and one more case of deletion of three base pairs in the somaclone OZR_1-S2. In only the somaclone WR_1-C3

of Waha variety an additional band corresponding to about 0.22kb was observed in locus *gwm427*. No genetic variation was detected in R_1 plants with the microsatellite *gwm135*.

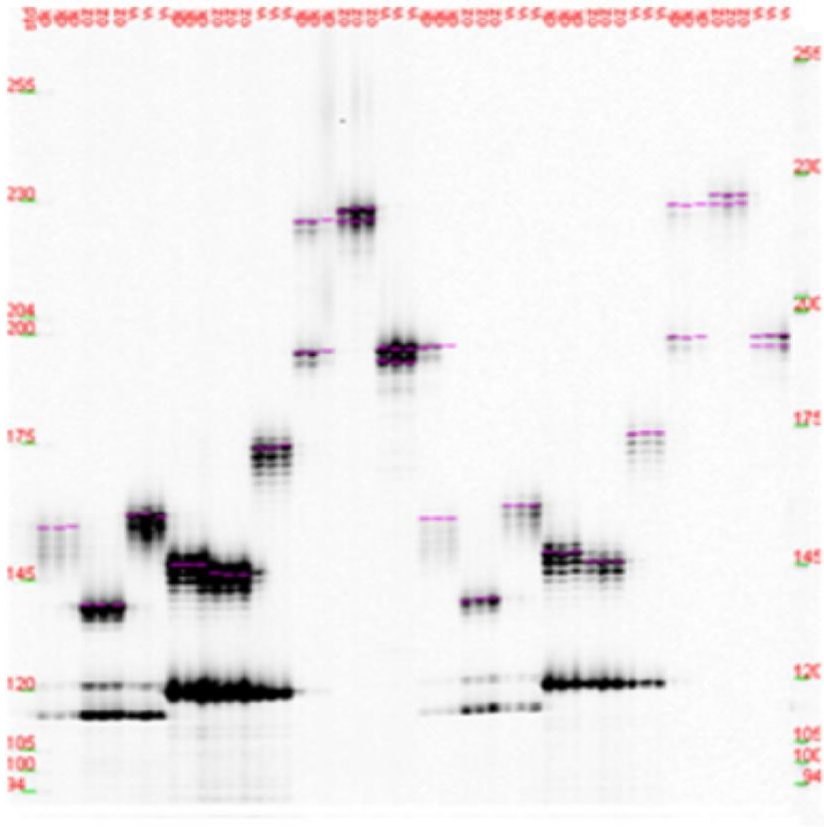

Figure 2. Microsatellites fragments, the amplification products are between 139 bp to 225bp. The DNA was amplified by PCR and migrated on Li-Cor DNA Sequencer. Dk (Djenah Khetifa), OZ (Oued Zenati), W(Waha)**.**

The frequencies of total variation detected by the three primers (*gwm131*, *gwm135* and *gwm427*) was 21.74%; the highest variation rate is recorded in the locus *gwm131* (17.39%) followed by *gwm427* (4.35%). However the locus *gwm135* did not reveal any genetic variation. 80% of these variations was revealed in plants originating from stressed callus (PEG20%) while 20% was observed in plants obtained from non-stressed callus (PEG0%) (Table 3).

Table 3. Characterization of genetic variation in the first generation (R_1) of durum wheat somaclones. Genetic variation was observed in five somaclones (DKR_1-S1, DKR_1-S2, OZR_1-S2, WR_1-S1 and WR_1-C3) in loci *gmw131* and *gmw427* in comparison to mother plants of 3 durum wheat cultivars (DK - Djenah Khetifa, OZ - Oued Zenati, W - Waha) cultivated in *vivo*.

Variety	*Code somaclones*	*Origin of plants*	*Fragment sizes (bp)* *gwm131*	*gwm135*	*gwm427*
DK		Mother Plant	155	148	223
Plant	DKR_1-C1	*Control Callus*	155	148	223
Plant	DKR_1-C2	*Control Callus*	155	148	223
Plant	DKR_1-C3	*Control Callus*	155	148	223
Plant	DKR_1-C4	*Control Callus*	155	148	223
Plant	DKR_1-C5	*Control Callus*	155	148	223
Plant	DKR_1-C6	*Control Callus*	155	148	223
Plant	DKR_1-S1	*Stressed Callus*	**151**	148	223
Plant	DKR_1-S2	*Stressed Callus*	**139**	148	223
Plant	DKR_1-S3	*Stressed Callus*	155	148	223
Plant	DKR_1-S4	*Stressed Callus*	155	148	223
Plant	DKR_1-S5	*Stressed Callus*	155	148	223
OZ		Mother Plant	139	146	225
Plant	OZR_1-C1	*Control Callus*	139	146	225
Plant	OZR_1-C2	*Control Callus*	139	146	225
Plant	OZR_1-C3	*Control Callus*	139	146	225
Plant	OZR_1-C4	*Control Callus*	139	146	225
Plant	OZR_1-C5	*Control Callus*	139	146	225
Plant	OZR_1-C6	*Control Callus*	139	146	225
Plant	OZR_1-S1	*Stressed Callus*	139	146	225
Plant	OZR_1-S2	*Stressed Callus*	**136**	146	225
W		Mother Plant	157	173	194
Plant	WR_1-C1	*Control Callus*	157	173	194
Plant	WR_1-C2	*Control Callus*	157	173	194
Plant	WR_1-C3	*Control Callus*	157	173	**222**
Plant	WR_1-S1	*Stressed Callus*	**156**	173	194

Genetic diversity

The results obtained showed high level of genetic diversity (71%) (inter-population polymorphism). The highest level of genetic diversity was recorded in locus *gmw131* (75.37%) followed by *gmw427* (70.76%) and *gmw135* (66.67%). In contradiction, the level of intra-population polymorphism was lower (0.145%). The highest level of intra-population polymorphism was observed in locus *gmw131* (31.06%) followed by *gmw427* (12.28%), whereas absence of polymorphism was observed in locus

gmw135. The number of alleles range from 1 to 3 within the population of Djenah Khetifa and from 1 to 2 for the rest two populations of cultivars. The number of alleles ranged from 3 in locus *gwm135* to 6 in the locus *gwm131*. The marker *gmw427* showed in total 4 alleles (Table 4).

Table 4. Allele frequency at 3 loci across 3 population of durum wheat (DK - Djenah Khetifa, OZ - Oued Zenati, W - Waha).

Locus	Alleles (bp)	Populations DK	OZ	W	Total
gwm131	**(N)**	**12**	**9**	**5**	
	136	0.000	0.111	0.000	0.037
	139	0.083	0.889	0.000	0.324
	151	0.083	0.000	0.000	0.028
	155	0.833	0.000	0.000	0.278
	156	0.000	0.000	0.200	0.067
	157	0.000	0.000	0.800	0.267
gwm135	**(N)**	**12**	**9**	**5**	
	146	0.000	1.000	0.000	0.333
	148	1.000	0.000	0.000	0.333
	173	0.000	0.000	1.000	0.333
gwm427	**(N)**	**12**	**9**	**5**	
	194	0.000	0.000	0.800	0.267
	222	0.000	0.000	0.200	0.067
	223	1.000	0.000	0.000	0.333
	225	0.000	1.000	0.000	0.333

Table 4 also presents the frequencies and sizes of alleles from different populations. The allelic frequencies calculated for each locus and each population ranged from 0 to 0.89. The locus *gwm131* is presented by six alleles, and was the most polymorphic. The predominance of the allele 139 is remarkable and its frequency was 0.324 in the three analysed populations. The *gwm135* locus indicates the presence of three alleles. For *gwm427* locus, the predominance of the allele 194 in Waha variety was remarkable, with a frequency of 0.8.

The matrix of genetic distances estimated between individuals (Table 5) indicated high values between each 2 populations. These distances ranged from 0.83 to 1.67. The two varieties Djenah Khetifa and Waha seem to be genetically more closer.

Table 5. Matrices of Nei genetic distances among populations of durum wheat.

	Djenah Khetifa	**Oued Zenati**	**Waha**
Djenah Khetifa	0		
Oued Zenati	1.16	0	
Waha	0.83	1.67	0

Discussion

The variations in the lengths of the amplification products at 2 of the tested microsattelite loci revealed somaclonal variation in R_1 plants of durum wheat. These variations suggest that such modifications were transmitted to descendants of regenerants. The transmission of genetic variations through mitosis and cellular heritability of variations is now well proven. According to Meins (1983) the changes resulting from permanent modifications of genome are genetic; these changes are transmissible in regenerating plants and their descendants according to the rules of the classic mendelian inheritance.

The obtained results are consistent with those conducted *in vitro* in *Artemisia* species in which two new alleles of 0.15 kb and 0.2 kb have been reported using ISSR markers (Khan et al., 2013). The finding here also are in agreement with earlier reports on application of SSR for identification of genetic variation among regenerated plants in several other plants such aspen (Rahman & Rajora 2001), rice (Khai and Lang 2005) and cotton (Jin et al. 2008).

Despite of the phenotypic similarities between somaclones and mother plants, the differences in genomic constituents of the regenerated plants originating from the same cell has been effectively determined by SSR markers. Phenotypic identification, based on a description of the morphological traits, can be used but some changes induced by *in vitro* culture cannot be easily observed. Similar findings on genomic variation in phenotypically normal regenerants have been well documented in some other plants (Rahman & Rajora 2001, Jin et al. 2008).

Based on our results, it seems that the presence of osmotic agent (20% PEG) in *in vitro* culture might be the reason of the observed genotypic variation. In this study the majority of variations were observed on plants obtained from stressed callus. The frequency of occurrence of somaclonal variation in regenerated plants is strongly affected by genotype, medium, duration of culture and the source of explants. Handro (1981) reported that when *in vitro* selective agents are added to the media, additional interactions are encountered. Various hypotheses have been put forward to explain this phenomenon. The best accepted hypothesis states that *in vitro* culture represents breakdown of normal, default cellular controls resulting in a cascade of genetic and epigenetic instabilities and hence lead to alterations in the gene expression and to the development of new phenotype (Kaeppler et al., 2000). Recent studies have indicated that the absence or presence of a specific gene or allele could cause a change in the drought resistant nature depending on the role of the genes affected (Nag et al., 2004, Arumingtyas et al., 2012).

The matrix of genetic distances estimated between individuals indicates high values between populations taken two by two. The genetic distances matrix indicates that the three populations showed a genetic dissemblance. The two varieties Djenah Khetifa and Waha seem to be the most genetically related. The monomorphic banding pattern confirmed the genetic uniformity of mother plants and certain clones of the same variety (Zhang et al. 2010; Manoj et al., 2012). Greater genetic diversity (inter-population genetic diversity) was observed among populations (71%) compared to the intra-population genetic diversity (14.5%), which translates an important genetic differentiation between the studied varieties of durum wheat. The locus *gwm131* was the most polymorphic, followed by the locus *gwm427*. Our results are in agreement with the studies of Medini et al. (2005) and those of Colomba and Gregorin (2011) conducted on different durum populations and showed the existence of a higher genetic diversity estimated on the base of the use of SSR and AFLP markers. Hamrick and Godt (1997) reported that intra-population genetic diversity of cross-pollinated species varies from 10.3% to 26.6%, whereas intra-population genetic diversity of self-pollinated species varies from 0.09% to 0.14%.

Conclusion

SSR markers can be efficiently used for assessment of genetic variation among regenerants derived from an *in vitro* culture. High level of genetic diversity in three durum wheat populations was observed. The frequency of somaclonal variation seems to be a consequence of the culture conditions, suggesting that the drought stress induced by PEG6000 is capable of altering DNA sequence. Somaclonal variation cannot always be detected at the gross morphological level. The genetic changes in somaclones (R_1 generation) are a result of segregation process during meiosis and assorted independently in the self fertilization. Performing stability tests at later generations and in water stress conditions would confirm the usefulness of this approach to select somaclonal variants tolerant to water deficit.

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