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DNA Stability Contrasts with Chromosome Variability in *Allium fistulosum* Calli

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RAPD analysis was applied to assess the degree of DNA polymorphism in *A. fistulosum* calli of high chromosomal instability. Nineteen of 24 randomly selected RAPD primers revealed scorable polymorphism between calli and seeds (reference material). Polymorphic band frequency was 55/237 in seeds and 36/233 in calli; variability on the DNA level was thus lower in calli than in seeds (15.4% vs. 23.2% of band positions). UPGMA analysis of Jaccard's coefficients confirmed the genetic similarity of the analyzed cultures. The most distinctive DNA changes in calli involved coincident loss of original bands or the appearance of novel bands. Seven such changes (4 losses, 3 gains) were observed. Our results suggest that changes on the chromosomal level and on the DNA level occurred independently of each other and that different callus lines underwent similar genetic changes during culture, presumably due to strong selection pressure effected by standard in vitro conditions.

Key words: *Allium fistulosum*, tissue culture, RAPD, DNA polymorphism, somaclonal variation.

INTRODUCTION

Tissue culture frequently causes various genetic and epigenetic abnormalities, which are key components of somaclonal variation in plants (Bayliss, 1980; Lee and Phillips, 1988; Kaeppler et al., 2000; Neelakandan and Wang, 2012; Kovarik et al., 2012). Genetic changes in cultured cells occur at genomic, chromosomal and DNA sequence levels and are generally considered disadvantageous in cloning and micropropagation of plants. The basis of tissue culture-induced variation is poorly understood, and any steps to understand it should be helpful in developing a more stable and manipulatable somatic cell system (Phillips et al., 1994).

Allium fistulosum (Welsh onion), a crop widely cultivated in Asia, is known for its extraordinary chromosomal variation in tissue culture (Joachimiak et al., 1995). Callus of this species provides an excellent model system for studies of chromosome alterations in cultured plant cells (Joachimiak et al., 1993, 1995; Lee and Ono, 1999; Joachimiak and Ilnicki, 2003; Gernand et al., 2007). The initial step of chromosomal destabilization of *A. fistulosum* callus is the formation of dicentric chromosomes, followed by breakage-fusion-bridge (BFB) cycles. As a result, acentric fragments, rearranged chromosomes, polyploidy, and disturbances in the quantity and location of heterochromatin are generated (Joachimiak et al., 1995).

Fluorescent in situ hybridization (FISH) on callus chromosomes of *A. fistulosum* revealed extensive transposition of specific, noncanonical telomeric 375-bp repeats and the presence of numerous rDNA clusters at unusual chromosome sites (Gernand et al., 2007). It was shown that massive rearrangements and copy number changes were not accompanied by changes in the sequence or methylation level of analyzed repeats. The absence of molecular changes in two investigated repeats does not necessarily imply a concomitant lack of such changes in other sequences of cultured cells. So far, however, there has been no research on this problem.

There are a number of strategies available for detecting molecular DNA variation in plant tissue cultures, of which randomly amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) analyses have proved particularly useful

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Abbreviations: AFLP – amplified fragment length polymorphism; BFB – breakage-fusion-bridge; CTAB – cetyl trimethylammonium bromide; dendroUPGMA – dendrogram construction utility; FISH – fluorescent in situ hybridization; ISSR – inter-simple sequence repeat; MS – Murashige and Skoog medium; RAPD – randomly amplified polymorphic DNA; UPGMA – unweighted pair group method with arithmetic mean.

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TABLE 1. Nucleotide sequences of tested random primers; RAPD – SAKAMOTO et al. 1995, OPA-I – Operon Technologies Inc

Primer	Sequence (5'-3')	Primer	Sequence (5'-3')
RAPD 1	GCAAGTAGCT	RAPD 15	GTCGTTACGA *
RAPD 2	TGGCTCAAAG	OPA-04	AATCGGGCTG
RAPD 3	CAGTGTGTGG	OPA-17	GACCGCTTGT
RAPD 4	GTGTCAGGCA	OPB-07	GGTGACGCAG
RAPD 5	ATACCATCCC	OPC-01	TTCGAGCCAG
RAPD 6	GATCCCCTGA	OPC-10	TGTCTGGGTG
RAPD 7	GATAACCGCA	OPD-12	CACCGTATCC
RAPD 8	ATCCGCGTTC	OPD-17	TTTCCCACGG *
RAPD 9	CCAGTGGTTC	OPE-05	TCAGGGAGGT
RAPD 10	TGACGATGCA	OPF-11	TTGGTACCCC
RAPD 11	ACGGCATATG	OPG-20	TCTCCCTCAG *
RAPD 12	TAACCATCCC	OPH-14	ACCAGGTTGG
RAPD 13	ATGTCCGCAC	OPI-03	CAGAAGCCCA
RAPD 14	GTGTGGATGG		

* – not used in further investigation

because of their simplicity and cost-effectiveness (Yang et al., 1996). In the present study we used RAPD technique for analysis of DNA variation among chromosomally unstable *A. fistulosum* calli. RAPD analyses can quickly screen a large portion of the genome and reveal even small genetic differences, and have been successfully used in *Allium* for different purposes (Wilkie et al., 1993; Friesen and Klaas, 1998; Tanikawa et al., 2002; Umehara et al., 2006).

MATERIAL AND METHODS

PLANT MATERIAL AND TISSUE CULTURE

Calli obtained from root meristem of *A. fistulosum* cv. Motokura (2n=16) seedlings were used. Callus induction and maintenance followed the protocol given by Joachimiak et al. (1993). Briefly, developing root tips were excised and placed on MS agar medium supplemented with 0.1 mg/l 2,4-dichlorophenoxyacetic acid and 0.1 mg/l kinetin. Induced calli were transferred at monthly intervals onto fresh culture medium. Every four months the callus samples were collected, fixed in a mixture of 96% ethanol and glacial acetic acid (v/v 3:1) for 24–48 h and stored in 70% ethanol at 4°C until used, DAPI-stained and analyzed cytologically to determine whether chromosomal changes occurred, according to the previously described scheme (Joachimiak et al., 1993; Joachimiak and Ilnicki, 2003). For RAPD analysis we used 3-year-old calli exhibiting a fully developed syndrome of karyotype instability, characterized by the presence of cell nuclei of very different size, micronuclei, and well-recognizable chromosome changes (polyploidy, aneuploidy, chromosome rearrangements) (Joachimiak et al., 1995). Seeds of *A. fistulosum* cv. Motokura served as reference material in these studies.

DNA ISOLATION

Total genomic DNA was extracted from 5 callus lines established from root tips of different seedlings and from 5 seeds using the CTAB method (Gavel and Jarret, 1991), with modifications. Fresh calli were ground in liquid nitrogen with a mortar and pestle. Approximately 100 mg homogenous powder was mixed with 700 µl extraction buffer [1% CTAB, 100 mM Tris-HCl (pH 8.0), 1.4 M NaCl, 20 mM EDTA(Na_2), 1% PVP, 0.1% β-mercaptoethanol]. After incubation at 65°C for 20 min, 600 µl chloroform: isoamyl alcohol (24:1 v/v) was added, and the samples were shaken for 10 min and centrifuged (15,000 × g, 10 min). DNA was precipitated by mixing the aqueous phase with 500 µl isopropanol and centrifuging (15,000 × g, 10 min, 4°C). The DNA pellet was washed with 500 µl 70% ethanol, dried, and dissolved in 25 µl H_2O. DNA was extracted from seeds in a similar way except for grinding, which was done in an Eppendorf tube immersed in liquid nitrogen, using a plastic pestle and in a lower volume of reagent [250 µl extraction buffer, 200 µl chloroform:isoamyl alcohol (24:1 v/v), 170 µl isopropanol, 170 µl 70% ethanol, 10 µl H_2O]. The DNA concentration was determined using a NanoDrop ND1000 spectrophotometer, and each sample was diluted to 10 ng/µl in sterile distilled water.

TABLE 2. RAPD primers and their products in seeds and calli

Primer	Seeds		Calli		Band length (bp)
	number of bands	polymorphic	number of bands	polymorphic	
RAPD 1	14	0	14	0	530 – 2250
RAPD 2	8	0	9	0	540 – 2140
RAPD 3	4	1	4	0	630 – 1600
RAPD 4	8	0	9	1	320 – 3300
RAPD 5	14	9	11	2	800 – 3000
RAPD 6	12	9	12	8	800 – 2920
RAPD 7	13	2	13	1	450 – 2750
RAPD 8	14	9	15	5	770 – 2800
RAPD 9	9	0	9	0	700 – 2320
RAPD 10	9	0	11	2	610 – 2490
RAPD 11	11	0	12	1	530 – 3370
RAPD 12	11	1	11	3	580 – 2190
RAPD 13	8	0	8	0	810 – 1670
RAPD 14	2	0	2	0	370 – 760
OPA-04	16	0	16	1	420 – 2380
OPA-17	11	2	11	1	1250 – 3470
OPB-07	6	0	8	1	910 – 2130
OPC-01	4	0	4	0	990 – 2100
OPC-10	9	1	9	0	410 – 1530
OPD-12	16	11	10	2	360 – 3190
OPE-05	8	1	6	4	590 – 1800
OPF-11	12	5	12	2	740 – 2680
OPH-14	11	3	9	0	700 – 3250
OPI-03	7	1	8	2	580 – 1360
	237	55	233	36	320 – 3470

DNA AMPLIFICATION

Twenty-seven RAPD primers were tested for amplification with *A. fistulosum* DNA; 24 of them generated reproducible RAPD profiles and were employed for our purposes (Tab. 1). The RAPD reaction mixture (10 µl total volume) contained 1 × Taq buffer with KCl (Fermentas), 2 mM $MgCl_2$, 0.25 mM dNTPs, 1 µM primer, 1 U Taq DNA Polymerase (Fermentas) and 10 ng DNA.

Amplifications were performed in an Eppendorf Mastercycler. The RAPD program consisted of initial denaturation of the DNA at 94°C for 1 min, followed by 39 cycles of 30 s at 93°C, 1 min at 34°C, and 2.5 min at 68°C. Amplification was terminated with an additional 68°C step for 5 min. DNA amplifications were performed simultaneously in the same thermocycler. The amplification products were separated on 1% agarose gel by electrophoresis, stained with ethidium bromide and photographed under ultraviolet light. Profiles that included the appearance of bands typical for seeds or calli were repeated to ensure that all bands were in the same position.

DATA ANALYSIS

RAPD data were compiled as a binary matrix of the presence (1) or absence (0) of a band at a particular position. Faint and ambiguous bands were not included in the statistical analysis. Pairwise comparisons of samples were done to generate Jaccard's coefficients. Coefficients of similarity were used to construct a phenogram using the UPGMA method in DendroUPGMA (http://genomes.urv.es/UPGMA/).

RESULTS

The total number of scored amplification products yielded by all primers was 248; of these, 237 bands were observed in seeds and 233 in calli. The number of bands generated by RAPD ranged from 2

TABLE 3. Summarized frequency of RAPD bands in the analyzed material

Primer	Bands in seeds	Present in calli, absent in seeds	Bands in calli	Present in seeds, absent in calli
RAPD 1	14		14	
RAPD 2	8	1	9	
RAPD 3	4		4	
RAPD 4	8	1	9	
RAPD 5	14		11	3
RAPD 6	12	1	12	1
RAPD 7	13		13	
RAPD 8	14	1	15	
RAPD 9	9		9	
RAPD 10	9	2	11	
RAPD 11	11	1	12	
RAPD 12	11	1	11	1
RAPD 13	8		8	
RAPD 14	2		2	
OPA-04	16		16	
OPA-17	11		11	
OPB-07	6	2	8	
OPC-01	4		4	
OPC-10	9		9	
OPD-12	16		10	6
OPE-05	8		6	2
OPF-11	12		12	
OPH-14	11		9	2
OPI-03	7	1	8	
Sum	237	11	233	15
	248		248	

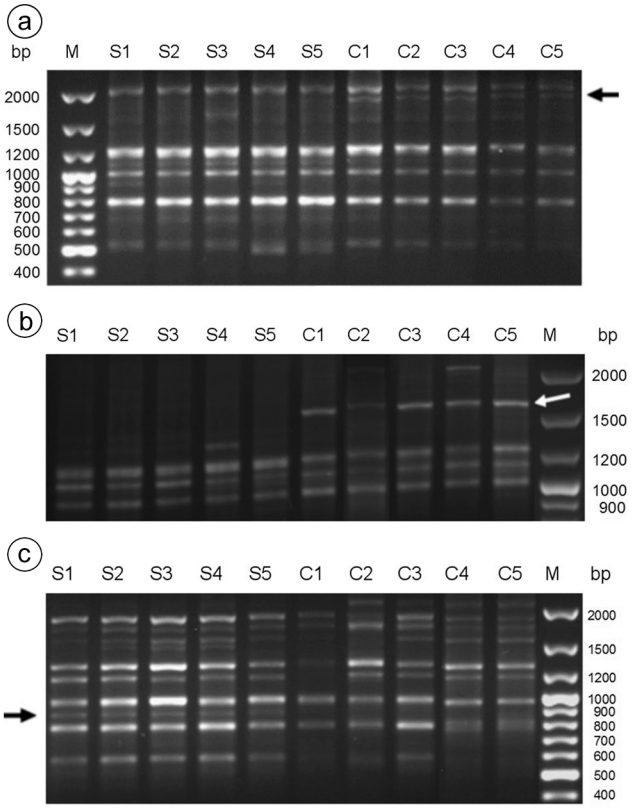

Fig. 1. RAPD pattern obtained with RAPD 2 in seeds (S1–S5) and calli (C1–C5) (**a**). M – 100 bp molecular size marker. Arrow indicates additional 1970 bp band observed in calli, (**b**) OPB-07 in seeds (S1–S5) and calli (C1–C5). M – 100 bp molecular size marker. Arrow indicates additional 1670 bp band observed in calli, (**c**) RAPD 12 in seeds (S1–S5) and calli (C1–C5). M – 100 bp molecular size marker. Arrow indicates additional 890 bp band observed in seeds.

(RAPD 14) to 16 (OPA-04 and OPD-12) (Tab. 2). Primers RAPD 1, 9, 13, 14 and OPC-01 generated similar band patterns in seeds and calli (Tabs. 1, 2), with some differences in band intensity. Such differences were not considered in further analyses. Other primers showed differences in the presence/absence of some bands between and within analyzed groups (Tabs. 2, 3).

The majority of band positions appeared to be common to seeds and calli. Some bands, however, were present in calli and absent in seeds (3 bands) (Fig. 1a, b), or vice versa (4 bands) (Fig. 1c). Bands that were found only in callus were 1970 bp (RAPD 2), 2920 bp (RAPD 6), and 1670 bp (OPB-07) in size. Bands that were found only in seeds were 1800 bp and 680 bp (OPE-05), 920 bp (RAPD 5), and 890 bp (RAPD 12) in size.

There were also some differences involving band polymorphism within seed and callus samples. The frequency of scorable polymorphic bands was 55 out of 237 in seeds and 36 out of 233 in calli. The share of variable loci in the total material analyzed was 29.4% (73 loci out of a total 248). The frequency of bands presented in both seeds and calli but variable only in callus was 6.07% (19 loci out of 248), and variable only in seeds 15% (38 out of 248).

The data on the presence/absence of RAPD bands were used to calculate values of genetic distance between all samples studied. The results are given in Table 4. The genetic distance values in seeds (0.080–0.154), calli (0.059–0.114) and between seeds and calli (0.133–0.208) suggested low genetic divergence in the analyzed material. The

TABLE 4. Genetic distance between analyzed seed (S1–S5) and callus (C1–C5) samples, based on Jaccard's distance coefficient

	S1	S2	S3	S4	S5	C1	C2	C3	C4	C5
S1	0	0.102	0.123	0.123	0.154	0.182	0.207	0.183	0.196	0.200
S2		0	0.101	0.134	0.140	0.169	0.208	0.162	0.190	0.186
S3			0	0.080	0.103	0.150	0.190	0.150	0.186	0.175
S4				0	0.111	0.133	0.181	0.134	0.186	0.174
S5					0	0.140	0.149	0.140	0.170	0.158
C1						0	0.097	0.062	0.101	0.072
C2							0	0.114	0.059	0.090
C3								0	0.093	0.072
C4									0	0.077
C5										0

average distance between seeds (0.117) and between calli (0.084) indicates greater genetic uniformity of calli.

The phenogram showing genetic similarities among the analyzed samples consisted of two separate clusters: one containing exclusively seeds, and the other containing calli (Fig. 2).

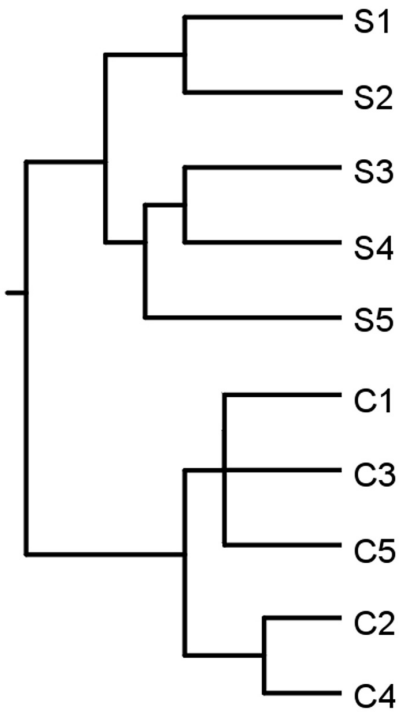

Fig. 2. Phenogram of seed (S1–S5) and callus (C1–C5) samples based on Jaccard's coefficient of similarity, obtained by UPGMA cluster analysis of markers generated with 24 primers.

DISCUSSION

The mutagenic nature of tissue culture has been known and studied for a long time (Bayliss, 1980; Lee and Phillips, 1988). Micropropagation techniques are of practical importance; many currently published papers on in vitro-induced genetic changes relate to the variability of cloned plants (somaclonal variation). Most of them show a rich array of genetic variants (Bednarek et al., 2007; Jin et al., 2008; de la Puente et al., 2008; Aversano et al., 2009; Mo et al., 2009) though only a small part of the genetic variability is transferred from cultured tissues to micropropagated plants. Genetic variation in vitro arises from chromosome alterations and/or changes on the DNA level (Lee and Phillips, 1988; Kaeppler et al., 2000; Kwasniewska et al., 2012), but it remains unclear whether these phenomena occur independently.

In this study, polymorphisms within seeds were revealed by 13 primers, and within calli by 15 of the 24 primers. Polymorphic band frequency was 55/237 in seeds and 36/233 in calli; variability on the DNA level was thus higher in seeds than in calli (23.2% vs. 15.4% of band positions). This seems surprising because *A. fistulosum* cells cultured in vitro showed extremely high cytogenetic variation. They differ in chromosome number, the presence of aberrant chromosome types, and the location/amount of repeated sequences (Joachimiak et al., 1995; Lee and Ono, 1999; Joachimiak and Ilnicki, 2003; Gernand et al. 2007). Our results would suggest that changes on the chromosomal and DNA levels occurred independently of each other.

On the other hand, the analyzed tissue samples contained thousands of cells, hence the resulting RAPD profiles do not reflect the actual diversity of cells within callus. Instead, they provide general information about the DNA templates available in a mixture of DNA from different cells. From this per-

spective, the stable bands observed exclusively in seeds or calli deserve special attention. The 5+/5- or 5-/5+ band distributions are unlikely to have arisen from natural variation because the probability of such an event is negligible. It achieves the highest value ($p < 0.001$) under the assumption of equal frequency of (+) and (–) alleles in a population. It can be assumed that both situations (5+/5– and 5–/5+) were due to mutations in tissue culture resulting in the loss of an original band or the appearance of a novel one. Seven such changes were observed (four losses and three gains). This does not mean that all tissue-cultured cells contained a particular mutation but indicates that it was present in at least some cells in each callus sample. A similar phenomenon was observed in rye, where variation in the same RAPD or AFLP products was detected in plants regenerated from different initial calli (Linacero et al., 2000; de la Puente et al., 2008). Those authors showed that such independent mutational events affected exactly the same sequences.

In the case of bands found only occasionally in calli, it is difficult to rule out that their sources were seeds with less frequent alleles. The likelihood of such situations depends on the allele frequency and, under a binomial probability distribution, may be relatively high (data not shown). It is worth mentioning that our UPGMA analysis revealed high similarity among calli, despite the fact that the Jaccard's similarity coefficient does not consider negative matches (Vierling and Nguyen, 1992).

CONCLUSIONS

Our study demonstrated that DNA variation is apparent both in seeds and in tissue cultures of *A. fistulosum*. Unlike seeds, calli are characterized by extraordinary chromosomal variation, but polymorphism on the DNA level was higher in seeds. This suggests that these two types of genetic changes may arise independently in plant tissue cultures. If so, studies of the genetic variability of cultured tissues and derived plants (somaclones) should take both components into account.

The occurrence of DNA changes common to all callus lines, together with the separated UPGMA clustering of seeds and calli, indicate that the different callus lines underwent similar genetic changes during culture in vitro, presumably due to the strong selection pressure effected by standard in vitro conditions.

AUTHORS' CONTRIBUTION

PM, DK, TI study conception and design; TI callus culture and cytological screening; PM acquisition of data; PM, DK analysis and interpretation of data; PM, DK drafting of manuscript; TI critical revision of manuscript. The authors declare that there are no conflicts of interests.

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