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Full Length Research Paper

Organelles genome stability of wheat plantlets produced by anther culture

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Plantlets derived from *in vitro* culture might exhibit somoclonal variation which is often heritable, and molecular variations may be generated *in vitro.* Since the direction of most studies is toward nuclear genome, there is a little known about the DNA of organelles. This study was conducted to test the genetic stability of wheat organelles genomes for plantlets produced by anther culture using restriction fragment length polymorphism (RFLP) analyses. One of the intergenic regions of cpDNA and one of mtDNA introns were amplified with polymerase chain reaction (PCR). The PCR products were then sequenced and digested with four restriction endonucleases (EcoR1, BamH1, Ndel and Sac1). The amplified product from cpDNA was 1000 bp in size, and digested only with Ndel into two bands with 650 and 350 bp. The amplified product from mtDNA was 1550 bp in size, and digested only with Sac1 into two bands with 1220 and 330 bp. The results obtained showed that no noticeable difference can be detected between doubled haploid plantlets and parental plants at the level of ctDNA and mtDNA organization. It can be concluded that *in vitro* culture by itself does not systematically generate a cytoplasmic variation in plant cells.

Key words: RFLP analysis, wheat plantlets, wheat anther culture, doubled haploids, genetic stability, mitochondria and chloroplast genome.

INTRODUCTION

Wheat (*Triticum aestivum* L.) is one of the most important staple food crops of the family *Poaceae.* Among the food crops, wheat is a common source of energy and proteins for the world population (Keresa et al., 2000). It is characterized by a large genome size (approximately 17000 Mb) thus making the improvement process by any method genetically challenging.

Haploid plants are very important in various research disciplines such as plant biotechnology, molecular genetics and traditional plant breeding; they provide useful information regarding recombination and genetic control of chromosomal pairing (Basu et al., 2011). Haploid plants have many uses in basic plant research such as cytogenetics, crop evolution, induced mutagenesis and on genetic transformations (Chawla, 2002; Folling and Olesen, 2002; Cuthbert et al., 2008; Touraev et al., 2009). In breeding programmes of wheat cultivars, *in vitro* anther culture technique has been utilized to obtain haploid plantlets in the F_1 generation. However, as is the case for many members of the monocot species, not all wheat species respond to the *In vitro* conditions (Yasmin et al., 2009).

An efficient doubled-haploid production technology, inducing homozygosity, can greatly reduce the time and cost of cultivar development (Liu et al., 2002). The spontaneous duplication of chromosomes often occurs within anther culture-derived callus cells, resulting in the production of fertile, doubled haploid plantlets (Kyung-Moon and Baenziger, 2005; Smith, 2000). This technique speeds up the process of development of new cultivars by several years, in addition to simplifying and making the selection process more efficient (Ramos et al., 2000).

Wheat cultivars developed from doubled haploid technology have been released as dominant cultivars (Guzy-Wróbelska and Szarejko, 2003; Thomas et al., 2003; Humphreys et al., 2007a, b; Baenziger and

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Table 1. DNA sequences of the primers used in this study.

Primer	Sequence	Reference
cp-rbcL	5'-TTC GAG TTC GAG CCG GTA GAT A	Unlu and Sumer, 2005
cp-Psal	5'-CTA AGC CTA CTA AAG GCA CGA	
mt-nad1 exon B	5'-GCA TTA CGA TCT GCA GCT CA	Sun, 2002
mt-nad1 exon C	5'-GGA GCT CGA TTA GTT TCT GC	

DePauw, 2009; Touraev et al., 2009). These cultivars could form a basis for exploitation in molecular mapping, cytogenetics and transfer of genes of interest (Mujeeb-Kazi, 2005; Ceoloni and Jauhar, 2006; Touraev et al., 2009; Basu et al., 2010). Doubled haploids have been employed in releasing several barley cultivars (Muñoz-Amatriaín et al., 2008; Gomez-Pando et al., 2009) and oat lines (Kiviharju et al., 2005).

The chloroplast genomes vary in size from 35 to 217 kb, but the vast majority from photosynthetic organisms is between 115 and 165 kb. The chloroplast genome of wheat is 134.5 Kb (Ogihara et al., 2002). Most of the genes in chloroplast genome that code for proteins are mostly involved in photosynthesis or gene expression, with the remainder being transfer RNA or ribosomal RNA genes (Martin et al., 2002). The small amount of diversity observed within chloroplast genomes, relative to nuclear genomes, led some workers to suggest that chloroplast DNA (cpDNA) restriction fragment variants could be useful in constructing molecular phylogenies for studying inter and intraspecific relationships in plants (Amane et al., 2000). The mitochondrial genome is important in plant development, as well as in productivity (Siculella and Palmer, 1988), and extensive studies have been achieved to investigate its functions (Cooper, 2000). The wheat mitochondrial genome structure is a 452.5 Kb circular molecule that revealed a nucleotide-level evidence of intramolecular recombination (Ogihara et al., 2005).

The genetic information present in the plant cpDNA and mitochondrial DNA (mtDNA) is of great interest in phylogeny and in population genetics, largely because of their characteristics of non-Mendelian inheritance. The coding regions of chloroplast genomes of higher plants are highly conserved across species and genera. However, the non-coding regions of the chloroplast genome have been described as highly variable (Ogihara et al., 1992). Therefore, by amplification and direct sequencing of these non-coding sequence regions, or simply by using a variety of restriction enzymes to explore the polymorphism, this information can be both used for phylogenetic and population genetic studies (Matyas and Sperisen, 2001).

Plantlets derived from *in vitro* culture might exhibit somoclonal variation, which is often heritable. The use of wheat *in vitro* culture could create genetic variability at the somatic cell level. This biotechnological approach requires a deeper study of the variability of nuclear, chloroplast, and mitochondrial DNA sequences (Wang and Yen, 2005) and the genetic stability of cells cultured under the conditions of isolated growth and in artificial growth media.

Several strategies have been used to find the rearrangements of wheat DNA nucleotide sequences during *in vitro* tissue culture, to assess the genetic integrity, and to detect genetic stability of plantlets produced during *in vitro* tissue culture such as restriction fragment length polymorphism (RFLP), random amplified polymorphism DNA (RAPD) and polymer chain reaction (PCR) especially inter simple sequence repeat (ISSR) and simple sequence repeat (SSR) methods have been used (Gerashchenkov et al., 2000; Leroy et al., 2000, 2001).

The aim of this study was to investigate the genetic stability of wheat plantlets produced via anther culture by testing the stability of organelles genomes of the produced plantlets using RFLP technique.

MATERIALS AND METHODS

Development of doubled haploid plantlets

The doubled haploid plantlets used throughout this study were derived from anther culture of wheat, *T. aestivum*, cv. 'Acsad 65' (Hassawi et al., 2005). The regenerated plantlets were maintained by selfing for three generations (F_1, F_2, and F_3).

Isolation of genomic DNA

Total DNA was isolated from the control plant (Acsad 65) and from five replicates of each F1, F_2, and F_3. Leaf tissues were taken from two-week old seedlings, after two days etiolation in the dark following micro CTAB method (Khan et al., 2004).

PCR analysis

Amplifications were conducted in a total volume of 25 µl that contained: 25 ng of template DNA, 0.5 µl of 10 mM dNTPs, 2.5 µl of 25 mM $MgCl_2$, 2.5 µl of 1X buffer, 1 unit of Taq polymerase, 1.0 µl of 10 µM of each forward and reverse primer (primers are listed in Table 1), and doubled distilled water to the final volume. PCR was used to amplify the chloroplast intron lies at the end of rbcL gene and beginning of psal gene, and to amplify the mitochondrial intron located between nad1 exon B and nad1 exon C using the following program: One cycle of 2 min at 94°C, 30 cycles of 50 s at 94°C, 40 s at 64°C, and 80 s at 72°C, followed by 7 min at 72°C. The

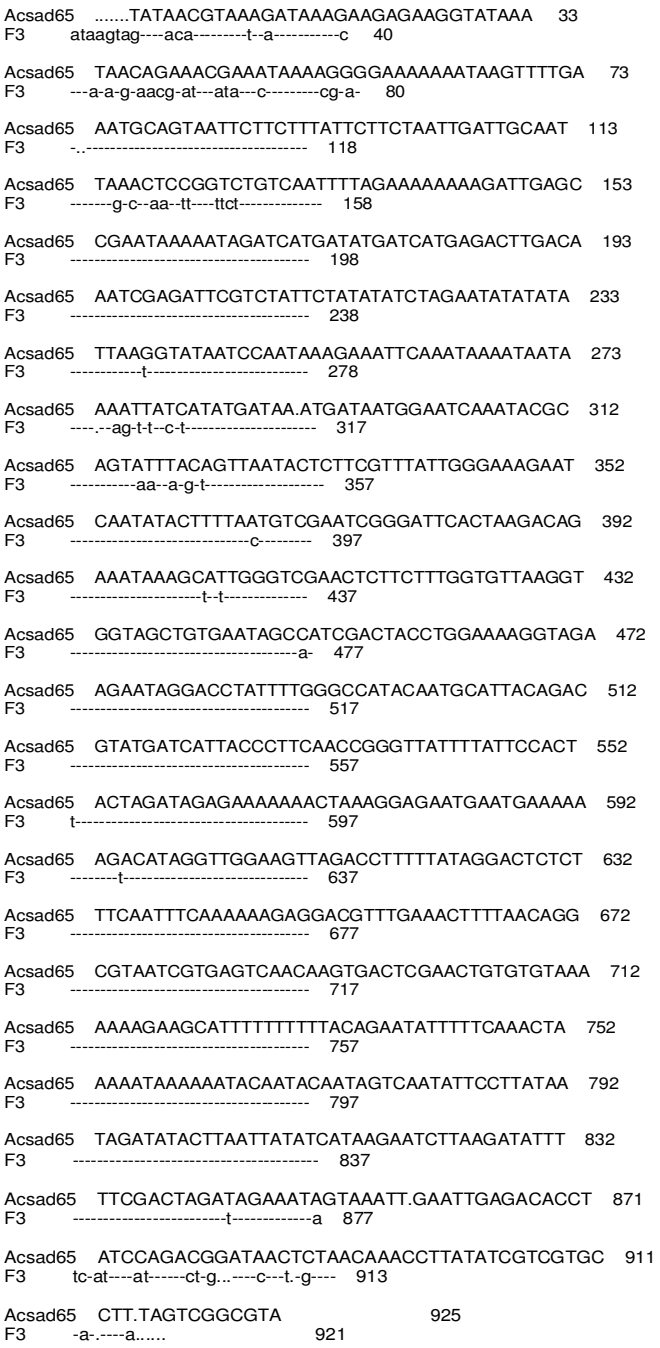

Figure 1. Alignment of chloroplast DNA sequence between mother plants and F_3 plantlets. F_1 and F_2 plantlets are not shown.

reactions samples were detected by agarose gel electrophoresis using 10 µl of the PCR products stained by ethidium bromide, and photographed under UV light.

Sequencing of PCR products

To ensure the right isolation of cpDNA and mtDNA regions, the PCR products were subjected for sequencing (Macrogen, Seoul, South Korea). Analysis of the sequences was performed using the DNAMAN™ software (lynnon Biosoft, Quebec., Canada and Version 5.2.9) and the BLAST service provided by the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/).

Digestion of PCR products

DNAMAN™ software was used to digest DNA fragments sequences for both mitochondria and chloroplast fragments amplified

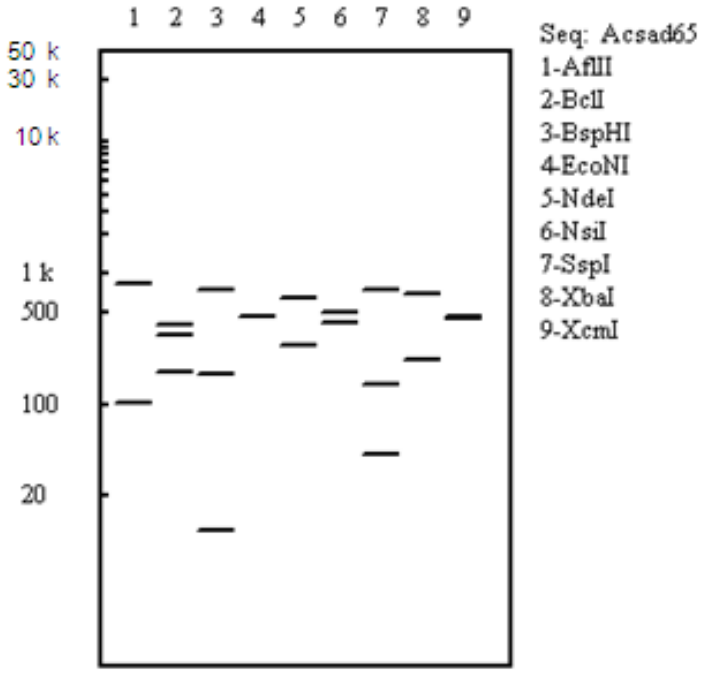

Figure 2. Digestion pattern of chloroplast DNA sequence for mother plant with restriction enzymes using DNAMAN software.

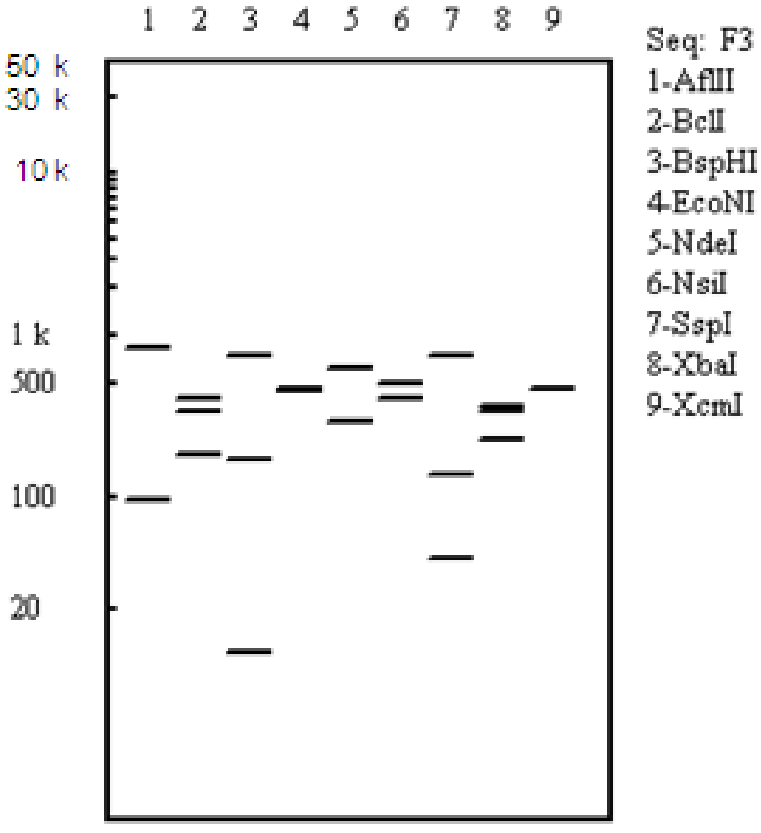

Figure 3. Digestion pattern of chloroplast DNA sequence for F3 plantlets with restriction enzymes using DNAMAN software.

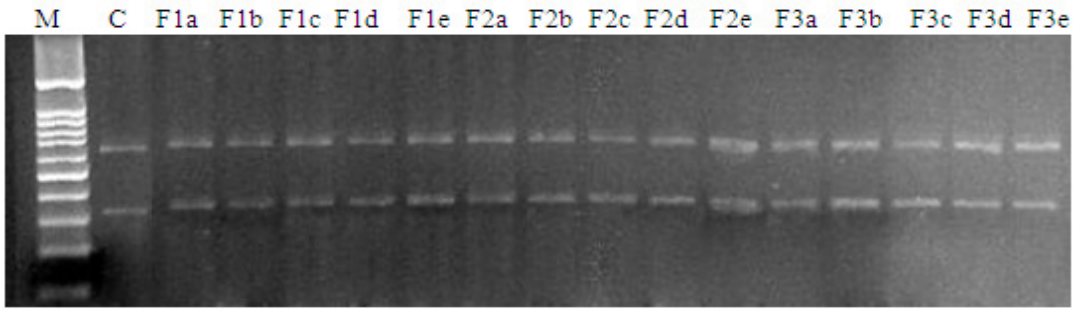

Figure 4. Restriction fragment patterns of cpDNA by NdeI. Lane M: 100 bp ladder. Lane C: Mother plant. Lane F1a-F1e: First generation replicates. Lane F2a-F2e: Second generation replicates. Lane F3a-F3e: Third generation replicates.

amplified by PCR with 117 restriction enzymes available in this software. The restriction patterns were then printed to detect the differences between the mother plants (Acsad 65), F_1, F_2, and F_3 plantlets.

DNA fragments amplified by PCR were digested with the following four restriction enzymes: NdeI, EcoRI, BamHI, and SacI. The restriction reactions were performed in 20 µl total volume that contained: 1 µl of amplification product, 2 µl of 10X restriction buffer, 10 U restriction enzyme, 1 µl BSA, and doubled distilled water to the final volume. After incubation at 37°C for 24 h, restriction fragments were separated by agarose gel electrophoresis in 1.5% agarose gels and detected by staining with ethidium bromide.

RESULTS

The region of the chloroplast genome bounded to rbcL and psaI primers was successfully amplified by PCR reaction. The size of the amplified fragments for the mother plant, F_1, F_2, and F_3 plantlets was approximately 1000 bp; these fragments were sequenced (Figure 1, shows the sequence of mother plant and F_3) and digitised with DNAMAN software by 117 restriction enzymes. One hundred and eight (108) of these enzymes revealed no restriction sites within the sequenced fragments, while 9 of them showed restriction sites. The digestion patterns of the mother plant and F_3 are shown in Figures 2 and 3. To confirm these findings, restriction digestion reactions for the PCR products of the cpDNA for mother plant, F_1, F_2, and F_3 were conducted in the laboratory with four available restriction enzymes (EcoRI, BamHI, SacI, and NdeI). No restriction sites were found for EcoRI, BamHI, and SacI, while one restriction site observed for NdeI resulted in two bands with approximately 650 and 350 bp (Figure 4); these results are in agreement with the digestion patterns represented in Figures 2 and 3 for NdeI.

The mitochondrial nad1 exon B and nad1 exon C primers are anchored to the b and c exons; these primers amplify a fragment of approximately 1550 bp. The fragments of the mother plant, F_1, F_2, and F_3 plantlets were sequenced (Figure 5, shows the sequence of mother plant and F_3) and digitised with DNAMAN software by 117 restriction enzymes. Eighty-one (81) of these enzymes revealed no restriction sites within the sequenced fragments, while 36 of them showedrestriction sites. The digestion patterns of the mother plant and F_3 are shown in Figures 6 and 7. To confirm these findings, restriction digestion reactions for the PCR products amplified from mtDNA were digested in the laboratory with four available restriction enzymes (EcoR1, BamH1, Sac1, and NdeI). No restriction sites were found in this region for EcoRI, BamHI, and NdeI; one restriction site observed for SacI resulted in two bands with approximately 1220 and 330 bp. These results are in agreement with the digestion patterns represented in Figures 6 and 7 for SacI.

DISCUSSION

The genetic information present in the plant cpDNA and mtDNA is of great interest in molecular genetics studies. Therefore, by amplification and direct sequencing of the non-coding sequence of these regions, or simply by using a variety of restriction enzymes to explore the polymorphism, this information can be used both for genetic investigation. This research was carried out in order to determine the usefulness of restriction site analysis of a PCR amplified products of cpDNA and mtDNA for measuring the stability of wheat plantlets produced via anther culture technique. For the examined genetic material, no visually detectable changes were observed among the chloroplast and mitochondrial PCR products when comparing the patterns of the mother plant (Acsad 65) to those of doubled haploid plantlets (F_1, F_2, and F_3) following separation on agarose gel. This is in agreement with the results of Rode et al. (1985) who mentioned that no noticeable difference can be detected between doubled haploid lines and parental line at the level of ctDNA and mtDNA organization; that is *in vitro* culture by itself does not systematically generate a cytoplasmic variation in plant cells.

The PCR fragment amplified with rbcL and psaI primers was 1000 bp in size; this size was obtained from each of the mother plant, F_1, F_2 and F_3 plantlets. When the

```
Acsad_65  ..CCAGGATATA.CAATCGAGCT..ATTCTATTGTGCGCC    35
F3        ...-c-ac----.--cag-----..-----t---------    34

Acsad_65  TTGTGAGCACGTTTGGATCCGCGAAGGCAATCGCTCGGAT    75
F3        ----------------------------------------    74

Acsad_65  CTTCCCC.TAACCCAACCCGGGAACGGACCGGAGGGAACC   114
F3        -------.-------------------------------   113

Acsad_65  GCAGCATGAGGAATGTCCGCGTCTCGTCGCAAGGCTCATT   154
F3        ----------------------------------------   153

Acsad_65  TTGAGTTTTGGGTCATAGGGCGGGCAGTGCAGTCCGGGGC   194
F3        ----------------------------------------   193

Acsad_65  ACAAGGGTCCTGGTACTACCCAGGTGCGAAGAACCCCGGA   234
F3        ----------------------------------------   233

Acsad_65  GGCGACTGCAATGAGCAAAAATGTCACTCACCGGCCTAAA   274
F3        ----------------------------------------   273

Acsad_65  CGACGAGCAAACACTCGAACGTGAGAGCAAGGGATCACCC   314
F3        ----------------------------------------   313

Acsad_65  AACGAATGGACGAGCTCCAAGGAGGGAGGAGAGAGGGAGG   354
F3        ----------------------------------------   353

Acsad_65  CAAGAACCATGCTTTCAGAGAAGTGGCGGTCCGAATCCAC   394
F3        ----------------------------------------   393

Acsad_65  TCTGACAAAATAAAATAACAGACTAAGCCGTGCCGTAAGG   434
F3        ----------------------------------------   433

Acsad_65  GGGGCATTCTCCACACGGGACGGGGCCAAGGCCTTCATGT   474
F3        ----------------------------------------   473

Acsad_65  ATGGGTGACAGATCGGCCATAGGAGTACTCCGGGATATAC   514
F3        ----------------------------------------   513

Acsad_65  ACCAGGGCAACTAATGTCGAGCATACGATGATGCCGCCCG   554
F3        ----------------------------------------   553

Acsad_65  TTTTCATTTCGTGAAAGTCCCCGGCAGAGGAAAGGGCTGT   594
F3        ----------------------------------------   593

Acsad_65  AGGTGATGGCGCGTTCTGCTTCTTAGGGCGATGAAATCGT   634
F3        ----------------------------------------   633

Acsad_65  TCCTATGGGATCGTGCGTGGCAGCTGGTATAGATGATGAT   674
F3        ----------------------------------------   673

Acsad_65  GAAAGGCGGGCCGCTTGAACCGGGACCTATTCTCATAATA   714
F3        ----------------------------------------   713

Acsad_65  GGCAGCAAGCAAAGCTAAATAGGAAAGGGGGCGACTGATG   754
F3        ----------------------------------------   753

Acsad_65  ACTGCCTTTTTCGTCAGAAATCAAAAAGGGTGGAATGTGG   794
F3        ----------------------------------------   793

Acsad_65  AGCTAATATGGCTGG.CTACAAGTATAGCCAAAGAAAGAT   833
F3        ---------------.------------------------   832
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Figure 5. Alignment of mitochondrial DNA sequence between mother plants and F3 plantlets. F1 and F2 plantlets are not shown.

restriction enzyme NdeI was used to digest the 1000 bp fragment, the restriction pattern of cpDNA shows that this enzyme had a single restriction site within this fragment resulted in 2 bands with approximately 350 bp and 650 bp. This is in agreement with Unlu and Sumer (2005) and Kucuk et al. (2006) who clarified that region of chloroplast genome bounded by *rbcL* and *psaI* genes was amplified by PCR reaction and produced 1000 bp with *T. aestivum*.

The PCR fragment amplified from mitochondrial genome by the primers nad1 exon B and nad1 exon C

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Acsad_65  GAGACGAGACGGACTGTCAGAGGACGCAGCGGGACTACCA  873
F3        ----------------------------------------  872

Acsad_65  GGGGAAAG...CCCGCCCCCGCTAGCTAA.CATAGATATC  909
F3        --------...----------.---------  908

Acsad_65  TATTCT.CGGTGCGCATATTCGAG.ATTTAGAATCTCTTT  947
F3        ------.----------.----------  946

Acsad_65  CC.GACCAGGCCAGGCCGAATCGGGCCACCACTTGGGATG  986
F3        --.----------------------------------  985

Acsad_65  GG.AATGGCTCAGCCCACATGCATCATTTGATGAAAGCAC  1025
F3        --.----------------------------------  1024

Acsad_65  TCCAGTCCAGTCGCCTCCGATCAGTGGCTTGTCAACCACC  1065
F3        ----------------------------------------  1064

Acsad_65  GGACCGTAGCTCCTCACCAAATGCCCCTGCGTTGGGGCAA  1105
F3        ----------------------------------------  1104

Acsad_65  CACAGCACATGACTAGTTCGCTCAAGGACCACACCCTCTC  1145
F3        ----------------------------------------  1144

Acsad_65  GAGAGCAGGATGCCGGCCGAGATGGAGCGCCAACCTAGAC  1185
F3        ----------------------------------------  1184

Acsad_65  TTTCCTTGGGCTTGCCTTGCCCCAACATCTAAATAAAGGG  1225
F3        ----------------------------------------  1224

Acsad_65  CGGGCGCCGAAAAGGAAGCAGTGACGCCTTTTCGACGAAA  1265
F3        ----------------------------------------  1264

Acsad_65  GCTTAGCTTGGTAGGCGCAGCTTACTCACCCTACTCCCTT  1305
F3        ----------------------------------------  1304

Acsad_65  AGACAATGCAATGCTCTGAACACGAAAGTTTGCAGTTCAG  1345
F3        ----------------------------------------  1344

Acsad_65  CCCTCTTCTCCCATGCAGAGTCGCAGGCAGCGCCTCGGAT  1385
F3        ----------------------------------------  1384

Acsad_65  AAAAGCACGGACGAGCCACATGCAGGGAAACTTGCACGTG  1425
F3        ----------------------------------------  1424

Acsad_65  TGGTTCTGGCCGGGGACCCCGGTATACTGTACTAATATGT  1465
F3        ----------------------------------------  1464

Acsad_65  GTAGGTCCCTGTAATTCGAGTGAGATTGTCATGGCGCAAA  1505
F3        ----------------------------------------  1504

Acsad_65  AGCAGATATGGTCCTG.ATTCCCTGTGCCCCGTCATAATC  1544
F3        ------------g-t--cc-----t------ttgt---  1544

Acsad_65  C.TCGGGGGGG                    1553
F3        ta----t--.                    1553
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Figure 5. Contd.

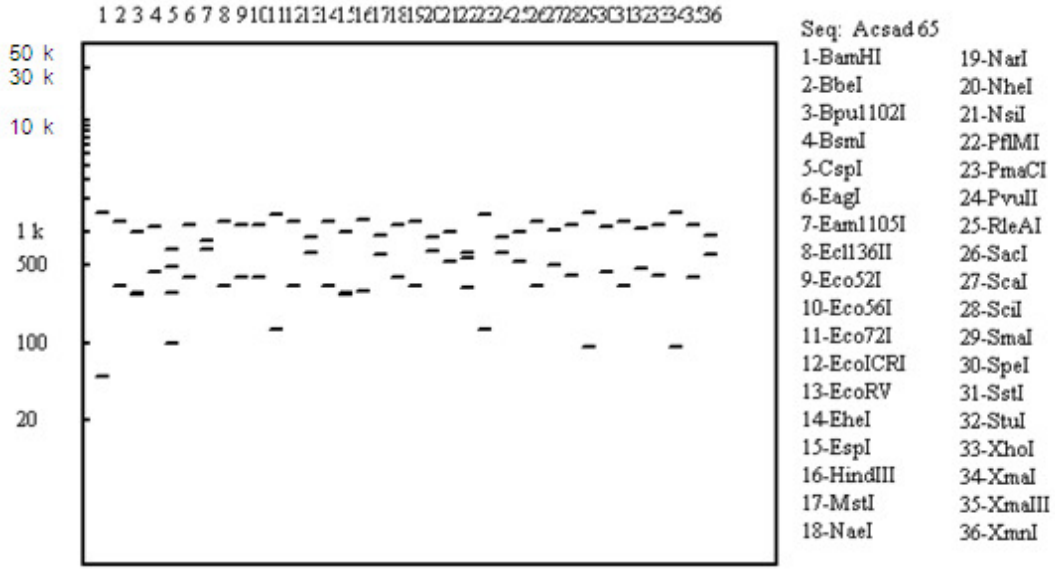

Figure 6. Digestion pattern of mitochondrial DNA sequence for mother plant with restriction enzymes using DNAMAN software.

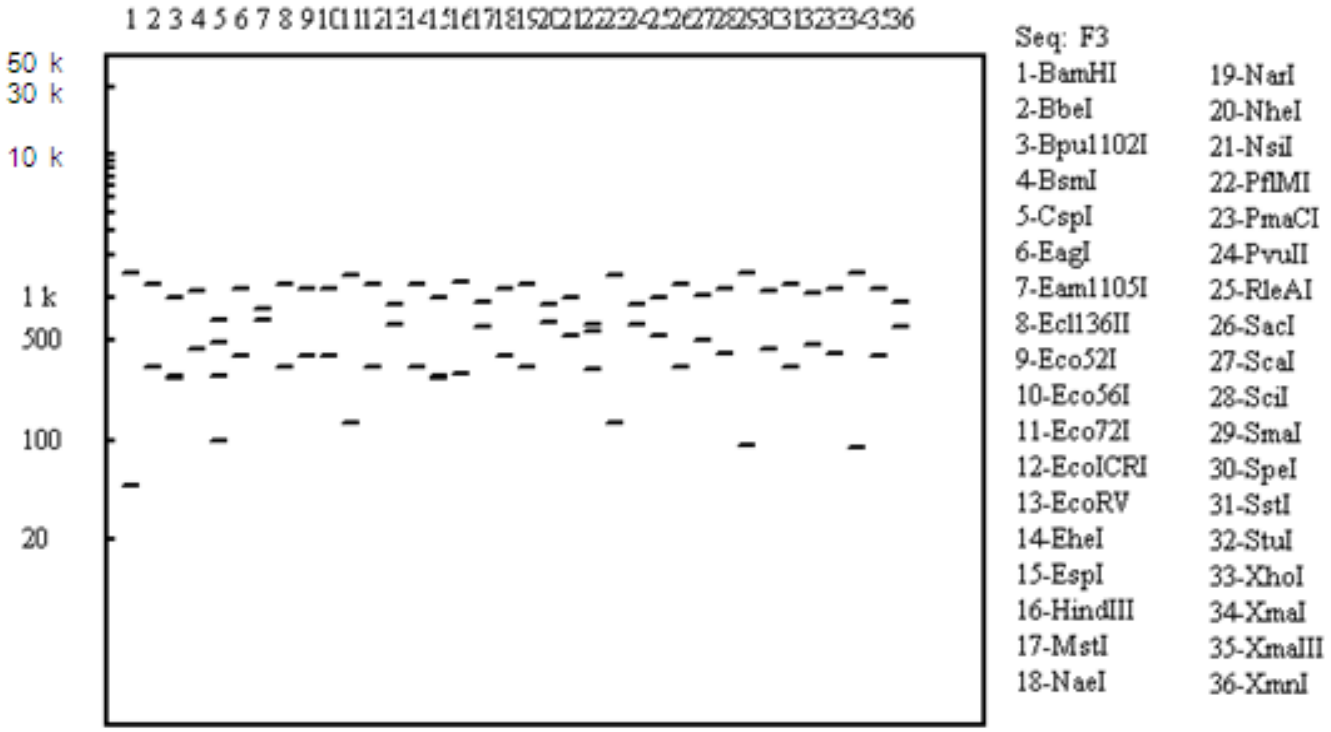

Figure 7. Digestion pattern of mitochondrial DNA sequence for F3 plantlets with restriction enzymes using DNAMAN software

was 1550 bp in size in the mother plant and also in the three plantlets generations. When using SacI to digest this fragment, a single restriction site for SacI existed, this resulted in two bands of 1220 bp and 330 bp. This is in agreement with Sun (2002) who stated that mitochondrial nad1b and nad1c primers amplify a fragment of 1600 bp in *Elymus* species. Sequence analysis revealed no variations within the sequences of the amplified fragments of both cpDNA and mtDNA. These findings indicate that F_1, F_2, and F_3 plantlets produced from anther culture remain stable and their genetic material showed no modifications compared to the mother plant.

In conclusion, the present study was based on PCR-RFLP analysis of two cpDNA fragments, and one mitochondrial nad gene DNA fragment. They have been shown to be informative for the analysis of stability of wheat plantlets generated by anther culture. Four restriction enzymes were used to digest these regions; one restriction site was found for each of NdeI with cpDNA and SacI with mtDNA. The cpDNA and mtDNA of wheat remain stable after anther culture, and the high rate of multiplication in callus stage did not cause notable changes within their DNA sequences. Anther culture can be used as an efficient method for the propagation of large number of wheat plantlets that are typical to their parent in their cpDNA and mtDNA. Several coding and noncoding regions within cpDNA and mtDNA of wheat plantlets need to be studied to confirm their genetic stability; also, it is important to test the stability of several coding and noncoding regions within the nuclear genome of wheat plants after anther culture propagation. *In vitro* anther culture by itself does not involve systematically cytoplasmic variability, it can thus be concluded that the induction of cytoplasmic variability consecutive to *in vitro* anther cultures is not a general phenomenon. Never the less, plant cell culture by itself may sometimes generate genetic variability in cultured sub-clones as well as in regenerated plantlets; therefore, further studies are needed to confirm our findings.

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