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Non-radioactive organization and transcript analysis of the ATPase subunit 6 gene region in the mitochondrial genome from fertile and sterile (CMS) forms of wheat and triticale

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Abstract In sterile triticale forms [with cytoplasmic male-sterile (CMS)-inducing *timopheevi* cytoplasm], fertile forms (with normal cytoplasm) and the corresponding wheat cross parents mitochondrial DNA (mtDNA) and RNA (messenger mtRNA) were characterized using total DNA and RNA material for Southern and Northern blots. A novel non-radioactive technique was applied by marking the probes with digoxenin. The fertile and sterile *Triticum* and triticale forms were analysed in three genes, *atp6*, *coxIII* and *rps13*. These forms can be distinguished in the *apt6* gene at the mtDNA and mtRNA levels.

Key words Triticale · Cytoplasmic male sterility · Analysis of the mitochondrial *atp6* gene region

Introduction

Cytoplasmic male sterility (CMS) is a widely occurring phenomenon in higher plants that is found in many species (Edwardson 1956, 1970) including important crops such as maize, rice, sorghum and sunflower. The CMS phenotype is essential for the commerical production of hybrid seed because self-pollination of the female parent is prevented. In alloplasmic situations the CMS phenotype is probably caused by a nuclear-mitochondrial incompatibility (Lonsdale 1987). This type of incompatibility can be investigated by comparing plants with identical cytoplasms but different nuclear backgrounds, and *Triticum* and the man-made cereal crop triticale (× *Triticosecale* Wittmack) are good material for this type of research.

Triticale is an intergeneric hybrid that combines the genomes of *Triticum* and *Secale*. Different alloplasmatic lines of wheat that derive from the cytoplasm of *Triticum timopheevi* exhibit a stable pollen sterility (Baier et al. 1978) This cytoplasm is able to induce male sterility in triticale and the expression of this sterility is dependent on the rye part (Cauderon et al. 1985; Pfeiffer-Schad 1989).

CMS systems have been studied in detail using both genetic and molecular methods (Hanson and Conde 1984). In all of these systems the fertile/sterile phenotype is correlated with differences in the organization and expression of the mitochondrial genes.

In this paper we present an analysis of the wheat and triticale *atp6* gene region, which demonstrates differences in fertile- and male-sterile plants. In the fertile cytoplasm the ATPase subunit 6 gene is located on a 1.4-kb repeated element that generates four different arrangements (Bonen 1987). On the other side the *timopheevi* cytoplasm indicates a completely different stituation: here a single-copy gene codes for the ATPase subunit 6 (Mohr 1991; Mohr et al. 1993). In the past such investigations had to rely almost exclusively on the use of DNA probes labeled with [^{32}P]. The use of radioactive probes encompasses several problems including laboratory safety, disposal of radiocative waste and the short half-life of [^{32}P]. Therefore, we have developed a non-radioactive DNA labeling and detection method using Boehringer's kit (1989).

Materials and methods

Plant material

In this study the following wheat and triticale genotypes were used: *Triticum durum* with *durum* cytoplasm (D8), *Triticum durum* with *timopheevi* cytoplasm (cms D8), and fertile and sterile triticale of cross number 228. The triticale forms are primary types. The fertile type was produced by crossing *Triticum durum* with *durum* cytoplasm (D8), the sterile type by crossing *Triticum durum* with *timopheevi* cytoplasm (cms D8), with in both cases the rye inbred line L 305 being the rye parent.

Preparation of total DNA and RNA

Total DNA and RNA were isolated using a modification of a procedure described by Hoge et al. (1982). Etiolated shoots (approximately 2 g) were harvested after 20 days, ground in a mortar with

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liquid nitrogen and the powder dissolved in equal volumes of boiling lysis buffer (0.2 *M* boric acid, 30 m*M* EDTA, 1% SDS, pH 9.0) and saturated phenol. The mixture was centrifugated for 10 min at 12000 *g* and 4 °C. The supernatant was extrated twice with phenol/chloroform/isoamylalcohol (25:24:1). After precipitation of the nucleic acids with 3 *M* sodium acetate the pellet was dissolved in bidistilled water. The RNA was selectively precipitated by adding lithium chloride (4 *M*) and incubation for 2–4 h at 0 °C. The RNA precipitate was washed twice with 70% ethanol and dissolved in bidistilled water. After dialysis [in 0.1 TE buffer (50 m*M* TRIS, 20 m*M* EDTA, pH 8.0)] of the DNA-containing supernatant for 12 hours the DNA was precipitated with 0.3 *M* sodium acetate, washed twice with 70% ethanol and dissolved in bidistilled water.

Heterologous probe

The probe used for DNA/DNA and DNA/RNA hybridization corresponds to a *Eco*RI fragment from *Oenothera* that contains one of the four possible recombination products from the *atp6* gene region together with the 5'- and 3'-flanking genes for cytochrome C oxidase subunit 3 (*cox III*) and the ribosomal protein 13 (*rps 13*). The fragment was subcloned into plamid pBR328 by S. Mohr and E. Schulte-Kappert (Ruhr-Universität Bochum, Institut für Botanik, Bochum Germany) and named pTae8.

Restriction endonuclease digestion

Total DNA was digested with *Eco*RI according to Sambrook et al. (1989).

Electrophoresis and Southern blotting

The separation of the DNA fragments was effected in 0.6% agarose gels in TRIS-borate-EDTA (1 *M* TRIS, 1 *M* boric acid, 20 m*M* EDTA, pH 8.3). The DNA in the gel was denatured by successfully soaking the gel for 30 min in NaOH-NaCl (1.5 *M* NaCl, 0.5 *M* NaOH, pH 5.5). The capillary transfer of the denatured DNA fragment onto nylon membranes (Hybond-N, Amersham) was standarized according to Southern (1975). The filter was baked for 2 h at 80 °C.

Electrophoresis and Northern Blotting

All solutions are free of RNAse using pretreatment with 0.05% diethylpyrocarbonate (DEPC). Total RNA was fractionated in 1.0% agarose gels according to Sambrook et al. (1989). The overnight transfer of the RNA onto nylon membranes (Hybond-N, Amersham) was carried out as described by Sambrook et al. (1989). Subsequently the filter was baked for 2 h at 80 °C.

DNA labeling with digoxigenin (Dig)

DNA was labeled with Dig according to a modified (Hoeltke et al. 1990) random-primed DNA labeling protocol (Feinberg and Vogelstein 1983, 1984). An aliquot of 1 µg DNA was diluted with H_2O to 10 µl and denatured for 10 min at 100 °C. After rapid cooling on an ice-ethanol mixture for 4 min, 2 µl of the hexanucleotid mixture, 2 µl of the dNTP labeling mixture and 2 U Klenow enzyme were added. The mixture was incubated at 37 °C for 24 h, and then the reaction was stopped by the addition of 2 µl EDTA (0.2 *M*, pH 8.0). The labeled DNA was precipitated with 2.5 µl LiCl (4 *M*) and 75 µl ethanol (100% v/w, −20 °C). The mixture was left for at least 2 h at −40 °C. The precipitate was then centrifuged at 8000 rpm for 30 min, washed with cool ethanol (70% v/w), dried and dissolved in 50 µl TE (10 m*M* TRIS, 1 m*M* EDTA, pH 8.0).

DNA labeling with ^{32}P

The DNA probe was labeled by random priming using [α-^{32}P] dATP (the specific activity was 110 TBq/mM) according to Feinberg and Vogelstein (1983).

Southern hybridization with Dig-labeled probes

The prehybridization was carried out in a hybridization flask for 2 h at 50 °C in Dig hybridization buffer [50% (v/v) formamide, 50% (v/v) 5 × SSC (3 *M* NaCl, 0.3 *M* sodium citrate, pH 7.0), 0.1% (w/v) lauroylsarcosine, 0.02% (w/v)SDS, 2% Boehringer-blocking reagents, 50 µg/100 ml denaturated herring sperm DNA]. For hybridization, the prehybridization buffer was replaced by 10 ml hybridization solution per 100 cm^2 filter, and 0.1 µg of DIG-labeled DNA per milliliter hybridization was used. The hybridization was performed for 16 h at 50 °C. After removal of the hybridization solution the membrane was washed twice in 2 × SSC, 0.1% (w/v) SDS at room temperature and twice in 0.1 × SSC, 0.1% (w/v) SDS at 68 °C.

Northern hybridization with Dig-labeled probes

Northern prehybridization and hybridization was performed at 50 °C in Dig hybridization buffer [50% (v/v) formamide, 50% (v/v) 5 × SSC (3 *M* NaCl, 0.3 *M* sodium citrate, pH 7.0), 50 m*M*/1 sodium phosphate, 2% (w/v) Boehringer blocking reagents, 7% (w/v) SDS, 0.1% (v/v) diethylpyrocarbonate]. The filter was washed after hybridization by the same procedure as that used for Southern hybridization.

Northern hybridization with [^{32}P]-labeled probes

Prehybridization was performed for 1 h at 37 °C followed by hybridization for 16 h at 37 °C. The hybridization medium contained 50% (v/v) formamide, 5 × SSPE (0.9 *M* NaCl, 5 m*M* EDTA, 50 m*M* sodium phosphate, pH 7.4), 0.5% (w/v) SDS, 100 µl/100 ml herring sperm DNA. After hybridization the filter was washed in 5 × SSPE, 0.2% (w/v) SDS for 5 min at 60 °C. After this procedure the membrane was dried at 37 °C. The filter was finally autoradiographed in X-ray film at −20 °C for 20 days.

Immunological detection of DNA and RNA

Except for the equilibration, the colour reaction and the last two wash steps all of the incubations were performed in hybridization flasks at room temperature. The membranes were briefly washed for 5 min with 100 ml buffer 1 [0.1 *M* maleic acid, 0.15 *M* NaCl, 0.3% (w/v) Tween 20, pH 7.5], incubated for 30 min in 100 ml buffer 2 [1% (v/v) blocking reagents in buffer 1], incubated for 30 min in 20 ml diluted Dig-AP solution, equilibrated for 2 min with 100 ml buffer 3 (0.1 *M* TRIS, 0.1 *M* NaCl, 50 m*M* $MgCl_2$, pH 9.5 and finally incubated with 100 ml freshly prepared colour solution [100 µl nitro-blue tetrazolium salt (NBT), 75 µl 5-bromo-4-chloro-3-indolyl phosphate (BCIP), 100 ml buffer 3] in a plastic box in the dark for 8–20 h.

Results and discussion

Comparative hybridization analysis of total DNA from fertile and male-sterile *Triticum* and triticale genotypes

To study structural differences in the *Triticum* and triticale mitochondrial genomes which are possibly re-

lated to the CMS trait we attempted to determine the location of the atp6 gene using the pTae8 probe, which contains three mitochondrial genes: ATPase subunit 6 (*atp6*), cytochrome C oxidase subunit (*coxIII*) and ribosomal protein 13 (*rps13*). Figure 1 shows the results of the hybridization analysis of total DNA from *Triticum durum* with either *durum* or *timopheevi* cytoplasm. The pTae 8 probe hybridized strongly with four *Eco*RI fragments (12.0 kb, 11.0 kb, 10.0 kb and 9.0 kb) from the mtDNA of *Triticum durum* with *durum* cytoplasm and also with two fragments (12.2 kb and 3.0 kb) in the mitochondrial genomes of the sterile lines (*Triticum durum* with *timopheevi* cytoplasm). The same result was also observed for the location of the *Eco*RI fragment in the different triticale genotypes (Fig. 2).

According to Bonen (1987) in the fertile *Triticum aestivum* the *atp6* gene is located on a repetitive sequence which exists in two copies. Four different recombination products are possible by homologous recombination of the two copies. It is also known from the results of Bonen (1987) that two genomic copies contain the *rps13* gene in the 3′ position of the *atp6*, while the *coxIII* gene is induced in the other two copies in the 5′ position of the *atp6* gene. In our investigation the four genomic environments were also detectable in *Triticum durum* and triticale with *durum* cytoplasm although two copies were in a lower stoichiometric quantity. In plants with *timopheevi* cytoplasm the situation is completely different: the *atp6* exists as a single-copy gene including the *rps13* gene (Mohr 1991; Mohr et al. 1993). This copy is located on the 3.0-kb *Eco*RI fragment. The 12.2-kb fragment contains the *coxIII* gene. In contrast to the fertile cytoplasm in *timopheevi* the *coxIII* gene is not placed in the genomic environment of the *atp6* but upstream of the *orf25* gene (Mohr 1991; Mohr et al. 1993).

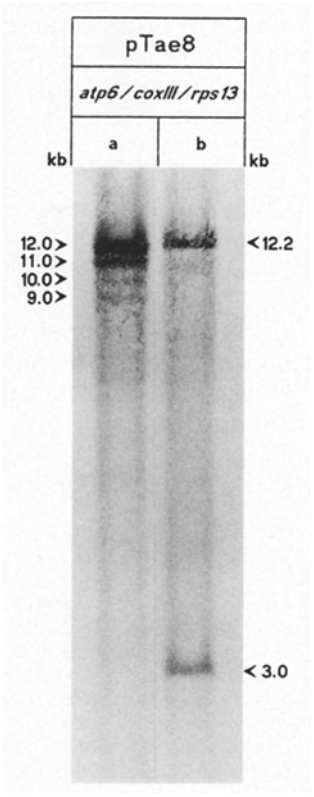

Fig. 2 Southern hybridization of a Dig-labeled DNA probe with total DNA isolated from triticale of cross number 228 (*line a*: with *durum* cytoplasm, *line b*: with *timopheevi* cytoplasm). The Dig-labeled probe contains the *atp6* gene region (*atp6*, *coxIII* and *rps13*). The *numbers* on both sides of the electropherogram show the sizes of the fragments in kb

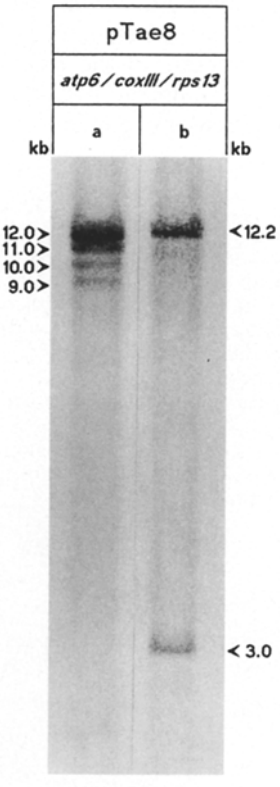

Fig. 1 Southern hybridization of a Dig-labeled DNA probe with total DNA isolated from *Triticum durum* with *durum* cytoplasm (*line a*) and total DNA from *Triticum durum* with *timopheevi* cytoplasm (*line b*). The Dig-labeled probe contains the *atp6* gene region (*atp6*, *coxIII* and *rps13*). The *numbers* on both sides of the electropherogram show the sizes of the fragments in kb

Transcription of the mitochondrial genes in fertile and sterile *Triticum* and triticale genotypes

Northern blots were hybridized with the three mitochondrial genes *atp6*, *coxIII* and *rps13* in order to analyse the transcriptional patterns of the protein coding the mitochondrial genes. The pTae 8 probe detected differences in the transcripts of sterile and fertile *Triticum* and triticale: the transcripts in the fertile cytoplasms were 1.2 kb and 1.4 kb in size, while male-sterile cytoplasm contained transcripts of 1.2 and 1.6 kb (Figs. 3 and 4).

The 1.2 kb fragment is identical with the *coxIII* transcript in *Triticum* (Gualberto et al. 1990). In the fertile cytoplasm the larger fragment of 1.4 kb (*atp6* mRNA) corresponds approximately to the recombinationally active repeated DNA (Bonen and Bird 1988); in the sterile cytoplasm (*timopheevi*) the *atp6* transcript was 1.6 kb in size. According to Mohr (1991) extensive sequencing analysis of the 3′ and 5′ flanking regions of the *atp6* gene indicates that the termination sequence is located in both cytoplasms at the same position. Therefore, the different size of the transcripts is possibly

caused by distinct transcript initiation in the 5′ region of the *atp6* gene.

In both cytoplasms the *rps13* gene showed no stable transcript.

Comparison of the non-radioactive Dig hybridization method and the conventional radioactive [^{32}P] method

In Northern blots of total RNA the sensitivity of the non-radioactive detection technique was compared with that of the radioactive detection method. Figures 3 and 5 show the results of an experiment in which the pTae8 probe was labeled with either digoxigenin or [^{32}P] and both probes were then used to detect *atp6* and *coxIII* transcription patterns. Fragments of 1.2, 1.4 and 1.6 kb were detected on both filters. The non-radioactive method (Fig. 3) demonstrated a sensitivity at least equal to that of the one using radioactively labeled probes (Fig. 5) while lacking such problems as laboratory safety, disposal of radioactive waste and the short half-life of [^{32}P]. Furthermore, the Dig detection required a shorter time of filter exposure (8 h in comparison to 20 days). In contrast to the [^{32}P]-technique the filter exposure is always verifiable and can be interrupted for a short time and continued afterwards. Another advantage is the fact that Dig-labeled probes can be synthesized in large quantities and then stored for at least 1 year (Kessler et al. 1990). Boehringer (1989) proposes an increase in the volume of the prehybridization buffer and the addition of heterogenic nucleic acids for a reduction in the colour background. In the course of our investigations these methodical suggestions for improvement were tested but without success. In this connection it was found that doubling of the prehybridization time and increase in the concentration of the block reagents showed possitive effects.

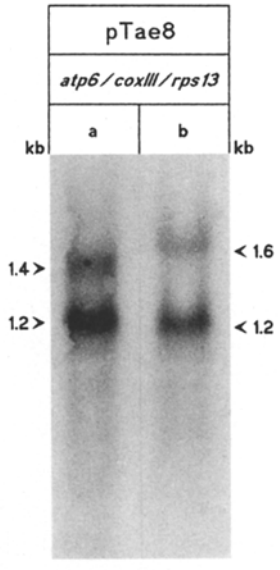

Fig. 3 Norhern hybridization of a Dig-labeled DNA probe with total RNA isolated from *Triticum durum* (*line a*: with *timopheevi* cytoplasm, *line b*: with *durum* cytoplasm). The Dig-labeled probe contains the *atp6* gene region (*atp6*, *coxIII* and *rps13*). The *numbers* on both sides of the electropherogram show the sizes of the fragments in kb

Future directions

Recently a few papers have been published that are connected with our own investigations. A research group in France has shown a great variability in the construction of the mitochondrial genome the DNA level in euplasmic wheat plants regenerated from different in vitro-cultured explants of a single variety (Morere-Le Paven et al. 1992b). Further results of this group are also very informative about the relationship between the structure of the mitochondrial genome and characters of the tissue cultures: "The presented data strengthen the hypothesis of an association between a particular organization of the mitochondrial genome in

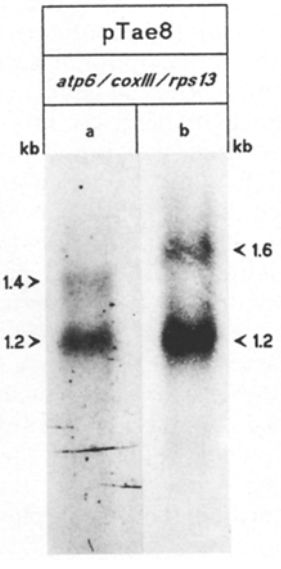

Fig. 4 Norhern hybridization of a Dig-labeled DNA probe with total RNA isolated from triticale (*line a*: with *timopheevi* cytoplasm, *line b*: with *durum* cytoplasm). The Dig-labeled probe contains the *atp6* gene region (*atp6*, *coxIII* and *rps13*). The *numbers* on both sides of the electropherogram show the sizes of the fragments in kb

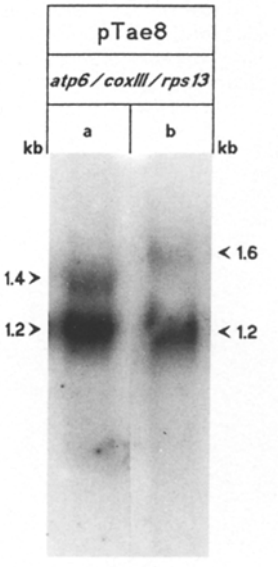

Fig. 5 Norhern hybridization of a [^{32}P]-labeled DNA probe with total RNA isolated from *Triticum durum* (*line a*: with *timopheevi* cytoplasm, *line b*: with *durum* cytoplasm). The [^{32}P]-labeled probe contains the *atp6* gene region (*atp6*, *coxIII* and *rps13*). The *numbers* on both sides of the electropherogram show the sizes of the fragments in kb

tissue culture and its regeneration capacity" (Morere-Le Paven et al. 1992a).

In special consideration of the results of the above-mentioned group the future investigations of our own research team will be directed towards the extent of somaclonal variation using different triticale forms that were regenerated from tissue or suspension cultures and a larger number of mitochondrial probes at the DNA and RNA level. In the course of our investigations attention will be directed to the possible connection between the organization of the mitochondrial genome and regeneration capacity in tissue cultures of triticale froms.

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References

Baier WH, Keydel F, Odenbach W (1978) Pollensterile Winterweizenlinien unter verschiedenen Umweltbedingungen. Z Pflanzenzuecht 80:134–141

Boehringer Mannheim (1989) DIG DNA labeling and detection non-radioactive. Applications manual. Boehringer Mannheim GmbH Biochemica

Bonen L (1987) The mitochondrial S13 ribosomal protein gene is silent in wheat embryos and seedlings. Nucleic Acids Res 15:10393–10404

Bonen L, Bird S (1988) Sequence analysis of the wheat mitochondrial atp 6 gene reveals a fused upstream reading frame and markedly divergent N termini among plant ATP 6 proteins. Gene 73:47–56

Cauderon Y, Cauderon A, Gay G, Roussel J (1985) Alloplasmatic lines and nucleo-cytoplasmatic interactions in triticale. In: Bernard M, Bernard S (eds) (1985) Genetics and Breeding of Triticale. Clermont-Ferrand. INRA Paris, pp 177–192

Edwardson JR (1956) Cytoplasmatic male sterility. Bot Rev 22: 696–738

Edwardson JR (1970) Cytoplasmatic male sterility. Bot Rev 36: 341–420

Feinberg AP, Vogelestein B (1983) A technique for radioactive labeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6–13

Feinberg AP, Vogelstein B (1984) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 137:266–267

Gualberto JM, Domon C, Weil J-H, Grienenberger J-M (1990) Structure and transcription of the gene coding for subunit 3 of cytochrome oxidase in wheat mitochondria. Curr Genet 17:41–47

Hanson MR, Conde MF (1985) Functioning and variation of cytoplasmatic genomes: lessons from cytoplasmatic-nuclear interactions affecting male fertility in plants. Int Rev Cytol 94:213–267

Hoelkte HJ, Seibl R, Burg J, Mühlegger K, Kessler C (1990) Non-radioactive labeling and detection of nucleic acids. II. Optimization of the digoxigenin system. Biol Chem Hoppe-Seyler 371:929–938

Hoge JHC, Springer J, Zantige B, Wessels JHG (1982) Absence of differences in polysomal RNAs from vegetative monokaryotic and dikaryotic cell of the fungus Schizophyllum commune. Exp Mycol 6:225–232

Kessler C, Höltke HJ, Seibl R, Burg J, Mühlegger K (1990) Non radioactive labeling and detection of nucleic acids. I. A novel DNA labeling and detection system based on digoxigenin: anti-digoxigenin ELISA principle (digoxigenin-system). Biol Chem Hoppe-Seyler 371:917–927

Lonsdale DM (1987) Cytoplasmatic male sterility: a molecular perspective. Plant Physiol Biochem 25:265–271

Mohr S (1991) Einfluss von Kern und Zytoplasma auf die Organisation und Expression mitochondrialer Gene bei *Triticum*, Triticale und *Secale*. Dissertationes botanicae, vol 180. J. Cramer in der Gebr. Bornträger Verlagsbuchhandlung, Berlin Stuttgart

Mohr S, Schulte-Kappert E, Odenbach W, Oettler G, Kück U (1993) Mitochondrial DNA of cytoplasmic male-sterile *Triticum timopheevi*: rearrangement of upstream sequences of the *atp6* and *orf25* genes. Theor Appl Genet 86:259–268

Morere-Le Paven MC, De Buyser J, Henry Y, Corre F, Hartmann C, Rode A (1992a) Multiple patterns of mtDNA reorganization in plants regenerated from different in vitro-cultured explants of a single wheat variety. Theor Appl Genet 85:9–14

Morere-Le Paven MC, Henry Y, De Buyser J, Corre F, Hartmann C, Rode A (1992b) Organ/tissue-specific changes in the mitochondrial genome organization of in-vitro cultures derived from different explants of a single wheat variety. Theor Appl Genet 85:1–8

Pfeiffer-Schad B (1989) Untersuchungen über die Herstellung männlich steriler primärer Triticale und deren Fertilitätsrestauration. PhD thesis. University of Hohenheim, Stuttgart, FRG

Sambrook J, Fritsch FF, Maniatis T (1989) Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98: 503–517