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Molecular and morphological evaluation of doubled haploid lines in maize. 1. Homogeneity within DH lines

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Abstract. The homogeneity of anther culture-derived lines of maize has been evaluated by means of field observations and molecular markers. The homogeneity of the doubled haploid (DH) lines was shown by the absence of segregation for morphological oligogenic traits. The intravariance for polygenic traits for 42 DH and two conventionally derived lines was similar, which confirmed the homozygosity of the DH lines. More than 100 RFLP markers were tested on 189 DH lines derived from two crosses, DH5 × DH7 and A188 × DH7, and 60 single-seed descent (SSD, F_6) lines derived from A188 × DH7. The overall rate of heterozygosity for all of the DH lines was approximatively 1% and pertained to 6 lines out of 189, while it was 8.5% for the SSD lines after four selfings. A precise description of the material used suggested that the events which led to this unexpected heterozygozity in DH lines were more likely to have occurred after rather than during the androgenetic process. Nine duplicated pairs of genotypes were found within the DH lines, indicating that a single microspore-derived structure can fragment to give two identical plantlets. Despite the extensive screening with more than 100 markers, only 2 lines showed unexpected banding profiles, and these were probably gametoclonal variants. The use of a direct regeneration system that avoids any callus phase might explain this low frequency of gametoclonal variation.

Key words: Androgenesis – RFLP – Maize – Agronomic evaluation – Gametoclonal variation

Introduction

The potential of doubled haploid (DH) lines in maize breeding programmes has long been recognized (Chase 1969), and anther culture is fast becoming a promising system for obtaining a large number of DH lines (Petolino et al. 1988; Barloy et al. 1989). The large-scale use of this technique for the release of pure lines in a breeding programme, however is dependent on the ability to derive such DH lines from elite germ plasm. The degree of homogeneity, stability and conformity of DH plant progenies with respect to parental progenitors must also be checked.

Several investigators have characterised anther-derived DH plants for their homogeneity and the presence of unexpected phenotypes. Heterogeneity within progenies of a DH line is the consequence of retaining heterozygous loci in the genome. In a recent study Bentolila et al. (1992) reported the total absence of heterozygous loci in 72 DH lines of maize tested with 87 probes. The influence of the tissue culture process on genetic variability, so-called somaclonal variation, has been documented in numerous species (Larkin and Scowcroft 1981; Evans 1987). In maize, tissue culture-induced variations, assumed to occur preferentially during the callus culture phase, have been studied by evaluating agronomic traits, and cytological or qualitative variations. Lee et al. (1988) reported a lower grain yield and moisture content for tissue culture-derived lines. Beckert et al. (1983) also evaluated agronomic traits and concluded that the variability induced

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by the in vitro process was low. On the other hand, cytological changes in chromosome number or structure and single-gene mutations seem to be frequent (Edallo et al. 1981; Zehr et al. 1986; Lee and Phillips 1987a, b; Armstrong and Phillips 1988). Using restriction Fragment length polymorphism (RFLP) markers on rice plants regenerated from callus culture, Müller et al. (1990) found a correlation between a higher degree on genetic instability (DNA rearrangement and methylation change) and the length of the culture period. In maize it has been reported that when DNA extracted from calli of line A188 are probed with RFLP markers, the banding profiles that appear are different from those normally found in seed-grown plants (Brown et al. 1991). However, differentiation and organogenesis seem to act as a screen in selecting against novel DNA rearrangements, and the high rate of DNA variation observed during the callus phase falls in regenerated plants.

The studies reported so far that characterise DH lines at the molecular level either used a limited number of markers or provided no details on the origin of the unexpected genotypes. The objective of the investigation reported here was to evaluate the degree of homogeneity and conformity of maize DH lines obtained through an anther culture procedure. The protocols included field observations and characterisation with a large set of molecular markers displaying a complete coverage of the genome. We were able to acquire precise information on the homogeneity of DH lines and consequently to check the reliability of androgenesis for the production of homozygous lines. The potential consequences of in vitro culture, such as the occurrence of duplicated progenies or somaclonal variants, were also investigated.

Materials and methods

Experimental material and production of DH plants

DH plants were obtained using methods previously described (Dieu and Beckert 1986) and recent improvements thereof (Barloy and Beckert 1993). DH lines such as DH5 and DH7 were derived directly from original synthetic populations (Barloy et al. 1989) or through a second step of anther culture from the crosses DH5 × DH7 or A188 × DH7. The A188 line has been maintained since 1968 at INRA Clermont-Ferrand through manual selfing. Regenerated plants were grown in the greenhouse, and fertile ones were selfed. The subsequent steps of seed multiplication involved a classical ear-to-row procedure from the R_1 to the R_3 generation.

A sample of single-seed descent (SSD) lines were developed from the A188 × DH7 cross to the F_6 generation using the SSD method. Eighty-three F_6 lines were obtained from 85 F_2 plants.

Morphological and agronomical evaluation

Classical maize nursery conditions were used for the field trials. A total of 41 different DH lines (including the original and second-generation ones) were analysed. The control lines were F2 and F546, and these were maintained through manual selfing. They were, respectively the 22nd and 12th selfing generations since line release. The trial was conducted with two replications in one location for 1 year.

Four agronomical characters – plant height, height of ear insertion, number of leaves, number of lateral branches on tassels – were measured. Plant to plant measurements were done, and 5–10 plants were scored per nursery row (30 plants). Altogether about 200 plants were measured per control line and 100 per DH line. Other plant characters were also evaluated, including plant morphology and miscellaneous characters such as tassel shape and flower pigmentation.

For the four agromorphological characters, an estimation of the intravariance of each DH line and the two control lines was obtained. This parameter enables unfixed material to be evaluated for such polygenic controlled characters. A global evaluation of the intravariances of the DH and the control lines was also performed, and these intravariances were then compared using the F statistic. For the other characters, off-types were recorded.

RFLP analysis

In total, 71 DH lines derived from the DH5 × DH7 cross, 118 DH lines and 60 SSD lines derived from the A188 × DH7 cross and the three parental lines, DH5, DH7 and A188, were studied for their RFLP genotype. The seed lots used were the R_3 (three selfings after plant regeneration) for the DH5 × DH7 DH lines, the R_2 for the A188 × DH7 DH lines and the F_6 for the SSD lines. Per line, 8–12 seedlings grown in the dark were collected to obtain five grams of fresh material.

DNA extraction and blotting

After the fresh material was ground in liquid nitrogen, DNA extraction was performed using cetyl-trimethyl-ammonium-bromide (CTAB) as previously described by Rogers and Bendich (1988) except that an additional RNAse and Proteinase K treatment was done after the first chloroform isoamyl alcohol extraction. DNA was digested with five restriction enzymes, *Eco*RI, *Hin*dIII, *Bgl*II, *Xho*I and *Bam*HI, according to the manufacturer's instructions (Amersham) with 5 units of enzyme per microgram of DNA over 6 h at 37 °C. Aliquots of 3 µg of digested and purified DNA were loaded onto a 0.7% agarose gel in TBE buffer (Sambrook et al. 1989). After electrophoresis, the gel was denaturated in 0.4 *N* NaOH, and the DNA was blotted onto a Hybond N + membrane (Amersham).

Preparation of the probe and hybridization

The RFLP probes used were kindly provided by B. Burr (Brookhaven National Laboratory, Upton) and D. Hoisington (University of Missouri, Columbia). They were prepared either with low-melting-point agarose or with the polymerase chain reaction (Sambrook et al. 1989; Saiki et al. 1988) and were labelled with the multiprime DNA labelling kit (Amersham) for 2 h with [^{32}dCTP] to a specific activity of 1–3 × 10^9 cpm per microgram. Prehybridization and hybridization were carried out in a buffer containing 6 × SSC, 0.5% SDS, 5 × Denhart (Sambrook et al. 1989) and 25 µg/ml of sonicated and denatured salmon sperm DNA. The blots were prehybridized for several hours at 68 °C in 4 ml buffer per 100 cm^2 blot; the prehybridization solution was then removed and replaced with 2.5 ml buffer per 100 cm^2 blot containing denatured radioactive probe at 1–3 × 10^6 cpm per milliliter; hybridization was then performed overnight at 68 °C. The blots were then washed at 68 °C with preheated buffers twice

for 30 min each in 2 × SSC, once for 30 min in 2 × SSC, 0.1% SDS and finally once for 30 min in 0.1 × SSC, 0.1% SDS. The blots were wrapped in Saran Wrap and exposed for 4 days to Hyper film MP (Amersham) with two intensifying screens at −80 °C. For reuse, boiling 1% SDS was poured on the blots, which were shaken until the SDS solution reached room temperature.

Results

The general observations of the DH lines under field conditions revealed the very high homogeneity of the material derived from anther culture in vitro. This homogeneity was evident for a large number of different morphological characters such as anther pigmentation or general shape of plants (erect leaves). No off-type was observed for more than 10000 plants. One line, DH96, was not included in the field trial since it segregated for several traits, such as endosperm colour or anther pigmentation.

A comparison of the intravariance of each DH line with the intravariance of the F2 control did not reveal any large difference between the different types of material. The two control lines, F2 and F546, were in fact different. F2 was theoretically more fixed (22 selfings after line release) but showed larger intravariances for all of the characters. The global comparison of the intravariances of both of these types of lines is given in Table 1 for the four analysed characters. A significant difference (at $P <= 0.05$) was found only for number of leaves.

One hundred and twenty probes spread evenly over the genome according to the published map (Coe et al. 1990 MNL 64: pp 154–163) and showing clear polymorphism between DH5 and DH7 were tested on the DH and parental lines. For DH lines and SSD lines derived from A188 × DH7 106 and 102 probes, respectively, were tested. Maps of each of the three populations were constructed (data not shown) so as to obtain a better estimation of the tested parameters. For example, a better evaluation of the level of residual heterozygosity is achieved by using regularly spaced loci and avoiding clustered loci. In all of the calculations (percentage of heterozygosity or non-parental alleles), each pair of segregating bands, corresponding to one locus, was considered separately. In other words, the unit of calculation is the pair of allelic bands and not the band pattern.

Of the 71 DH lines from the DH5 × DH7 cross, 68 showed one of the expected parental bands for every probe, while 3 showed a number of unexpected patterns. A hybrid profile (presence of the two parental bands) was observed for 31 different loci for line DH96. Lines DH195 and DH241 showed non-parental banding profiles for 21 and 17 different loci, respectively. The hybridization of the probe UMC 139 (Fig. 1a) showed the presence of the expected pattern for all DH lines, the hybridization of the probe UMC 128 (Fig. 1b) showed both the hybrid profile for DH96 and the non-parental band for DH195 and DH241.

The rate of abnormality can be calculated as the number of loci with unexpected banding profiles divided by the total number of loci evaluated for all plants. For this calculation 118 loci and 71 DH lines were considered. The rate is 69/8281 (97 missing values) or 0.83%. These observations were repeated with fresh DNA extracted from newly germinated seedlings, and the original result was confirmed in all cases.

Within the 118 DH lines derived from A188 × DH7, 9 duplicated pairs of lines were found. Identical RFLP bands were found for all of the loci evaluated (more than 100). Five other lines showed numerous loci with hybrid profiles (between 30 and 41 over 105 loci tested). The ratio of the number of loci showing a hybrid banding profile (171), over all of the loci characterised (12115 : 105 loci, 118 DH lines and 275 missing values) was 1.4%. However, no unexpected bands were observed in either the DH or SSD lines derived from this cross A188 × DH7. The 60 SSD lines showed a residual heterozygosity rate of 8.5% for the 99 loci considered in this calculation.

Discussion

The homogeneity of the anther-derived material is clearly shown by the discrete traits under oligogenic control, such as anther pigmentation or general shape of the plant (erect leaves). Since these agromorphological traits confirmed that DH lines are clearly homo-

Table 1. Comparison of intravariances between DH lines and control lines for four agromorphological characteristics

Characteristics	Number of individual plants measured (DH/control)	*F* value	SD[a]	Trial CV[b]
Plant height	4025/419	0.88	NS	12.1%
Height of ear insertion	4099/419	0.94	NS	10.3%
Number of leaves	3680/383	1.18	*	8.4%
Number of tassel branches	3148/419	0.96	NS	11.5%

[a] Significance of difference between DH and control lines: $*P < 0.05$; NS, not significant
[b] Coefficient of variation within the trial

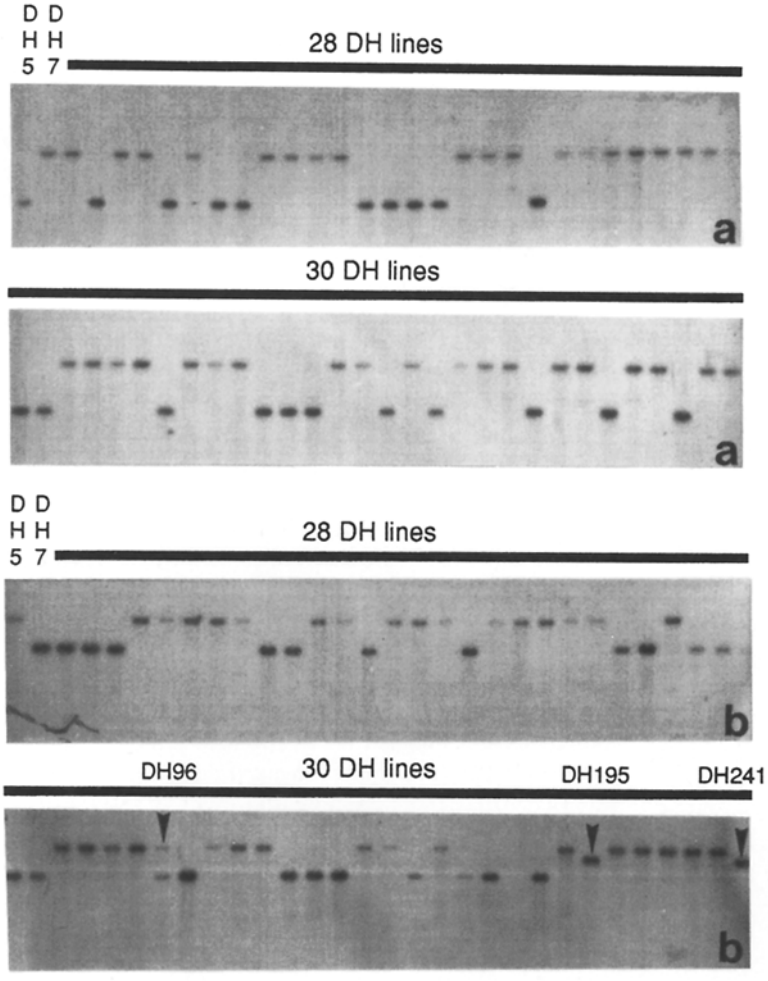

Fig. 1a, b. Autoradiogram from Southern hybridization of DH5, DH7 and 58 doubled haploid lines derived from the cross DH5 × DH7. **a** Hybridization with the probe UMC 139 shows one of the parental bands for each DH line; **b** hybridization with the probe UMC 128 shows unexpected patterns for DH 96 (presence of both parental bands), DH 195 and DH 241 (presence of a non-parental band)

zygous, the intravariance parameter can be considered to be more an evaluation of the general homeostasis of this material. A larger intravariance could have been predicted for DH lines since no selection for adaptation to the environment was made during their production, as opposed to the control lines. The fact that the DH material is closer to F2 than to F546 might be a reflection of its origin. The DH material is derived from Chinese local ancestry and F2 is derived from French local ancestry, while F546 is a more advanced selected line. It is possible that lines that have undergone several cycles of selection might be more adapted and consequently less influenced by environmental microvariation within a field trial.

In the cross DH5 × DH7, line DH96 which had shown segregations in several morphological characteristics showed hybrid banding profiles for about 25% of the loci tested. The heterozygosity of this line could be explained by several different hypotheses. If a non-reduced pollen grain or a diploid cell from the anther wall is able to regenerate into a plant, the R_0 plant would be a hybrid. Because subsequent seed multiplication steps involve three selfings (from the R_0 plant to R_3 seed lot), 12.5% of the original heterozygozity of the R_0 plant would remain. Since at least 8–12 R_3 seedlings were used for DNA extraction the level of heterozygosity evaluated relative to the R_2 level is what we actually observed: 25%. This is in accordance with the hypothesis that the DH96 line derived from a diploid heterozygous cell (maternal diploid cell or unreduced pollen grain) and that the alleles at some loci have been fixed through seed multiplication. However, the R_0 plant did not show all of the characteristics of a hybrid: if a segregation was found in endosperm colour it was not typical segregation of F_2 kernels (DH5 and DH7 are yellow and white kernel lines, respectively), and therefore the regeneration of a diploid cell can not be clearly demonstrated.

The percentage of heterozygous loci observed in DH lines derived from A188 × DH7 (between 28% and 39% compared to an expected one of 50% since pooled R_2 seedlings are used) does not seem to be in agreement with the hypothesis of the regeneration of a diploid cell. If the occurrence of 1 plant with 39% of heterozygous

loci after one selfing is possible, the fact that all 5 lines showed percentages lower than those expected is in contradiction with this hypothesis. Another possibility might be based upon the chimeric structure of the regenerated plant, which might be formed through the fusion of two different pro-embryos during the first steps of microspore division. According to the number of differences between the microspores from which the two embryos derived, different heterozygosity rates would be observed. But, on average, two different gametes have common alleles at 50% of their loci and upon fusing would give rise to a R_0 plant with 50% heterozygous loci. It is therefore possible to discriminate between regeneration of the sporophytic cell and fusion of the pro-embryos when the number of selfings after regeneration is known. The percentage observed for lines showing heterozygous loci does not really fit in with this hypothesis. Heterozygosity at 12.5% of the loci for DH96 and 25% for DH lines derived from A188 × DH7 should be observed.

In addition to these hypotheses, contamination is another possibility. Contamination of the R_0 plant with foreign pollen (other than R_0) can be excluded since all allelic forms are characteristic of either one of the two parents. But when the regenerated plants are grown in a greenhouse contamination between R_0 plants could occur. A discrimination between pollen contamination and the pro-embryo fusion described above is not possible since both consist of mixing the contents of two different gametes. Pollen contamination might also occur during the last step of seed mutiplication. The hybridization of the DNA extracted from 10 progeny seedlings derived from the selfing of a plant that is heterozygous at 1 locus would lead to an equal intensity of both bands. Inequal band intensity would be found if homozygous plant was partially contaminated by pollen of other DH lines during the last step of seed multiplication. Female gametes are homogeneous, and male ones can be a mix of both allelic forms. The band intensities were very similar for all of the heterozygous loci of DH96, but not for lines derived from A188 × DH7. Therefore, the heterozygosity found in the 5 lines derived from A188 × DH7 is more likely to be the consequence of contamination during the last step of seed multiplication rather than contamination between R_0 plants or pro-embryo fusions.

Problems might also arise at several subsequent steps of the experiment, such as mixing seed lots or DNA samples. These would give the same results as pollen contamination during the last step of seed multiplication. At least for lines derived from DH5 × DH7 this is very unlikely since the experiment was carried out a second time using fresh DNA extractions and consistent results were obtained.

The theoretical rate of residual heterozygosity for F_6 SSD lines is 3.12%, but since an average of 10 seedlings were used for DNA extraction, the F_5 level was actually evaluated and the theoretical rate is 6.25%. The observed rate of 8.5% is quite close to the expected one and confirms similar results obtained with other recombinant inbred populations (Burr and Burr 1991).

The occurrence of nine pairs of identical DH lines within lines derived from A188 × DH7 is quite surprising. The very high number of markers used clearly shows that they arise from the regeneration of the same gamete. Despite the fact that our protocol does not involve a callus phase, fragmentation of the plantlet seems to be possible during regeneration.

With respect to the occurrence of unexpected RFLP banding profiles, the absence of non-parental bands in both SSD and DH lines derived from the A188 × DH7 cross shows the very good quality of the material in terms of purity. It also shows that at least for this cross, no variation at the DNA level occurred during the in vitro process. The unexpected bands observed for DH lines 195 and 241 are not a consequence of a residual heterogeneity of the parental lines since both parents (DH5 and DH7) are themselves doubled haploid lines. Contamination of the R_0 plants by foreign pollen or during subsequent generations can be definitively excluded since it would lead to a heterozygous pattern (foreign band and DH5 or DH7 band). All of the non-parental bands observed are in a homozygous state. One possibility would be pollen contamination during seed production of the F_1 used as a donor plant for anther culture. The evaluation of the genome ratio at more than 100 loci (data not shown) showed a large excess of DH7 bands in these 2 lines: on average, 72% DH7 bands, 12% DH5 bands, 16% non-parental bands). This ratio shows that contamination with foreign pollen at this step is very unlikely, since DH7 was the male parent for F_1 seed production.

The more probable hypothesis to explain these non-parental banding profiles is a modification of the DNA during the anther culture process, either methylation changes that modify restriction enzyme behaviour or mutations that modify restriction sites, DNA translocations, inversions or duplications. Recombination between the two restriction enzyme sites can also lead to a non-parental band, but there is no reason that it would occur in only 2 plants and at so many loci. The mutations are obviously not randomly distributed within the lines, as only 2 out of 189 were affected. This is in agreement with results obtained in rice (Müller et al. 1990) and beet (Sabir et al. 1992), where with a low number of markers more than one variation was found in a single plant.

Out of the 189 plants evaluated, only 17 have some form of aberrant genotype. The heterozygozity at some loci is probably the consequence of a problem that

occurred after androgenesis and not during the tissue culture process. It would also be easy to avoid duplicated genotypes by transferring only discrete plantlets. Therefore, for only 2 lines, DH195 and DH241, does the unexpected RFLP genotype seem to be a consequence of an androgenetic process that could not be controlled. This rate of 2/189 seems to be acceptable for line production. The regeneration protocol used for obtaining the DH lines minimizes the tissue culture phase, which takes less than 5 weeks from the plated anthers to the regenerated plantlets in a tube. The very short period of time required to obtain plants and the avoidence of any callus formation using direct androgenetic embryo formation may partially explain the low level of gametoclonal variation revealed here.

Our investigation has shown that any evaluation of the efficiency of androgenesis (recovery of homozygous lines) and any evaluation of the frequency of gametoclonal variation with molecular markers requires the use of a large set of markers and a clear description of the plant material used. The availability of these tools enabled us to avoid any erroneous conclusions due to contamination in the material used. But the most important conclusion is that, according to our agromorphological and molecular observations, anther culture is a reliable process for the production of lines in terms of homogeneity. Since line production takes only 7 months from the plated anthers to the R_1 seeds, this method shows potential promise as soon as it is applicable to a wider range of germ plasm.

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References

Armstrong CL, Phillips RL (1988) Genetic and cytogenetic variation in plants regenerated from organic and friable, embryogenic tissue cultures of maize. Crop Sci 28:363–369

Barloy D, Beckert M (1993) Improvement of regeneration ability of androgenetic embryos by early anther transfert in maize. Plant Cell Tissue Org Cult 33:45–50

Barloy D, Denis L, Beckert M (1989) Androgenetic aptitude of DH lines obtained through anther culture. Maydica 34:303–308

Beckert M, Pollacsek M, Caenen M (1983) Etude de la variabilité génétique obtenue chez le maïs aprés callogénèse et régénération de plantes in vitro. Agronomie 3:9–18

Bentolia S, Hardy T, Guitton C, Freyssinet G (1992) Comparative genetic analyses of F_2 plants and anther culture-derived plants of maize. Genome 35:575–582

Brown PTH, Göbel E, Lörz H (1991) RFLP analysis of *Zea mays* callus cultures and their regenerated plants. Theor Appl Genet 81:227–232

Burr B, Burr FA (1991) Recombinant inbreds for molecular mapping in maize: theoretical and practical considerations. Trends Genet 7:55–60

Chase SS (1969) Monoploids and monoploid derivatives of maize. Bot Rev 35:117–167

Coce E, Hoisington D, Chao S (1990) Gene list and working maps. MNL 64:154–163

Dieu P, Beckert M (1986) Further studies of androgenetic embryo production and plant regeneration from in vitro-cultured anther of maize. Maydica 31:245–259

Edallo S, Zuccinali C, Perenzin M, Salamini F (1981) Chromosomal variations and frequency of spontaneous mutation associated with the in vitro culture and plant regeneration in maize. Maydica 26:39–56

Evans DA (1987) Somaclonal variation. In: Nevins DJ, Jones RA (eds) Tomato biotechnology. (Plant biology vol 4). Alan R. Liss, New York, pp 59–69

Larkin PJ, Scowcroft (1981) Somaclonal variation – a novel source of variability from cell cultures for plant improvement. Theor Appl Genet 60:197–214

Lee M, Phillips RL (1987a) Genomic rearrangements in maize induced by tissue culture. Genome 29:122–128

Lee M, Phillips RL (1987b) Genetic variants of regenerated maize plants. Genome 29:834–838

Lee M, Geadelmann JL, Phillips RL (1988) Agronomic evaluation of inbred lines derived from tissue cultures of maize. Theor Appl Genet 75:841–849

Müller E, Brown PTH, Hartke S, Lörz H (1990) DNA variation in tissue-culture-derived plants. Theor Appl Genet 80: 673–679

Petolino JF, Jones AM, Thompson SA (1988) Selection for increased anther culture response in maize. Theor Appl Genet 76:157–159

Rogers SO, Bendich AJ (1988) Extraction DNA from plant tissues. Plant Mol Biol Manual A6:1–10

Sabir A, Newbury HJ, Todd G, Catty J, Ford-Lloyd BV (1992) Determination of genetic stability using isozymes and RFLPs in beet plants regenerated in vitro. Theor Appl Genet 84: 113–117

Saiki RK, Gelfand DH, Stoffel S, Schaft SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 29:487–491

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Zehr BE, Williams ME, Duncan DR, Widholm JM (1986) Somaclonal variation in the progeny of plant regenerated from callus culture of seven inbred lines of maize. Can J Bot 65:491–499