CHAPTER 7

GENETIC FIDELITY ANALYSES OF *IN VITRO* PROPAGATED CORK OAK (*QUERCUS SUBER* L.)

C. SANTOS, J. LOUREIRO, T. LOPES AND G. PINTO

Laboratory of Biotechnology and Cytomics, Department of Biology & CESAM, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

1. INTRODUCTION

In vitro propagation methods, such as somatic embryogenesis (SE), are very interesting approaches when compared with traditional propagation, which presents serious drawbacks. SE is frequently regarded as the best system for propagation of superior genotypes (Kim, 2000) mostly because both root and shoot meristems are present simultaneously in somatic embryos. Cork oak (*Quercus suber* L.), as other woody species, is recalcitrant concerning somatic embryogenesis (SE). Most of the successful studies regarding SE within this species used juvenile materials (Gallego et al., 1997; Hernandez et al., 1999; Toribio et al., 1999; Hornero et al., 2001a; Pinto et al., 2001). Only recently, SE was successfully and reproducibly induced from adult cork oak trees (Pinto et al., 2002; Lopes et al., 2006), opening perspectives for breeding programmes of selected genotypes of this species.

Plantlets derived from *in vitro* culture might exhibit somaclonal variation (Larkin & Scowcroft, 1981), which is often heritable (Breiman et al., 1987). Some reports claim that morphological, cytological, and molecular variations may be generated *in vitro* (Larkin et al., 1989) due to the genotypes (Breiman et al., 1987) and to the protocols used in *in vitro* culture and plant regeneration. Main genetic variations may be divided in changes in chromosome structure and number and in changes involving DNA structure.

Flow cytometry (FCM) has increasingly been chosen for analysis of major ploidy changes in genetic stability assays. It thereby replaces other methods such as chromosome counting being that FCM provides unsurpassed rapidity, ease, convenience and accuracy. Until this moment very few reports used this technique to assay somaclonal variation in woody plants (e.g. Bueno et al., 1996; Endemann et al., 2002; Conde et al., 2004; Pinto et al., 2004). In *Q. suber*, only recently the first study on the assessment of ploidy stability of the SE process using FCM was presented (Loureiro et al., 2005).

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This delay may be related, besides the difficulty of establishing the SE process, with the difficulty of analysing woody plant species using FCM. These species, usually present some compounds (e.g. tannins) that are released during nuclear isolation procedures, and that are known to interact with nuclei, hampering in some situations the FCM analysis (Noirot et al., 2000; Loureiro et al., 2006a).

Other genetic variations can be detected by molecular techniques such as RFLPs (restriction fragment length polymorphisms), RAPDs (randomly amplified polymerphic DNAs), AFLPs (amplified fragment length polymorphisms) or microsatellites/ SSRs (simple sequence repeats). Both RFLPs and AFLPs are highly reproducible techniques but more costly and time-consuming than SSRs, while RAPDs show a lack of reproducibility either within or between laboratories (Jones et al., 1997). In addition, microsatellites have high levels of polymorphism (Glaubitz & Moran, 2000), being extremely useful for fine-scale genetic analyses. There are several reports concerning the use of these molecular markers in micropropagated plants. With respect to the *Quercus* genus, RAPD markers have been used in *Q. serrata* Thunb. (Ishii et al., 1999; Thakur et al., 1999) and *Q. robur* L. (Sanchez et al., 2003) somatic embryos and no aberrations were detected in the banding pattern. In *Q. suber*, no somaclonal variation in several embryogenic lines obtained from zygotic embryos has been detected by RAPD analyses (Gallego et al., 1997). This result was later confirmed by AFLP markers (Hornero et al., 2001a), while in embryogenic lines from leaves of mature trees, the same authors were able to detect somaclonal variation (Hornero et al., 2001a).

Until recently, *Quercus suber* combined recalcitrance in *in vitro* plant regeneration and in genetic analysis (e.g. this species was reported as being very difficult to analyse by FCM, at least when nuclear DNA content of mature leaves was estimated, see Loureiro et al., 2005). We have optimized both methodologies and this species is now an excellent model for genetic stability assessment within woody species micropropagation studies. The protocols here presented are focused on the successful genetic analysis (using FCM and microsatellites) of this species, highlighting strategies to overcome putative troubleshooting that may arise when dealing with this or other recalcitrant species.

2. PROTOCOL

2.1. Flow Cytometry

Methods for FCM measurement of DNA content have been developed for individual plant cells, protoplasts, and intact plant tissues, the latter being the most successful approach. The basis of the protocol here presented was developed by Galbraith et al. (1983) and consists in a rapid and convenient method for isolation of plant nuclei by chopping plant tissues in a lysis buffer.

Several recommendations on the behalf of best practices should be taken in consideration. Major issues are reported above and several advices are referred in each protocol step. A troubleshooting section is also provided.

Plant material. Intact plant tissues should be disease and stress-free if this is not the interest of the study (e.g. toxicological assays). Young and rapidly growing tissues usually give the best results. *Ex vitro* leaves should be transported or sent by post enclosed in moistened paper and kept at low temperatures.

Flow cytometers. The flow cytometers currently used in plant DNA flow cytometry can be divided in three major brands: Partec®, Beckman-Coulter® and Becton-Dickinson®. According with the FLOWER database presented by Loureiro et al. (2007a), cytometers of these brands were used in 94.1% of the publications analysed in this database, with Partec®, being the leading brand in plant science with 44.1% of publications. The prominent position of this brand was justified as being due to its suitability for analysis of plant materials and/or to a relatively low price of their products. When acquiring a flow cytometer attention is needed to the wavelength of the laser source (for details on fluorochromes characteristics see section below).

Nuclear isolation buffers. Due to different chemical composition and diversity of plant tissues, no single nuclear isolation buffer is universally optimal. This was clearly shown by Loureiro et al. (2006b), who systematically compared four of the most common lysis buffers for DNA analysis by FCM. Also, Loureiro et al. (2006a) studied the effect of tannic acid on plant nuclei and estimation of DNA content, and found that different nuclear isolation buffers granted samples with different resistance to the negative effect of this compound. Based on the results of these two studies, a new buffer was developed by the authors (Loureiro et al. 2007b). This buffer provided very satisfying results with more than 30 plant species, most of them woody plants, and was therefore called "woody plant buffer" (WPB). Nuclei isolated with this buffer are stabilised with **$MgCl_2$** (chromatin stabilizer) and **EDTA** (chelator agent), the pH of the solution is maintained at 7.5 with **TRIS buffer**, **NaCl** ensures proper ionic strength, and a high concentration of **Triton X-100** (surfactant) reduces adhesion of cellular debris and removes and hinders cytoplasmic remnants. **Metabisulfite**, as reducing agent of phenolic compounds and polyvinyl pyrrolidone (10 000 MW, **PVP-10**), as a competitor with amide groups were also added to the buffer. Phenols and other compounds are very common in woody plants, like *Q. suber*, and the addition of these protectants is mandatory for obtaining good results (Loureiro et al. 2007b).

Standardization. The fluorescence units of a FCM DNA histogram are presented in arbitrary units of channel numbers. Therefore for genome size and DNA ploidy analyses, a reference standard with known nuclear DNA content and/or ploidy level should be added. The nuclear DNA content estimation is made by comparing sample and standard G_0/G_1 peak positions. From the two types of standardization (**external**, nuclei from sample and standard are analysed separately; and **internal**, nuclei from sample and standard are isolated, stained and analysed simultaneously), internal standardization is considered the most reliable method as nuclei from sample and standard are exposed to the same conditions. An ideal DNA reference standard should have a genome size close but not overlapping to the targeted species (Greilhubu et al. 2007). A set of reference standards with genome size distributed at appropriate intervals is already available from some sources. We recommend those provided free of

charges by the Laboratory of Molecular Cytogenetics and Cytometry, Olomouc, Czech Republic (Doležel & Bartoš, 2005, Table 1). These standards are genetically stable with constant genome size, seed propagated, easy to use and available in sufficient quantities as elite lines from breeders. For DNA ploidy level analyses, reference standards can also be an individual from the same species with known ploidy level (e.g. tetraploid). Following the recommendations of the Genome Size Workshop, held at Kew Royal Botanical Gardens in 1997 (see http://www.rbgkew.org.uk/cval/pgsm/), the use of chicken red blood cells (CRBCs) as internal standard is discouraged.

Table 1. *DNA reference standards available from the Laboratory of Molecular Cytogenetics and Cytometry, Olomouc, Czech Republic (adapted from Doležel & Bartoš, 2005).*

Species	***Cultivar***	***2C DNA Content (pg)****	***1C Genome Size (Mbp)*****	***Reference***
Allium cepa	Alice	34.89	17,061	(Doležel et al., 1998)
Vicia faba ssp. *faba* var. *equina*	Inovec	26.90	13,154	(Doležel et al., 1992)
Secale cereale	Dankovske	16.19	7,917	(Doležel et al., 1998)
Pisum sativum	Ctirad	9.09	4,445	(Doležel et al., 1998)
Zea mays	CE-777	5.43	2,655	(Lysák & Doležel, 1998)
Glycine max	Polanka	2.50	1,223	(Doležel et al., 1994)
Solanum lycopersicum	Stupické	1.96	958	(Doležel et al., 1992)
Raphanus sativus	Saxa	1.11	543	(Doležel et al., 1992)

* Nuclear DNA content was established using human male leukocytes (2C = 7.0 pg DNA; Tiersch et al., 1989) as a primary reference standard.
** 1 pg DNA = 978 Mbp (Doležel et al., 2003).

Fluorochromes. The fluorochromes used to stain DNA must be chosen according to their resolution, stability, DNA stoichiometry and most importantly the excitation wavelength available on the flow cytometer. DAPI (4',6-diamidino-2-phenylindone) and propidium iodide (PI) are the most popular fluorochromes used in plant DNA FCM. Whereas DAPI is AT-specific, inexpensive, staining is readable in 5 min and excitation occurs at 340 nm, PI intercalates with double stranded DNA, stains in 10 min and is excited at 535 nm. The excitation wavelength of both fluorochromes restricts their use; DAPI needs lamp-based machines (UV excitation) whereas PI requires laser-based flow cytometers. It should also be noticed that DAPI, due to its binding properties, should not be used for absolute nuclear DNA content estimations, at least if the AT:GC ratio of the sample and standard DNA is unknown (which is often the case) (Doležel et al., 1992). When PI is chosen, RNAse should

be added to samples, as this fluorochrome also binds to double stranded RNA. The protocol here presented describes the use of the simplest instrument configuration, i.e. an argon laser operating a 488 nm, with one fluorochrome, PI.

Quality assessment. The quality and precision of DNA histograms obtained by FCM is usually evaluated by the coefficient of variation (CV) value, which should be <5% (Galbraith et al., 2002). Excellent analyses with high degree of resolution will have CV = 1–2%, and routine analyses CV values of 3%. Also, recent literature (Loureiro et al., 2006a) has alerted for the importance of measuring CV values of scatter parameters (in this case in the form of full peak coefficient of variation, FPCV), as higher values (>50%) may be an indication of the negative effect of cytosolic compounds, released upon chopping. It should be noticed that the CV value does not tell anything about the reproducibility of the DNA content estimation. It is therefore important to perform sufficient number of independent measurements. For genome size estimations, it is suggested that each measurement is repeated at least three times on three different days to uncover any unexpected variation (e.g. instrumental shifting).

2.1.1. Materials

Plant material for analysis. Intact plant tissues (leaves of *in vitro* and *ex vitro* plants) and plant tissue culture or callus (somatic embryos at different stages, embryogenic/organogenic/non-differentiated callus tissue). In *Q. suber,* plants were grown according to the protocols developed by Lopes et al. (2006) and Pinto et al. (2002).

Internal reference standard. Plant with known nuclear content. In the case of *Q. suber*, *Glycine max* cv. Polanka (2C = 2.50 pg of DNA, Doležel et al., 1994) was used as an internal reference standard.

Nuclear isolation buffer. WPB (Woody Plant Buffer) – 0.2 M Tris.HCl pH 7.5, 4 mM $MgCl_2.6H_2O$, 2 mM EDTA $Na_2.2H_2O$, 86 mM NaCl, 10 mM metabisulfite, 1% (w/v) PVP-10, 1% (v/v) Triton X-100. Store at 4°C.
Prepare 1 mg/ml propidium iodide (PI) stock solution with caution because it may cause health risks. Store in 1.5–2.0 ml aliquots at –20°C.
Prepare 1 mg/ml RNAse stock solution. Store in 1.5–2.0 ml aliquots at –20°C.
Sheath fluid: use either distilled water or commercial sheath fluid solution.
Fluorescent microspheres (Flow-Check®, Beckman-Coulter).
Ice in a polystyrene box.

Glass Petri dishes
New double-edged razor blades
50 μm nylon filters
Micropipettes and tips (200 μl and 1 ml)
Cytometer sample tubes
Flow cytometer with 488 nm light source

2.1.2. Methods

1. Weigh approximately 50 mg of plant material and place it in a glass Petri dish. A leaf of the internal reference standard should also be weighed (approx. 50 mg) and added to the same Petri dish. Some initial experiments should be performed to determine the weight of sample and internal standard necessary to obtain similar amounts of isolated nuclei. At a low speed flow cytometer configuration, a flow rate of 40–80 particles/s should be obtained.
2. Add 1.0 ml of WPB nuclear isolation buffer and chop tissues using a new double-edged razor blade.
3. Filter nuclear suspension through 50 μm nylon filters into an ice-cold cytometer sample tube. This step will remove large debris. Cut the 1 ml micropipette tip to help suction of the nuclear suspension liquid.
4. Add 1 mg/ml PI stock to a final concentration of 50 μg/ml and 1 mg/ml RNase stock to a final concentration of 50 μg/ml.
5. Incubate sample on ice for 10 min. The time of incubation should be sufficient for a stable fluorescence staining.
6. Turn the computer and flow cytometer on. This process should take approximately 15 min. In the meantime, follow instrument starting instructions, fill the sheath fluid tank with commercial sheath fluid solution (or distilled water) and empty the waste tank.
7. Load a protocol for analysis of fluorescent beads. Run a sample tube with approximately 100 μl. Collect at least 5,000 beads. If the flow cytometer is correctly aligned, CV values must be <2% for fluorescence and scatter parameters.
8. Load the protocol for nuclear DNA content analyses. The discriminator should be set at a fluorescence signal of 50. The FCM analysis should include the following graphics (Figure 1): histogram of PI fluorescence (in our cytometer, using the photomultiplier tube nº 3, PMT3, Figure 1A), cytograms of forward-angle light scatter (FS) vs. side-angle light scatter (SS) both in logarithmic scale (Figure 1B), PI fluorescence vs. time (to monitor fluorescence stability of nuclei, Figure1C), SS in logarithmic scale vs. PI (to monitor for possible effect of tannic acid, for details see Loureiro et al., 2006a, Figure 1D) and PI fluorescence pulse integral vs. PI fluorescence pulse height (Figure 1E). The discriminator is used to eliminate particles with autofluorescence and/or low fluorescence values.
9. Run samples at room temperature at a data rate of 40–80 particles/s (it usually stands for the lowest flow rate configuration).
10. Define the necessary regions for obtaining statistics from the flow cytometric data. Usually the following regions are defined: in PI fluorescence histogram (Figure 1A), linear regions are defined for each peak; in FS log. vs. SS log. cytogram (Figure 1B), a region is defined around the population of nuclei; in fluorescence pulse integral vs. fluorescence pulse height (Figure 1E), a region is defined around individual nuclei. By this way doublets of 2C that can be erroneously assess as 4C are eliminated.

11. Determine the mean channel number of the sample G_0/G_1 nuclear DNA peak and that of internal reference standard.
12. Estimate the nuclear DNA content of the sample using the formula:

$$\textit{sample 2C DNA content (pg)} = \frac{\textit{sample}\, G_0/G_1 \;\; \textit{peak mean}}{\textit{standard} \;\; G_0/G_1 \;\; \textit{peak mean}} \times \textit{standard 2C DNA content} \quad (1)$$

If needed, the DNA content values can be converted in number of base pairs (bp), taking in consideration that 1 pg DNA = 978 Mbp (Doležel et al., 2003). The genome size in base pairs is usually shown in terms of haploid size (1C) of the genome, whether genome size in mass values are typically shown per 2C value.

13. Using the regions that were defined, record the full peak coefficient of variation (FPCV) of both FS and SS and the half peak coefficient of variation (HPCV) of sample and standard G_0/G_1 peaks.
14. Carefully and critically interpret the results. Small differences in the peak position of both samples and internal standard should be interpreted with caution as they may be due to instrumental drift or to the possible effect of cytosolic compounds. Whether in the first case, these differences may be reduced by planning the experience in at least three different days, to eliminate the second hypothesis and ascertain the real occurrence of DNA differences, a new sample containing the tissues where the differences were obtained may be prepared. If only one peak is observed it is a strong indication that the observed differences were probably artefactual.

2.1.3. Troubleshooting

1. Empirical experience has strongly advised the chopping procedure instead of slicing for obtaining a high output of nuclei and little debris. Before the chopping step it is important that the plant tissue does not dry. Also, the chopping procedure should be fast (60 s of chopping should be enough). However, in some plant tissues containing compounds that affect histograms quality, chopping should be less intense. If only few nuclei are isolated, the amount of plant material can be increased. As an example, in *Q. suber* leaves chopping must consist of only several cuts and nuclear suspension should be quickly filtered. Callus tissue and somatic embryos of *Q. suber* are easier to be analysed than leaves. Friable callus (e.g. from *Pinus pinaster*) may be difficult or in some cases inappropriate for FCM analysis, as the method is only able to isolate a reduced number of nuclei.
2. After using the suggested buffer, if the obtained histograms present low resolution, other lysis buffers should be tested. A review on nuclear isolation buffers can be found in Greilhuber et al. (2007).

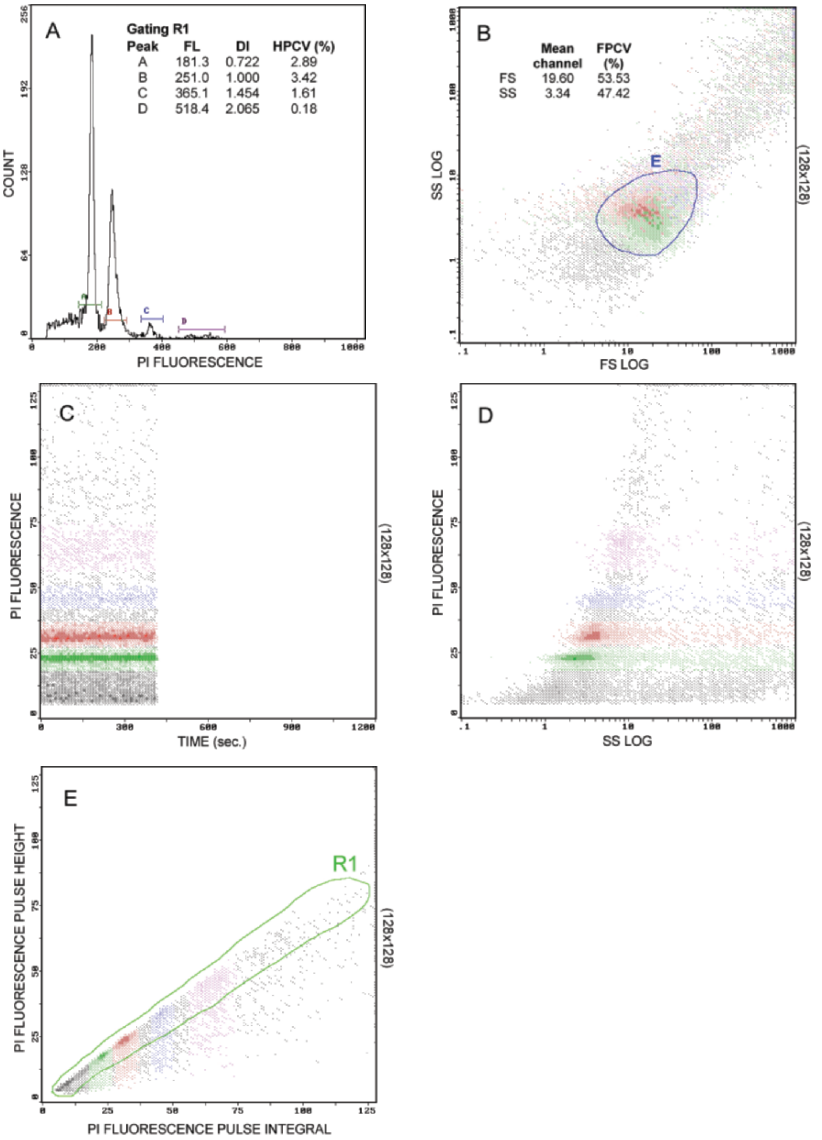

Figure 1. *A) Histogram of PI fluorescence and B) cytograms of forward-angle light scatter (FS LOG) vs. side-angle light scatter (SS LOG) both in logarithmic scale, C) PI fluorescence vs. time, D) SS LOG vs. PI and E) PI fluorescence pulse integral vs. PI fluorescence pulse height of nuclei of* Quercus suber *(peaks: A – G_0/G_1 nuclei, coloured in green, and C – G_2 nuclei coloured in blue) and* Glycine max *(as internal reference standard with peaks B – G_0/G_1 nuclei, coloured in red, and D – G_2 nuclei, coloured in purple) simultaneously isolated in WPB and stained with PI. Mean FL channel (FL), DNA index (DI=mean channel of* Q. suber*/mean channel of* G. max*) and HPCV % of each peak are given in the histogram A and mean channel of FS and SS and (Mean channel) FPCV % of each parameter are given in cytogram B.*

3. In addition to testing various buffers, selection of tissues with lower or no phenolic compounds may enable unbiased estimations (Suda, 2004). If no tissue/buffer provides acceptable results, changing the type of buffer additives (in WPB metabisulfite acts as an antioxidant, and PVP-10 as a tannin-complexing agent) or their concentration may reduce the negative effect of cytosolic compounds, and is therefore recommended.

However, the absence of major ploidy changes does not exclude the possible existence of genetic differences such as DNA polymorphism. Molecular methods like microsatellite marker analysis can be the ideal tool to assess DNA polymorphism, and a protocol developed for *Q. suber* by Lopes et al. (2006) is presented thereafter.

2.2. Microsatellite Markers

Genotyping (PCR analysis) of SSR fragments can be achieved in various ways: radioactive detection (incorporation of labelled nucleotides and end-labelling of one of the PCR primers), non-radioactive detection (high resolution gel electrophoresis, silver staining, blotting and hybridization and fluorescent dyes on automated sequencers).

The protocol given here is for genotyping using fluoro-labelled primers. The forward primers are synthesized with a fluorescent label attached to the 5′ end (ABI dyes: 6-FAM, JOE, HEX and TET) to allow detection of the polymerase chain reaction (PCR) products (see Table 2). About 0.01 μmol of each primer are ordered, which is sufficient to perform at least 2,500 PCR runs under the standard conditions described in this protocol. Primers can generally be purified by standard liquid chromatography (e.g. the HPSF® method, MWG Biotech) or by high performance liquid chromatography (HPLC). For this individual application, we recommend the use of standard liquid chromatography for unlabelled primers and the use of HPLC purification for labelled primers, as it is more sensitive. We resuspend the primers in sterile deionized water or sterile buffer (i.e. TE; 10 mM Tris pH 8, 1 mM EDTA). The standard concentration for stock solution of PCR primers is 100 μM. To obtain a concentration of 100 μM the synthesis report of the vendor should provide you with the appropriate diluent volume.

The protocol described below refers only to genotyping with previously known markers and was adapted from Lopes et al. (2006). Protocols for isolation of microsatellite markers in *Quercus* species can be found elsewhere (Isagi & Suhandono, 1997; Steinkellner et al., 1997a; Kampfer et al., 1998). From the available nuclear microsatellites (nSSRs) developed in the *Quercus* genus that have been transferred with success to *Q. suber*, eight were chosen for study according to their degree of polymorphism (heterozygosity and number of alleles) and the quality of the PCR product. Briefly, of the eight nSSRs assayed, QM58TGT and QM50–3M were first described by Isagi & Suhandono (1997) in *Q. myrsinifolia* Blume, and QpZAG9, QpZAG15, QpZAG36 and QpZAG110 were first described in *Q. petraea* (Matts.) Liebl. (Steinkellner et al., 1997a,b). The transferability of these loci to *Q. suber* has previously been reported (Gomez et al., 2001; Hornero et al., 2001b). The other two nSSRs, QrZAG7 and QrZAG11 were first described in *Q. robur* (Kampfer et al., 1998) and their transferability to *Q. suber* was reported by Hornero et al. (2001b).

To amplify the selected microsatellites by PCR, the primers designed by the authors are used. It should be referred that more recently Borges et al. (2003, GenBank: http://www.ncbi.nlm.nih.gov) have developed SSR markers specifically for *Q. suber*.

Table 2. *Characteristics of the microsatellite loci amplified in* Q. suber *that were primarily developed for* Q. myrsinifolia *(Isagi & Suhandono, 1997),* Q. robur *(Steinkellner et al., 1997a, b) and* Q. petraea *(Kampfer et al., 1998). Repeat structure, primer sequences and variable PCR conditions used.*

Locus	***Repeat structure***	***Primer sequences***	***Annealing temp. (°C)***	***Primer conc. (μM)***
QM58TGT	$(CAA)_{11}$	GGTCAGTGTATTTTGTTGGT AAATGTATTTTGCTTGCTCA	55	0.5
QM50-3M	$(CCT)_3(CCG)(CCT)_2(CCA)(CCT)_2 + (CCA)_7$	CCCGATTTCCCTTCCCTGCT CGGGCTTTGGATACGGATTC	55	0.3
QpZAG9	$(AG)_{12}$	GCAATTACAGGCTAGGCTGG GTCTGGACCTAGCCCTCATG	55	0.3
QpZAG15	$(AG)_{23}$	CGATTTGATAATGACACTTGG CATCGACTCATTGTTAAGCAC	55	0.5
QpZAG36	$(AG)_{19}$	GATCAAAATTTGGAATATTAAGAGAG ACTGTGGTGGTGAGTCTAACATGTAG	50	0.2
QpZAG110	$(AG)_{15}$	GGAGGCTTCCTTCAACCTACT GATCTCTTGTGTGCTGTATTT	50	0.2
QrZAG7	$(TC)_{17}$	CAACTTGGTGTTCGGATCAA GTGCATTTCTTTTATAGCATTCAC	55	0.5
QrZAG11	$(TC)_{22}$	CCTTGAACTCGAAGGTGTCCTT GTAGGTCAAAACCATTGGTTGTTGACT	55	0.5

2.2.1. Materials

Plant material for analysis: leaves of *in vitro* and *ex vitro* plants, embryogenic and undifferentiated calli and somatic embryos at different stages of development. In *Q. suber* and for comparative purposes, the same plant material was used for microsatellite and FCM analyses. This is a recommended practice.

Other materials:

Taq polymerase (5 units/μl). Store at –20°C

10× PCR buffer (75 mM Tris-HCl pH 9, 50 mM KCl, 20 mM $(NH_4)_2SO_4$, 0.001% BSA-bovine serum albumin). Store at –20°C

Sterile ultra pure water

Forward primer. Store at –20°C in aliquots of 10 μM

Reverse primer. Store at –20°C in aliquots of 10 μM

dNTP mix: 2 mM of each dATP, dCTP, dGTP and dTTP. Store at –20°C

GeneScan internal size standards: 400 HD/500 labelled with ROX or 2500 labelled with TAMRA. Store at 4°C
De-ionized formamide. Store at –20°C

Thermal cycler
Automated sequencer
Micropipettes and sterile tips (2 µl, 20 µl, 200 µl and 1 ml)
Sterile thin-walled PCR tubes (200 or 500 µl)

2.2.2. Methods

1. Label thin-walled PCR tubes and add 1 µl (10–20 ng) of genomic DNA to each tube. Keep the samples on ice.
2. Preparation of master mix. Add the following components of the reaction to the bottom of a 1.5 ml tube kept on ice (where *n* denotes the number of PCR reactions):

   n × 2.5 µl 10× PCR buffer
   n × 1.25 µl $MgCl_2$ (2.5 mM)
   n × 2 µl dNTP mix (0.2 mM each)
   n × 0.5 µl each primer
   n × 0.2 µl *Taq* polymerase (1 unit)
   Sterile ultra pure water up to *n* × 24

   Remember to include a volume of master mix with no DNA for a negative control of the PCR reaction. Also, when a large number of samples need to be prepared, an extra 10% of master mix should be prepared to remedy pipetting errors.

3. Mix gently. Pulse spin for a maximum of 5 s.
4. Add 24 µl of master mix to each labelled thin-walled tube. If necessary pulse spin for a maximum of 5 s.
5. Place the tubes in a thermal cycler and perform the PCR amplification with the following profile (according to Hornero et al., 2001b):

   5 min at 94°C, as initial denaturing step
   followed by 10 cycles of:
   – 94°C for 15 s
   – 65 to 56°C (decreasing 1°C per cycle) for 30 s
   – 72°C for 30 s
   followed by 25 cycles of:
   – 94°C for 15 s
   – variable annealing temp ºC for 30 s (see Table 2)
   – 72°C for 30 s
   final extension step at 72°C for 5 min.

6. Optional: it may be useful to run some if not all PCR products on an agarose gel prior to analysing them on an automated sequencer (see 2.2.3, Trouble-shooting section). In this way one can check if the amplification was successful and even try to quantify the PCR product.
7. After PCR amplification, mix 1 µl of PCR product with 0.5 µl of GeneScan internal size standard and 25 µl of formamide. Vortex the mixture briefly, spin it down for a few seconds, then incubate it at 95°C for 3–5 min and finally place it on ice for 3–5 min.
8. The PCR products can then be visualized by capillary electrophoresis on an automated sequencer.
9. Remember to run each sample about three times to minimise error, this is particularly important when trying to detect rare mutations.
10. Following capillary electrophoresis the computer generates a gel image showing the bands that were detected. DNA fragment size (in base pairs) can be estimated with precision for all the peaks in a lane, either by the use of internal (e.g. Applied Biosystems) or external lane standards (e.g. PHARMACIA and LI-COR systems). The computer programs recognize the standard peaks and construct a standard curve. The sizes of the products are then estimated based on their migration relative to the known standard. All peaks are labelled, including microsatellite alleles (the most intense peaks; intensity is measured in fluorescence units), stutter bands, and stray bands arising from non-specific PCR amplifications. Stutter bands are usually smaller than the original alleles and are most likely the result of *in vitro* DNA slippage during PCR amplification.
11. Figure 2 depicts a series of electropherograms of *Q. suber* microsatellite data analysed on an Apllied Biosystems 310 system where fragment sizes were automatically calculated to two decimal places using the Local Southern Method option of the GeneScan v.3.1 software. Data were then imported into Genotyper program where the peaks were filtered to remove stutter bands. Since SSRs are co-dominant markers, a homozygous individual is represented by the presence of only one allele and a heterozygous individual is represented by two alleles, in the case of a diploid species such as *Q. suber* (see Figure 2). The differences in allele sizes between individuals are indicative of polymorphism/genetic variation.
12. A careful manual analysis should be done by the investigator to ignore stray peaks arising from non-specific PCR amplifications and to check the possibility of allelic deletion. Also, the investigator has to keep in mind that identical alleles can generally migrate within 0.5 bp of each other on the same sequencing run (gel). Larger variations could be observed when comparing data from the same sample on different sequencing runs (gels). Therefore, allele binning is of utmost importance.

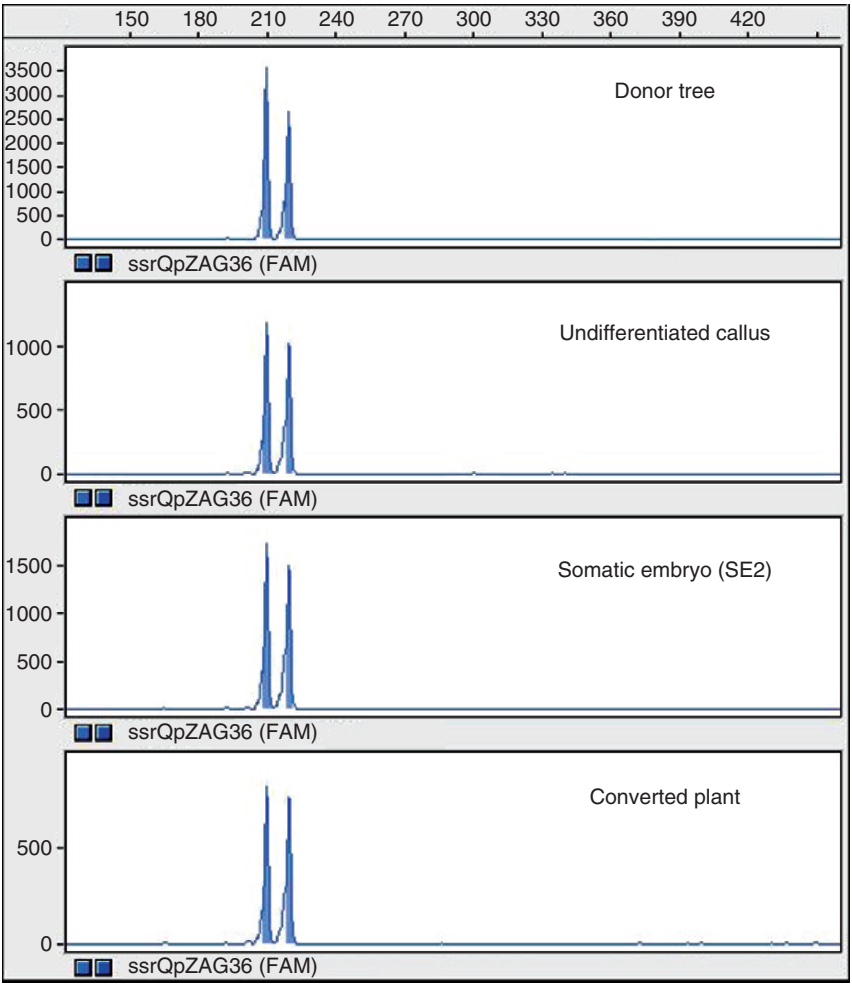

Figure 2. *Amplification of the dinucleotide ssrQpZAG36 (FAM) locus in* Quercus suber *(from top to bottom): donor tree, an undifferentiated callus, a normal dicotyledonary somatic embryo (SE2) and a converted plant. All electropherograms show heterozygous individuals with two alleles of approximately 209 and 219 bp. Top scale indicates fragment size in nucleotides. Left scale indicates fluorescence intensity measured in relative fluorescence units (adapted from Lopes et al., 2006).*

Microsatellite markers/loci that can be amplified with minimum non-specific annealing, and that even have overlapping size ranges, can be separated efficiently and simultaneously in automated sequencers as there are dyes that fluoresce at different wavelengths (Karp et al., 1997). In this case, several PCR products can be pooled

(i.e. multiple loading/multiloading). Alternatively, several loci/markers can be co-amplified during PCR (i.e. multiplexing); this can be particularly useful when a great number of loci are to be amplified and/or when limited amounts of DNA are available. Optimizing a multiplex PCR system may present many difficulties, although there are now some multiplex PCR commercial kits available. For oak species PCR multiplexing procedures are available in the literature.

2.2.3. Troubleshooting

1. Although rare with this protocol, the problem of disappearing microsatellites, whereby previously scorable samples fail to appear when a PCR is repeated, can sometimes be avoided by pipetting the master mix onto each sample individually and ensuring it is mixed with the template DNA.
2. When analysing the fragment size in the automated sequencer, the fluorescence intensity of a specific sample may change dramatically when amplifying different microsatellite markers (using different fluoro-labels). Therefore, after PCR it is advisable to run samples on an agarose gel to try and quantify the PCR product. Alternatively, at first it is better to run only a few samples on the automated sequencer to estimate whether scorable readings are obtained or not. If fluorescence is too intense that it prevents the obtaining of a result, a water-dilute must be done to the PCR sample. Conversely, if fluorescence intensity is too low, it is very likely that the PCR was not successful. In this case a repetition of the PCR run and/or optimization of PCR conditions are needed.
3. Many microsatellites show, besides stutter bands, an additional band above the expected allele size. This is due to the activity of the terminal transferase of *Taq* DNA polymerase which adds an adenine to the PCR product (the plus-A phenomenon described by Clark, 1988). Note that this terminal transferase activity is polymerase- and PCR primer-dependent and if it cannot be surpassed by PCR optimization, the microsatellite in question must be discarded.

3. CONCLUSION

Once a micropropagation protocol is well established and performed routinely (as is the case of *Quercus suber* somatic embryogenesis) it is of utmost importance to verify the fidelity of the *in vitro* derived material, particularly, when the objective is to use the genetic material in a plant breeding programme. Due to the existing constraints of the current genetic screening tools and to the different types of genetic modifications, the combined use of different techniques able to provide complementary data is recommended.

In the present chapter, detailed protocols for the application of the highly sensitive and reproducible techniques of flow cytometry and nuclear microsatellites are given. Also, some best practices concerning plant fidelity using the FCM and microsatellite analysis in *Q. suber* are highlighted here, and can be transposed to regeneration processes of other species. Both techniques can be used at different stages of any

micropropagation protocol, requiring reduced amounts of plant material and their combination provides more accurate information on the fidelity of the obtained plants.

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