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Determination of genetic stability in long-term somatic embryogenic cultures and derived plantlets of cork oak using microsatellite markers

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Summary Microsatellites were used to test genetic stability in somatic embryos (SE) of *Quercus suber* L. The SE were obtained by a simple somatic embryogenesis protocol: leaf explants from two adult plants (QsG0, QsG5) and from two juvenile plants (QsGM1, QsGM2) were inoculated on Murashige and Skoog (MS) medium with 2,4-dichlorophenoxyacetic acid and zeatin. Calluses with primary embryogenic structures were transferred to MSWH (MS medium without growth regulators) and SE proliferated by secondary somatic embryogenesis. High morphological heterogeneity was found among cotyledonary SE. However, converted plants looked morphologically normal with well-developed rooting systems and shoots. The genetic stability of the plant material during the somatic embryogenesis process was evaluated by using six to eight nuclear microsatellites transferred from *Q. myrsinifolia* Blume, *Q. petraea* (Matts.) Liebl. and *Q. robur* L. Five of eight microsatellites distinguished among the genotypes analyzed, and for QsG0, QsGM1 and QsGM2, uniform microsatellite patterns were generally observed within and between SE and the respective donor genotypes. For genotype QsG5, the same pattern was observed in all samples analyzed except one, where the mutation percentage was 2.5%. We conclude that microsatellite markers can be used to assess genetic stability of clonal materials and to determine genetic stability throughout the process of somatic embryogenesis. The simple somatic embryogenesis protocol described has potential for the commercial propagation of *Q. suber* because it results in a low percentage of mutations.

Keywords: *genetic variability, Quercus suber, somatic embryogenesis, SSR markers.*

Introduction

Quercus suber L. is of great economic importance in the Mediterranean region. However, survival of the species is threatened in Portugal, where it has been classified as a threatened species since 1996 (FAO 1996). In recent years, there have been efforts to develop methods for the conservation and sustainable management of *Q. suber* genetic resources, with special attention focused on micropropagation, particularly somatic embryogenesis, as a strategy for the maintenance of economically valuable genes and genotypes.

In *Q. suber*, somatic embryogenesis has been induced in leaves of seedlings (Fernandez-Guijarro et al. 1995) and of 3-year-old plants (Pinto et al. 2001), in nodal segments (El Maâtaoui and Espagnac 1987) and in zygotic embryos (Bueno et al. 1992, Manzanera et al. 1993). Recently, somatic embryogenesis was obtained from leaf explants of mature plants (Hernandez et al. 1999, 2003*a*, 2003*b,* Toribio et al. 1999, Hornero et al. 2001*a*, Pinto et al. 2002).

Plantlets derived from in vitro culture might exhibit somaclonal variation (Larkin and Scowcroft 1981), which is often heritable (Breiman et al. 1987). Somaclonal variation could result from either genetic (mutation) or epigenetic modifications; the latter due mainly to changes in DNA methylation (Kaeppler et al. 2000). Some reports claim that morphological, cytological and molecular variations may be generated in vitro (Larkin et al. 1989) as a result of several factors (Vasil 1987, 1988), such as the genotypes (Breiman et al. 1987) and the protocols used for in vitro culture. On the other hand, the detection of these variations may depend on the parameters used, such as gross morphology and cytology (Swedlund and Vasil 1985, Saieed et al. 1994, Högberg et al. 2003), field assessment and molecular studies (Shenoy and Vasil 1992, Chawdhury et al. 1994).

Genetic variations can be detected by several molecular techniques such as RFLPs (restriction fragment length polymorphisms), RAPDs (randomly amplified polymorphic DNAs), AFLPs (amplified fragment length polymorphisms) and microsatellites, also known as simple sequence repeats (SSRs). Both RFLPs and AFLPs are highly reproducible techniques, but they are more costly and time-consuming than SSRs (once microsatellite loci and their flanking primers are available for use in a given species), whereas RAPDs show a lack of reproducibility both within and between laboratories (Jones et al. 1998). In addition, microsatellites are highly polymorphic (Glaubitz and Moran 2000), making them useful for fine-scale genetic analyses. Furthermore, the search for "mic-

rosatellite instability" (MSI; Frayling 1999) has broad applications in genetic instability diagnosis of dividing populations of cells such as cancers (e.g., Hirose et al. 2002, Kabbarah et al. 2003).

There are several reports on the use of molecular markers in micropropagated plants. In the *Quercus* genus, RAPD markers have been used in *Q. serrata* Thunb. somatic embryos (SE) (Ishii et al. 1999, Thakur et al. 1999) and in *Q. robur* L. (Sanchez et al. 2003) and no aberrations were detected in the banding pattern. In *Q. suber*, no somaclonal variation in several embryogenic lines obtained from zygotic embryos was detected by RAPD analyses (Gallego et al. 1997). This result was later confirmed by AFLP markers (Hornero et al. 2001*a*), whereas in embryogenic lines from leaves of mature trees, somaclonal variation was detected (Hornero et al. 2001*a*). Recently, Loureiro et al. (2005), using flow cytometry, found no ploidy or significant DNA content variations in embryogenic tissues or among SE. Working with *Q. robur*, Wilhelm et al. (2005) found microsatellite instability among SE, but no genetic instability was found among the regenerated plants.

We report the results of molecular analyses, based on six to eight nuclear microsatellite (nSSR) loci, undertaken to assess: (1) genetic stability/fidelity of the derived clones (both SE and derived plants) from two juvenile plants and two mature trees of *Q. suber*; (2) the usefulness of SSR markers for determining genetic stability of in vitro derived clones; and (3) the reliability of a simple somatic embryogenesis protocol for true-to-type cloning.

Materials and methods

Somatic embryogenesis

Cuttings were collected during May and June from two adult *Q. suber* trees (QsG0 and QsG5) in northern Portugal and from 3-year-old plants (QsGM1 and QsGM2) supplied by Viveiros do Furadouro, Óbidos, Portugal. The cuttings were disinfected with commercial bleach as described by Pinto et al. (2001, 2002). Somatic embryogenesis was induced according to the protocol of Pinto et al. (2002). Briefly, young leaf explants were placed on Murashige and Skoog (MS; 1962) medium with 30 g l^{-1} sucrose, 3 g l^{-1} gelrite, pH adjusted to 5.8 and supplemented with 4.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 9.0 μM zeatin, in the dark at 24 ± 1 °C to induce primary somatic embryogenesis. After 3 weeks, cultures were transferred to a 16-h photoperiod at an irradiance of 98 ± 2 μmol m^{-2} s^{-1}. Whenever primary SE appeared (1 month for genotypes QsGM1 and QsGM2; 3 months for genotypes QsG0 and QsG5), they were isolated and transferred to fresh MS medium without growth regulators (MSWH) for proliferation by secondary somatic embryogenesis. Simultaneously, undifferentiated callus lines were maintained in vitro as described by Pinto et al. (2002) for SSR stability assessment (Figure 1).

Every four weeks, non-embryogenic calluses and SE were subcultured on fresh MSWH medium. Embryogenic lines used for the stability studies were 4 years old for the QsG0 genotype and 1 year old for the QsG5, QsGM1 and QsGM2 genotypes.

Plant material used in molecular studies

To assess variation within clones of the same origin and between these clones and their mother plant, the following samples were analyzed per genotype: (1) two juvenile leaves from each mother tree; (2) 10 embryogenic calluses; (3) 10 undifferentiated calluses; (4) 20 normal dicotyledonary somatic embryos (SE2); (5) 10 abnormal somatic embryos with one cotyledon (SE1); (6) 10 abnormal somatic embryos with more than two cotyledons (SE3); and (7) two juvenile leaves from each converted plant. For each of the somatic embryo morphotypes (SE1, SE2 and SE3), all of the plant material used was chosen randomly.

Molecular methods

Total genomic DNA was extracted with the DNAeasy Plant Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. Following extraction, DNA concentration and purity were estimated by 0.8% agarose gel electrophoresis with ethidium bromide staining, and comparison with a standard molecular mass marker (lambda *Hind*III, NEB).

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 of the quality of the PCR product. Briefly, of the eight nSSRs assayed, QM58TGT and QM50–3M, were first described by Isagi and Suhandono (1997) in *Q. myrsinifolia* Blume, and QpZAG9, QpZAG15, QpZAG36 and QpZAG110 were first described in *Q. petraea* (Matts.) Liebl. (Steinkellner et al. 1997*a*). The transferability of these loci to *Q. suber* has previously been reported (Lopes 2000, Gomez et al. 2001, Hornero et al. 2001*b*). 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. (2001*b*). To amplify the selected microsatellites by polymerase chain reaction (PCR), we used the primers designed by the authors.

The forward primers were synthesized with a fluorescent label attached to the 5′ end (ABI dyes: 6-FAM, JOE, HEX and TET) to allow detection of the PCR products. For each DNA sample, PCR amplification of each of the eight microsatellite loci was performed in a 25 μl PCR reaction mixture containing 25 ng of genomic DNA, reaction buffer (75 mM Tris-HCl pH 9, 50 mM KCl, 20 mM $(NH_4)_2SO_4$, 0.001% BSA-bovine serum albumin), 2.5 mM $MgCl_2$, 0.8 mM dNTPs mix, 1 unit of Biotools DNA polymerase and varying primer concentration, according to Hornero et al. (2001*b*). Details of the studied loci and respective primers used to test for genetic stability are given in Table 1. Amplification of DNA was carried out in an Eppendorf Mastercycler programmed as follows: 5 min at 94 °C as the 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 and 72 °C for 30 s, followed by 25 cycles of 94 °C for 15 s,

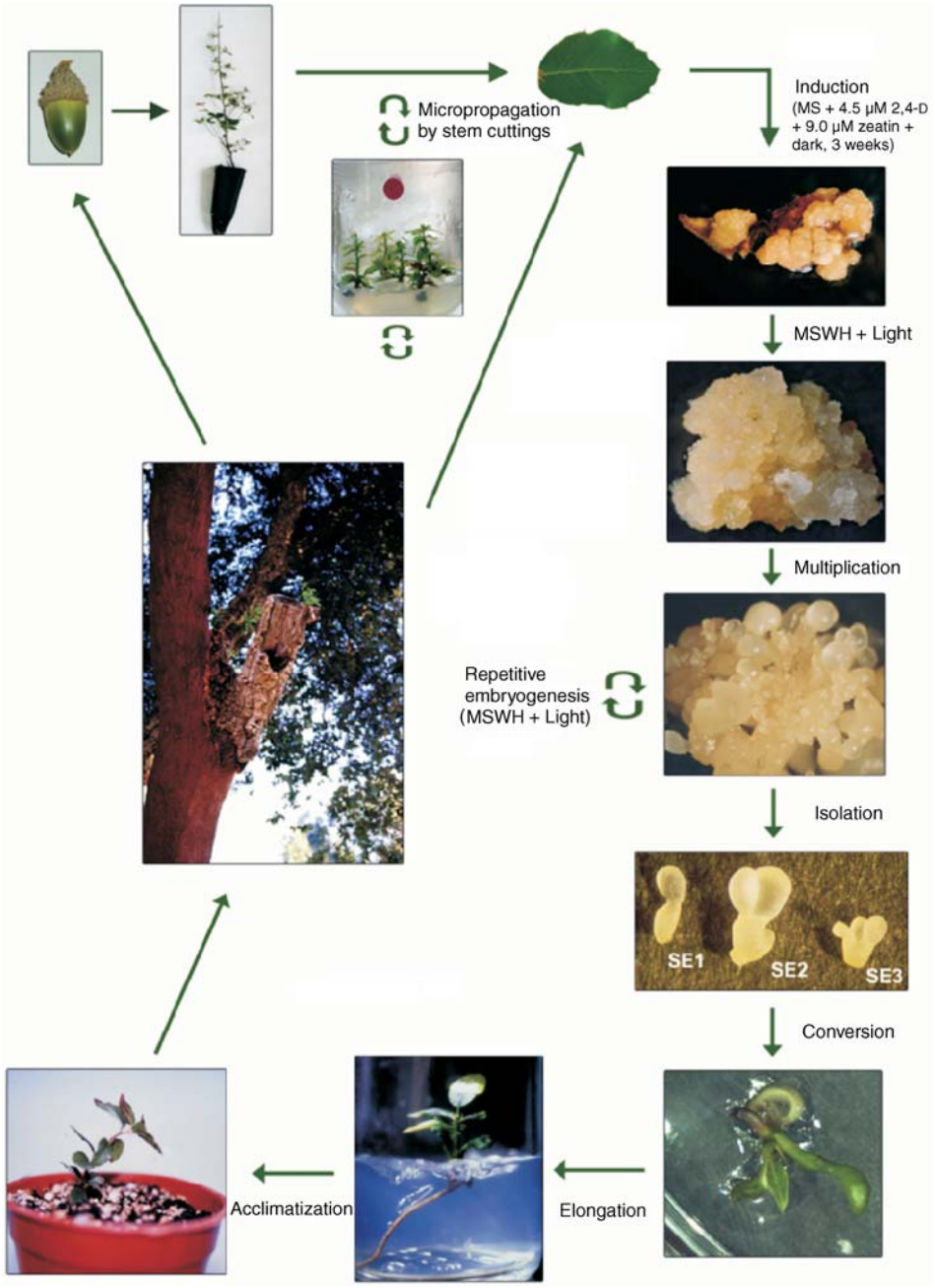

Figure 1. Schematic diagram illustrating the embryogenic process for obtaining somatic embryos (SE) in *Quercus suber*. Three somatic embryo morphotypes are shown: an abnormal SE with one cotyledon (SE1), a normal dicotyledonary embryo (SE2) and an abnormal SE with more than two cotyledons (SE3). Abbreviations: MS = Murashige and Skoog medium; and MSWH = MS medium without growth regulators.

55 °C for 30 s and 72 °C for 30 s. A final extension step was carried out at 72 °C for 5 min.

After PCR amplification, 1 µl of water-diluted PCR product was mixed with 0.5 µl of GeneScan internal size standard labeled with either ROX or TAMRA (PE Biosystems, Foster City, CA) and with 25 µl of formamide. The mixture was briefly vortexed, incubated at 95 °C for 3–5 min and finally placed on ice for 3–5 min. The PCR products were then visualized by capillary electrophoresis on an ABI PRISM 310 Genetic Analyzer (PE Biosystems). After electrophoretic analysis, all fragment sizes were scored and each donor plant was compared with its cloned embryos and somatic embryo-derived plantlets. Fragment sizes were automatically calculated to two decimal places with Local Southern Method option of the GeneScan v.3.1 software.

Results

Undifferentiated calluses were whitish, highly friable and with large and vacuolated cells. Embryogenic calluses in which primary SE formed (in all genotypes) were pearly-white, compact and had embryogenic formations at different evolutionary stages (asynchronism). In secondary somatic embryogenesis (Figure 1), cotyledonary SE had large, compact and, when mature, green cotyledons. Three morphotypes of embryos were observed: (1) normal dicotyledonary embryos (SE2); (2) with

Table 1. Characteristics of the microsatellite loci amplified in this study that were primarily developed for *Quercus myrsinifolia* (Isagi and Suhandono 1997), *Q. robur* (Steinkellner et al. 1997*a*) and *Q. petraea* (Kampfer et al. 1998). Repeat structure, primer sequences and variable PCR conditions used are present. Abbreviation: *T* = temperature.

Locus	Repeat structure	Primer sequences	Annealing *T* (°C)	Primer concentration (μM)
QM58TGT[1]	$(CAA)_{11}$	GGTCAGTGTATTTTGTTGGT AAATGTATTTTGCTTGCTCA	55	0.5
QM50–3M[1]	$(CCT)_3(CCG)(CCT)_2$ $(CCA)(CCT)_2+(CCA)_7$	CCCGATTTCCCTTCCCTGCT CGGGCTTTGGATACGGATTC	55	0.3
QpZAG9[2]	$(AG)_{12}$	GCAATTACAGGCTAGGCTGG GTCTGGACCTAGCCCTCATG	55	0.3
QpZAG15[2]	$(AG)_{23}$	CGATTTGATAATGACACTTGG CATCGACTCATTGTTAAGCAC	55	0.5
QpZAG36[2]	$(AG)_{19}$	GATCAAAATTTGGAATATTAAGAGAG ACTGTGGTGGTGAGTCTAACATGTAG	50	0.2
QpZAG110[2]	$(AG)_{15}$	GGAGGCTTCCTTCAACCTACT GATCTCTTGTGTGCTGTATTT	50	0.2
QrZAG7[3]	$(TC)_{17}$	CAACTTGGTGTTCGGATCAA GTGCATTTCTTTTATAGCATTCAC	55	0.5
QrZAG11[3]	$(TC)_{22}$	CCTTGAACTCGAAGGTGTCCTT GTAGGTCAAAACCATTGGTTGTTGACT	55	0.5

[1] From Isagi and Suhandono (1997) for *Q. myrsinifolia.*
[2] From Steinkellner et al. (1997*b*) for *Q. petraea*.
[3] From Kampfer et al. (1998) for *Q. robur.*

one cotyledon (SE1); and (3) with three or more cotyledons (SE3) (Figure 1). Nevertheless, plants converted from normal and abnormal cotyledonary SE looked morphologically normal (Figure 1).

Amplification of the SSR markers was performed with six to eight primer pairs that produced 40 reproducible fragments/alleles that ranged from 106 to 292 bp in size (Table 2). Allele size was well within the values described in the literature for the given species. Most loci resulted in banding patterns with two bands (alleles) corresponding to heterozygous individuals, with the exceptions of locus QM58TGT, which resulted in a profile with only one band (allele) for genotype QsG0, and loci QM50–3M and QpZAG110, which both resulted in profiles with only one band for genotype QsGM1, representing homozygous individuals.

All primer pairs used produced amplification products (alleles) that were polymorphic within the four genotypes tested as donor explants (Table 2). For genotypes QsG0 (Figure 2), QsGM1 and QsGM2 (Figure 3), these primers were monomorphic across all the embryos from each genotype, its derived plantlets and its respective donor tree. For genotype QsG5 the pattern was similar, but for the primer pair corresponding to locus QM50–3M, one mutant heterozygote allele out of 40 QsG5 SE was detected that had a size range of 285/292 compared with a size range 285/287 for the initial allele (Figure 4).

To test the reproducibility of the technique, duplicates were run for five of the samples. These assays were complete reruns, i.e., all procedures from DNA extraction up to PCR amplification took place on different occasions for each of the duplicates. All sets of duplicates returned exactly the same banding patterns.

Table 2. Characteristics of the microsatellite loci amplified in *Q. suber.* Allele size found in this study and allele size range and number of alleles (in parenthesis) found in other publications are also given: values for genotypes QrZAG7 and QrZAG11 are from Hornero et al. (2001*b*) and values for the six remaining loci are from Lopes (2000). Abbreviation: nd = no data.

Locus	Allele size (bp)				Allele size (bp) in other publications (No. of alleles)
	QsG0	QsG5	QsGM1	QsGM2	
QM58TGT	201	185/211	203/210	203/210	193–229 (5)
QM50–3M	276/286	285/287/292	278	282/288	272–300 (7)
QpZAG9	223/238	223/233	218/223	223/233	224–250 (6)
QpZAG15	108/123	106/120	nd	nd	120–136 (4)
QpZAG36	209/219	216/220	207/209	207/209	208–228 (7)
QpZAG110	223/233	220/238	221	221/233	218–266 (14)
QrZAG7	106/119	115/127	106/121	106/121	115–134 (7)
QrZAG11	229/263	261/273	nd	nd	261–275 (6)

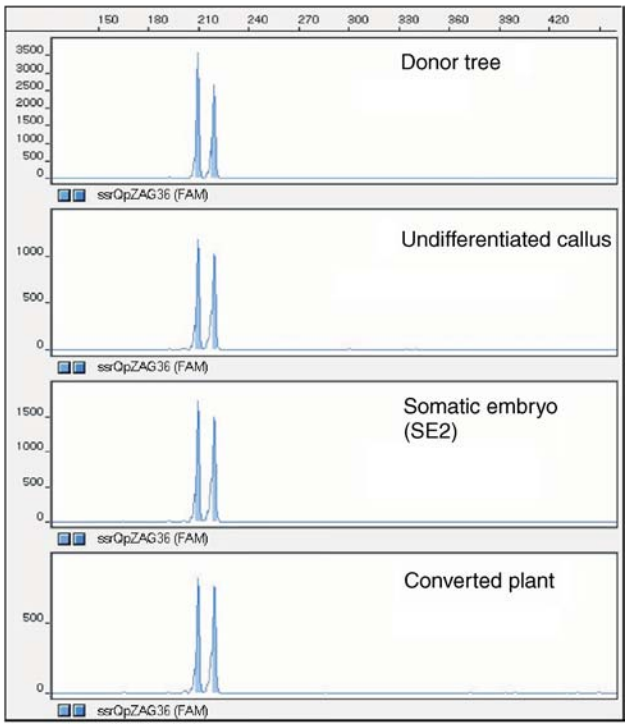

Figure 2. Amplification of the ssrQpZAG36 (FAM) locus in genotype QsGO (from top to bottom): donor tree, an undifferentiated callus, a normal dicotyledonary somatic embryo (SE2) and a converted plant. All electropherograms show heterozygous individuals with two alleles of about 209 bp and 219 bp. Top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

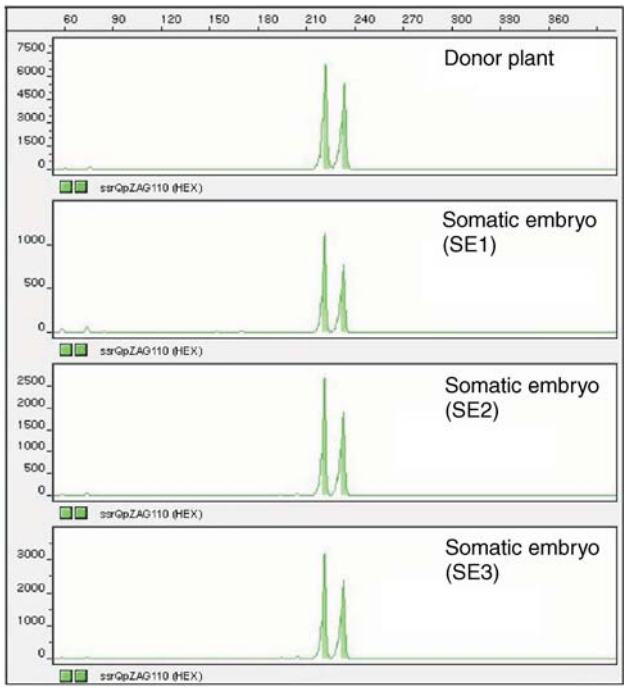

Figure 3. Amplification of the ssrQpZAG110 (HEX) locus in genotype QsGM2 (from top to bottom): donor plant, an abnormal somatic embryo (SE) with one cotyledon (SE1), a normal dicotyledonary SE (SE2) and an abnormal SE with more than two cotyledons (SE3). All electropherograms show heterozygous individuals with two alleles of about 221 bp and 233 bp. Top scale indicates fragment size in nucleotides. Left scale indicates fluorescence intensity measured in relative fluorescence units.

Discussion

The use of secondary somatic embryogenesis represents an efficient method of overcoming the low rates of induction of primary SE found for *Q. suber* species (e.g., Pinto et al. 2001, 2002, Hernandez et al. 2003*a*). Despite the occurrence of morphologically abnormal SE, which has been reported previously (Pinto et al. 2001, 2002, Pinto 2003, Loureiro et al. 2005), the plants derived from these SE showed no morphological variability when compared with each other or when compared with plants derived from axillary bud micropropagation (Pires 2004, Loureiro et al. 2005).

The usefulness of the microsatellite molecular markers selected for this work has been confirmed previously by other authors (Table 2). The finding that all the primers we used showed polymorphisms among the different donor genotypes confirms that the selected primers were efficient in detecting polymorphisms present in the cloned embryos, their derived plantlets and respective donor trees.

No microsatellite instability was found between the dicotyledonary (SE2) morphotype and the abnormal SE1 or SE3 morphotypes (Figures 1 and 3), as shown in Figure 2, which depicts electropherograms of individuals belonging to clone QsG0. However, a low percentage of allele size variation (locus QM50–3M) was found, but only in the QsG5 genotype. It is unlikely that this result is an artefact because the sample was reanalyzed several times to confirm the presence of the third band. This mutation could have arisen as a result of the addition of the repeat units at this locus. Genetic instability is frequently reported to occur in tissue culture and it is most likely caused by mitotic recombination as a result of inter-chromatid unequal crossing over or intra-chromatid exchange of repeats (Rahman and Rajora 2001).

There are several reports of the occurrence of somaclonal variation in SE: AFLP analyses performed by Hornero et al. (2001*a*) revealed the occurrence of somaclonal variation in *Q. suber* SE derived from leaf sprouts of adult trees (a maximum of 7.3% polymorphism). Endemann et al. (2001) found somaclonal variation (tetraploidy) in 8% of *Q. robur* somatic clones belonging to 7-year-old embryogenic lines, when using flow cytometry. Also, using another technique (stability of five microsatellite loci) the same group reported the occurrence of mutant SE clones in *Q. robur* (Wilhelm et al. 2005), with a high mutation frequency of 29.2–62.5%, depending on the genotype.

By contrast, the lack of somaclonal variation in somatic embryogenesis has been reported for *Q. robur* and other *Quercus* species: Hornero et al. (2001*a*), using AFLP markers, found no instability in SE derived from zygotic embryos of *Q. suber*; and both Thakur et al. (1999) and Gallego et al. (1997) reported no RAPD instability for *Q. serrata* and

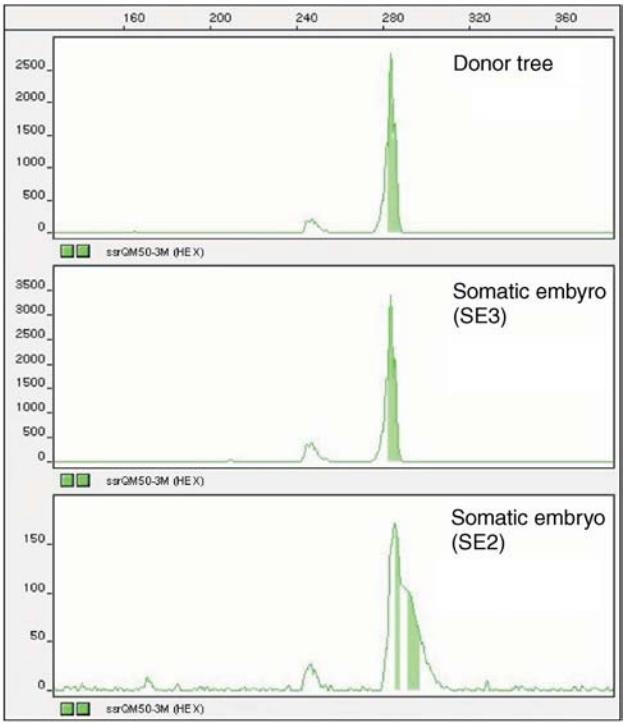

Figure 4. Amplification of the ssrQM50–3M (HEX) locus in genotype QsG5 (from top to bottom): donor tree, an abnormal somatic embryo (SE) with three cotyledons (SE3) and a normal dicotyledonary somatic embryo (SE2). Electropherograms corresponding to the donor plant and SE3 are similar and show heterozygous individuals with two alleles of about 285 bp and 287 bp. The lower electropherogram corresponding to SE2 shows a heterozygous individual with two alleles of about 285 bp and 292 bp. Top scale indicates fragment size in nucleotides. Left scale indicates fluorescence intensity measured in relative fluorescence units.

Q. suber SE, respectively. However, some authors (Isabel et al. 1996, Fourré et al. 1997) have concluded that RAPD markers fail to detect genetic variation (e.g., fine-scale genetic changes or alterations) and are inappropriate for this type of study. More recently, Helmersson et al. (2004) reported a high stability of three nuclear microsatellite loci during the early stages of somatic embryogenesis in *Picea abies* (Norway spruce) and embryo-derived plantlets.

We found a mutation only in genotype QsG5, but the low mutation frequency precludes any conclusion about the effect of the genotype. Nevertheless this aspect must be considered in further studies. Wilhelm et al. (2005) have suggested that the influence of genotype/species may play an important role in the frequency of genetic anomalies during somatic embryogenesis. Etienne and Bertrand (2003) observed large differences in somaclonal variation among genotypes of *Coffea arabica* L. plants produced from 12-month-old embryogenic cell suspensions.

Prolonged in vitro culture can cause genetic instability such as polyploidization (e.g., Endemann et al. 2001). Leroy et al. (2000) postulated that the number of genomic lesions is greater in calluses of a more advanced stage of maturation if the occurrence of such alterations increases with cellular proliferation. Therefore, analyses of embryogenic calluses may aid in the prediction of whether genetic instability could be initiated at an early stage of callus formation or proliferation and thus contribute to the understanding of different rates of genetic instability among genotypes (Leroy et al. 2000).

Given the long period that our SE and calluses have been maintained in culture (1–4 years), and considering the high rates of variance previously reported for other long-term cultures of somatic embryogenic lines of *Q. robur* (Wilhelm et al. 2005), we predicted that our cultures would exhibit mutations at a high frequency, but this was not observed. One of the microsatellites that proved to be useful in detecting genetic instability in somatic embryogenic culture lines of *Q. robur* by Wilhelm et al. (2005) was also used in this study (QpZAG9), but it detected no variation in *Q. suber* SE. Besides the species/genotype effect, we offer two possible explanations for this apparent discrepancy: (1) marker QpZAG9 was developed for *Q. petraea*, which is more closely related to *Q. robur* than to *Q. suber* and, therefore, may be able to detect more variation in *Q. robur* species/genotypes than in cork oak (Steinkellner et al. 1997*b*); and (2) the protocol used for *Q. robur* SE induction from zygotic embryos required a primary culture of 3 weeks in the presence of 6-benzyladenine (BA) and 2,4-D and then transfer to a medium supplemented with BA for maintenance. In contrast, for *Q. suber* SE induction, explants were exposed for 1–3 months (1 month for genotypes QsGM1 and QsGM2; 3 months for genotypes QsG0 and QsG5) to 2,4-D and zeatin and then transferred to a growth regulator free medium where the cultures remained repetitively embryogenic and therefore, putatively less mutagenic.

The age of the donor genotype may influence genetic stability. The only mutation we found was in SE derived from leaves of a mature field tree (QsG5). Similarly, Hornero et al. (2001*a*) reported mutations in SE only when using leaves from adult trees. It is known that differentiated cells have to proceed through a dedifferentiation process (Mo and von Arnold 1991, Goto et al. 1998); however, this aspect deserves further study because there are also reports of SE from leaf explants of mature trees that appear to show no genetic instability (e.g., Hornero et al. 2001*a*), as was the case for genotype QsG0 in our study.

In conclusion, we developed a simple protocol to induce somatic embryogenesis in *Q. suber* that was reproducible in young and mature genotypes, and led to little or no genetic instability even in long-term cultures. We found that: (1) the cork oak morphotypes observed were not correlated with the genetic variability assessed (the influence of DNA methylation to assess epigenetic changes is being presently studied); and (2) the nuclear microsatellites proved to be a useful tool for screening somaclonal variation in *Q. suber* SE and their derived plantlets. Nevertheless, reliable analysis of genetic stability in micropropagated processes requires, whenever possible, complementary approaches (e.g., SSH-PCR, AFLPs, RAPDs, flow cytometry).

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