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# High-efficiency *Agrobacterium*-mediated transformation in *Quercus robur*: Selection by use of a temporary immersion system and assessment by quantitative PCR

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## Abstract

An efficient protocol for genetic transformation of somatic embryos of *Quercus robur* by selection in a temporary immersion system is reported. The transformation frequency was 5 times higher than achieved by conventional culture on semi-solid medium, ranging between 6 and 26 % for the four genotypes evaluated. Clumps of globular or torpedo somatic embryos were precultured for 7-10 days, inoculated with *Agrobacterium tumefaciens* strain EHA105:p35SGUSINT and cocultivated for 4 days before being cultured for 4 weeks on semi-solid selection medium supplemented with 25 mg $L^{-1}$ kanamycin. Explants were transferred to RITA® bioreactors and subjected to a two-step selection protocol involving immersion in liquid medium supplemented with 25 mg $L^{-1}$ kanamycin, for 18 weeks, and then with 75 mg $L^{-1}$ kanamycin. Putatively transformed explants appeared after serial transfer to selection medium over 12-16 weeks. The presence of neomycin phosphotransferase II and β-glucuronidase genes in the plant genome was confirmed by histochemical and molecular analysis, and the copy number was determined by Southern blotting and real-time quantitative polymerase chain reaction. Transformed somatic embryos were germinated and transferred to soil for acclimatization, approximately 8 months after inoculation of the original tissue with bacteria. As the limiting factor for recovery of plants from oak embryogenic lines is the low embryo conversion rate, axillary shoot lines were established from transformed germinated embryos. Transformed embryos and shoots were cultured in medium with or without kanamycin and the responses to several morphogenetic processes (recovery after cryopreservation, germination, shoot proliferation, and rooting) were evaluated.

**Keywords:** pedunculate oak, improved genetic transformation, temporary immersion, transgene copy number, quantitative PCR

**Introduction**

Pedunculate oak (*Quercus robur* L.) is a slow-growing long-lived tree that is native to Europe, Western Asia, and Northern Africa. Although it is economically and ecologically one of the most important hardwood tree species in European forests, it has been greatly affected by industrialization and the expansion of cropland and urban areas. It is also currently threatened by oak decline, a disorder caused by interactions between biotic and abiotic factors (Thomas et al. 2002) or by the effects of individual biotic factors, such as the green oak leaf roller moth (Schroeder and Degen 2008), oak powdery mildew (Hajji et al. 2009), and lesions caused by *Phytophthora* sp. at the root or collar level (Jung et al. 2000).

Biotechnological tools are used to modify oak species with the aim of enhancing the productivity of the species and increasing their resistance to biotic and abiotic stressors, thus complementing conventional breeding and selection programmes (Vieitez et al. 2012). Efficient in vitro regeneration systems based on somatic embryogenesis (San-José et al. 2010; Mallón et al. 2012) can facilitate classical breeding programmes by providing large amounts of clonal material (for propagation) and explants (for transformation and cryopreservation). Genetic transformation mediated by *Agrobacterium tumefaciens* is a powerful biotechnological tool of potential use in pedunculate oak for introducing important resistance traits.

However, information about the genetic transformation of pedunculate oak is scarce. In a preliminary study, we inoculated somatic embryogenic cultures derived from pedunculate oak trees with *A. tumefaciens* and selected transformed embryos on the basis of GUS expression and kanamycin resistance (Vidal et al. 2010). Although transformed oak embryos were derived from both juvenile and mature trees, the transformation frequency was low (Vidal et al. 2010). Another drawback of the protocol was the slow regeneration of transgenic embryogenic material from infected explants, which delayed the production of genetically modified embryogenic lines and made the transformation protocol time-consuming (the process took more than 1 year to produce transgenic plantlets). The available transformation system for pedunculate oak is therefore of limited application and improvements must be made before further efforts to transform this material with constructs that confer disease resistance. We have already demonstrated that use of a temporary immersion system significantly improves the proliferation rates and quality of oak somatic embryos (Mallón et al. 2012). Therefore, we applied this system in the present study, with the aim of increasing the efficiency of the genetic transformation of pedunculate oak.

Transgenic material must be maintained in vitro prior to analysis, evaluation and eventual use. However, maintenance is labour-intensive and time-consuming and the process is prone to

contamination and inter-batch variability. For the long-term preservation of transgenic material, it is important to develop techniques that are capable of ensuring the genetic integrity of transgenes (Wang et al. 2012). Although a cryopreservation protocol has been reported for *Q. robur* somatic embryos (Sánchez et al. 2008), it remains to be tested with genetically transformed material.

Characterization of regenerated lines (i.e. accurate measurement of the transgene copy number) is another important aspect common to all transformation studies. Although this is usually done by Southern blotting, the technique is time-consuming as it requires large amounts of genomic DNA and continuous culture and maintenance of transgenic lines. Use of real-time quantitative polymerase chain reaction (RT-qPCR), a sensitive technique that enables specific and reproducible quantification of nucleic acids, has led to the development of high-throughput molecular diagnostics (Arya et al. 2005). This methodology requires less input of DNA and enables more efficient assessment of numerous putative transgenic lines than Southern blotting (Casu et al. 2012).

Once transgenic lines are characterized, multiple vigorous plantlets from different transgenic events are required to test transgene performance throughout plant growth and development. However, one of the main problems associated with oak somatic embryogenesis is the low rate of conversion of somatic embryos into plants, which limits plant production (Vieitez et al. 2012). To overcome this, regenerated somatic plantlets could be used to initiate shoot culture lines that can be proliferated and rooted by use of the micropropagation protocols described for pedunculate oak (Vidal et al. 2003; Martínez et al. 2012).

The main objectives of the present study were as follows: 1) to define an *Agrobacterium*-mediated transformation system for pedunculate oak based on selection by temporary immersion of putative transformed embryogenic material, with the aim of increasing transformation frequencies and reducing the time required to produce transgenic lines; 2) analysis of transgenes by RT-qPCR to enable the efficient assessment of numerous potentially transgenic lines; 3) cryopreservation of transgenic lines to allow the establishment of long-term preservation of transgenic materials; and 4) improvement in the quantity and quality of transgenic plantlets acclimatized to soil by combining plant conversion of transgenic embryos and micropropagation of the somatic plantlets. Accomplishment of all four objectives will enable high-throughput production of transgenic pedunculate oak.

## Materials and methods

Plant material and culture conditions

Four embryogenic lines were used in the transformation experiments. Line T2H was of juvenile origin (Cuenca et al. 1999) and lines B13, B17, and Sainza were initiated from *Q. robur* trees of between 100 and 300 years old (Toribio et al. 2004; Martínez et al. 2008). These embryogenic lines were maintained by secondary embryogenesis with serial subculture at 5-week intervals (Valladares et al. 2006). Embryo clumps were cultured in Petri dishes (90 mm in diameter) with 25 mL of proliferation medium (PM) consisting of Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 0.27 µM $\alpha$-naphthaleneacetic acid (NAA), 0.44 µM $N^6$-benzyladenine (BA), 500 mgL$^{-1}$ casein hydrolysate, 30 g L$^{-1}$ sucrose, and 6 g L$^{-1}$ agar (Vitroagar, Hispanlab, Spain). The embryogenic lines were also cultured in a temporary immersion system (TIS), in 1 L RITA® bioreactors, with 45 embryo clumps (approximately 500 mg) per bioreactor and 150 mL of liquid PM (without agar), under an immersion cycle of 1 min every 12 h (Mallón et al. 2012). All culture media were adjusted to pH 5.7 before being autoclaved at 121 °C for 20 min. The cultures were incubated under a 16 h photoperiod, provided by cool-white fluorescent lamps at 50–60 µmol m$^{-2}$ s$^{-1}$, at 25 °C (light) and 20 °C (dark) (standard conditions).

Effect of antibiotics on the growth of embryogenic cultures

Embryo clumps (4-10 mg) of non-transformed embryogenic lines T2H and Sainza were cultured in liquid or semi-solid medium supplemented with 0, 10, 25, 50, or 75 mg L$^{-1}$ of kan plus 300 mg L$^{-1}$ carbenicillin and 200 mg L$^{-1}$ cefotaxime, or without these antibiotics. All antibiotics were filter-sterilized and added after autoclaving. Embryo clumps from stable transformed embryogenic lines previously obtained from T2H and Sainza material (Vidal et al. 2010) were cultured in liquid or semi-solid medium supplemented with 0, 25, or 75 mg L$^{-1}$ of kan plus 300 mg L$^{-1}$ carbenicillin and 200 mg L$^{-1}$ cefotaxime. The cultures were transferred to fresh medium every 2 weeks and symptoms of necrosis were recorded. The data (percentage of necrotic explants and the number of secondary embryos developed per explant) were recorded after 6 and 12 weeks. The percentage necrosis was also determined after 16 weeks of culture.

Transformation and selection: comparison of temporary immersion system and semi-solid medium

*Agrobacterium tumefaciens* strain EHA105:p35SGUSINT, which contains the neomycin phosphotransferase II (*npt*II) gene and the intron-containing *uid*A reporter gene (GUS) driven by CaMV 35S, was used for transformation (Vidal et al. 2010). Explants consisting of small clumps of two to three globular or early-torpedo stage somatic embryos were precultured for 7-10 days on semi-solid PM. The explants were immersed for 30 min in the *Agrobacterium*

suspension ($OD_{600}$=0.6), blot-dried on sterile filter paper, and transferred to PM. After 4 days of cocultivation at 25 °C in the dark, the embryos were washed for 30 min with sterile distilled water containing 500 mg $L^{-1}$ cefotaxime, blot-dried on sterile filter paper, and transferred to Petri dishes (nine explants per dish) containing PM supplemented with 300 mg $L^{-1}$ carbenicillin, 200 mg $L^{-1}$ cefotaxime, and 25 mg $L^{-1}$ kan (selection medium CCK25, hereafter CCK25). After 4 weeks of co-culture, half of the explants were transferred to RITA® bioreactors (one immersion every 12 h) and the other half were cultured on semi-solid medium, to compare the transformation efficiency after selection by TIS and plate culture. The selection medium consisted of CCK25 with and without agar. After 18 weeks on CCK25, the amount of kanamycin in both liquid and semi-solid selection media was increased to 75 mg $L^{-1}$, whereas carbenicillin and cefotaxime levels were maintained as before (selection medium CCK75); this was designated as the standard selection scheme. Additional selection schemes, in which a new selection medium with 10 mg $L^{-1}$ kan (CCK10) was combined with CCK25 and CCK75 and applied at different intervals, were also used. Explants were transferred to fresh selection medium at 2-week intervals throughout the experiment.

After 12-14 weeks from the start of the experiments, necrotic explants with newly emerging somatic embryos or nodular embryogenic structures were isolated from bioreactors or Petri plates and proliferated on semi-solid CCK75 medium. A single somatic embryo from each independent transformation event was used to establish a putatively transgenic line.

The transformation efficiency was defined as the percentage of initial explants that developed GUS-positive embryogenic cultures, and selection efficiency was defined as the percentage of kan-resistant explants producing embryos that yielded GUS-positive embryogenic cultures.

### β–Glucuronidase (GUS) assay

GUS activity was determined by use of the histochemical assay described by Jefferson (1987). All kan-resistant embryogenic lines and samples of leaves and roots from germinated plantlets derived from transformed lines were tested for stable GUS expression.

### Molecular analysis

#### PCR and Southern blot

PCR and Southern blot methods were used to confirm the presence of the *npt*II and *uid*A genes in kan-resistant and GUS-positive embryogenic lines. DNA was isolated from transformed and untransformed somatic embryos with the DNeasy Plant Mini Kit (Qiagen), using the following primer sequences: 5´-GTCATCTCACCTTGCTCCTGCC-3´ and 5´-AAGAAGGCGATAGAAGGCGA-3´

for the *npt*II gene; 5’-CAAGGCACTAGCGGGACT-3’ and 5’-GCGAAGCGGGTAGATGTC-3’ for the *uid*A gene and 5´-ATGATTTGTAGCGGACT-3´ and 5´-AGCTCAACCTGCTTC-3´ for the *virC* gene. The reaction mixture (25 µl) contained 10 ng DNA, 200 µM dNTPs, 0.6 µM of each primer, 2 mM $MgCl_2$ and 0.5 U of Taq DNA polymerase (Bioline). PCR conditions were 35 cycles of 30 s at 94 °C, 30 s at 60 °C, and 42 s at 72 °C for *npt*II amplification, 35 cycles of 1 min at 94 °C, 1 min at 57 °C, and 1.5 min at 72 °C for *uid*A detection and 30 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1.5 min at 72 °C for *virC* detection. All cycling profiles were followed by a termination step of 72 °C for 7 min followed by 4 °C in a MJ Mini™ Personal Thermal Cycler (Bio-Rad). PCR products were expected to be 472 bp for *npt*II, 123 bp for *uid*A, and 730 bp for virC gene. For Southern blotting, genomic DNA (20-30 µg) was digested with *Eco*RI, which recognized a single site within the T-DNA upstream of the promoter of the 2.8 kbp *uid*A construction (Vidal et al. 2010), thus indicating that positively hybridizing fragments should be larger than 2.8 kbp. The fragments were electrophoresed on a 1 % agarose gel in half-strength Tris-borate buffer (TBE). After depurination, denaturation and neutralization treatments, DNA was transferred onto a positively-charged nylon membrane (Roche Applied Science, Mannheim, Germany) by capillarity. A Digoxigenin (DIG)-labelled *uid*A probe was produced by use of a PCR DIG Probe Synthesis kit (Roche Applied Science) (with primers 5´-GGTGGGAAAGCGCGTTACAAG-3´ and 5´-TGGATCCCGGCATAGTTAAA-3´; 589 bp). The probe was hybridized and detected by use of a DIG luminescent Detection kit (Roche Applied Science) according to the manufacturer’s instructions. Genomic DNA extracted from a non-transformed plant and plasmid DNA served as negative and positive controls, respectively.

Quantitative real-time PCR

The copy number of the *uid*A gene in putatively transformed lines was estimated by real-time quantitative PCR (RT-qPCR). RT-qPCR assays were carried out with an Applied Biosystems StepOne™ Real-Time PCR System. Primer sequences were 5’-CAAGGCACTAGCGGGACT-3’ and 5’-GCGAAGCGGGTAGATGTC-3’ for the *uid*A gene. The size of the amplified product was 123 bp.

All reactions were carried out in a final volume of 20 µl comprising 2 µl sample DNA (a total of 1.1 ng), 10 µl of 2× SYBR Green I Master PCR buffer (Roche), 1 µl of each primer (0.5 µM), and 6 µl sterile Type I water. Samples were replicated three times, and experiments were repeated twice. A standard curve was prepared by diluting a sample of DNA from a transformed line with only one transgene copy (confirmed by Southern blotting). Serial dilutions of the DNA of the transformed line were carried out to final concentrations of 10, 3.3, 1.1, 0.37, 0.12, and 0.04 ng per reaction and subjected to triplicate RT-qPCR assays, with four independent

repetitions each time. Quantitative RT- PCR conditions were as follows: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. At the end of each cycle, the level of SYBR-specific fluorescence was measured and the accompanying software was used to record and analyze the data. Following thermal cycling, melting curve analysis was performed to verify the sequence specificity of amplified products. A standard curve was constructed to study the linear relationship between the level of fluorescence and DNA input . The analytical method used threshold cycle (Ct) values to extrapolate the initial concentration of target DNA in each sample. The copy number in each sample was determined by comparison of the Ct-extrapolated concentrations against an in-run standard curve derived from the one-copy-containing line used as external control. The amplification efficiency (E) was calculated as $E=10^{(-1/slope)}$ (Ginzinger 2002).

Cryopreservation

Transformed lines from T2H and Sainza genotypes were cryopreserved using a vitrification-based protocol (Sánchez et al. 2008). Briefly, 4-6 mg clumps of globular or heart-shaped secondary embryos were isolated from transgenic lines maintained on semi-solid medium and in bioreactors. The embryo clumps were then precultured for 3 days on basal medium (BM; PM without plant growth regulators) with 0.3 M sucrose (102.7 g $L^{-1}$) and without casein hydrolysate. The explants (15-20 embryo clumps) were placed in PVS2 vitrification solution in cryovials (2 mL), which were maintained for 60 min at room temperature (Sakai et al. 1990) and then immersed in liquid nitrogen (LN). After a period of at least 24 h, 3-4 cryovials per treatment were removed from the LN and thawed (2 min 40 °C). The samples were washed twice with liquid BM supplemented with 1.2 M sucrose (410.8 g $L^{-1}$), blot-dried on filter paper sterilized by autoclaving, transferred to Petri dishes and cultured on PM with or without kan. After 6 weeks, embryo recovery was assessed as the percentage of explants that showed resumption of secondary embryogenesis. Stability of the inserted genes after cryopreservation was evaluated by histochemical assays. The germination and plant conversion ability of cryopreserved embryos was also evaluated.

Germination and plant regeneration

Somatic embryos from transformed lines were germinated following the protocol developed by Martínez et al. (2008). The resulting somatic plantlets were transferred to pots containing gamma sterilized peat:perlite (3:1), for acclimatization in a phytotron for 4-8 weeks, and were then grown in the greenhouse. Alternatively, somatic plantlets derived from germinated embryos were used to initiate shoot culture lines, which proliferated by axillary branching

(Martínez et al. 2012). The axillary lines were maintained and rooted by the protocol previously described for oak micropropagation (Vidal et al. 2003). Briefly, shoot proliferation medium consisted of Gresshoff and Doy (GD) medium (Gresshoff and Doy 1972) supplemented with 30 g $L^{-1}$ sucrose, 6.5 g $L^{-1}$ agar (Vitroagar, Hispanlab, Spain) and 0.44 μM BA. GD medium consisted of macronutrients (1.51 mM $(NH_4)_2SO_4$, 9.89 mM $KNO_3$, 1.02 mM $CaCl_2 \cdot 2H_2O$, 1.01 mM $MgSO_4 \cdot 7H_2O$, 0.21 mM $Na_2HPO_4$, 0.65 mM $NaH_2PO_4 \cdot H_2O$, 4.02 mM KCl), micronutrients (0.1 mM FeNaEDTA, 48.5 μM $BO_3H_3$, 59.16 μM $MnSO_4 \cdot H_2O$, 10.43 μM $ZnSO_4 \cdot 7H_2O$, 1 μM $CuSO_4 \cdot 5H_2O$, 1.03 μM $MoO_4Na_2 \cdot 2H_2O$, 1.05 μM $CoCl_2 \cdot 6H_2O$, 4.52 μM KI) and vitamins (2.96 μM Thiamine HCl, 0.81 μM Nicotinic acid, 0.48 μM Pyridoxine HCl, 5.33 μM Glycine, 56 μM myo-Inositol). For rooting, shoots (around 20 mm in length) were harvested at the end of the multiplication step and placed in 100 mL jars sealed with Magenta™ B-caps and containing 30 mL of GD medium with 1/3-strength macronutrients supplemented with 5.5 g $L^{-1}$ agar, 30 g $L^{-1}$ sucrose and 120 μM indole-3-butyric acid (root induction medium). After 24 h, shoots were transferred to auxin-free medium of the same composition but supplemented with 6.5 g $L^{-1}$ agar (root expression medium). Shoot proliferation rates and rooting capacity were evaluated in media with and without 75 mg $L^{-1}$ kan.

Sampling and data analysis

The effect of antibiotics on the growth of embryogenic cultures was tested on 45 embryo explants (five replicate Petri dishes with nine explants per dish) and one bioreactor (45 embryo clumps) per treatment, and each experiment was repeated twice. For transformation experiments, 90 explants per treatment (semi-solid medium or TIS) were used per embryogenic line, and 45 uninfected explants were cultured on PM with or without antibiotics (negative and positive controls, respectively). Each experiment was performed at least twice. At least three replicate 10-embryo clumps per treatment and line were used in each cryopreservation experiment, and the experiments were repeated twice. To evaluate shoot multiplication rates and growth, six replicate 500 mL jars (six explants per jar) were used per shoot line, and the experiment was repeated twice. For rooting experiments, 30 explants were used per treatment (six explants per 100 mL jar), and the experiment was repeated three times. The data were examined by analysis of variance followed by the least significant difference (LSD) test for comparison of means (at $P$=0.05). Percentage data were subjected to arcsine transformation prior to analysis.

RESULTS

Effect of antibiotics on the growth of embryogenic cultures

The addition of carbenicillin and cefotaxime to semi-solid medium (plates) or liquid medium (bioreactors) did not negatively affect the proliferation of non-transformed oak somatic embryos. Growth of previously transformed lines cultured with 0, 25, or 75 mg $L^{-1}$ kan plus 300 mg $L^{-1}$ carbenicillin and 200 mg $L^{-1}$ cefotaxime was similar. The number of newly developed somatic embryos was genotype and culture system dependent, ranging from 23 (T2H line) to 48 embryos (Sainza line) under TIS, and from 12 (T2H) to 18 (Sainza) for semi-solid cultures.
The proliferation of non-transformed oak embryogenic cultures decreased substantially after the addition of kan. Similar levels of growth inhibition were obtained with 25 mg $L^{-1}$ kan in plate cultures and 10 mg $L^{-1}$ kan in liquid medium (Fig. 1). However, full necrosis was only observed in the first weeks of culture in the explants proliferated in medium supplemented with 75 mg $L^{-1}$ kan. As the identification of new (putatively transformed) tissues is difficult without the necrosis of original explants, a two-step selection protocol was adopted. This selection scheme consisted in culturing the explants for 18 weeks in CCK25 (25 mg $L^{-1}$ kan) and then transferring them to CCK75 medium (75 mg $L^{-1}$ kan).

Transformation and selection: temporary immersion system compared with culture on semi-solid medium

After cultivation for 6-8 weeks on selection medium, explants gradually turned brown and some showed signs of necrosis (Fig. 2a, b, c). Necrosis was more evident in *Agrobacterium*-inoculated explants than in control explants, probably because of the physiological state induced by the bacterial infection. Newly emerging embryos or structures were observed in otherwise necrotic explants after 12-14 weeks in explants cultured in liquid medium (bioreactors) and after 18-20 weeks in those cultured on semi-solid medium. These putatively transformed explants were removed and proliferated in plates with CCK75 until individual embryos were obtained and independent transgenic lines were established. To avoid problems with the assessment of independent transformation events in bioreactors, only those kan-resistant embryos or embryogenic structures still attached to an original necrotic explant were recorded as independent transformed events and were used to initiate transgenic lines (Fig. 2d, e). All newly developed structures not attached to any necrotic tissue were discarded to avoid replication of scoring. Selection of putatively transformed explants was performed at 2-week intervals and continued for up to 30 weeks. All kan-resistant embryogenic lines were evaluated on the basis of GUS activity, and most showed a strong positive reaction (Fig. 2f, 5a,

e). Selection efficiencies (average 87 %, ranging from 83 to 100 % depending on the experiment) were not influenced by either genotype or culture system.

Use of the TIS reduced the time required for detection of putatively transformed material by 2-8 weeks, and higher transformation rates were obtained by the use of this system than by plate culture for all four genotypes tested (Fig. 3 a-d). In line B13, the transformation rate was significantly higher (P=0.035) in explants cultured in bioreactors (26 %) than in those maintained on plates (4 %) (Fig. 3a). Significant differences between the methods (P=0.011) were also observed with line T2H (transformation efficiency of 23 % in bioreactors compared with 4 % in plates: Fig. 3b). In each experiment performed with these genotypes, an average of 10 transformed explants were isolated after 20 weeks of selection in bioreactors, whereas only 2.4 kan-resistant explants were yielded on semi-solid medium in the same period, which represents a fourfold increase in the establishment of transgenic lines. Although selection in RITA® bioreactors also provided better results with Sainza and B17 (Fig. 3c, d), the transformation frequencies were low and there were no significant differences between systems (P=0.510 and 0.173, respectively). To increase the transformation frequency, lower selection pressures were applied to these recalcitrant genotypes during the first 4-6 weeks after co-cultivation (Table 1). RITA®-based selection significantly improved the efficiency of transformation relative to plate-based selection (P=0.007 for B17 and P=0.005 for Sainza line) although there were non-significant differences between the three selection protocols (P=0.817 for B17 and P=0.052 for Sainza).

Molecular analysis

Genomic DNA from 24 putative transgenic embryogenic lines was extracted and analyzed by PCR, and the transgenic nature of the lines was confirmed (Fig. 4a, b). No bands were amplified when the *virC* gene primers were used, and therefore the presence of contaminating *A. tumefaciens* cells in oak tissues was discounted (data not shown). Four PCR-positive lines were chosen at random for Southern blotting, which verified the stable integration of the transgene *uid*A. A DIG-labelled probe consisting of a 589 bp fragment of the *uid*A gene revealed that all of these lines contained the target gene at different integration patterns from one to three copies since all the hybridizing fragments were larger than the expected size of 2.8 kbp of the *uid*A construction (Fig. 4c).

The transgene copy number was estimated by RT-qPCR in the 24 transgenic lines previously subjected to PCR. The Ct values were plotted against the log-transformed total DNA (ng) of each dilution. The correlation coefficient of the standard curve ($R^2$) was 0.996 and the slope was -3.378. Correlation analysis from extrapolated Ct values in transgenic lines revealed that

the copy number varied from one to 15 (Table 2). Most of the transgenic lines (approximately 61 % of the lines analyzed) had a single copy of the *uid*A gene in the genome. Integration of the different copies in the genomes was not constant among the different oak genotypes used in this study. Although the genotypes with the highest percentage of transformation usually contained one copy of the foreign gene (71 % for T2H or 89 % for B13), the most recalcitrant genotypes contained more than one copy. Thus, 75 % of transgenic lines derived from B17 and up to 100 % of the Sainza transgenic lines possessed more than one copy (Table 2).

Cryopreservation of transformed lines

The effect of adding kan to the recovery medium was studied in T2H and Sainza embryogenic lines (Table 3). The recovery of cryopreserved transgenic lines of both genotypes without kan was not significantly different from that of untransformed controls ($P= 0.306$ for T2H and $P= 0.186$ for Sainza). However, the addition of kan to the medium significantly affected ($P< 0.001$) the recovery of transgenic cryopreserved explants. Embryos recovered on medium without kan were subjected to GUS assay or re-cultured on PM with kan to further verify maintenance of kan-resistance. All transgenic embryos were GUS positive (Fig. 5a) and all of the explants cultured with kan grew successfully. All embryos were plated on PM without kan in subsequent experiments.

The recovery rates of cryopreserved T2H and Sainza transgenic lines previously proliferated in RITA® or plates were compared. Cultures proliferated in bioreactors contained fewer first-stage embryos (i.e. globular, heart, and torpedo) than cultures on semi-solid medium, although the embryos were isolated from the clumps more easily. Differences between transgenic lines derived from genotype T2H were non-significant ($P=0.569$), and a slight, but non-significant ($P=0.155$), increase in embryo recovery percentage was obtained in RITA® cultures (Fig. 6). The new embryos developed from the cryopreserved clumps had a similar appearance in both culture systems, with 5.8 embryos per explant for T2H lines and 7.8 for Sainza lines (Fig. 5b). Four additional transgenic lines from T2H, and two from the Sainza genotype, were proliferated in bioreactors before being cryopreserved. Recovery was 92 % ± 2 in line T2H, and 70 % ± 15 in the Sainza line. The lines were evaluated on the basis of GUS activity (Fig. 5a), and cryopreserved embryos were proliferated and germinated before successful acclimatization of plants (Fig. 5c).

Germination and plant regeneration

Successful conversion into plants took place only occasionally in kan-treated explants (less than 1 %), and this antibiotic was therefore not used in further germination experiments. Plant

conversion was similar in transgenic embryos and non-transformed embryos (data not shown). More than 85 % of the recovered plantlets were successfully transferred to soil (Fig. 5d). Sainza, T2H, and B17 germinated plantlets were also used to initiate axillary shoot cultures that were subsequently rooted to increase the propagation of transformed material.

Shoots derived from control and transgenic embryos of Sainza line were proliferated with or without kan. Although shoot number and shoot length were similar in transformed and control material in medium devoid of kan, the addition of 75 mg $L^{-1}$ kan arrested growth of control shoots and even decreased proliferation rates in transformed shoots (Table 4). After four subcultures, the number and length of shoots differed significantly ($P<0.001$) between kan-treated and non-treated transformed cultures.

There were no significant differences in the rooting frequencies, root number, or root length in either the transformed or non-transformed shoots when kan was not added to rooting medium (Table 5). This antibiotic did not affect rooting of transformed shoots when applied only to the induction medium (24h), whereas it completely inhibited rooting in non-transformed shoots. When kan was added to the expression medium, the rooting frequency of transformed shoots decreased from 90 to 10 % and root length was halved (Table 5).

Kan was not included in the proliferation or rooting media used for the other transgenic lines. The rooting ability of transformed T2H, B17, and Sainza cultures was approximately 90-94 %. Leaves from the rooted plantlets were positive for GUS expression (Fig. 5e). Transgenic oak plants derived from rooted explants were transferred to soil, acclimatized in a phytotron for 1-2 months, and grown on in the greenhouse (Fig. 5f).

**DISCUSSION**

The goal of this study was to develop an efficient protocol for *Agrobacterium*-mediated genetic transformation of *Q. robur* somatic embryos. Use of the TIS increased the proliferation rates and quality of pedunculate oak somatic embryos and reduced explant manipulation (Mallón et al. 2012). This system was therefore adopted for selecting putatively transformed embryos, as an alternative to the conventional method of selection on semi-solid medium. Although the use of TIS for mass clonal propagation has increased (Etienne and Berthouly 2002; Watt 2012), reports of its application for the selection phase of genetic transformation studies are scarce and involve genetic transformation based on adventitious shoot regeneration in pineapple (Espinosa et al. 2002) and strawberry (Hanhineva and Kärenlampi 2007), or adventitious root formation in sage (Marchev et al. 2011). However, none of these studies compared the transformation efficiency of TIS and semi-solid medium. To the best of our knowledge, TIS was

applied for the first time in the present study for genetic transformation involving somatic embryo production.

The increased transformation frequencies achieved with TIS yielded high-throughput production of transgenic pedunculate oak embryos, by overcoming the main bottleneck of the genetic transformation of this species. In general, use of TIS resulted in at least a fivefold increase in transgenic events in all embryogenic lines, although genotypical differences were observed. These differences cannot be related to the proliferation rates of the lines, all of which are highly proliferative (Cuenca et al. 1999; Sánchez et al. 2008). The importance of genotype is well known and frequently reported for forest tree species (Nehra et al. 2005). Genetic transformation of mature trees is difficult because of the recalcitrance of adult tissues to both *Agrobacterium* infection and transformation, and also because of the low regeneration ability of transgenic mature tissues (Cervera et al. 2008). However, three out of four genotypes used in our study were of mature origin, and in one of them (B13) the transformation efficiency reached 26 %, which allowed the generation of 78 independent transgenic lines. The use of TIS also increased the transformation frequency of the other mature lines, Sainza and B17, which proved to be more recalcitrant to *Agrobacterium*-mediated transformation on semi-solid medium, whereas transformed lines were always obtained in the TIS. The transformation frequencies obtained in the present study are sufficient for transformation of recalcitrant species (Matsunaga et al. 2012). Within the genus *Quercus*, *Q. suber* is the only other species in which genetic transformation on semi-solid medium has been investigated (Álvarez and Ordás 2007). In the present study, the transformation efficiencies achieved with TIS were similar to those reported for transformed embryogenic lines of cork oak, although transformation of some lines of the latter species was not successful (Álvarez and Ordás 2007).

Use of the TIS also reduced the time required for initiation of transformed lines. Transgenic tissue grown on semi-solid medium appeared 2-8 weeks later and mainly comprised pro-embryogenic masses (PEMs), which require further subculture before giving rise to suitable somatic embryos for the initiation of transgenic lines. However, the TIS yielded torpedo-cotyledonary somatic embryos in the bioreactor (Fig. 2d, e), and therefore transformed lines were produced 12-16 weeks earlier. The number of transformed explants obtained in TIS in the first 20 weeks was sufficient for the selection of useful lines, thus simplifying and speeding up the process.

The use of liquid medium to culture cells in suspension has proved advantageous for the transformation of several woody plants. In citrus (Dutt and Grosser 2010), sandalwood (Shekhawat et al. 2008), and yew (Zhang et al. 2011), cell suspension cultures have been used to generate more responsive target explants, although putatively transformed explants were

selected on semi-solid medium. Cell suspension cultures have been used during the selection phase in mango (Mathews et al. 1992), avocado (Cruz-Hernandez et al. 1998; Raharjo et al. 2008) and olive (Torreblanca et al. 2010), although no clear comparison was made with selection on semi-solid medium. An efficient transformation protocol involving the use of embryogenic suspension cultures throughout the whole procedure has been established for American chestnut (Fagaceae) (Andrade et al. 2009). In addition to the increased proliferation rates and the stringent selection conditions provided by liquid medium, use of the TIS provides other advantages, such as easier handling of large amounts of starting material, and removal of phenolic compounds released by the explants after transformation. Removal of these compounds prevents the destructive effects on the newly developed tissue, as proposed for *A. rhizogenes*-mediated transformation of sage (Marchev et al. 2011).

A further advantage of the TIS was that it enabled the origin of each transformation event to be traced, because the original explant with the putatively transformed tissue attached was removed from the RITA® vessel. The difficulty in identifying each transformation event within all culture flasks has been identified as a limiting step in transformation experiments involving cell suspension cultures. All of the transformed material developed in a flask must be considered as a unique event because of the characteristics of the culture (Andrade et al. 2009). Therefore, the TIS potentially yields more transformed lines with the same starting material, which considerably reduces the space, labour and costs involved in the process.

In contrast to direct gene transfer methods, in which several copies of the foreign DNA are often transferred, *Agrobacterium*-mediated transformation has the advantage that only a small number of copies are integrated into the plant genome (Gelvin 2003). However, quantification of the transgene copy number is necessary because the spectrum of transgenic insertions is occasionally quite large, with reports of up to three copies in olive (Torreblanca et al. 2010), four copies in cork oak (Álvarez et al. 2009), avocado (Palomo-Ríos et al. 2012), and plum (Wang et al. 2013), and as many as 10 copies in hybrid poplar (Yevtushenko and Misra 2010). Insertion of a large number of transgene copies can lead to unpredictable performance, ranging from excessive to undetectable expression levels (Gelvin 2003). Although hybridization analysis by Southern blotting of genomic DNA was previously considered the most reliable way of estimating the transgene copy number, this method requires large amounts of genomic DNA. The higher sensitivity of RT-qPCR enables estimation of the transgene copy number from small quantities of plant material (Bubner and Baldwin 2004). In the present study, the usefulness of RT-qPCR for analyzing the transgene copy number was confirmed by comparison of the copy number of *uid*A derived from RT-qPCR analysis and Southern blotting. The transgene copy numbers identified by both methods were perfectly correlated, for all lines

analyzed (T2H, B13, and B17), which demonstrates the feasibility of using RT-qPCR analysis in pedunculate oak. Integration of the transgene in the genomic DNA was genotype-dependent, probably as the result of a direct relationship between recalcitrance and transgenic copy number. However, these findings must be considered cautiously because of the scant number of transgenic regenerants of the recalcitrant genotypes.

Transformed embryogenic lines were proliferated and maintained in RITA® vessels, taking advantage of the high proliferative levels accomplished in oak embryos with this system (Mallón et al. 2012). To save labour costs and minimize contamination risks, the transformed embryogenic lines of forest tree species must be stored (e.g. by cryopreservation) until proper evaluation. The transformed lines of pedunculate oak were successfully cryopreserved, and the embryo recovery rates and embryo morphology were similar to those obtained with the untransformed material used in this study and to those previously reported for the same genotypes (Sánchez et al. 2008), thus confirming the stability of these embryogenic lines for cryopreservation.

The recalcitrance of pedunculate oak embryos to plant conversion, a feature shared with many other hardwood trees (Pijut et al. 2007), may seriously limit the application of genetic transformation. However, this can be overcome by initiating transgenic shoot lines in plant material derived from somatic embryos, which exhibited similar proliferation and rooting capacities as the non-transformed material. In addition, a certain level of rejuvenation was achieved in shoot lines derived from somatic embryos, in which rooting was more successful than in shoot cultures established from epicormic shoots (Martínez et al. 2012). Thus, this method yielded numerous plants that could be acclimatized and grown on in the greenhouse.

Transgenic material derived from woody species has been reported to be susceptible to antibiotics. Thus, paramomycin decreased the percentage of transgenic olive embryos that underwent maturation (Torreblanca et al. 2010), and kanamycin inhibited embryo development in transgenic cells of papaya (Kung et al. 2010) and also decreased the germination rate of transgenic white spruce embryos (Le et al. 2001) and hampered growth, differentiation, and rooting in transgenic black pepper (Varghese and Bhat 2011). Plant susceptibility to antibiotics varies widely among species, genotypes and plant tissues (Padilla and Burgos 2010), and there is no general rule about including selective agents in media when regenerating transgenic woody plants. Torreblanca et al. (2010) maintained the selection pressure during the entire regeneration process (including embryo maturation and germination), to prevent the formation of chimaeric olive transgenic embryos, and kanamycin has been used both in proliferation and rooting medium of transgenic green ash (Du and Pijut 2009) and poplar (Yevtushenko and Misra 2010). However, transgenic embryos of other woody

plants have also been matured and germinated in the absence of selective agents, as reported for avocado (Palomo-Ríos et al. 2012), rubber tree (Leclercq et al. 2010), and other species of Fagaceae such as European and American chestnut (Corredoira et al. 2012, Andrade et al. 2009) and cork oak (Álvarez and Ordás 2007, Álvarez et al. 2009). Although no chimaeric oak transgenic embryos have been detected with the selection pressure described in this study, this problem may be more common than originally thought (Flachowsky et al. 2008, Padilla and Burgos 2010), and establishing the concentrations of antibiotics that can be applied to transgenic materials in each step of their development is of a practical interest. In transgenic pedunculate oak, kanamycin affected embryo recovery after cryopreservation, plant conversion, shoot proliferation and rooting to different degrees, although the effects were not as detrimental as observed in non-transformed tissue. Therefore, this antibiotic could be used at all developmental stages during the regeneration of plants to distinguish between transformed and non-transformed material.

In conclusion, this is the first report of an efficient protocol for selecting transgenic *Q. robur* somatic embryos by temporary immersion in liquid medium. The transformation frequency ranged from 6 to 26 % (depending on the genotype), representing a fivefold increase over that achieved with the conventional protocol based on selection on semi-solid medium. Transgenic embryogenic lines were established 12-16 weeks earlier than with the conventional protocol; therefore, numerous transgenic plantlets could be produced for acclimatization 8 months after inoculation of the tissue with *Agrobacterium*, i.e. about 4 months earlier than with the previous method. RT-qPCR proved to be a suitable alternative to Southern blotting for quantifying the transgene copy number, and it enabled molecular characterization of many transgenic lines. Transformed embryogenic lines maintained their ability to be cryopreserved and germinated for plant conversion, and both plantlets and rooted shoots from axillary shoot cultures were successfully transferred to soil and acclimatized.

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Table 1. Genetic transformation of *Quercus robur* somatic embryos cultured in Petri plates or RITA® systems under low selection pressure.

| Genotype | Selection protocol | Transformation frequencies (%) | |
|---|---|---|---|
| | CCK 10; CCK25; CCK 75 (weeks) | PLATE culture | RITA® culture |
| B17 | 0;18;12 | 0 b | 5.0 + 3.93 a |
| | 4;14;12 | 0 b | 7.5 + 5.31 a |
| | 6;12;12 | 0 b | 3.8 + 2.27 a |
| SAINZA | 0;18;12 | 0.5 + 0.70 b | 1.5 + 0.71 ab |
| | 4;14;12 | 0 b | 6.1 + 3.93 a |
| | 6;12;12 | 0 b | 0.4 + 0.50 ab |

Percentage of inoculated explants that produce kan-resistant and GUS-positive embryogenic lines (data shown are mean values + standard errors from two experiments). Explants were submitted to three selection protocols in which three levels of kan were applied during different intervals. CCK10, CCK25, and CCK75 stand for 10, 25, and 75 mg $L^{-1}$ kan, respectively. CCK10 was applied for 0, 4, or 6 weeks after *Agrobacterium* inoculation, followed by CCK25 during 12, 14, or 18 weeks, and for CC75 for the last 12 weeks of the experiment. Total selection time was 30 weeks for all the treatments. For each genotype, mean values followed by the same letter were not significantly different at P=0.05 (LSD test).

Table 2. Estimated copy numbers in transformed somatic embryogenic lines of *Quercus robur* determined by quantitative real-time PCR.

| Genotype | Transgenic line | Ct[a] | DNA [ ][b] | Estimated copy number | |
|---|---|---|---|---|---|
| | | | | Real-time PCR | Southern blot |
| T2H | 35–1 | 23.783 + 0.035 | 3.142 + 0.210 | 3 | 3 |
| | 35–6 | 25.783 + 0.015 | 0.775 + 0.008 | 1 | |
| | 35–7 | 24.915 + 0.007 | 1.401 + 0.006 | 2 | |
| | 35–10 | 25.583 + 0.020 | 0.888 + 0.012 | 1 | |
| | 35–15 | 25.406 + 0.092 | 1.003 + 0.064 | 1 | |
| | 35–20 | 25.333 + 0.135 | 1.056 + 0.095 | 1 | |
| | 35–25 | 26.630 + 0.014 | 0.435 + 0.004 | 1 | |
| B13 | 37–1 | 26.170 + 0.030 | 0.595 + 0.012 | 1 | 1 |
| | 37–4 | 25.620 + 0.141 | 0.868 + 0.083 | 1 | |
| | 37–7 | 25.380 + 0.113 | 1.022 + 0.078 | 1 | 1 |
| | 37–14 | 25.403 + 0.058 | 1.005 + 0.039 | 1 | |
| | 37–22 | 25.336 + 0.098 | 1.052 + 0.069 | 1 | |
| | 43–3 | 24.196 + 0.070 | 2.288 + 0.109 | 2 | |
| | 43–5 | 25.450 + 0.034 | 0.973 + 0.023 | 1 | |
| | 43–6 | 25.905 + 0.091 | 0.714 + 0.044 | 1 | |
| | 43–8 | 25.446 + 0.101 | 0.976 + 0.068 | 1 | |
| Sainza | 36–2 | 22.975 + 0.007 | 5.258 + 0.025 | 5 | |
| | 51–3 | 24.380 + 0.091 | 2.020 + 0.127 | 2 | |
| | 51–6 | 21.413 + 0.035 | 15.248 + 0.364 | 15 | |
| B17 | 39–1 | 24.770 + 0.127 | 1.549 + 0.134 | 2 | |
| | 39–2 | 24.616 + 0.015 | 1.717 + 0.018 | 2 | 2 |
| | 40–2 | 25.870 + 0.069 | 0.731 + 0.034 | 1 | |
| | 40–3 | 24.230 + 0.098 | 2.238 + 0.148 | 2 | |
| | NT | 34.376 + 0.971 | 0.002 + 0.001 | 0 | |

[a] Mean values of threshold cycle (Ct) + standard deviation derived from three replicate samples. [b] Mean DNA concentrations + standard deviation extrapolated from mean Ct values.

Table 3. Embryo recovery frequencies from control and transgenic lines (TL) of T2H and Sainza *Quercus robur* genotypes after cryopreservation.

| Embryogenic line | Embryo recovery (%) (Mean ± standard error) | |
|---|---|---|
| | Kan + | Kan - |
| T2H control | 0 b | 83 ± 4.9 a |
| T2H TL 1 | 22 ± 5.4 b | 85 ± 4.6 a |
| T2H TL 2 | 15 ± 8.2 b | 70 ± 10.5 a |
| Sainza control | 0 b | 62 ± 4.1 a |
| Sainza TL 1 | 4 ± 2.8 b | 66 ± 2.8 a |
| Sainza TL 2 | 27 ± 8.b3 b | 50 ± 9.3 a |
| Sainza TL 3 | 30 ± 8.5 b | 80 ± 7.4 a |

Embryo recovery was assessed 6 weeks after thawing and plating on PM with or without 75 mg $L^{-1}$ kan (kan +, kan -). PM consisted of MS supplemented with 0.44 µM BA, 0.27 µM NAA, 500 $mgL^{-1}$ casein hydrolysate, 30 g $L^{-1}$ sucrose, and 6 g $L^{-1}$ agar. Within the same row, values followed by the same letter are not significantly different at P=0.05 (LSD test).

Table 4. Shoot number and length of axillary shoot cultures derived from control and transgenic somatic embryos of the *Quercus robur* Sainza line.

| Sainza line | kan | No shoots per explant | No shoots > 14 (mm) per explant | Longest shoot length (mm) per explant |
|---|---|---|---|---|
| Control | - | 4.9 $\pm$ 0.88 a | 1.8 $\pm$ 0.56 a | 23.2 $\pm$ 2.75 a |
| | + | 0 c | 0 b | 0 c |
| Transgenic | - | 4.8 $\pm$ 1.13 a | 2.0 $\pm$ 0.59 a | 24.8 $\pm$ 2.36 a |
| | + | 2.6 $\pm$ 0.30 b | 0.4 $\pm$ 0.12 b | 13.4 $\pm$ 1.35 b |

Data shown are mean values $\pm$ standard errors from two replicate experiments, with 36 shoots per replicate (six shoots per jar). For transgenic shoots, data were recorded after four successive subcultures (each lasting 4 weeks) in shoot proliferation medium with or without 75 mg $L^{-1}$ kan. For kan-treated control shoots, the data were recorded after a single cycle of treatment. Within each column, values followed by the same letter are not significantly different at P=0.05 (LSD test).

Table 5. Rooting ability of shoot cultures derived from control and transgenic somatic embryos of the *Quercus robur* Sainza line.

| Sainza line | kan | | Rooting (%) | No roots per rooted shoot | Longest root length (mm) per rooted shoot |
|---|---|---|---|---|---|
| | RIM | REM | | | |
| Control | - | - | 90.5 ± 4.25 a | 4.0 ± 0.46 a | 24.4 ± 5.17 ab |
| | + | - | 0 b | - | - |
| | - | + | 0 b | - | - |
| Transgenic | - | - | 93.8 ± 3.02 a | 3.3 ± 0.32 ab | 29.1 ± 2.49 ab |
| | + | - | 88.7 ± 9.75 a | 2.1 ± 0.17 bc | 30.3 ± 5.32 a |
| | - | + | 11.3 ± 5.56 b | 1.1 ± 0.09 c | 12.0 ± 3.87 b |

Data shown are mean values ± standard errors from three replicate experiments, with 30 shoots per replicate (six shoots per jar). Shoots were isolated after 4 weeks on shoot proliferation medium and transferred onto root induction medium (RIM) with or without 75 mg $L^{-1}$ kan. After 24h, shoots were transferred to the rooting expression medium lacking auxin (REM) with or without 75 mg $L^{-1}$ kan. Data were recorded after 30 days of auxin treatment. RIM consisted of GD medium with 1/3-strength macronutrients supplemented with 5.5 g $L^{-1}$ agar, 30 g $L^{-1}$ sucrose, and 120 μM indole-3-butyric acid. REM stands for auxin-free medium of the same composition but supplemented with 6.5 g $L^{-1}$ agar. Within each column, values followed by the same letter are not significantly different at P=0.05 (LSD test).

**Figure legends**

**Fig 1**. Effect of kanamycin on secondary embryo production in non-transformed explants from line T2H cultured in RITA® or plate systems. Number of secondary embryos per initial explant were recorded after 12 weeks (data shown are mean values+ standard error).

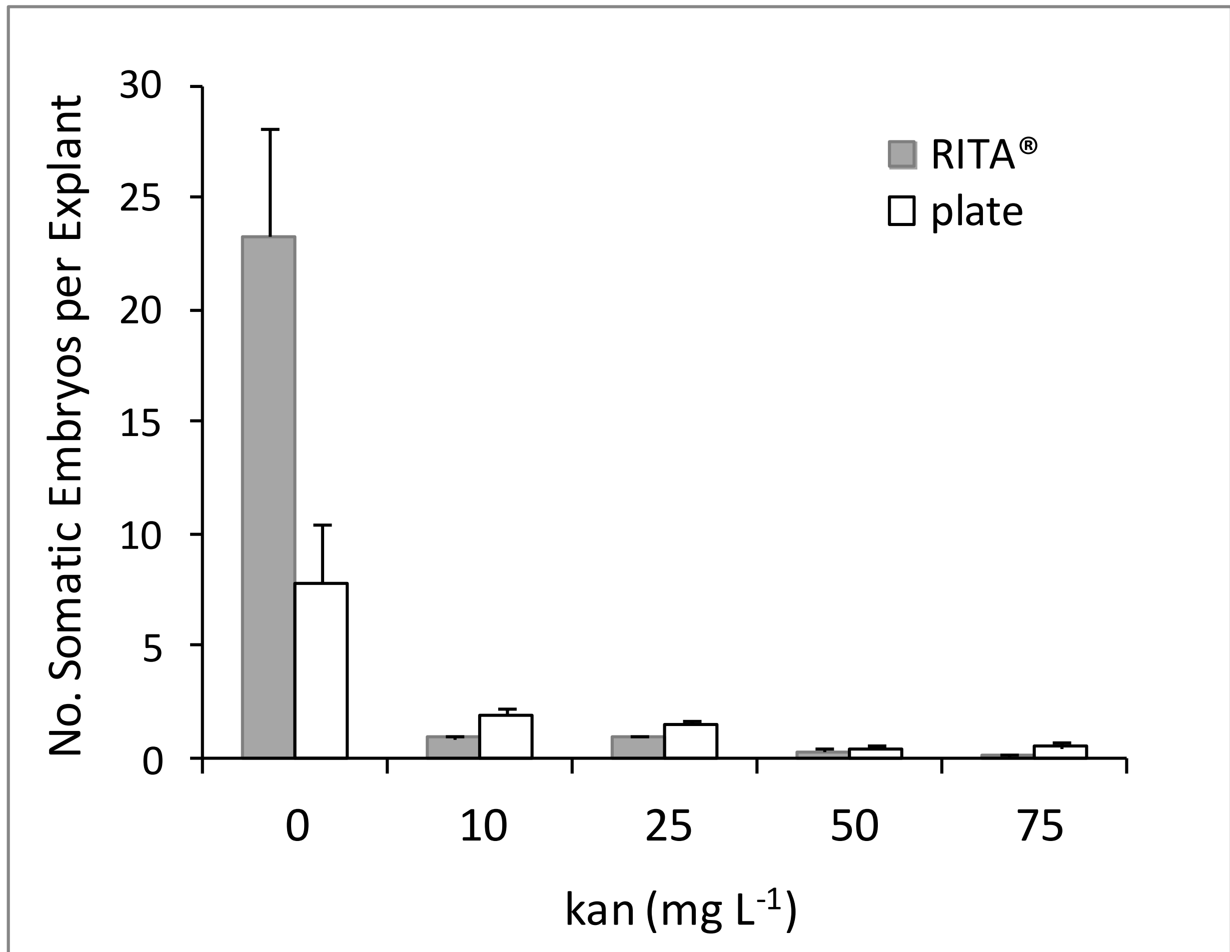


**Fig 2**. Explant selection in RITA® bioreactors: **a** Explants of line B17 after 6 weeks without kan. **b**, **c** Appearance of explants of line B17 after 8 (b) and 26 weeks (c) of kan selection (first 18 weeks 25 mg $L^{-1}$ kan and 75 mg $L^{-1}$ kan thereafter). **d** Enlarged view of a putative kan-resistant B17 explant. **e**, **f** Kan-resistant explant growing on a necrotic explant of B13 embryogenic line and histological test for stable GUS expression.

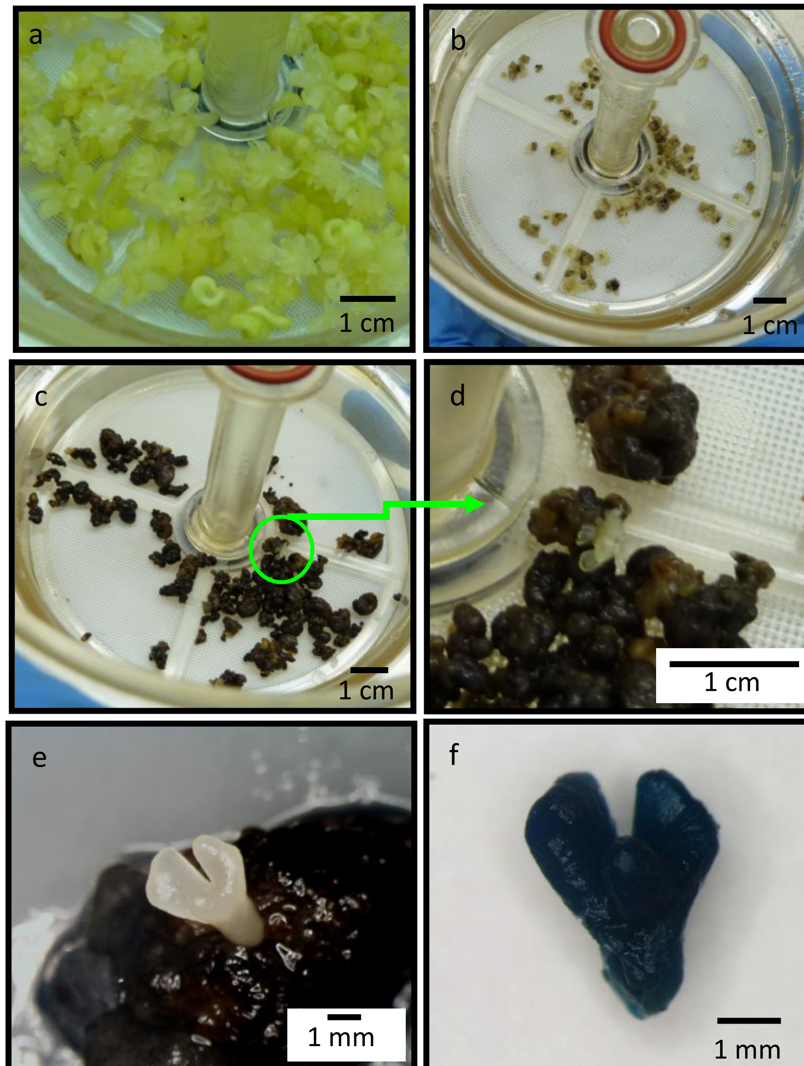


**Fig 3**. Genetic transformation of oak embryos of lines B13 (**a**), T2H (**b**), Sainza (**c**) and B17 (**d**) cultured in plates or in RITA® bioreactors (selection protocol: 18 weeks in CCK25 medium followed by 12 weeks in CCK75 medium). Data (mean values ± standard errors from three independent experiments) represent the percentage of inoculated explants that produced kan-resistant and GUS-positive embryogenic lines during the selection process. Within the same sampling date values marked with an asterisk are significantly different at P<0.05.

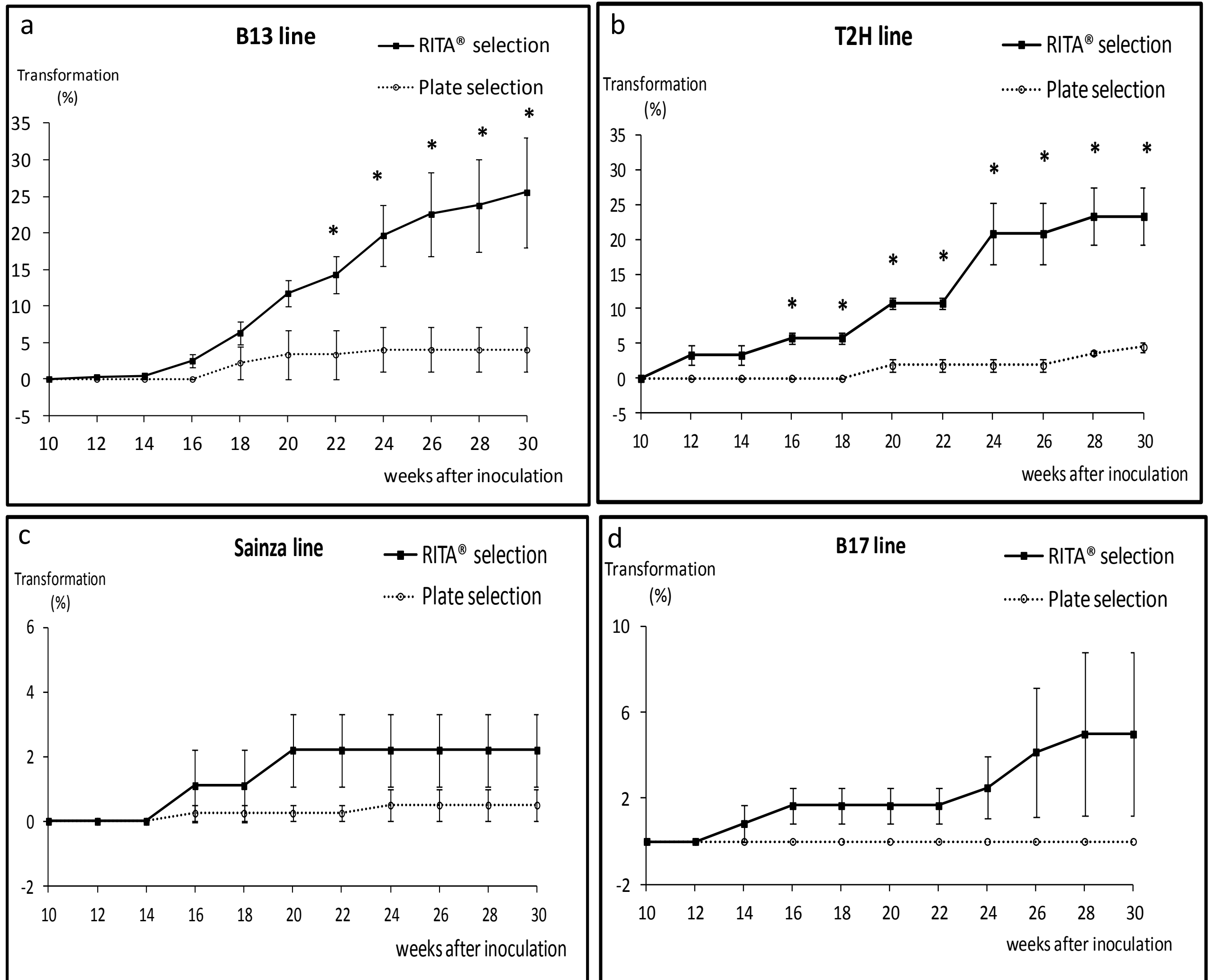
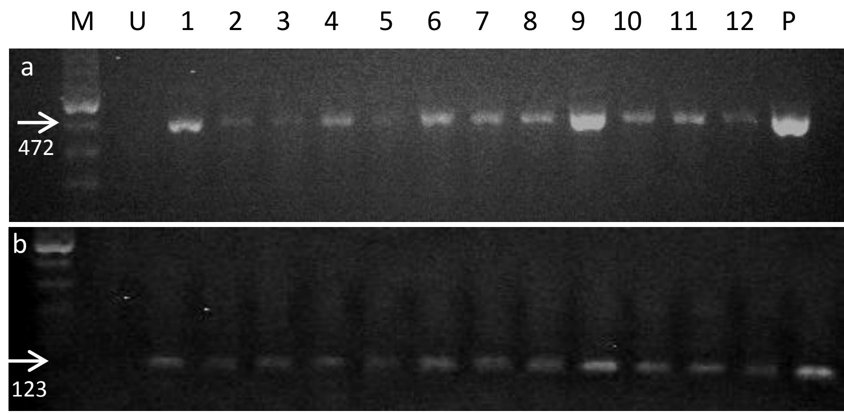


**Fig 4**. Molecular analysis of transgenic somatic embryos: **a**, **b** PCR analysis of transformed somatic embryogenic lines of *Q. robur* selected in bioreactors. Detection of the *npt*II (a) and the *uid*A (b) genes of kan-resistant embryogenic lines. M Molecular ladder, P plasmid (positive control), U untransformed oak somatic embryos (negative control), lanes 1–12 putative transgenic lines of T2H (1-3), B13 (4-6), Sainza (7-9), and B17 (10-12) genotypes. **c** Southern blot analysis of transgenic somatic embryos. Genomic DNA was digested with *Eco*RI and hybridized with probes derived from *uid*A gene. Lanes 1, 2, 3 and 4, GUS-positive transgenic lines of B17 39-2 (1), B13 37-7 and B13 37-1 (2, 3), and T2H 35-1 (4) genotypes, Lane U untransformed embryogenic line (negative control), Lane P positive control (plasmid).



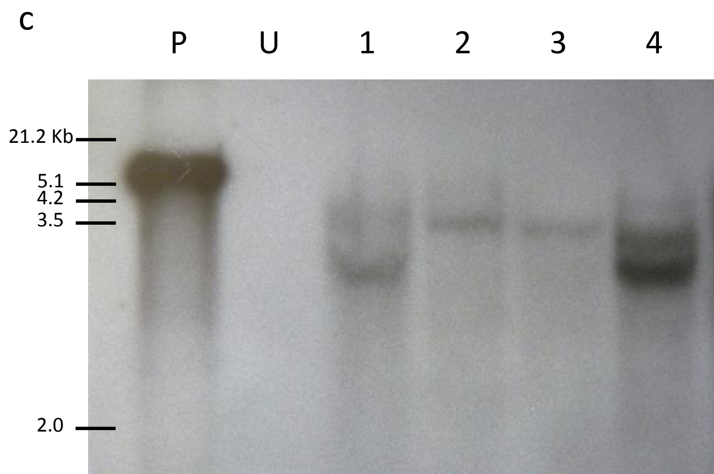



**Fig 5**. GUS expression and plant acclimatization after cryopreservation of transformed T2H and Sainza lines: **a** GUS expression in a transformed explant (left) and a control explant (right) of T2H after cryopreservation and recovery in medium without antibiotics. **b** Embryo recovery from a cryopreserved explant of a Sainza transformed line proliferated in a RITA® bioreactor after 6 weeks in recovery medium. **c** Germinated embryo from a cryopreserved explant of T2H line proliferated in a RITA® bioreactor. **d** Transformed (right) and control (left) acclimatized plantlets of B17 and T2H genotypes grown for 1 month in the greenhouse. **e** GUS expression in shoot apices and leaves of shoots derived from germinated plantlets of T2H and Sainza genotypes. **f** Rooted plants derived from shoot cultures from a transgenic Sainza line 1 year after transfer to soil.

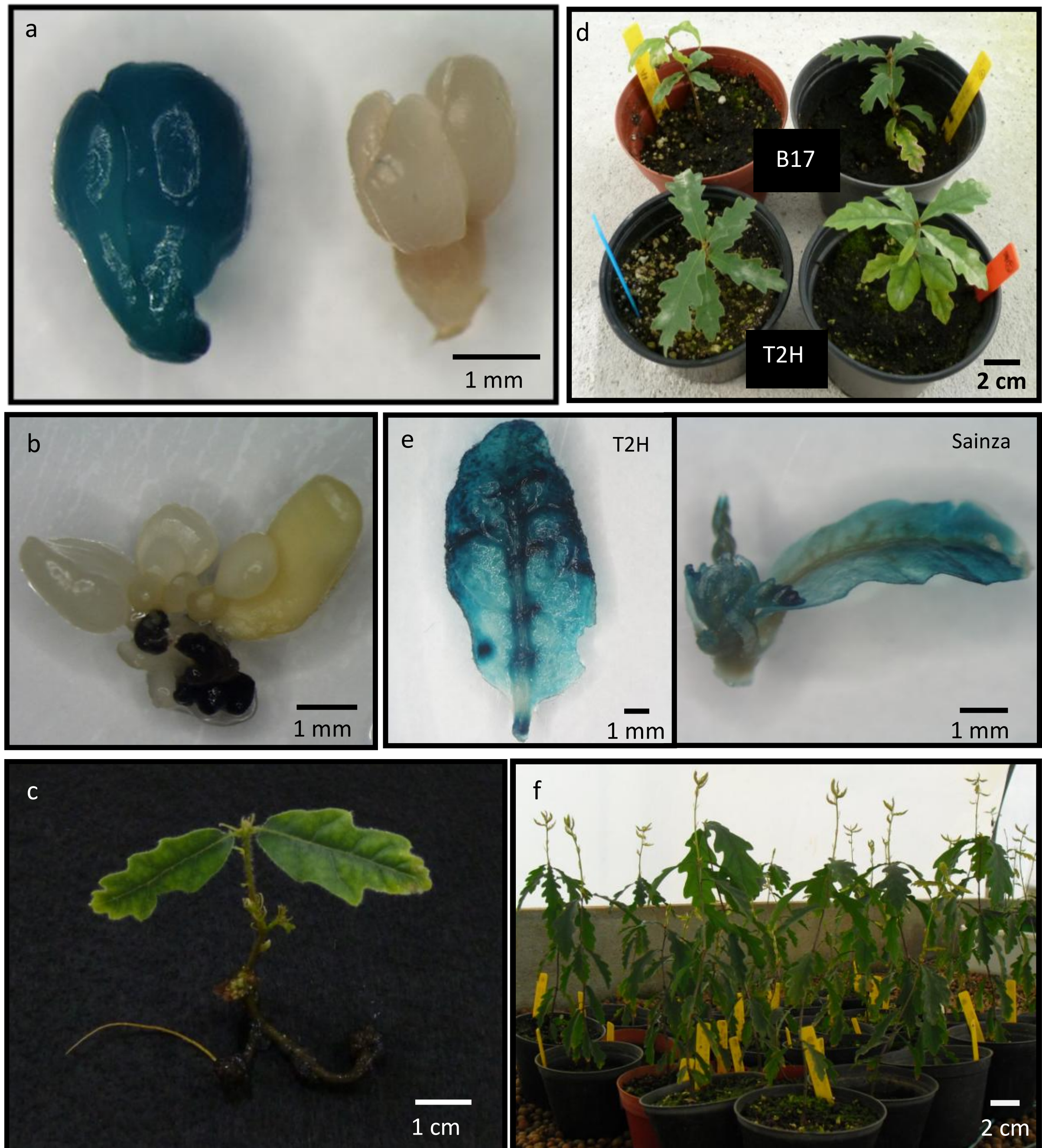


**Fig 6**. Embryo recovery (percentage) after cryopreservation of somatic embryos of three different transgenic lines (TL) derived from the T2H genotype. Embryos were proliferated in RITA® or plates for 5 weeks before cryopreservation. Recovery was assessed 6 weeks after thawing. Bars represent standard errors. Main effects analysis showed non-significant P values for transgenic line (P=0.596) and culture system (P=0.155).