Genetic transformation of European chestnut somatic embryos with a native thaumatin-like protein (CsTL1) gene isolated from Castanea sativa seeds

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The availability of a system for direct transfer of antifungal candidate genes into European chestnut (Castanea sativa Mill.) would offer an alternative approach to conventional breeding for production of chestnut trees tolerant to ink disease caused by Phytophthora spp. For the first time, a chestnut thaumatin-like protein gene (CsTL1), isolated from chestnut cotyledons, has been overexpressed in three chestnut somatic embryogenic lines. Transformation experiments have been performed using an Agrobacterium tumefaciens Smith and Townsend vector harboring the neomycin phosphotransferase (NPTII) selectable and the green fluorescent protein (EGFP) reporter genes. The transformation efficiency, determined on the basis of the fluorescence of surviving explants, was clearly genotype dependent and ranged from 32.5% in the CI-9 line to 7.1% in the CI-3 line. A total of 126 independent transformed lines were obtained. The presence and integration of chestnut CsTL1 in genomic DNA was confirmed by polymerase chain reaction (PCR) and Southern blot analyses. Quantitative real-time PCR revealed that CsTL1 expression was up to 13.5-fold higher in a transgenic line compared with its corresponding untransformed line. In only one of the 11 transformed lines tested, expression of the CsTL1 was lower than the control. The remaining 115 transformed lines were successfully subjected to cryopreservation. Embryo proliferation was achieved in all of the transgenic lines regenerated and the transformed lines showed a higher mean number of cotyledonal stage embryos and total number of embryos per embryo clump than their corresponding untransformed lines. Transgenic plants were regenerated after maturation and germination of transformed somatic embryos. Furthermore, due to the low plantlet conversion achieved, axillary shoot proliferation cultures were established from partially germinated embryos (only shoot development), which were multiplied and rooted according to procedures already established. Transgenic plants were acclimatized and grown in a greenhouse. No phenotypic differences were found with control plants, suggesting no potential cytotoxic effects of the green fluorescent protein. The results reported in the present work could be considered as a first step toward the production of fungal-disease tolerant cisgenic chestnut plants.

Keywords: Agrobacterium tumefaciens, cisgenesis, CsTL1, cryopreservation, green fluorescent protein, ink disease, pathogenesis-related proteins, qPCR.

Introduction

European chestnut or sweet chestnut (Castanea sativa Mill.) is a tree species with a wide distribution and an important economic role in Europe. It is distributed in all Mediterranean basin countries and the forest spread from Spain and Portugal to the Caucasus, covering an area of over 2 million hectares.
Ink disease is one of the most destructive diseases affecting sweet chestnut. It was initially observed in Spain and Portugal during the middle of the 19th century (Fernández de Ana 2002) and has since been reported in many European countries (Vannini and Vettraino 2001, Vettraino et al. 2005). Ink disease is mainly caused by the fungi Phytophthora cinnamomoni Rand, widely prevalent in Spain, Portugal and France as well as by Phytophthora cambivora (Petri) Buis, which is the main cause of the disease in Italy and Greece (Vannini and Vettraino 2001, Vannini et al. 2010). The fungus attacks the phloem tissue and the cambium of the roots and root collars causing specific symptoms such as necrosis of feeder and main roots, which can spread to the collar and the trunk resulting in cortical lesions with black exudates. Chlorosis, small fruits, microphyll and wilting followed by a quick or a progressive death of susceptible trees are the responses to the fungus attack. The disease spreads rapidly by zoospores and chlamydospores with the flow of water through the soil, and the movement of soil particles (Zentmyer 1980) and both human practices and site factors represent key factors in its progression. The association disease–human activities was demonstrated in chestnut plantations (Martins et al. 2007) but the disease distribution and gradient in natural chestnut forest was also studied (Vannini et al. 2005). The expansion of this and other fungal diseases would increase in the future under predicted conditions of climate change (Desprez-Lostau et al. 2007, Sturrock et al. 2011), as expected with P. cinnamomoni according to simulation models (Bergot et al. 2004).

The production of ink-resistant trees by conventional breeding is based on the Asian species Castanea mollissima Blume and Castanea crenata Sieb. and Zucc. both of which exhibit natural tolerance/resistance to the disease. Programs for developing inter-specific hybrids with European chestnut have been carried out (Vieitez et al. 1996) but this approach, although successful in developing ink-tolerant chestnut plants, has been slowed by the lack of genetic tools. Programs aimed at identifying resistant individuals within natural chestnut stands are underway in France (Ramos Guedes-Lafargue et al. 2005) and in Spain (Rodriguez et al. 2005). The rapid development of molecular genetics associated with physiological and taxonomic studies, gene mapping and the identification of genes and alleles linked to specific traits will provide new opportunities for chestnut improvement (Barakat et al. 2009). An integrated web-based resource for members of the Fagaceae family, including Castanea (http://www.fagaceae.org), disseminates genomic data which are being posted as they become available.

Limitations of conventional breeding have spurred development of transgenic plants as a complementary strategy for obtaining resistant chestnut trees. Specific genes for resistance to ink disease are not yet identified but genetic studies suggest that at least two genes (which are incompletely dominant) are responsible for resistance (Guedes-Lafargue and Salesse 1999). Alternatively, the use of pathogenesis-related (PR) proteins, which play a major role in natural defense against pests and pathogens, would be an interesting alternative approach. The recognized PRs have been extensively reviewed and currently comprise 17 families of induced proteins (Van Loon et al. 2006, Veluthakkel and Dasgupta 2010). Among these, the PR-5 family of proteins (thamatin-like proteins, TLP) are generally of low molecular weight (below 35 kDa) and cause transmembrane pores on fungal plasma membranes promoting osmotic rupture (Roberts and Selitrennikoff 1990). Chitinases, belonging to the PR-3 family of proteins, hydrolyze the β-1,4 glycosidic bonds that link the N-acetylgalactosamine residues of chitin and play a direct role in plant defense by hydrolyzing chitin (Veluthakkel and Dasgupta 2010). Finally, cystatins target cysteine proteinases and they also exhibit antifungal properties (Martínez and Díaz 2008).

The isolation of PR proteins from the seeds of European chestnut allows an interesting opportunity to produce transgenic plants with native genes encoding these proteins. These plants would be close to the concept of ‘cisgenic’ if intact native genes derived from the same or sexually compatible relatives are used (Schouten et al. 2006, Jacobsen and Nataraja 2008, Jacobsen and Schouten 2008). A 23-kDa TLP (CsTLP1), PR-5 family of proteins, has been purified from mature C. sativa cotyledons and was shown to possess in vitro antifungal activity against Trichoderma viride Pers. and Fusarium oxysporum Schlecht. The protein shows strong synergistic effects with CsCh3, the most abundant chestnut cotyledon endochitinase (Collada et al. 1992, Alloña et al. 1996, García-Casado et al. 2000). In addition, a cystatin (CsC) has also been characterized from chestnut seeds (Pernas et al. 1998) and the purified protein inhibited the growth of phytopathogenic fungi, contributing to plant defense (Pernas et al. 1998).

An efficient Agrobacterium-mediated genetic transformation protocol by using somatic embryogenic cultures as target material and marker genes has been described for European chestnut, a recalcitrant in vitro species (Corredoira et al. 2004). The transgenic lines were successfully cryopreserved and the stable integration of the genes into the transgenic chestnut plants that were regenerated following cryopreservation was demonstrated (Corredoira et al. 2007). In addition, suitable transformation protocols have also been reported for American chestnut (Castanea dentata (Marsh) Borkh). This species, once a great canopy tree, has been converted to no more than an early-succession shrub today due to the chestnut blight fungus Cryphonectria parasitica (Murrill) Barr., which attacks the stems and branches but does not infect the roots of the trees.
American chestnut plantlets engineered with a candidate antifungal gene oxalate oxidase (OxO) were obtained via transformation of somatic embryos with Agrobacterium tumefaciens (Polin et al. 2006, Maynard et al. 2008, Zhang et al. 2011). Furthermore, sexually matured transgenic American chestnut trees via embryogenic suspension-based transformation procedures with marker genes were also reported (Andrade et al. 2009).

The aim of the present work was the study of the over-expression of the native thaumatin-like protein CsTL1 gene in embryogenic cultures of European chestnut (C. sativa) via Agrobacterium-mediated transformation, as the first step to achieve tolerant chestnut plants to ink disease. We use the green fluorescence protein (GFP) as visual marker gene which simplifies and improves the evaluation of transformation events in real time.

Materials and methods

Plant material

Transformation experiments were carried out with an embryogenic culture line originated from leaf explants obtained from shoot multiplication cultures (C12-H1) and with embryogenic lines derived from zygotic embryos (Cl-3 and Cl-9) of European chestnut (Castanea sativa Mill.) (Corredoira et al. 2003, 2006). These embryogenic cultures have been maintained by secondary embryogenesis with subcultures at 6-week intervals onto standard embryo proliferation medium consisting of MS (Murashige and Skoog 1962) mineral salts (half-strength macronutrients and vitamins), 3 mM glutamine, 0.44 mM benzyladenine (BA), 0.49 mM 1-naphthaleneacetic acid, 3% sucrose (w/v) and 0.8% Sigma-agar (w/v) (Sigma-Aldrich, St. Louis, MO, USA). The pH was adjusted to 5.7 before sterilization by autoclaving at 121 °C for 20 min. The embryogenic lines were grown under a 16-h photoperiod (provided by cool-white fluorescent lamps at a photon flux density of 50–60 µmol m⁻² s⁻¹) and 25 °C light/20 °C dark temperatures (standard conditions).

Construction of the binary vector and Agrobacterium transformation

The chestnut gene encoding a thaumatin-like protein, termed CsTL1, purified from mature European chestnut cotyledons (Garcia-Casado et al. 2000), was used in this study. The CsTL1 gene was cloned into pK7WG2D vector (Karimi et al. 2002) under the CaMV35S promoter using the Gateway cloning system (Invitrogen, Life Technologies, NY, USA). This construct also contains the neomycin phosphotransferase (NPTII) selectable marker and green fluorescence protein (EGFP) reporter genes. This binary vector was obtained from the Functional Genomics Unit (Department of Plant Systems Biology, VIB Ghent University, Belgium). Briefly, pBluescript SK(−) plasmid harboring the cDNA sequence coding for CsTL1 was amplified by PCR using the proofreading Pfx DNA polymerase (Invitrogen). This PCR product was inserted into the pENTR™/D-TOPO® vector (Invitrogen) as recommended by the manufacturer. Plasmids pENTR™/D-TOPO® containing cDNA insert in the correct orientation were selected by PCR and the complete insert was subsequently sequenced. Correct inserts were transferred into the Gateway-compatible vector pK7WG2D using an LR clonase reaction (Invitrogen) carried out according to the manufacturer’s instructions. The binary vector, designated as the pK7WG2D-TAU (Figure 1), was transferred into A. tumefaciens strain EHA105 (Hood et al. 1993) by the freeze–thaw method (Xu and Li 2008) and used in the transformation studies.

Transformation procedures

For the transformation experiments, explants consisting of small clumps (4–7 mg) of two to three somatic embryos, at globular or early-torpedo stages, were dissected from embryogenic cultures 4 weeks after the last subculture. Somatic embryo clumps were pre-cultured on growth regulator-free proliferation medium for 1 day.

Cultures of A. tumefaciens strain EHA 105 pK7WG2D-TAU were initiated from a glycerol stock and grown overnight at 28 °C with shaking (200 rpm) in liquid Luria-Bertani (LB, 1% tryptone, 0.5% yeast extract and 1% NaCl, pH 7.0) medium containing kanamycin (50 mg l⁻¹). A single colony was inoculated into 2 ml of LB medium (Sambrook et al. 1989) containing 50 mg l⁻¹ kanamycin and nalidixic acid and this culture was incubated overnight at 28 °C at 150 rpm in darkness. One milliliter of the bacterial suspension was used to inoculate 600 ml of LB liquid medium with the appropriate antibiotics, and this bacterial suspension was incubated at 28 °C at 100 rpm until an OD₆₀₀ = 0.6 was achieved. The bacterial

![Figure 1. Schematic representation of the T-DNA region of the binary vector pK7WG2D-TAU.](http://www.treephys.oxfordjournals.org)
culture was subsequently centrifuged at 6500 rpm for 10 min at 10 °C and resuspended in 200 ml of MS liquid medium plus 5% sucrose.

Pre-cultured embryo clumps were immersed in the bacterial culture for 30 min, blot-dried on sterile filter paper and transferred to proliferation medium with growth regulators. After a cocultivation period in the dark at 25 °C, the explants were washed with sterilized water containing 500 mg l\(^{-1}\) cefoxitaxime for 30 min, blot-dried on sterile filter paper and then transferred to Petri dishes (10 explants per dish) containing selection medium consisting of proliferation medium supplemented with 300 mg l\(^{-1}\) carbenicillin, 200 mg l\(^{-1}\) cefoxitaxime and 150 mg l\(^{-1}\) kanamycin, and incubated under standard conditions. This kanamycin concentration was selected on the basis of previous results (Corredoira et al. 2004). After 2, 4, 6, 8 or 12 weeks on selection medium, kanamycin-resistant explants were recorded. The kanamycin-resistant embryos recorded after 8 weeks were then transferred to fresh selection medium for a further 4 weeks. At the end of this period (12 weeks in total), putative transformants identified by growth on selection medium were evaluated using GFP-specific fluorescence (GFP+).

In a first experiment, the effects of embryogenic genotype (Cl-3, CI-9 and C12-H1) and the length of cocultivation period (4 or 5 days) were evaluated. For each line, 240 embryogenic explants in total) were infected with Agrobacterium explants in total). The kanamycin-resistant embryos recorded after 8 weeks were then transferred to fresh selection medium for a further 4 weeks. At the end of this period (12 weeks in total), putative transformants identified by growth on selection medium were evaluated using GFP-specific fluorescence (GFP+).

To study the effect of antioxidants (cysteine and ascorbic acid) included in either the cocultivation or selection media on the transformation efficiency, embryo clumps of lines C12-H1 or CI-3 were infected with Agrobacterium and cocultured during 5 days. Cysteine (100, 200 and 400 mg l\(^{-1}\)) and ascorbic acid (80 mg l\(^{-1}\)) were dissolved in deionized water and filter sterilized before addition to autoclaved medium. In these experiments, 120 explants were used per treatment.

In each transformation experiment, 20 non-inoculated (wild type, wt) embryo explants were cultured on proliferation medium with or without antibiotics (negative and positive controls).

**Evaluation of GFP fluorescence and development of transformants**

After 12 weeks of culture in selection medium, kanamycin-resistant somatic embryos were observed for GFP-specific fluorescence, which was evaluated using a Zeiss SV11 epi-fluorescence stereomicroscope equipped with a light source consisting of a 100-W mercury bulb and an FITC/GFP filter set with a 480-nm excitation filter and a 515-nm long-pass emission filter (Chroma Technologies Corp., Bellows Falls, VT, USA). The transformation efficiency was defined as the percentage of initial explants that developed GFP-positive (GFP+) embryogenic cultures. Cotyledonary-stage embryos were isolated from GFP+ lines and subcultured on selection medium to proliferate and to establish different embryogenic transgenic lines. These transgenic lines were isolated from different transformation events and were routinely maintained by secondary embryogenesis with sequential subcultures at 6-week intervals according to the conditions previously defined (Corredoira et al. 2004). The proliferation ability of different embryogenic lines was determined by recording the number of somatic embryos produced by explant and compared with those of the corresponding non-transformed (control) embryogenic line.

**Molecular analysis of both transgenic embryogenic lines and transgenic plants**

**Polymerase chain reaction analysis**

Chestnut genomic DNA was extracted from somatic embryos and from leaves of untransformed and putative transgenic embryo lines and regenerated plants using Qiagen DNeasy® Plant Mini Kit (Qiagen, Hamburg, Germany), as recommended by the manufacturer. The presence of the transgenes, CsTL1, NPTII and EGFP was confirmed by PCR analysis. Reactions were carried out in a 50 µl volume containing 1 x supplied Taq buffer, 2.5 mM MgCl\(_2\), 200 µM dNTPs, 0.6 µM each primer, 1 U Taq DNA polymerase (Qiagen) and 100–200 ng of genomic DNA. Polymerase chain reaction analysis was carried out employing gene-specific primers: CsTL1 (forward: 5′-AGGTC ACTGGATTITGTGAT-3′; reverse: 5′-CACCATGATGAAACCGTCG-3′), NPTII (forward: 5′-GTCATCTCTGCTTCCGCC-3′; reverse: 5′-AGAGAAGCCGATAGAACGA-3′) and EGFP (forward: 5′-CAC CGGGGTGGTGGCCCATT-3′; reverse: 5′-CTAGTGGATCCCCCGGC-3′). The expected sizes of PCR fragments were 981 bp for CsTL1, 472 bp for NPTII and 740 bp for EGFP. Amplifications were carried out in an MJ Mini™ thermal cycler (Bio-Rad, Hercules, CA, USA) using the following programs after initial polymerase activation of 95 °C, 3 min: 40 cycles at 94 °C for 40 s, 56 °C for 1 min and 72 °C for 40 s for CsTL1 gene, 35 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 42 s for NPTII gene, and 40 cycles at 94 °C for 15 s, 56 °C for 30 s and 72 °C for 1 min for EGFP gene. The amplified products were resolved on 1.2% (w/v) agarose gel and confirmed as only one single band of the expected size.

**Southern blot**

Genomic DNA of transgenic somatic embryos was isolated using the Qiagen DNeasy® Plant Maxi Kit. DNA (20–30 µg) was EcoRI digested and then separated on a 0.8% (w/v) agarose gel in 0.5x Tris-borate buffer (TBE). Following gel electrophoresis of digested genomic DNA and subsequent depurination, denaturation and neutralization treatments, DNA was transferred onto a positively charged nylon membrane (Roche Applied Science, Mannheim, Germany) by capillary transfer. A DIG-labeled CsTL1 probe was produced by PCR using a PCR DIG Probe Synthesis kit (Roche Applied Science). Hybridization
and detection of the probe were carried out using DIG luminescent Detection kit (Roche Applied Science) according to the manufacturer’s instructions. Hybridization bands were detected using X-ray film (Kodak, Rochester, NY, USA) autorography. Genomic DNA extracted from untransformed somatic embryos was used as negative control. The number of bands reflects the number of T-DNA insertions.

**Quantification of CsTL1 overexpression by real-time PCR**

Total RNA was isolated from early cotyledonary somatic embryos of the three chestnut somatic embryonic lines (CI-9, CI-3 and C12-H1) as well as 11 putatively transgenic lines using Qiagen RNeasy Plant Mini Kit. Total RNA was subjected to On-column DNase I Digestion Set (Sigma-Aldrich, St Louis, MO, USA), according to the manufacturer’s instruction. cDNA was prepared from 1 µg total RNA using the Quanta cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD, USA). Quantitative real-time PCR (qPCR) was performed in an optical 48-well plate with a Step One Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The PCR reactions were performed in a final volume of 20 µl. The assays contained 1× FastStart DNA Master SYBR Green I (Roche Applied Science), 450 nm of each primer and 1.5 µl of cDNA mixture. The following standard thermal profile was used for all PCRs: 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The reactions were run for 40 cycles and at the end of each run melt curves were generated to ensure product uniformity. The CsTL1 primers used for qPCR analysis were designed with Primer3 software (www.primer3.sourceforge.net) (CsTL1 forward: 5′-GTCCAAGCTCCATGGAAAGG-3′; CsTL1 reverse: 5′-ACCTGACCGTGCTACAATC-3′). The expression data were normalized against the expression of three reference genes, ACTIN (ACT), POLYMERASE ELONGATION FACTOR (EF) (Soler et al. 2008) and TUBULIN (TUB) (Porth et al. 2005), previously selected based on their stability during embryo development using the geNorm software (Biogazelle NV, Belgium) (Vandesompele et al. 2002). Primer efficiency was tested using a standard curve for each gene. Three or four independent biological replicate samples were assessed and samples were added to the plates in triplicates.

Relative expression values for CsTL1 were expressed as fold-change using the comparative Ct method (Livak and Schmittgen 2008). All calculations and normalizations were done using DataAssist™ v3.0 software (Applied Biosystems).

**Germination and plant regeneration of transgenic somatic embryos**

Embryo germination was carried out according to Corredoira et al. (2003, 2008). Cotyledonary somatic embryos (4–6 mm) were isolated from transgenic cultures and transferred to maturation medium consisting of PGR-free MS (half strength macronutrients) medium supplemented with maltose (3%). After 4 weeks of culture on maturation medium, somatic embryos were transferred to MS medium with 3% sucrose and stored at 4 °C for 2 months, and then cultured for 8 weeks on germination medium (MS with 1/2 strength macronutrients, 0.44 µM BA, 0.49 µM indole-3-butyric acid (IBA) and glutamine 200 mg l−1). Although conversion frequency was normally low, a number of embryos responded by producing only shoots. Shoots from these germinating embryos may be multiplied by axillary shoot proliferation with subsequent rooting, according to the procedure defined by Vieitez et al. (2007) which allows the production of unlimited number of transgenic rooted plantlets. Elongated shoots (15–20 mm) were rooted in GD medium (Gresshoff and Dow 1972) with 1/3 strength macronutrients and supplemented with 122.5 µM IBA for 24 or 48 h and then transferred to an auxin-free medium (Vieitez et al. 2007). Plantlets were transplanted to pots containing sterilized peat : perlite (3 : 1) and acclimatized in a phytootron at 25 ± 1 °C and 90% relative humidity under a 16-h photoperiod (100 µmol m−2 s−1) for 6–8 weeks. The plants were then transferred to a greenhouse.

**Fluorescence analysis of plants**

Young leaves of transgenic and untransformed plants were laid on slides with 10% glycerol and squashed with coverslips. They were observed with laser scanning spectral confocal and multiphoton system, TCS SP2 microscope (Leica, Wetzlar, Germany). The 488 nm excitation and 500–555 nm emission filters were used.

**Cryopreservation of transgenic lines**

Due to the great number of transformed embryogenic lines achieved and in order to minimize labor costs and contamination risks, embryos were subjected to cryopreservation, awaiting proper evaluation. For cryopreservation experiments, somatic embryos were collected from the embryogenic transgenic lines and their corresponding wt lines. Explants for cryopreservation experiments consisted of small clumps (6–8 mg) of 2–3 somatic embryos in globular or early torpedo stages isolated from 4-week old embryogenic cultures of wt and transgenic lines. Cryopreservation using vitrification procedure was performed according to Corredoira et al. 2007 and Vieitez et al. 2011. Briefly, somatic embryo clumps were pre-cultured on MS medium with 0.3 M sucrose for 3 days, treatment with PVS2 solution for 60 min at 0 °C followed by rapid immersion in liquid nitrogen. After at least 24 h, samples were removed from liquid nitrogen and thawed by placing the vials in a water bath at 40 °C for 2 min and then cultured in proliferation medium. In each embryogenic line (wt or transformed lines), 40 embryo clumps (eight explants per Petri dish) were cryopreserved.

**Statistical analysis**

Two-way analysis of variance (ANOVA) was applied to examine the influence of each of the two main factors (cocultivation length × genotype) and their possible interaction in the
Table 1. Effect of genotype and coculture length (4 or 5 days) on kanamycin-resistant explants, transformation efficiency and selection efficiency following 12 weeks of culture in selection medium of somatic embryos transformed with the strain EHA105pK7WG2D-TAU

<table>
<thead>
<tr>
<th>Embryogenic line</th>
<th>Kanamycin-resistant explants (%)&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Transformation efficiency (%)&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Selection efficiency (%)&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 days</td>
<td>5 days</td>
<td>Mean</td>
</tr>
<tr>
<td>C12-H1</td>
<td>9.2 ± 2.2</td>
<td>8.3 ± 2.0</td>
<td>8.8 ± 1.4a</td>
</tr>
<tr>
<td>CI-3</td>
<td>5.0 ± 3.9</td>
<td>10.0 ± 2.5</td>
<td>8.8 ± 1.3a</td>
</tr>
<tr>
<td>CI-9</td>
<td>39.1 ± 5.2</td>
<td>37.5 ± 7.8</td>
<td>38.3 ± 0.8b</td>
</tr>
<tr>
<td>F-test</td>
<td>Kanamycin-resistant (%)</td>
<td>Transformation efficiency (%)</td>
<td></td>
</tr>
<tr>
<td>Cocultivation length (A)</td>
<td>P = 0.206 ns</td>
<td>P = 0.577 ns</td>
<td></td>
</tr>
<tr>
<td>Genotype (B)</td>
<td>P = 0.002**</td>
<td>P = 0.0001***</td>
<td></td>
</tr>
<tr>
<td>A × B</td>
<td>P = 0.094 ns</td>
<td>P = 0.494 ns</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE of three replicates with 40 explants each. ns, not significant.
<sup>1</sup>Percentage of initial explants that are kanamycin resistant after 12 weeks in selection medium.
<sup>2</sup>Percentage of initial explants that are GFP<sup>+</sup> after 12 weeks in selection medium.
<sup>3</sup>Percentage of kanamycin-resistant explants which also are GFP<sup>+</sup>. Within each variable, values with the same letter are not statistically significant at P = 0.05 level (LSD test).

**P ≤ 0.01; ***P ≤ 0.001.

Table 2. Effect of cysteine in cocultivation or selective media on the transformation efficiency of the embryogenic line C12-H1 transformed with the strain EHA105spK7WG2DTAU and using a cocultivation period of 5 days.

<table>
<thead>
<tr>
<th>Cysteine (mg l&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Kanamycin-resistant explants (%)&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Transformation efficiency (%)&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Selection efficiency (%)&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>In the cocultivation medium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>10.0 ± 2.6</td>
<td>8.3 ± 1.7</td>
<td>83.3</td>
</tr>
<tr>
<td>100</td>
<td>8.3 ± 2.1</td>
<td>5.0 ± 3.0</td>
<td>60.2</td>
</tr>
<tr>
<td>200</td>
<td>5.0 ± 2.3</td>
<td>3.3 ± 2.1</td>
<td>60.2</td>
</tr>
<tr>
<td>400</td>
<td>0.0 ± 0.0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>400</td>
<td>0.0 ± 0.0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Source of variation</td>
<td>Kanamycin-resistant (%)</td>
<td>Transformation efficiency (%)</td>
<td></td>
</tr>
<tr>
<td>Cysteine in the cocultivation medium</td>
<td>df</td>
<td>χ²</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>6.372</td>
<td>0.095 ns</td>
</tr>
<tr>
<td>Cysteine in the selection medium</td>
<td>3</td>
<td>12.414</td>
<td>≤0.006**</td>
</tr>
</tbody>
</table>

Values are means ± SE of six Petri dishes with 10 explants each. ns, not significant.
<sup>1</sup>Percentage of initial explants that are kanamycin-resistant after 12 weeks in selection medium.
<sup>2</sup>Percentage of initial explants that are GFP<sup>+</sup> after 12 weeks in selection medium.
<sup>3</sup>Percentage of kanamycin-resistant explants which also are GFP<sup>+</sup>. **P ≤ 0.01.

experiment shown in Table 1. Levene’s test for homogeneity of variances was performed prior to ANOVA and least significant difference (LSD) test at P = 0.05 level was used to determine significant differences among means. Percentages were subjected to square-root transformation prior to analysis to normalize the data. Non-transformed data are presented in Table 1. In Table 2, the significant differences between cysteine concentrations were estimated using G-test. Statistically significant differences between transgenic lines and their wt counterparts on CsTL1 overexpression analysis by qPCR were revealed by using a Student’s t-test. The SPSS 19.0 statistical package for Windows (Chicago, IL, USA) was used for all the statistical analysis.

**Results**

**Transformation and selection of transgenic lines**

Initial experiments focused on optimizing variables associated with *Agrobacterium*-mediated transformation including the coculture duration and the inclusion of cysteine and
ascorbic acid as antioxidants into the cocultivation or selection media.

After culture initiation in selection medium, the original creamy-yellowish embryo clumps became brownish/blackish, especially in the embryogenic lines CI-3 and C12-H1. The response of the three embryogenic lines to transformation following 4 or 5 days of cocultivation with the strain EHA105pK7WG2D-TAU is shown in Figure 2. The response was clearly genotype dependent. New growth of the most responsive embryogenic line (CI-9) was visually recorded after only 2 weeks of culture, and the surviving explants (putative kanamycin-resistant) increases in number by increasing the time spent in selection medium. For the lines CI-3 and C12-H1, at least 8 weeks of culture were required to visually detect kanamycin-resistant explants with the two cocultivation periods evaluated (Figure 2). There were no significant differences for the number of resistant explants following 4 or 5 days of cocultivation. After 8 weeks, kanamycin-resistant explants were isolated and subcultured for 4 weeks more on selection medium to continue embryogenic growth.

After a further 4 weeks of growth in selection medium, the percentages of kanamycin-resistant and GFP+ explants as well as the transformation efficiency were recorded. The transformation efficiency was determined in basis to the fluorescence (GFP+) of the surviving explants. In order to prevent the development of potential chimeras, only completely fluorescent embryos (Figure 3a–d) were considered as GFP+. Those exhibiting partial fluorescence were discarded. The genotype significantly affected the percentage of kanamycin-resistant explants ($P \leq 0.002$) and the transformation efficiency ($P \leq 0.0001$) with the highest frequencies observed by CI-9 genotype (Table 1). The percentage of GFP+ explants was, on average 8.3% in line C12-H1, 7.1% in line CI-3 and 32.1% in line CI-9. Apparently, these different responses, attributed in principle to the genotype, could not be related to the origin of the explant from which derived the embryogenic line, as those lines originated from immature zygotic embryos (lines CI-3 and CI-9) exhibited the lowest and highest efficiencies, respectively, whereas an intermediate value was obtained in the line C12-H1, which has been originated from leaf explants isolated from shoot multiplication cultures. Although in line CI-3, higher percentages of kanamycin-resistant explants and transformation efficiency were recorded following 5 days of cocultivation, the results were not significantly different with respect to those obtained with the 4-day period. In the lines CI-9 and C12-H1, data of these parameters showed small differences between the two coculture treatments, resulting in no significant differences (Table 1). No significant interaction between the cocultivation length and genotype was observed for the percentage of kanamycin-resistant explants or the transformation efficiency.

The selection efficiency ranged from 80 to 100% (Table 1), indicating that kanamycin and GFP double selection appear to be appropriate for this transformation system. These results demonstrated that GFP fluorescence is an effective and non-destructive marker for identifying transgenic somatic embryos in chestnut.

In order to protect target explants from their excessive brownish occurring in selection medium and to optimize the transformation efficiency, the effect of antioxidants was evaluated in the two embryogenic lines exhibiting lower transformation responses. The addition of ascorbic acid (80 mg l$^{-1}$) in either cocultivation or selective media did not improve the selection efficiency of the line CI-3 (data not shown). The inclusion of 100 mg l$^{-1}$ or 200 mg l$^{-1}$ cysteine into the cocultivation medium significantly influenced neither the frequencies of kanamycin-resistant nor GFP+ explants of the line C12-H1 (Table 2), whereas the addition of this antioxidant to the selection medium was clearly detrimental ($P \leq 0.006$). Consequently with these results, no antioxidants were added to the media in further experiments.

To ensure that each putative transformed line derives from a unique transformation event only one cotyledonary embryo was isolated from each GFP+ explant and independently multiplied by secondary embryogenesis on selection medium to establish and to proliferate different transgenic embryogenic lines (Figure 3e, d). Following this procedure, 126 transformed lines were obtained: 18 for CI-3, 27 for C12-H1 and 81 for CI-9, demonstrating the efficiency of the present transformation protocol. All GFP+ embryogenic lines obtained exhibited a

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Figure 2. Percentage of kanamycin-resistant explants of the three embryogenic chestnut lines tested after 2, 4, 6 and 8 weeks of growth in selection medium following cocultivation for 4 or 5 days with the strain EHA105pK7WG2D-TAU.
Figure 3. Transformation and regeneration of chestnut using the vector pK7WG2D-TAU containing CsTL1 and EGFP genes. (a) Globular transformed somatic embryos observed under white light. Bar: 1 mm. (b) The same globular somatic embryos observed under blue light showing green fluorescence. Bar: 1 mm. (c) GFP expression in secondary embryos formed from a globular embryo. (d) Histodifferentiated transgenic cotyledonal-ary embryo showing GFP expression. (e, f). Transgenic plants derived from embryo germination (e) and from rooted shoots derived from axillary shoot cultures (f). (h) Confocal image of GFP fluorescence captured on leaf obtained from a chestnut transgenic plant. (g, i, j). The GFP expression on leaf (g), apex (i) and root (j) of transgenic plant (right) and untransformed plant (left) visualized with an epi-fluorescence stereomicroscope. Untransformed tissues appear red under blue light due to autofluorescence of chlorophyll.
normal phenotype, as the embryos are morphologically very similar to those of their wt counterparts. Successful embryo proliferation was achieved in all of the transgenic lines regenerated. To estimate the multiplication capacity of the transgenic lines, the number of secondary somatic embryos produced per embryo explant was recorded in eight of the transgenic lines obtained, after 6 weeks of culture onto standard embryo proliferation medium. Although the competence for secondary embryogenesis in the evaluated transgenic lines (Table 3) differed between genotypes, generally, and within each genotype, the transformed lines showed a higher mean number of cotyledary stage embryos and total number of embryos per embryo clump than their corresponding untransformed lines. Embryo development was asynchronous, embryos at different developmental stages were observed.

Molecular characterization of transgenic lines
To verify gene integration and expression, transgenic lines were tested using PCR, qPCR and Southern blot (Figures 4–6). A total of 32 transgenic lines, corresponding to 25% of GFP-positive lines isolated, were evaluated by PCR. In the analysis, fragments of 472, 740 and 981 pb, corresponding to the predicted size of the NPTII, EGFP and CsTL1 genes, respectively, were amplified from the putative transgenic somatic embryos, but not from untransformed somatic embryos (Figure 4a–c). Leaves from transgenic shoots derived from transgenic somatic embryos were evaluated by PCR and the amplification of the NPTII, EGFP and CsTL1 genes was also observed (data not shown).

Total RNA of 11 PCR-positive transgenic lines (CI-3 L1, L2; CI-9 L1-L7; C12-H1 L1, L2) and their wt counterparts was isolated from early cotyledonary embryos and was analyzed with qPCR in order to verify CsTL1 transgene expression. The CsTL1 transcripts were detected in all the tested transgenic lines (Figure 5). The CsTL1 expression was 3–4-fold higher in the CI-3 transformants (CI-3 L1 and L2) compared with the

Table 3. Secondary embryogenesis ability of three wt chestnut embryogenic lines and their different transgenic lines (L). Data are based on the total number of somatic embryos per explant and the number of embryos per developmental stage per explant, recorded after 5 weeks of culture in proliferation medium.

<table>
<thead>
<tr>
<th>Line</th>
<th>Embryo stage</th>
<th>Total number of somatic embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Globular</td>
<td>Heart-torpedo</td>
</tr>
<tr>
<td>C12-H1-wt</td>
<td>6.9 ± 0.5</td>
<td>3.6 ± 0.6</td>
</tr>
<tr>
<td>L1</td>
<td>8.8 ± 0.8</td>
<td>3.5 ± 0.4</td>
</tr>
<tr>
<td>L2</td>
<td>8.3 ± 0.1</td>
<td>5.0 ± 0.8</td>
</tr>
<tr>
<td>CI-3-wt</td>
<td>8.7 ± 1.0</td>
<td>8.1 ± 0.2</td>
</tr>
<tr>
<td>L1</td>
<td>10.0 ± 0.7</td>
<td>6.3 ± 0.8</td>
</tr>
<tr>
<td>L2</td>
<td>7.0 ± 0.9</td>
<td>5.0 ± 0.4</td>
</tr>
<tr>
<td>CI-9-wt</td>
<td>7.1 ± 0.4</td>
<td>4.5 ± 0.3</td>
</tr>
<tr>
<td>L1</td>
<td>9.5 ± 1.0</td>
<td>7.3 ± 1.0</td>
</tr>
<tr>
<td>L2</td>
<td>7.7 ± 0.2</td>
<td>5.2 ± 0.3</td>
</tr>
<tr>
<td>L7</td>
<td>6.7 ± 0.2</td>
<td>5.2 ± 0.3</td>
</tr>
<tr>
<td>L8</td>
<td>8.0 ± 0.5</td>
<td>4.2 ± 0.2</td>
</tr>
</tbody>
</table>

Values are means ± standard error of 3 Petri dishes with 8 explants each.

Figure 4. Polymerase chain reaction analysis of chestnut transgenic lines. (a) Polymerase chain reaction amplification using primers specific for production of a 740-bp EGFP fragment. (b) Polymerase chain reaction amplification using primers specific for production of a 472-bp NPTII fragment. (c) Polymerase chain reaction amplification using primers specific for production of a 981-bp CsTL1 fragment. M, DNA ladder; U, non-transgenic somatic embryos (negative control); P, corresponding plasmid DNA (positive control); lanes 1–17, transgenic lines; 600 pb indicates DNA marker size in base pairs.
control line (CI-3 wt). In the line CI-9, CsTL1 is expressed at the highest level in L1 and L2 (11- and 13.5-fold, respectively) compared with the wt line, to a slightly lesser extent in L5 and L6 (5- and 6.3-fold), and at a relatively low level in L3 and L7. In contrast, the expression of CI-9 L4 was lower than that of the wt line which may have been due to silencing/deletion of the CsTL1. The CsTL1 mRNA transcript levels in the C12-H1 line were lower compared with CI-3 and CI-9 transgenic lines, but still remained significantly higher compared with C12-H1 wt.

To estimate the transgene copy number, DNA from five randomly selected transgenic lines and a non-transgenic line was analyzed by Southern blot using a DIG-DNA probe corresponding to the CsTL1 gen (Figure 6). T-DNA sequence has a unique EcoRI restriction site and was used to determine insert copy number. Four of them presented two transgene insertion sites (line L7, L10 and L11 from original line CI-9, and L3 from line CI-3). The line L6 showed one transgene insertion site. DNA isolated from untransformed somatic embryos did not hybridize with the CsTL1 probe. These results could explain the variable CsTL1 gene expression observed following qPCR. Among the lines analyzed by Southern blot and qPCR, L6 (one copy transgene) had more expression than L7 (two transgene copies).

Germination and plantlet regeneration

Currently, the conversion of somatic embryos into plantlets is a limiting step for European chestnut embryogenic systems (Corredoira et al. 2006, 2008), and the same applies for transgenic embryos (Maynard et al. 2008). Following maturation and stratification of somatic embryos, embryos were cultured in germination medium for 8 weeks. The germination response (conversion into plantlets and/or shoot or root only development, Figure 3 e) occurred in all transgenic lines evaluated, although the conversion frequencies were very low (3.3–20%). However, all these lines also produced a number of embryos responding by producing only shoots, which were used to establish shoot cultures from each embryogenic line. Shoot cultures were multiplied by axillary branching, and both the multiplication and rooting rates were very similar in both transgenic and wt shoots (data not shown). Moreover, these transgenic plants were normal phenotypically. Overall, a total of seven shoot axillary lines were stabilized from germinated somatic embryos of different transgenic derived lines CI-3 and CI-9. Rooting rates and performance were very similar in both transgenic (70–80%) and wt shoots (90%) (Figure 3f). Fluorescence was observed in the leaves, shoots and roots from rooted shoots after their transfer to soil, confirming the non-toxicity of GFP in chestnut plantlets (Figure 3g, i, j). High GFP fluorescence was also observed in the cells of leaf samples of transgenic plants, as observed by confocal microscopy (Figure 3h). Rooted shoots were acclimatized in a phytotron.
for 6–8 weeks and then transferred to greenhouse for further growth. At this stage, no phenotypic differences were observed in the transgenic plants in comparison with non-transgenic plants.

**Cryopreservation of transgenic lines**
Chestnut transgenic lines selected were successfully cryopreserved using the vitrification procedure. Cryopreserved somatic embryos had good viability with percentages of embryo surviving ranging from 56 to 92% (Table 4). In both, wt and transgenic lines, new somatic embryos developed 3–4 weeks after thawing. No morphological differences were observed between transgenic somatic embryos and untransformed embryos. Embryogenesis recovery was observed in all transgenic lines evaluated, with values ranging between 56 and 84%. The recovered transgenic somatic embryos continued to proliferate normally on proliferation medium.

**Discussion**
The goal of this study was to examine the response of chestnut embryogenic cultures for constitutive expression of an endogenous gene (CsTL1) encoding a TLP. This is the first report in which a chestnut species has been transformed with its own genetic material and with a gene which, at least theoretically, could enhance the tolerance of the species to ink disease. Overexpression of TLP foreign genes enhanced disease resistance, among others, against *Rhizoctonia solani* Khun and *Sclerotinia homoeocarpa* F.T. Bennett in bentgrass (Fu et al. 2005), *Fusarium graminearum* Schwabe in wheat (Mackintosh et al. 2010), *Alternaria alternata* (Fr.) Keissl. in tobacco (Velazhahan and Muthukrishnan 2003/04), *Verticillium dahliae* Kleb. also in tobacco (Munis et al. 2010), *Erysiphe necator* Schw. in grapevine (Dhekney et al. 2011) and *F. oxysporum* in banana (Mahdavi et al. 2012).

In the present work, we have used an *Agrobacterium*-mediated transformation procedure already successfully developed for European chestnut (Corredoira et al. 2004) using somatic embryos as target material and marker genes. The former selectable marker gene GUS was replaced in this study by the GFP gene, which allows having a direct visual selection of successful transformants, and the CsTL1 gene which has been incorporated into the binary vector. The genotype and the components of the vector clearly influence the rates of the transformation achieved. In the present study, the transformation efficiency, measured as the percentage of GFP+ explants, reached 8.3% for the line C12-H1, 7.1% for Cl-3 and 32.5% for Cl-9. When the transformation rates were measured as the percentage of GUS+ explants, the values recorded for the same genotypes were, respectively, 21.7, 5.0 and 10.0% (Corredoira et al. 2007). The high selection efficiencies achieved (80–100%) in the present work indicate that kanamycin and GFP double selection imposed high stringent selection, minimizing escapes and avoiding losses of samples, as it may occur when the GUS histochemical assay is applied. The selection of whole fluorescent embryos facilitates the proliferation of transgenic embryos, limiting the subculturing of escape tissues. In American chestnut (*C. dentata*), substantial amount of non-transformed material escaped the selection, when the transformation procedure was carried out on semisolid culture media and GUS expression was used as selection marker (Andrade et al. 2009). The advantages of using the green fluorescent protein as an efficient selection marker have been highlighted in *Hevea brasiliensis* Muell. Arg. (Leclercq et al. 2010). These authors recognize that, in their working system, GFP selection is less time-consuming in terms of callus subculturing and offers the possibility of producing antibiotic resistance marker-free transgenic plants. The absence of antibiotics in the selection medium would be an interesting approach for accomplishing recommendations of official agencies (specifically in the European Union) and for simplifying the establishment of field trials of transgenic trees. However, lack of antibiotics in cocultivation and/or selection media may reduce the transformation efficiency, as demonstrated in clementine (*Citrus clementina* Hort. ex Tanaka) (Cervera et al. 2008) and in rubber tree (Leclercq et al. 2010).

Browning and necrosis of transformed tissues are common in *Agrobacterium*-mediated transformation processes which severely reduce transformation efficiency. Research in this topic indicates that the reactive oxygen species may be playing an important role in tissue browning and the application of antioxidants to cocultivation and/or selection media as to prevent/diminish the problem is a common practice in transformation experiments, as reviewed by Dan (2008). The application of these treatments to European chestnut did not increase the

**Table 4.** Embryo survival and recovery frequencies of chestnut somatic embryo clumps of untransformed (wt) and transgenic lines (L) after cryopreservation in liquid nitrogen for 24 h.

<table>
<thead>
<tr>
<th>Line</th>
<th>Survival (%)</th>
<th>Embryo recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C12-H1-wt</td>
<td>67.6 ± 2.0</td>
<td>65.6 ± 3.0</td>
</tr>
<tr>
<td>L1</td>
<td>56.3 ± 5.6</td>
<td>56.3 ± 5.6</td>
</tr>
<tr>
<td>L2</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>CI-3-wt</td>
<td>77.2 ± 13.1</td>
<td>72.3 ± 11.1</td>
</tr>
<tr>
<td>L1</td>
<td>92.5 ± 5.1</td>
<td>77.5 ± 7.4</td>
</tr>
<tr>
<td>L2</td>
<td>72.5 ± 4.8</td>
<td>65.0 ± 6.2</td>
</tr>
<tr>
<td>CI-9-wt</td>
<td>80.0 ± 8.6</td>
<td>80.0 ± 8.6</td>
</tr>
<tr>
<td>L1</td>
<td>85.0 ± 4.8</td>
<td>77.5 ± 7.4</td>
</tr>
<tr>
<td>L2</td>
<td>83.3 ± 8.5</td>
<td>79.1 ± 11.2</td>
</tr>
<tr>
<td>L7</td>
<td>62.5 ± 7.4</td>
<td>58.4 ± 4.3</td>
</tr>
<tr>
<td>L8</td>
<td>84.4 ± 3.1</td>
<td>84.4 ± 3.1</td>
</tr>
</tbody>
</table>

Values are means ± standard error of five Petri dishes with eight explants each. nd, not determined.
survival of the explants, probably due to the high content of polyphenols in chestnut material, as it occurred in other woody species such as Camellia sinensis L. (Mondal et al. 2001).

Different strategies have been followed for transformation of American chestnut. An Agrobacterium-mediated co-transformation procedure of somatic embryos with a wheat oxalate oxidase gene has been proposed (Zhang et al. 2011). Two vectors containing OxO and GFP were introduced to the bacteria strain separately so each Agrobacterium had only one vector and the authors conclude that having GFP on a separate plasmid provided a useful tool for removing of the marker gene. In addition, high transformation efficiencies, as evaluated by GUS assay, were reported for American chestnut employing size-fractionated proembryogenic masses via an embryogenic suspension-based transformation procedure (Andrade et al. 2009). The results obtained were, as in European chestnut, also genotype dependent. The suspension culture-based transformation/regeneration system is very interesting for the uniformity of the target material by filtration of suspension cultures and for its great potential for scalability. The culture of isolated transgenic embryos is an advantage in the subsequent steps of maturation, germination and plantlet conversion. On the contrary, the difficulties for the identification of separate transformation events within each culture flask should be considered a limitation of the whole procedure, which seems more complex and time consuming than the culture in semisolid medium. Interesting, Andrade et al. (2009) reported that staminate inflorescences were produced by transgenic somatic seedlings during third season in the greenhouse and anthers and filaments of some stamens expressed the GUS transgene. In European chestnut, untransformed somatic embryo-derived plants showed symptoms of precocious maturation, developing male catkins after only 3 years, and beginning to regularly bear chestnuts the following year (Vieitez and Merkle 2005). Precocity of somatic plants is an extremely valuable character which may be useful in breeding programs.

Chestnut transformed embryogenic lines from different transformation experiments are maintained by secondary embryogenesis and for long-term preservation transgenic somatic embryos were preserved in liquid nitrogen. Cryopreservation may be a reliable alternative for facilitating the management of transgenic embryogenic lines and limit the risk of contamination, as well as to reduce labor and supply costs. In this study, cryopreservation procedures allow us to store for long periods of time transgenic lines while other lines are evaluated for disease resistance. From the 126 independent transgenic lines generated, 115 lines are being cryopreserved following the described procedure for chestnut transgenic lines. On the other hand, eleven lines were analyzed by qPCR, showing in all except for one overexpression of the CsTL1 transgene. This assay also demonstrated the constitutive nature of CsTL1 in C. sativa. Initially, only plants regenerated from these embryogenic lines will be subjected to analyses of tolerance/resistance to ink disease. Transgenic lines analyzed by Southern blot presented one or two copies of the CsTL1 gene. Agrobacterium-mediated transformation often allows low transgenic copy insertions (Kohli et al. 2003). Our results showed that in the transgenic line with two copies there was no gene silencing. According to Cervera et al. (2000), copy number, position effects and the organization of a given insert could collectively explain the variable levels of expression showed by the transgenic lines.

Fluorescence indicating overexpression of the transgene was observed not only in shoots and leaves but also in roots, which is the entrance point of the fungus causing ink disease (Vannini et al. 2010). Although there has been some evidence that GFP may be cytotoxic to plant cells (Haseloff and Amos 1995), the data on embryo proliferation, maturation and plantlet conversion, as well as visual appearance of transgenic and wt plants, suggest that GFP is not cytotoxic for European chestnut material. These results are in accordance with those of Escobar et al. (2000) working with walnut somatic embryos, Yancheva et al. (2006) working with ‘Spadona’ pear and Leclercq et al. (2010) working with rubber tree.

The genetically modified (GM) chestnut plants regenerated by expression of a gene isolated from the host genome indicate that they are close to the concept of cisgenesis. A GM product is called cisgenic when intact native genes are used (including all regulatory sequences, exons and introns in the sense direction) and derived from the proper or sexually compatible relative species. When modified versions of native genes also derived from sexually compatible relatives are used, the GM plants are called intragenic (Schouten et al. 2006, Jacobsen and Nataraja 2008, Jacobsen and Schouten 2008). In the last few years, attempts to produce cisgenic plants has increased and applied to different species such as grapevine (Dhekeny et al. 2011), Populus (Han et al. 2011) and barley (Holme et al. 2012). However, the first report of the generation of a true cisgenic plant considered apple as the model plant (Joshi et al. 2011, Vanlaere et al. 2011). The interest in the production of cisgenic plants should be linked to the regulatory systems designed to manage the risks of GM organisms and to respond to public concern about them. Myskja (2006) argued that the development of cisgenesis/intragenesis represents an approach to genetic modification and may involve lower levels of risk and uncertainty. However, other authors critically evaluate the arguments that have been made in favor of cisgenics over transgenics and argue that these arguments are insufficient to justify a lower standard of regulation of cisgenesis (Russell and Sparrow 2008). Although plants recovered in this study contain viral promoters and reporter/marker genes, this is the first report of a cisgenic approach to obtain fungal disease-resistant European chestnut plants.
Acknowledgments

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Conflict of interest

None declared.

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References


