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Genetic transformation of European chestnut

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Abstract Stable incorporation of the *nptII* gene into *Castanea sativa* Mill. has been achieved by *Agrobacterium*-mediated transformation. The transformation assays were performed by infecting wounded hypocotyls with a strain of *Agrobacterium tumefaciens*, LBA 4404 harbouring the plasmid p35SGUSINT. Although two schemes of selection were tested, many escapes were obtained. The best strategy to avoid this problem is the introduction of higher concentrations of kanamycin in the culture medium, immediately after coculture. PCR analysis showed of the selectable *nptII* gene integration in the plant genome. β-Glucuronidase histochemical assay revealed the expression of the *uid*A gene in shoots, regenerated from transformed explants.

Key words *Agrobacterium tumefaciens* · *nptII* · *Castanea sativa* · β-Glucuronidase

Abbreviations *BA* Benzylaminopurine · *GUS* β-Glucuronidase · *MS* Murashige and Skoog medium

Introduction

Castanea sativa Mill. has played an important role in Europe. Historically, this tree species served as a staple food in many regions. The cultivation of chestnut is strongly connected with typical characters of certain European regions. Chestnut trees are very important economically because they produce fruits and wood of high quality and have ecological and landscape importance. They also have potential for timber production.

During the past 100 years, both European and American chestnut species have suffered severely from root rot, known as ink disease, caused by the fungi *Phytophthora cinnamomi* and *P. cambivora*. In the beginning of this century, the disease called chestnut blight, caused by the fungus *Cryphonectria parasitica*, almost completely destroyed of the American chestnut. The disease was detected in Europe in 1938, although it has been less destructive than in North America. The lower level of disease losses may result from the probable greater blight resistance of European chestnut and the apparent widespread occurrence in Italy and neighbouring areas of cytoplasmically hypovirulent *C. parasitica* strains. (Vieitez et al. 1986)

Recently, all countries involved in chestnut production have become very committed to expanding their plantation areas. However, there are limitations to achieving this, due to the lack of superior plant material. Improvement of perennial crops through traditional breeding methods is a long-term effort because of their long generation time, their high level of heterozygosity and the fact that many backcrosses are necessary to eliminate undesirable traits (Laimer da Câmara Machado et al. 1992).

In the past 10 years, much work has been done on the micropropagation of chestnut and there is now sufficient experience to use this mode of propagation instead of traditional methods (Vieitez et al. 1986; Feijó and Pais 1991; Seabra and Pais 1993 a).

Genetic transformation is a powerful tool for improvement of woody plants, like chestnut species, which are very heterozygous and are propagated mainly vegetatively. Transformation procedures allow one to make small, specific changes in the genome of a cell, i.e. the addition of one or a few genes. This stands in contrast to conventional breeding, where entire sets of chromosomes are combined and desired parental genotypes are not necessarily reconstituted. Transformation of woody plants offers the potential to make relatively quick specific changes in proven cultivars without disrupting their desirable genetic constitution (Shuerman and Dandekar 1993).

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Nowadays chestnut reforestation is done, almost exclusively, with hybrids of *C. sativa*×*C. crenata* because of their higher resistance to fungal diseases. However, these hybrid plants produce lower-quality wood and fruits, so the production of transgenic plants of *C. sativa*, by genetic engineering, with higher resistance to pathogenic fungi, is of great importance for reforestation programmes. The reason for choosing the gene transfer strategy to produce transgenic plants of chestnut, instead of micropropagation of resistant clones, is simply because there are almost no resistant *C. sativa* clones remaining in Portugal.

The results constitute, to the best of our knowledge, the first report on the production of transformed plants using *Agrobacterium* mediated systems in any *Castanea* species. We report the stable incorporation of the *nptII* gene into *C. sativa*, by *Agrobacterium*-mediated transformation. This may be seen as an important step for further studies of transformation with interesting genes, such as chitinases, glucanases and ribosome-inhibiting protein genes, to produce in the future transgenic plants of *C. sativa* expressing pathogen-related proteins in order to obtain improved plant material with a broad resistance to fungal pathogens. (Jach et al. 1995)

Materials and methods

Bacterial strains

The *Agrobacterium tumefaciens* strain used was LBA 4404 harbouring the plasmid p35SGUSINT. This plasmid has a T-DNA region which contains the eukaryotic *nptII* gene which confers resistance to kanamycin, as well as an intron-containing β-glucuronidase (GUS) gene which is not expressed in the bacterium (Vancanneyt et al. 1990) (Fig. 1). The bacteria were grown for 3–4 days in YEB solid medium containing the following components per litre: 1 g yeast extract, 5 g beef extract, 5 g bactopeptone, 5 g sucrose, 0.5 g magnesium sulphate heptahydrate and 15 g agar, pH 7.2. The medium was supplemented with the appropriate antibiotics (50 mg/l kanamycin and 50 mg/l rifampicin). After 3–4 days, the bacteria were subcultured in YEB liquid medium on a shaker at 100 rpm overnight (16–18 h) until an OD 600 of 0.6 was reached. Then a dilution of 1:50 was made in 3% sucrose.

Plant material and transformation conditions

Hypocotyl segments were harvested from seedlings of *C. sativa* Mill. The seedlings were obtained from seeds germinated in vitro, on Murashige and Skoog (1962) (MS) basal medium. Hypocotyls were used in the transformation protocols due to their high efficiency for shoot regeneration.

Fig. 1 Map of the T-DNA region of the p35SGUSINT plasmid, used in the present study. The *npt*II gene is under control of the NOS promoter and the *uid*A gene is under the CaMV 35S promoter. *PIV2* is the plant intron

All media components were autoclaved at 120 °C at 1.0 kg · cm^{-2} except the antibiotics, which were filter sterilised before adding to the medium. Both the precultivation period, corresponding to the period between the wounding of the hypocotyls until the coculture with the bacteria, and the cocultivation period, comprised 2 days. The wounded hypocotyls were cultured on regeneration medium which consisted of MS medium with 1/2 nitrate concentration, containing the following components per litre: 1 mg thiamine, 1 mg pyridoxine, 1 mg nicotinic acid, 2 mg glycine, 1 mg calcium panthotenate, 100 mg myo-inositol and 1 mg benzylaminopurine (BA). The media were supplemented with 20 g/l sucrose and pH was adjusted to 5.7 before autoclaving and agar addition (8 g/l). The hypocotyls were gently shaken in the bacteria suspension for about 10 min and blotted on a sterile filter paper. Afterwards, they were transferred to the medium and cocultivated under the same light conditions of the precultivation period (16 h day photoperiod of 20–25 $\mu E \cdot m^{-2} \cdot s^{-1}$) for 2 days, at 22±2 °C. After coculture, the explants were washed in MS medium with 500 mg/l cefotaxime, blotted on a sterile filter paper and transferred to the regeneration medium with 300 mg/l carbenicillin and 200 mg/l cefotaxime. Three subcultures were usually needed for the elimination of the bacterial contamination. Once bacterial growth appeared to be controlled, the concentrations of carbenicillin and cefotaxime were reduced to 200 mg/l and 100 mg/l, respectively.

Screening of the transformants

The selection medium had the same composition as the regeneration medium except that the concentration of BA that was reduced to 0.5 mg/l, 3 weeks after coculture. The initial selection levels used in transformation experiments were based on previous studies on the tolerance of the non-transformed tissues to antibiotics (Fig. 2). Non-transformed shoots do not withstand more than 50 mg/l kanamycin. Two schemes of transformant selection were tested: selection with kanamycin immediately after coculture (scheme 1), and selection 3 weeks after coculture (scheme 2).

GUS histochemical assay

We used the GUS histochemical assay as a rapid way to detect the presence of the GUS *uid*A gene in the putative transformants. The shoots formed from explants previously cocultivated with *Agrobacterium*, were incubated overnight at 37 °C in X-Gluc (Jefferson et al. 1987). After the overnight staining, the explants were washed in 70% (vol/vol) ethanol and observed.

Molecular analysis

The shoots able to grow in media with 150 mg/l kanamycin for 6 months were used for DNA extraction and amplification by PCR analysis with specific primers for the eukaryotic *nptII* gene, to determine whether the gene had integrated in the plant genome. DNA extraction from in vitro tissues (apical meristems and leaves) was done according to the CTAB-MINI DNA extraction for PCR (H. Y. Yang and G. King personal communication). Plant material was ground with acid-washed sand. The homogenised powder was mixed with 500 µl prewarmed 2% CTAB buffer, was ground a little more, and incubated for 10 min at 65 °C to disrupt membranes. The tissue/buffer mixture was then emulsified with 500 µl dichloromethane:isoamyl alcohol (24:1) to separate protein from DNA and microfuged for 2 min at room temperature. The topphase was transferred to a fresh microfuge tube and 300 µl of isopropanol was added to precipitate DNA, and centrifuged again for 2 min at room tem-

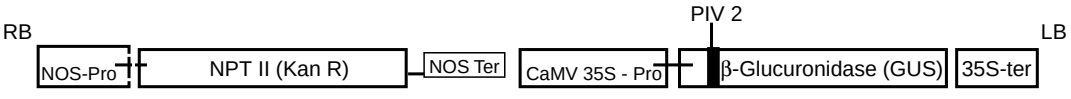

Table 1 Number and percentage of *npt*II-positive shoots, and escapes produced, using the two schemes of selection described in the text, and after 6 months of coculture. Values in parentheses are percentages

Number of hypocotyl segments	Number of segments with green shoots	Total number of shoots produced	Number of *npt*II-positive shoots	Number of escapes
Kanamycin selection immediately after coculture (scheme 1, three experiments)				
126	26 (20)	56	1 (1.8)	52 (92)
Kanamycin selection 3 weeks after coculture (scheme 2, five experiments)				
160	43 (27)	129	2 (1.5)	125 (97)

perature. The top phase was carefully removed and 500 µl of wash buffer was added to the pellet, left for 2 min and again centrifuged for 2 min at room temperature. The wash buffer consisted of 76% ethanol, 10 mM ammonium acetate and water. The top phase was removed and the pellet resuspended in TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). DNA quantification of the samples was done in 0.7% agarose gels by comparison with DNA markers of known concentration.

For each sample 0.4 µg of the plant genomic DNA was incubated in 1×PCR buffer (10×PCR buffer: 500 mM KCl pH 8.3 at 20 °C, 100 mM Tris HCl, 15 mM $MgCl_2$) 200 µM of dNTP mixture, 10 pM of each primer, 1 U Taq DNA polymerase (Boehringer Mannheim) and water for a total volume of 25 µl. The protocol used for plasmid extraction was the plasmid Midi protocol from Quiagen. The amplification products were analysed in 1% agarose gels stained with ethidium bromide. The primers used for the amplification of the *nptII* eukaryotic gene were, 5′GAG GCT ATT CGG CTA TGA CTG G3′, 5′ATC GGC TCC GTC GAT ACT AT3′, with an expected amplification product of 460 bp, and for the prokaryotic *nptII* gene, 3′AAT GCC ATA GCG GCG AGG CGT A 5′, 3′TGG TGG ATA CTA CAC CTT GC5′, with an expected amplification product of 700 bp.

Results and discussion

The main constraint for the successful genetic transformation of woody species is the availability of a good regeneration system. Production of adventitious shoots was achieved from stems of in vitro shoots, established from mature plant material (Seabra and Pais 1993 b). However, this regeneration system was not used for transformation purposes because of the low regeneration efficiency obtained to date, which determined the use of hypocotyl sections. Hypocotyl sections show a regeneration efficiency of 70% with an average of 6 shoots per hypocotyl, and are formed directly from epidermis without a callus phase (Seabra and Pais 1993 b). The selection with kanamycin reduced the hypocotyl regeneration efficiency to 20–27%, with an average number of 2.5 shoots per hypocotyl (Table1).

The sensitivity of the explants to kanamycin relative to the control (explants growing in media without kanamycin), was established first to determine the maximum concentration of kanamycin that permits the regeneration of transformed shoots, while preventing the formation of non-transformed shoots. (Fig. 2). A good selection procedure corresponds to a protocol, that permits only the production of transgenic shoots, which arise from one single transformed cell of the initial explant, used for transformation, and eliminates shoots that manage to escape this selection because the concentration of the selective agent used is not

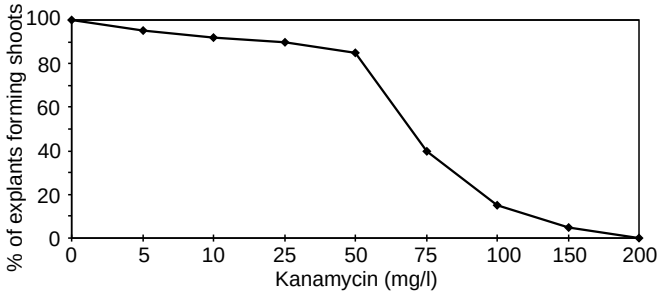

Fig. 2 Effect of kanamycin on the percentage of regeneration of shoots, related to the control

adequate. Data presented in Fig. 2 show that the concentration of 150 mg/l, reduces the regeneration by 98%. Using that concentration of kanamycin, we eliminated shoots from untransformed cells. However, we decided to use lower concentrations in the beginning, and then increase them to the maximum concentration of 150 mg/l, as we observed that when kanamycin was introduced to the regeneration medium immediately after co-cultivation, most of the explants became necrotic, and the shoots that regenerated, failed to elongate. According to some authors, it is not clear that applying higher concentrations of the selection agent is the best way to eliminate the non-transformed cells or organised tissues, since the selection pressure can also severely inhibit the differentiation of transgenic cells (Peña et al. 1995).

To identify a good selection procedure, two selection schemes were tested. In scheme 1, the explants were exposed to 50 mg/l kanamycin soon after the coculture, and the selection levels were sequentially increased in 3-week-subcultures intervals up to a maximum of 150 mg/liter for maintenance of transformants. In scheme 2, the explants were exposed to 50 mg/l kanamycin 3 weeks after cocultivation, and the selection levels were increased 2 weeks later, progressively from 50 to 150 mg/l. The results of both schemes of selection are presented in Table 1. The number of segments with green shoots was more or less the same in schemes 1 and 2 (20% and 27%, respectively) and the number of positive shoots was also identical for both schemes of selection. However, in scheme 1, selection immediately after coculture, less escapes were produced, but the difference does not seem to be significant (92% instead of 97%). Escapes are shoots that grow in media with high concentrations of kanamycin (150 mg/liter) for more than


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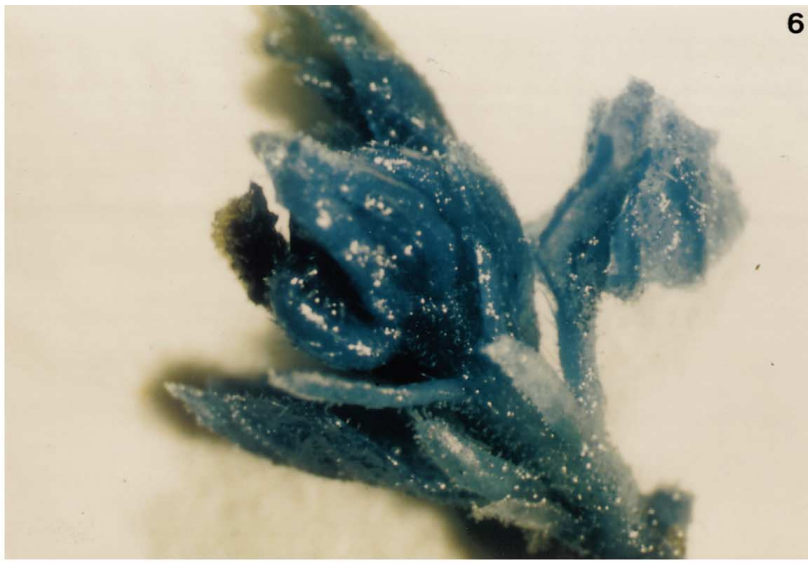
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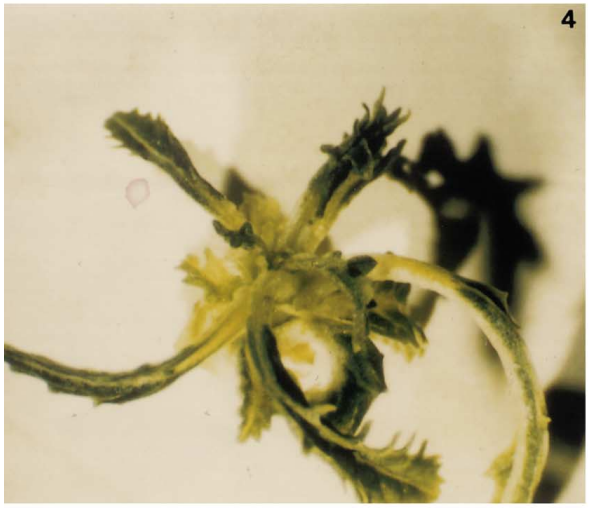
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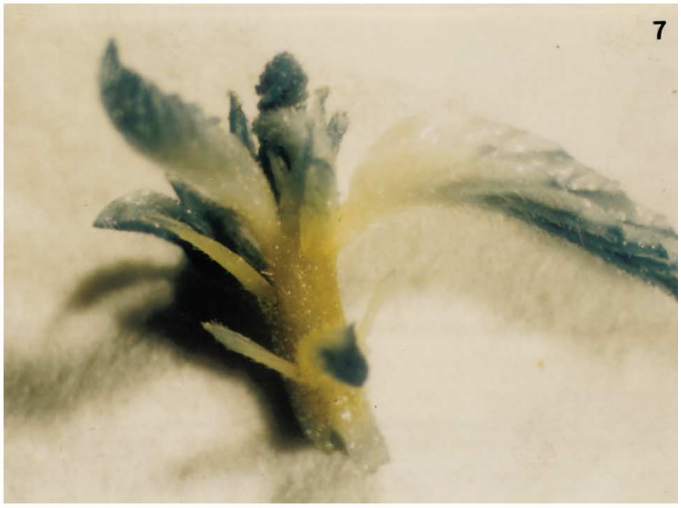
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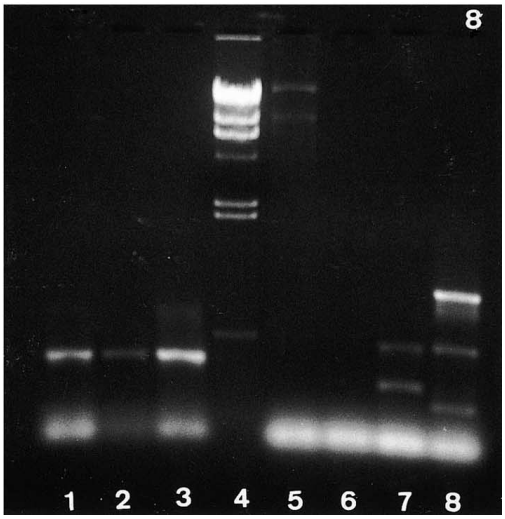
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6 months, but do not show integration of *nptII* gene. After a long time in culture they become vitrified (Fig. 4). 92.1% escapes were also obtained for sweet orange (Penã et al. 1995). The regeneration of escape shoots under kanamycin selection is not unique to *Castanea* and *Citrus*. It has been suggested that such escapes arise from non transformed cells that are protected from the selective agent by transformed cells in the explant (Dandekar et al. 1988; Jordan and McHughen 1988; Penã et al. 1995). In the procedure described by Peña et al. (1995), escapes were avoided by prolonged continuous exposure to kanamycin.

Chestnut tissues proved to be quite resistant to kanamycin, as are those of walnut, in contrast with other woody species which have shown extrem sensitivity (Yepes and Aldwinckle 1994). Kanamycin has been reported to completely inhibit morphogenesis in several fruit tree species e.g. in *Vitis* at 7 mg/l (Gray and Meredith 1992) and in *Rubus* at 10 mg/l (Fiola et al. 1990). In *Malus*, regeneration was inhibited with 20 mg/l kanamycin for the cultivar Greensleeves (James and Dandekar 1991).

Although delayed selection may permit cell division and the formation of multicell clusters that may withstand selection pressure better than single cells (Yao et al. 1995), our data suggest that for chestnut, that was not a good strategy. We may conclude from these results that applying kanamycin immediately after coculture seems to be better, even though the concentration of 50 mg/l proved to be low, allowing the production of many escapes. Concentrations start at 50 mg/l and then have to be increased at 3-week intervals. The high resistance to kanamycin is possibly due to the presence of endogenous non specific kanamycin phosphotransferase activity (Yepes et al. 1994). Others antibiotics, such as paromomycin and geneticin, were tested. It was observed that lower percentages of escapes were obtained when paromomycin was used instead of kanamycin (data not shown).

The transformation efficiency, defined as the number of transgenic shoots obtained per inoculated explant, was very low. However it was similar to the transformation efficiencies reported for the apple cultivar Greensleeves (approximately 1%; James et al. 1989), rootstock M26 (1.8–4%), Maheswaran et al. 1992) and for apple cultivar Royal Gala (0.4–2.8%); Yao et al. 1995). The transformed shoots elongated very well and had a normal phenoype (Fig. 5).

The regenerated shoots were subjected to a GUS assay. The *nptII* positive regenerants showed the typical indigo blue coloration of X-Gluc treatment (Fig. 6), while the negative ones did not (Fig. 3). However, we obtained shoots that showed a heterogeneous pattern of GUS staining and did not show a PCR-positive product for the *nptII* gene (Fig. 7). These shoots may be considered as chimeras, and may arise from a group of cells containing non transformed cells, as a result of the low initial concentration of kanamycin used for selection.

Molecular confirmation of integration of the *nptII* gene in the plant genome was done by PCR with specific primers. We decided to perform only PCR analysis because we intended to repeat the PCR assays at least four times during the growth of the plantlets. To eliminate false-positive amplification products due to bacterial contamination, the positive samples 18 and 25 were amplified with primers for the bacterial kanamycin gene, which is located outside the T-DNA borders. Figure 8 presents the results of the PCR assay. To the left of the standards (2300–560 bp) (lanes 1–3) are the products of the samples amplified with primers for the eukaryotic *nptII* gene, and at the right side (lanes 5 to 8) are the amplification products of samples amplified with primers for procaryotic *nptII* gene to detect contaminations. Lanes 1 and 2 show the amplification product of samples 18 and 25, respectively, which correspond to DNA-positive samples, extracted from plantlets growing on media with 150 mg/l kanamycin for more than 6 months. They show the same band (460 bp) corresponding to the relevant sequence, as the positive control, the plasmid preparation (lane 3). *Agrobacterium* contamination was not detected for the positive samples (lanes 5 and 6 in comparison with the plasmid preparation with a PCR product corresponding to a band of 700 bp in lane 8).

The regeneration system proved to be quick and efficient for the development of transgenic shoots. The first PCR analysis was performed from shoots that were growing in media with kanamycin, 6 months after transformation. The subsequent DNA extractions and PCR amplifications gave positive results. Although hypocotyls are considered to be undefined genetically material, it is very important to have improved fungus-resistant plant material to be used as rootstocks for reforestation programmes, as the roots are the main target of *Phytophthora* species.

These results constitute, to the best of our knowledge, the first report on the production of transformed chestnut plants using *Agrobacterium*-mediated systems. Although the transformation efficiency obtained was low, the avail-

Fig. 3 Negative Control of X-Gluc treatment (×5)

Fig. 4 Shoot with aberrant leaves growing on medium containing 150 mg/l kanamycin. This shoot displayed a PCR-negative reaction, and was considered as an escape (×4)

Fig. 5 Shoot with normal growth on medium with 150 mg/l kanamycin. This shoot showed integration of the *npt* II gene in the plant genome (×4)

Fig. 6 Blue shoot regenerant after X-Gluc treatment (×5)

Fig. 7 Shoot showing a heterogeneous pattern of GUS staining activity (×4)

Fig. 8 PCR products to show the integration of the *npt*II gene into the chestnut plant genome. Primers to the left side of the standards (*lanes 1–3*) are within the eukaryotic *npt* II gene. Primers to the right of the standards (*lanes 5–8*) are within the prokaryotic *npt*II gene, which is located outside the border regions of the DNA and help detect *Agrobacterium* contaminants (*lanes 1, 2* amplification products of the positive samples 18 and 25; *lane **3*** positive control – plasmid preparation for the eukaryotic *npt*II gene with an expected band size of 460 bp; *lane 4* standard - *Hin*dIII λ digest (2300–560 bp); *lanes 5 and 6* amplification products of samples 18 and 25, respectively; *lane 8* positive control – plasmid preparation for the prokaryotic *npt*II gene with an expected band size of 700 bp)

ability of well-established micropropagation and rooting protocols for chestnut enables the multiplication and acclimation of these transgenic plants and further study of the stability of the introduced genes. These data should be considered as preliminary, and we are already attempting to achieve higher transformation rates and fewer escapes using other selection pressure schemes, other constructs with different selection genes, such as the *bar* gene, and other explants such as stem segments, so that we can work with mature material. The availability of reliable and efficient genetic transformation and regeneration systems opens the possibility to transfer useful genes into *C. sativa* plants.

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