

Development of highly efficient genetic transformation protocols for table grape Sagraone and Crimson Seedless at low *Agrobacterium* density

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Abstract Highly efficient genetic transformation protocols and the regeneration of transgenic plants of Sagraone and Crimson Seedless grapevines (*Vitis vinifera* L.) were achieved from embryogenic calli co-cultured with low *Agrobacterium tumefaciens* densities. The sensitivity of embryogenic cultures to kanamycin, as well as the effect of *Agrobacterium* strains, C58(pMP90) or EHA105, and the bacterial concentration (0.06 or 0.2 at Optical Density OD₆₀₀) on transformation efficiency were studied. Embryogenic cultures showed different kanamycin sensitivities and the total suppression of embryo differentiation at 20 and 50 mg/l kanamycin for Crimson Seedless and Sagraone, respectively. *sgfp* gene expression was evaluated in callus co-cultured with each bacterial strain. Although GFP transient expression was higher with *A. tumefaciens* EHA105 in both cultivars at the beginning of the culture, there were no significant differences 28 days post-inoculation. However, the concentration of *Agrobacterium* did affected transformation efficiency: 0.06 OD₆₀₀ being more effective for the transformation of Crimson Seedless and 0.2 OD₆₀₀ for Sagraone. By following the optimised procedure, 21 and 26 independent transgenic plants were

generated from Sagraone and Crimson Seedless respectively, three to five months post-infection. PCR analyses were carried out to verify the integration of the *sgfp* and *nptII* genes into grapevine genome and the stable integration of the *sgfp* gene was confirmed by Southern blot.

Keywords *Vitis vinifera* · Transgenic plants · *Agrobacterium tumefaciens* · Kanamycin · Bacterial concentration

Abbreviations

2,4-D	2, 4-Diclorophenoxyacetic acid
BA	6-Benzyladenine
IAA	Indol-3-acetic acid
<i>sgfp</i>	Green fluorescent protein gene
<i>nptII</i>	Neomycine phosphotransferase II gene
PCR	Polymerase chain reaction
NOSpro/ter	Nopaline synthase promoter and terminator
35Spro	35S <i>Cauliflower mosaic virus</i> promoter
OD	Optical density

Introduction

Genetic engineering for grapevine improvement is a powerful tool for introducing disease resistance, product quality and production efficiency traits without altering the essential characters and identity of the cultivar in question (Kikkert et al. 2001). There

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are two main prerequisites for achieving an efficient *Agrobacterium*-mediated transformation system: (1) the availability of plant regenerative tissue and (2) an efficient transformation protocol.

Since grapevine embryogenic cultures appear to be the best cell source for transgenic plant regeneration (Martinelli 1997; Martinelli and Mandolino 2001), efforts increasingly focus on improving transformation efficiency using embryogenic calli as starting plant material. The efficiency of the transformation protocol depends on many factors, including the grapevine genotype used (Harst et al. 2000; Torregrosa et al. 2002), the selection strategies (Perl et al. 1996; Spielmann et al. 2000; Yamamoto et al. 2000; Legrand et al. 2003; Wang et al. 2005; Fan et al. 2008), the *Agrobacterium* strain (Baribault et al. 1989; Berres et al. 1992; Torregrosa et al. 2002), the culture method (Harst et al. 2000; Oláh et al. 2003a) and whether or not antioxidants are used (Mozsár et al. 1998; Perl et al. 1996; Li et al. 2006). The bacterial concentration is also an important factor affecting transformation efficiency although in few cases were comparisons of bacterial density made (Perl et al. 1996; Agüero et al. 2006). A low plant regeneration rate could also be due to a hypersensitivity-like reaction of the plant tissue to *Agrobacterium* in combination with the treatment used for selection (Perl et al. 1996; Bornhoff et al. 2005). The transformation and regeneration of transgenic grapevine plants from somatic embryos remains a problem (Iocco et al. 2001) at least for some economically important cultivars. Previously we developed an efficient method for somatic embryo recovery and plant regeneration from cvs Crimson Seedless and Sugaone (López-Pérez et al. 2005, 2006).

Crimson Seedless and Sugaone (Superior Seedless®) are two seedless table grape developed by David Ramming and Ron Tarailo (USDA Fruit Genetics and Breeding Research Unit, Fresno, CA) and Sun World International respectively. These two seedless grapes are among the most important and widely appreciated table grape cultivars for supermarkets worldwide because to its excellent eating characteristics. In many areas, young vines of Crimson Seedless and Sugaone have developed a disease condition caused by the presence of some viruses like Grapevine fanleaf virus (GFLV), Grapevine leafroll-associated virus 1, Grapevine leafroll-associated virus 2, Grapevine leafroll-associated virus 3 (GLRaV-1,

GLRaV-2, GLRaV-3), Grapevine virus A (GVA), Grapevine virus B (GVB), and Grapevine fleck virus (GFkV) (Prodan et al. 2003; Ahmed et al. 2004; Digiaro et al. 2006). Moreover, nearly all Crimson Seedless vineyards in warm regions require Ethrel (ethephon) in the véraison for optimum color development, while the application of GA at bloom reduces fruitset and increases berry length and berry weight but also reduces berry color, increases berry shatter, and decreases return fruitfulness (Dokoozlian and Peacock 2001; Avenant and Avenant 2006). The development of specific genetic transformation systems would allow the introduction of genes that confer resistance to biotic or abiotic stresses (Baldoni and Rugini 2002). Moreover, efficient genetic transformation systems are valuable tools for functional genomic studies, now that grape genome has been sequenced by the French-Italian Public Consortium for Grapevine Genome Characterization (Jaillon et al. 2007).

Our aim, therefore, was to determine the most suitable kanamycin concentration as well as the *Agrobacterium* strain and bacterial concentration best suited in order to establish efficient transformation protocols in the seedless table grapevine cultivars Crimson Seedless and Sugaone. To our knowledge, this is the first report that transgenic plants have been regenerated from Crimson Seedless.

Materials and methods

Plant material

Two seedless table grapevine cultivars (Sugaone and Crimson Seedless) were used. Embryogenic calli were induced from immature anthers as previously described by López-Pérez et al. (2005) and were maintained by monthly transfers onto fresh 4/1.3 culture medium.

Kanamycin sensitivity of embryogenic cultures

In order to establish a kanamycin concentration that completely inhibits somatic embryo differentiation, embryogenic calli were cultured onto petri dishes (50 × 15 mm) with 10 ml ½ MSAC medium (López-Pérez et al. 2005) with different concentrations of this antibiotic (5, 10, 20, 30, 40 and 50 mg/l).

As controls, embryogenic calli cultured onto $\frac{1}{2}$ MSAC medium, with or without cefotaxime (300 mg/l) were used. Twenty calli were used for each treatment and experiment was repeated twice. Cultures were maintained in the dark at 25°C. After 15 and 30 days, the percentage of calli that differentiated somatic embryo was scored for each treatment.

Bacterial strains and plasmid

Agrobacterium tumefaciens strains C58(pMP90) (Koncz and Schell 1986), a disarmed derivative of C58, and EHA105 (Hood et al. 1993), a disarmed derivative of A281, both carrying the binary plasmid pBin19-*sgfp* (Chiu et al. 1996), were used for in vitro transformation of grapevine embryogenic callus. The T-DNA of pBin 19-*sgfp* contains the NOSpro-*nptII*-NOSter cassette, which is used as a selectable marker, and the 35Spro-*sgfp*-35Ster cassette, which is used as a reporter marker (Ghorbel et al. 1999).

Effect of *Agrobacterium tumefaciens* strain and bacterial concentration

Bacteria were cultured overnight in an orbital shaker at 240 rpm and 28°C in LB medium with 100 μ M aceto-syringone (AS), 25 mg/l kanamycin and containing 25 mg/l nalidixic acid for EHA105/pBin19-*sgfp* or 20 mg/l gentamicin sulphate for C58 (pMP90)/pBin19-*sgfp*. Cells were centrifuged at 3,500 rpm for 10 min and resuspended to a final OD₆₀₀ of 0.2 or 0.06, in liquid MS medium plus AS (100 μ M). Twelve millilitre of *Agrobacterium* suspension were added to 0.08 g of embryogenic callus cultured on $\frac{1}{2}$ MSAC medium and incubated for 10 min. Excess bacteria were removed and calli were co-cultured for 48 h in dark at 26°C, washed with 12 ml of MS with cefotaxime (900 mg/l) for 20 min and cultured in a thin layer on sterile filter paper (Whatman N° 2) onto $\frac{1}{2}$ MSAC medium with cefotaxime (300 mg/l). At 10 d, the calli were transferred to the same culture medium with kanamycin. Six calli per table grapevine genotype and treatment were used and the experiments were repeated twice. The number of GFP spots (individual cells or multicellular aggregates) were counted periodically during a month and expressed as number of GFP spots per gram of callus (López-Pérez 2006).

Genetic transformation and plant regeneration

Embryogenic calli from the two cvs. were transformed with *Agrobacterium tumefaciens* EHA105/pBin19-*sgfp* at an OD₆₀₀ of 0.2 for Sugaone, and 0.06 for Crimson Seedless, following the protocol described above. Ten days post co-cultivation, calli were transferred to $\frac{1}{2}$ MSAC with 20 mg/l kanamycin for Crimson Seedless or 50 mg/l kanamycin for Sugaone. The cultures were maintained in the dark at 26°C and subcultured to fresh medium every 30 days.

The regeneration protocol was as described previously by López-Pérez et al. (2005). Putative transgenic somatic embryos (1–2 mm) from different transformation events, were isolated and transferred individually onto $\frac{1}{2}$ MSAC with cefotaxime (300 mg/l) but without kanamycin and maintained in the dark at 25°C for 20 to 30 days. Somatic embryos were then transferred onto germination medium (López-Pérez et al. 2006) with cefotaxime (300 mg/l) and maintained at 25°C and 16 h photoperiod with a photon flux density of 45 μ mol m⁻² s⁻¹ provided by GroLux fluorescent tubes (Sylvania). Germinated somatic embryos were cultured into test tubes with half strength MS medium and cefotaxime (300 mg/l) to regenerate plants. Plants were micropropagated on the same culture medium in 300 ml glass pots without cefotaxime.

Calli were examined periodically under a Leica MZ16F fluorescence stereomicroscope with a GFP2 filter. The light source was provided by a HBO 100.W high-pressure mercury lamp. The red autofluorescence from chlorophyll was not blocked with any interference filter. Photographs were taken using a digital camera Canon Power Shot S50.

For each condition and cultivar, six calli (0.08 g fresh weight per callus) were used. Number of GFP spots (expressed as number of GFP positive spots per gram of callus) and the number of differentiated transgenic somatic embryos were counted for each condition. The experiment was repeated twice. Data was transformed using \sqrt{x} before statistical analysis to stabilize the variance.

Acclimatization of plants: Well rooted and elongated plants were transplanted into 10 cm pots with a mixture of 50% peat and 50% perlite, covered with a plastic bag and incubated in a chamber under constant conditions: 16 h photoperiod, 27 \pm 1°C for 2 weeks and then, the plastic bag was gradually raised before being completely removed.

PCR and Southern blot analyses

Genomic DNA was isolated from young leaves according to Lodhi et al. (1994). Standard PCR techniques were employed to detect the presence of the *sgfp* and *nptII* genes in leaf samples from the regenerated putative transgenic plantlets. The primer pairs used were 5'-ATGGTGAGCAAGGGCGAGGA and 5'-GGACCATGTGATCGCGCTTC that amplified a 650 bp fragment of the *sgfp* gene and 5'-GACGAGGCAGCGCGCTAT and 5'-AAGAAGGC GATAGAAGGCGA that amplified a 600 bp fragment of the *nptII* gene (Ghorbel et al. 1999). PCR reactions were performed in 12 µl volume containing 4 ng DNA, 200 µM dNTPs, 3 mM MgCl₂, 50 mM KCl, 20 mM Tris-HCl pH: 9.0, 0.25 µM of each primer, and 0.24U of Taq DNA polymerase (Ecogen). Reactions were subjected to 35 cycles of 30 s at 95°C, 30 s at 55°C and 1 min at 72°C. Amplified DNA was detected by UV after electrophoresis on agarose gel (1% w/v) with ethidium bromide.

Southern blot analyses were performed to confirm the stable integration of the *sgfp* gene and to determine the number of integration events in the transgenic plants. DNA samples (20 µg) were digested with *Bam*HI, separated on 1% (w/v) agarose gels and blotted onto nylon membranes positively charged (Hybond, Roche). Following the transfer, the DNA was UV cross-linked to the membrane prior to overnight hybridization with a DNA DIG-labelled probe (20 ng/ml) from the coding region of the *sgfp* gene obtained after a PCR with the above described primers according to the manufacturer instructions.

Results and discussion

This report focuses on the effect of the *Agrobacterium* strain and bacterial concentration on the transformation efficiency and the optimum kanamycin concentration for selecting the transgenic cells using embryogenic calli of two table grape cultivars as starting material.

Sensitivity of embryogenic cultures to kanamycin

Different selection strategies have been followed to improve transgenic plant regeneration efficiency. Kanamycin is commonly used to select grapevine

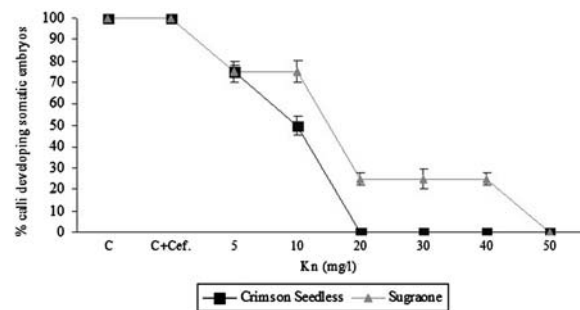


Fig. 1 Kanamycin sensitivity of embryogenic calli of Sugaone and Crimson Seedless at 30 d of culture. Percentages of calli with somatic embryo differentiation

transformed cells and plants but the sensitivity of grape tissues is highly variable to this antibiotic. Thus, it is important to determine the sensitivity of each particular species, cultivar or explant to this antibiotic to develop a new efficient transformation system.

In the present work, clearly differentiated somatic embryos for both cultivars could be observed in both control media (C and C + cef) after 15 and 30 d of culture with no differences among them. It was observed that inhibitory concentration of kanamycin depended on the cultivar. As Fig. 1 shows, Crimson Seedless embryogenic tissue was much more sensitive to kanamycin than Sugaone. Total suppression of embryo differentiation was achieved with 20 mg/l of kanamycin in calli of Crimson Seedless and with 50 mg/l in calli of Sugaone. These results are in contrast with those previously reported by Perl et al. (1996) who observed that 50–500 mg/l kanamycin was unable to inhibit embryo regeneration from Superior Seedless (Sugaone). Colby and Meredith (1990) inhibited callus formation, root initiation, and adventitious shoot formation from leaves and stem segments by using 20, 10 and 7 mg/l kanamycin, respectively but no differences were observed between the different genotypes. Vidal et al. (2003) used 15 mg/l kanamycin to select transgenic plants of *V. vinifera* Chardonnay. In contrast, it has been reported that only high concentrations of kanamycin (80–100–150 mg/l) were clearly lethal for somatic embryos from *V. rupestris* (Martinelli and Mandolino 1994), from seedless grapes (Gölles et al. 1997) or from other *V. vinifera* cvs. (Torregrosa et al. 2000).

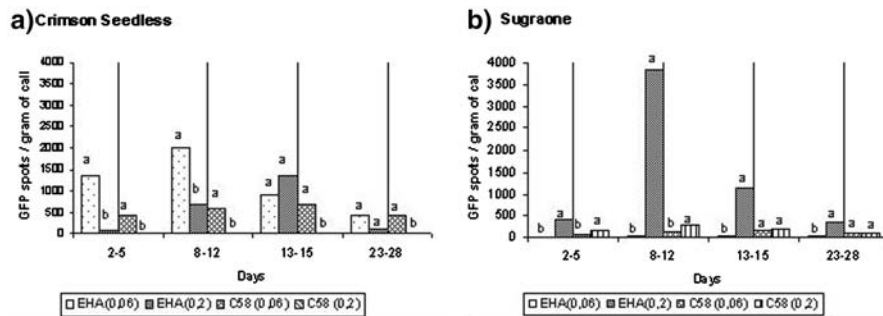


Fig. 2 Number of GFP spots per gram of callus in Crimson Seedless (**a**) and Sugaone (**b**) embryogenic calli transformed with two *Agrobacterium tumefaciens* strains (EHA105-pBin19-*sgfp* and C58(pMP90)-pBin19-*sgfp*) at two bacterial

concentrations (OD₆₀₀: 0.06 and 0.2). Per each bacterial strain and concentration the same letter means no statistical difference at $P < 0.05$ by LSD test

Effect of *Agrobacterium tumefaciens* strain and bacterial concentration on the transformation efficiency

Differences in the transient GFP expression were observed for each *Agrobacterium* strain, bacterial concentration and cultivar (Figs. 2, 3a shows GFP transient expression in calli of Crimson Seedless, 2–5 days post co-cultivation).

In general, GFP transient expression (number of spots expressing GFP) was higher in calli infected with EHA105 than with C58(pMP90) for both cultivars. Nearly 2,000 and 4,000 GFP spots per gram of callus being obtained 8–12 days post co-cultivation in Crimson Seedless and Sugaone, respectively. The number of GFP spots increased during the first 8–12 days and then gradually decreased. This could be correlated with the culture of calli on selective medium with kanamycin at 10 d post-infection as previously described with other reporter genes (Vidal et al. 2003).

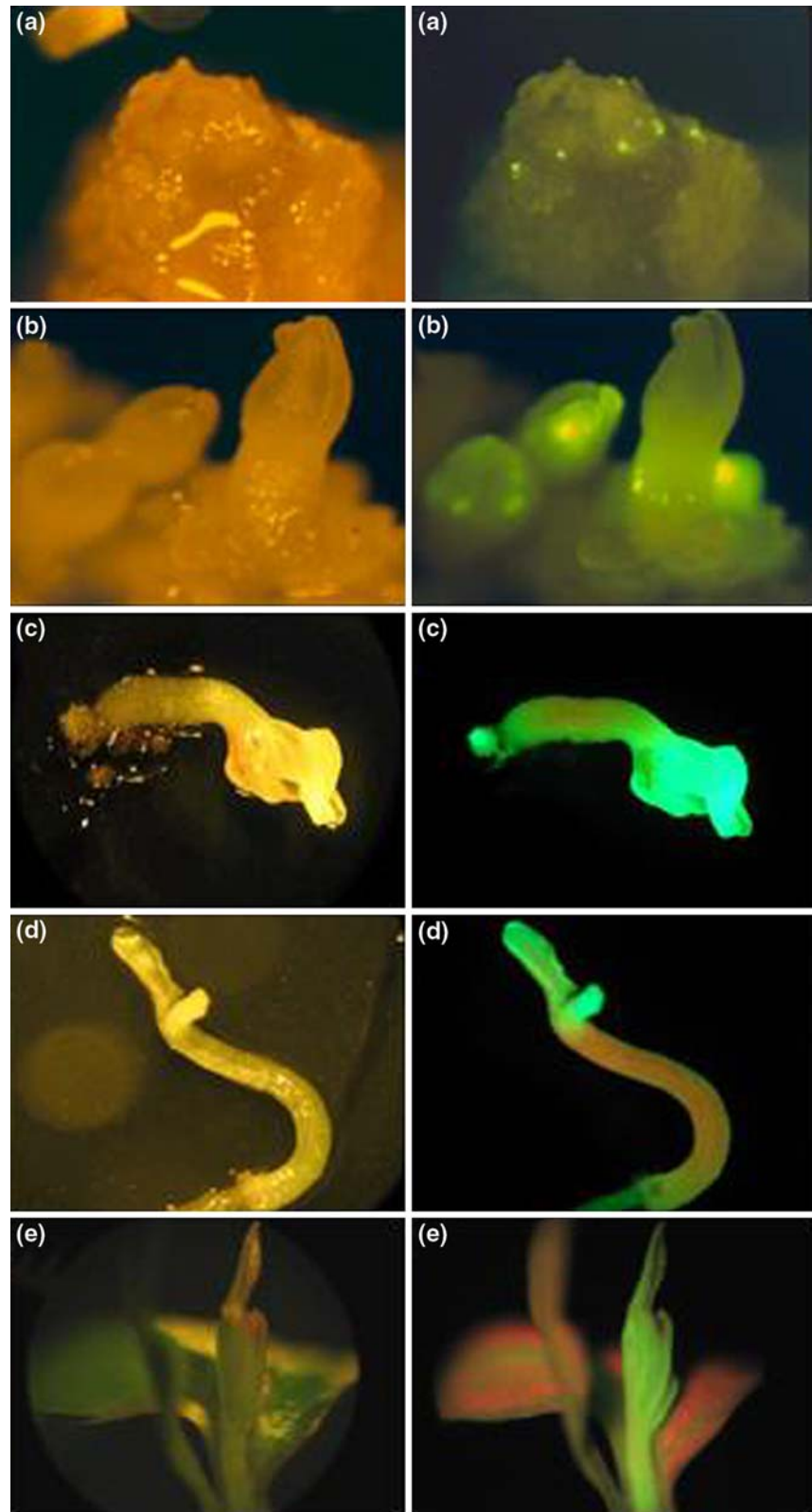
For Crimson Seedless, a bacterial concentration of 0.06 at OD₆₀₀ (independently of strain used) increased the number of GFP spots, while for Sugaone a concentration of 0.2 was the most suitable. This suggests that Crimson Seedless is a much more sensitive cultivar to the *Agrobacterium* concentration than Sugaone. At 28 d post co-cultivation (stable expression of GFP), the best results were observed with EHA105 at an OD₆₀₀ of 0.06 for Crimson Seedless (424.4 GFP spots/g) and at an OD₆₀₀ of 0.2 for Sugaone (349.7 GFP spots/g).

The effect of the *Agrobacterium tumefaciens* strain on the grapevine transformation has been described

previously (Baribault et al. 1989; Berres et al. 1992). Torregrosa et al. (2002) reported that the supervirulent strain EHA105 increased transformation efficiency (6.0 to 137.08 number of positive units/plate, 5wk after co-cultivation, depending on the grapevine cultivar used) compared to the widely used strain LBA4404 (2.42 to 37.0 positive units). These observations are in accordance with our results, since strain EHA105, showed better transformation efficiency than strain C58(pMP90) (although differences were not significant). The difference between both strains may be due to the different genetic background of the Ti plasmid from the *Agrobacterium* strains employed (pTiBo542 vs. pTiC58). Similarly, Torregrosa et al. (2002) also observed differences among *V. vinifera* cultivars.

Bacterial concentration is also an important factor affecting the transformation efficiency. Concentrations from 0.4, 0.6 or 1.8 measured as O. D. to 550, 600 or 630 nm (Torregrosa et al. 2002; Bornhoff et al. 2005; Wang et al. 2005) or 10⁸ cfu/ml (Oláh et al. 2003b; Nakajima et al. 2006) have been used, although in few cases were made comparisons of bacterial density. Perl et al. (1996) observed that bacterial cultures of *A. tumefaciens* with different optical densities (0.1–0.7 at O.D.₆₃₀) resulted in plant tissue necrosis and subsequent cell death and only after the inclusion of antioxidant mixtures in the transformation protocols it was possible to regenerate stable transgenic grape plants of Superior Seedless. Li et al. (2006) minimized *Agrobacterium*-induced tissue browning/necrosis by preculturing somatic embryos for 7 d in fresh medium or by adding 1 g/l of dithiothreitol to the post co-cultivation wash

Fig. 3 Crimson Seedless and Sugraone transgenic plants. **(a)** Transient GFP expression 2–5 d post co-cultivation. **(b–d)** Somatic embryos showing stable GFP expression at different stages of development. **(e)** Transgenic Crimson Seedless plant. Left: illuminated by incident light, Right: illuminated by blue light



media. In our case, the use of such low bacterial densities as 0.06 or 0.2 at OD₆₀₀ did not result in tissue necrosis and transgenic plants of both cvs. Sugaone (Superior Seedless) and Crimson Seedless could be regenerated. In preliminary experiments (data not shown) we observed necrosis by co-culturing embryogenic calli with higher concentrations of bacteria (0.5–0.6 at OD₆₀₀).

Agüero et al. (2006) reported that inoculation with 10⁹ cells ml⁻¹ *Agrobacterium* (EHA101) strain resulted in a higher number of selected calli than cultures inoculated with 10⁷ or 10⁸ cells ml⁻¹, independently of the cv. used (Chardonnay and Thompson Seedless). In our case, we observed an interaction between bacterial density and grape cultivar: the highest number of GFP spots was achieved with an optical density of 0.06 (approx. 0.3 × 10⁸ cells ml⁻¹) in Crimson Seedless and 0.2 (approx. 2.2 × 10⁸ cells ml⁻¹) in Sugaone.

Genetic transformation and plant regeneration

Taking into account the optimal bacterial concentration of *Agrobacterium tumefaciens* strain and optimum kanamycin concentration for each grapevine cultivar, we developed two cultivar-specific genetic transformation protocols.

One month after calli co-cultivation with EHA105-pBIN19-*sgfp* *Agrobacterium* strain at 0.06 OD₆₀₀ for Crimson Seedless and 0.2 for Sugaone, independent white somatic embryos of 5–8 mm (Fig. 3b) and with clearly visible apical roots (Fig. 3c, d) were isolated on medium with 20 or

50 mg/l kanamycin, respectively, although not all of them were GFP-positive. In each Petri dish, many brown and smaller somatic embryos without apical roots were observed, indicating that kanamycin selection was effective. The number of white somatic embryos isolated on kanamycin during the selection phase (1–2 months) was 204 for Sugaone and 422 for Crimson Seedless (Table 1). In the germination medium, 142 (69.6%) and 72 (17.1%) embryos from Sugaone and Crimson Seedless, respectively, were able to germinate. Torregrosa et al. (2002) observed that 28% of the isolated *gfp*-expressing embryos developed shoots. In our case, somatic embryos that failed germination, showed morphological abnormalities and no further development was observed as described by Bornhoff et al. (2005) and López-Pérez et al. (2006). From germinated embryos, 22 and 28 independent regenerated plants (15.5 and 38.9% plant regeneration) were obtained for Sugaone and Crimson Seedless respectively, in a period of 3–5 month after transformation (Fig. 3e).

Putative transgenic plants were initially analysed by PCR in order to check for the insertion of both *sgfp* and *nptII* genes (Fig. 4). Most of the regenerated plants were PCR positive for both *sgfp* and *nptII* genes (Table 1) while only 3 plants (one of Sugaone and two of Crimson Seedless) tested positive for the *nptII* gene but not for the *sgfp* gene. This result suggests that the integration of the T-DNA (two linked cassettes) was not always achieved, as mentioned by Yamamoto et al. (2000). Another three plants were negative for both genes and are considered as escapes. Several reasons could explain these

Table 1 Transgenic plant regeneration from embryogenic calli of Sugaone and Crimson Seedless^a

Cultivar	Somatic embryos selected on kanamycin	Germinated embryos	Regenerated plants ^b	Transgenic plants ^c
Sugaone	204	142 (69.6%)	22 (15.5%)	Total 21 (95.5%) gfp+/nptII + 20 (90.9%) gfp-/nptII + 1 (4.5%)
Crimson seedless	422	72 (17.1%)	28 (38.9%)	Total 26 (92.8%) gfp+/nptII + 24 (85.7%) gfp-/nptII + 2 (7.1%)

^a From a total of 1 g of embryogenic callus for each cultivar

^b Percentage with respect to germinated embryos

^c PCR-positive plants from the total number of regenerated plants

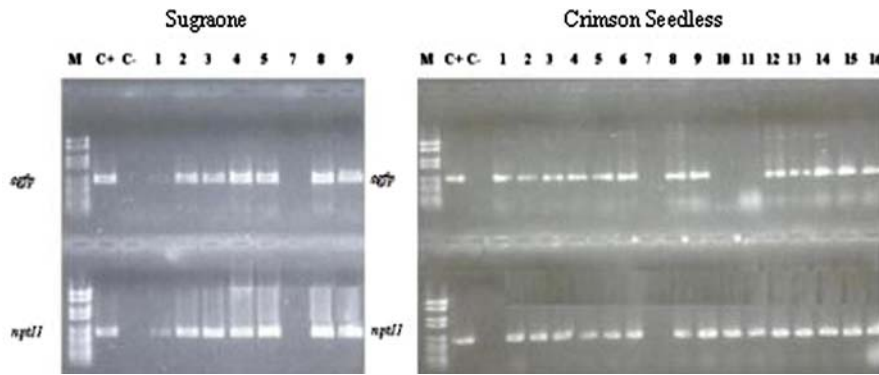


Fig. 4 PCR analysis of regenerated plants for positive signals of *sgfp* and *nptII* genes. Left samples 1–9 correspond to Sugraone regenerated plants and right samples 1–16 correspond to

Crimson Seedless regenerated plants. M: molecular weight marker VI (Roche), C+: positive control pBin19-*sgfp* plasmid, C–: negative control plant

results. As Iocco et al. (2001) described, non-transgenic embryos developing before 10–12 weeks could be attributed to the presence of some advanced multicellular embryos at the time of *Agrobacterium* inoculation, giving rise to untransformed or partly transformed multicellular embryos which may be resilient to the effects of kanamycin. In our case, it was possible that the starting material was a mixture of proembryogenic callus in various stages of early development. Torregrosa et al. (2002) reported that most of the putative structures isolated did not produce *gfp*-expressing shoots, probably because of their chimeric status or because the expression of *nptII* gene was ineffective.

Efficiency of transgenic plant regeneration (measured as the number of PCR positive plants/regenerated plants) was achieved at high levels: in Sugraone, 21 independent transgenic plants (95.5%) were PCR positive (20 with both genes and 1 plant with only *nptII* gene), and in Crimson Seedless 26 independent transgenic plants (92.8%) were positive (24 with both genes and 2 plants with only *nptII* gene).

The stable insertion of *sgfp* gene was confirmed by Southern Blot analysis (Fig. 5). Digestion with *Bam*HI, which has a unique restriction site between the 35S promoter and *sgfp* gene (Fig. 5b), revealed up to three integration events in three Sugraone transgenic plants (Fig. 5a, lanes 3, 4 and 5) and only one integration in the two Crimson Seedless transgenic plants analysed (Fig. 5a, lanes 8 and 9). No hybridization signals were detected in non-transgenic control

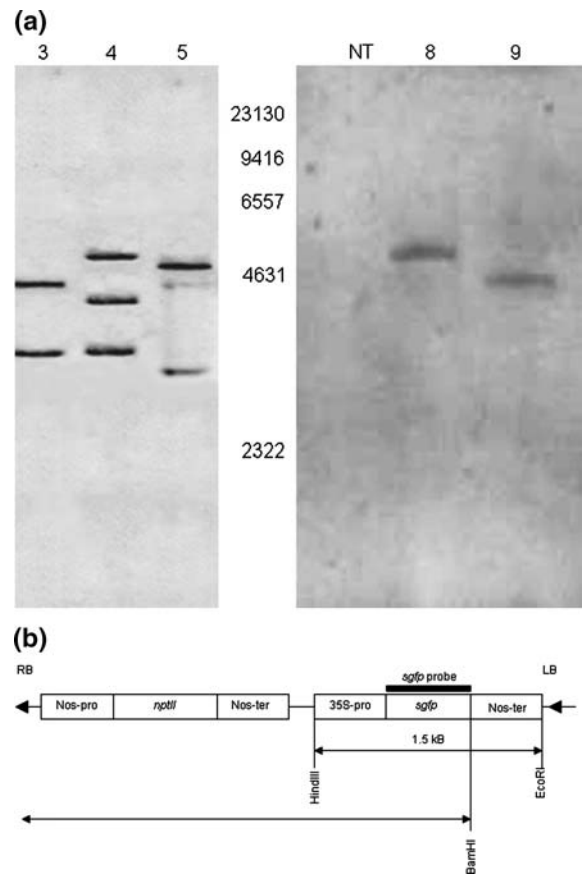


Fig. 5 Southern blot hybridisation for the *sgfp* gene. (a) Right: three transgenic Sugraone plants (3, 4, 5); left: two transgenic Crimson Seedless plants (8, 9). NT: non-transgenic plant. (M) Molecular weight marker II (Roche). (b) Map of the T-DNA of pBin19-*sgfp* with the restriction site for *Bam*HI used to digest plant DNA, and the position of the *sgfp* probe

plants. To our knowledge, this is the first time that transgenic plants of Crimson Seedless have been generated.

For each condition and cultivar, a total of 12 calli, with 0.08 g fresh weight per callus were used for transformation. In preliminary experiments, we used between 0.2 and 1 g fresh weight per embryogenic callus for co-cultivation but the degree of necrosis during the selection phase was high and very few plants were recovered (data not shown). In additional preliminary experiments (data not shown) we cultured the calli grouped in the centre of the Petri plate or spread in a thin layer all over the Petri plate surface. For the first case the necrosis was higher and the recoveries of somatic embryos lower than for the second case. Therefore the amount of tissue used in these experiments spread in a thin layer onto the filter paper over the culture medium was enough to reduce the necrosis and to efficiently recover transgenic somatic embryos. Thus, the efficiency of regenerated transgenic plants was high and ranged between 21 and 26 plants per gram of callus for Sagraone and Crimson Seedless respectively. These results were slightly higher than those obtained by Perl et al. (1996) who using 3 g of fresh weight embryogenic callus and an infection density of 0.6 at OD₆₃₀, were able to regenerate 14–20 plants per gram of embryogenic callus of Superior Seedless (on basta or hygromycin selective medium), but only after the inclusion of antioxidant mixtures in the transformation protocol. In our case, it was not necessary to add antioxidants because decreasing the amount of callus and the bacterial density at OD₆₀₀ to 0.2 or 0.06, for Sagraone and Crimson Seedless respectively, greatly reduced the necrotic effects of *Agrobacterium*, as it was also observed by Iocco et al. (2001). Recently, the transformation procedures developed by Fan et al. (2008) based on an appropriate selection method with hygromycine, allow the production of 72% GFP-positive germinated embryos and 38% of transformed embryos regenerated into normal plantlets, achieving 19 PCR-*HPT* II-positive transgenic lines, from which eleven were also positive for STS (stilbene synthase gene).

Transgenic plants were micropropagated, well rooted and elongated plantlets were transplanted to soil and acclimated progressively to phytotron conditions, where they grew normally. In our conditions, the percentage of acclimatization was high, up to 90–95%.

In conclusion, we present specific and highly efficient transformation protocols for two seedless table grapevine cultivars, Crimson Seedless and Sagraone, involving (1) the establishment of a suitable selective kanamycin concentration for each cultivar, 20 mg/l for Crimson Seedless and 50 mg/l for Sagraone; and (2) the use of the appropriate *Agrobacterium* strain (EHA105) and the optimal bacterial concentration for each grapevine cultivar: OD₆₀₀ at 0.06 or 0.2, respectively. Moreover, the use of a small amount of calli for co-cultivation and their culture on a thin layer reduced the necrosis of embryogenic tissues allowing a higher recovery of plants (data not shown). Following these protocols, in our experimental conditions, we achieved 21 (95.5%) and 26 (92.8%) independent transgenic Sagraone and Crimson Seedless plants from a total of 22 and 28 regenerated plants, respectively.

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References

- Agüero CB, Meredith CP, Dandekar AM (2006) Genetic transformation of *Vitis vinifera* L. cvs Thompson Seedless and Chardonnay with the pear PGIP and GFP encoding genes. *Vitis* 45(1):1–8
- Ahmed HMH, Digiario M, Martelli GP (2004) Viruses and virus diseases of grapevine in Egypt. *EPPO Bull* 34:395–398
- Avenant JH, Avenant E (2006) The effect of ethephon on berry colour of Crimson Seedless and Ebony Star table grapes. *Acta Hort* 727:381–388
- Baldoni L, Rugini E (2002) Genetic modification of agronomic traits in fruit crops. In: Valpuesta V (ed) *Fruit and vegetable biotechnology*. Woodhead Publishing Ltd., Cambridge, England, pp 23–113
- Baribault TJ, Skene KGM, Scott NS (1989) Genetic transformation of grapevine cells. *Plant Cell Rep* 8:137–140
- Berres R, Otten L, Tinland B, Malgarini-Clog E, Walter B (1992) Transformation of *Vitis* tissue by different strains of *Agrobacterium tumefaciens* containing the T-6B gene. *Plant Cell Rep* 11:192–195
- Bornhoff BA, Harst M, Zyprian E, Töpfer R (2005) Transgenic plants of *Vitis vinifera* cv. Seyval blanc. *Plant Cell Rep* 24:433–438
- Chiu W, Niwa Y, Zeng W, Hirano T (1996) Engineered GFP as a vital reporter in plants. *Curr Biol* 6:325–330

- Colby SM, Meredith CP (1990) Kanamycin sensitivity of cultured tissues of *Vitis*. *Plant Cell Rep* 9:237–240
- Digiario M, Fiore N, Tarricone L, Prodan S, Elbeaino T (2006) Influence of viruses on the performance and quality of cv. Crimson seedless. 15th Meeting of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine (ICVG Conf), Stellenbosch, South Africa, pp. 186–188
- Dokoozlian N, Peacock B (2001) Gibberellic acid applied at bloom reduces fruit set and improves size of ‘Crimson Seedless’ table grapes. *HortScience* 36:706–709
- Fan C, Pu N, Wang X, Wang Y, Fang L, Xu W, Zhang J (2008) *Agrobacterium*-mediated genetic transformation of grapevine (*Vitis vinifera* L.) with a novel stilbene synthase gene from Chinese wild *Vitis pseudoreticulata*. *Plant Cell Tiss Organ Cult* 92:197–206
- Ghorbel R, Juárez J, Navarro L, Peña L (1999) Green fluorescent protein as a screenable marker to increase the efficiency of generating woody fruit plants. *Theor Appl Genet* 99:350–358
- Gölles R, da Câmara-Machado A, Tsoleva V, Bouquet A, Moser R, Lopes MS, Mendonca D, Katinger H, Laimer da Câmara-Machado M (1997) Transformation of somatic embryos of *Vitis* sp. (grapevine) with different constructs containing nucleotide sequences from nepovirus coat protein genes. *Acta Hortic* 447:265–272
- Harst M, Bornhoff BA, Zyprian E, Töpfer R (2000) Influence of culture technique and genotype on the efficiency of *Agrobacterium*-mediated transformation of somatic embryos (*Vitis vinifera*) and their conversion to transgenic plants. *Vitis* 39:99–102
- Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993) New *Agrobacterium* helper plasmids for gene transfer to plants. *Transgenic Res* 2:208–218
- Iocco P, Franks P, Thomas MR (2001) Genetic transformation in mayor wine grape cultivars of *Vitis vinifera* L. *Transgenic Res* 10:105–112
- Jaillon O, Aury JM, Noel B et al (2007) The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. *Nature* 449(7161):463–467
- Kikkert JR, Thomas MR, Reisch BI (2001) Grapevine genetic engineering. In: Roubelakis-Angelakis KA (ed) *Molecular biology & biotechnology of the grapevine*. Kluwer Academic Publishers, The Netherlands, pp 393–410
- Koncz C, Schell J (1986) The promotor of TL-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. *Mol Gen Genet* 204:383–396
- Legrand V, Dalmayrac S, Latché A, Pech JC, Bouzayen M, Fallot J, Torregrosa L, Bouquet A, Roustan JP (2003) Constitutive expression of Vir-ERE gene in transformed grapevines confers enhanced resistance to eutypine, a toxin from *Eutypa lata*. *Plant Sci* 164:809–814
- Li ZT, Dhekney S, Dutt M, Van Aman M, Tattersali J, Kelley KT, Gray DJ (2006) Optimizing *Agrobacterium*-mediated transformation of grapevine. *In Vitro Cell Dev Biol Plant* 42:220–227
- Lodhi MA, Ye G-N, Weeden NF, Reisch BI (1994) A simple and efficient method for DNA extraction from grapevine cultivars, *Vitis* species and *Ampelopsis*. *Plant Mol Biol Rep* 12:6–13
- López-Pérez AJ (2006) Desarrollo de protocolos de regeneración de plantas vía embriogénesis somática y de transformación de vid (*Vitis vinifera* L.). PhD Thesis. Universidad de Murcia. Spain
- López-Pérez AJ, Carreño J, Dabauza M (2006) Somatic embryo germination and plant regeneration of three grapevine cvs: Effect of IAA, GA₃ and embryo morphology. *Vitis* 45:141–143
- López-Pérez AJ, Carreño J, Martínez-Cutillas A, Dabauza M (2005) High embryogenic ability and plant regeneration of table grapevine cultivars (*Vitis vinifera* L.) induced by activated charcoal. *Vitis* 44:79–85
- Martinelli L (1997) Regeneration and genetic transformation in the *Vitis* Genus. Ph. D. thesis for the award of the degree of Doctor of Agricultural and Environmental Sciences at the Agricultural University of Wageningen, The Netherlands
- Martinelli L, Mandolino G (1994) Genetic transformation and regeneration of transgenic plants in grapevine (*Vitis rupestris* S.). *Theor Appl Genet* 88:621–628
- Martinelli L, Mandolino G (2001) Transgenic grapes (*Vitis* species). In: Bajaj YPS (ed) *Biotechnology in Agriculture and Forestry* 47: 325–338 Springer-Verlag, Berlin
- Mozsár J, Viczián O, Süle S (1998) *Agrobacterium*-mediated genetic transformation of an interspecific grapevine. *Vitis* 37:127–130
- Nakajima I, Matsuta N, Yamamoto T, Terakami S, Soejima J (2006) Genetic transformation of ‘Kyoho’ grape with a GFP gene. *J Jpn Soc Hortic Sci* 75(2):188–190
- Oláh R, Szegedi E, Ruthner S, Korbuly J (2003a) Optimization of conditions for regeneration and genetic transformation of rootstock- and scion grape varieties. *Acta Hortic* 603:491–497
- Oláh R, Szegedi E, Ruthner S, Korbuly J (2003b) Thidiazuron-induced regeneration and genetic transformation of grapevine rootstock varieties. *Vitis* 42:133–136
- Perl A, Lotan O, Abu-Abied M, Holland D (1996) Establishment of an *Agrobacterium*-mediated transformation system for grape (*Vitis vinifera* L.): The role of antioxidants during grape-*Agrobacterium* interactions. *Nat Biotechnol* 14:624–628
- Prodan S, Montealegre J, Aballay E, Pino AM, Fernández P, Reyes R, Fiore N (2003) Report of new viral diseases in Chilean grapevines. 14th meeting of the international council for the study of virus and virus-like diseases of the grapevine (ICVG Conf), Locorotondo, p 145
- Spielmann A, Krastanova S, Douet-Orhant V, Gugerli P (2000) Analysis of transgenic grapevine (*Vitis rupestris*) and *Nicotiana benthamiana* plants expressing an Arabis mosaic virus coat protein gene. *Plant Sci* 156:235–244
- Torregrosa L, Péros JP, Lopez G, Bouquet A (2000) Effect of hygromycin, kanamycin and phosphinothricin on the embryogenic callus development and axillary micro-propagation of *Vitis vinifera* L. *Acta Hortic* 528:401–406
- Torregrosa L, Iocco P, Thomas MR (2002) Influence of *Agrobacterium* strain, culture medium, and cultivar on the transformation efficiency of *Vitis vinifera* L. *Am J Enol Vitic* 53:183–190
- Vidal JR, Kikkert JR, Wallace PG, Reisch BI (2003) High-efficiency biolistic co-transformation and plant regeneration of ‘Chardonnay’ (*Vitis vinifera* L.) containing *npt-II*

- and antimicrobial peptide genes. *Plant Cell Rep* 22:252–260
- Wang Q, Li P, Hanania U, Sahar N, Mawassi M, Gafny R, Sela I, Tanne E, Perl A (2005) Improvement of *Agrobacterium*-mediated transformation efficiency and transgenic plant regeneration of *Vitis vinifera* L. by optimising selection regimens and utilizing cryopreserved cell suspensions. *Plant Sci* 168:565–571
- Yamamoto T, Iketani H, Ieki H, Nishizawa Y, Notsuka K, Hibi T, Hayashi T, Matsuta N (2000) Transgenic grapevine plants expressing a rice chitinase with enhanced resistance to fungal pathogens. *Plant Cell Rep* 19:639–646