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Genetic transformation of *Vitis vinifera* L. cvs Thompson Seedless and Chardonnay with the pear PGIP and GFP encoding genes

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Summary

Transgenic plants of *Vitis vinifera* L. cvs. Chardonnay and Thompson Seedless expressing the β-glucuronidase gene (*GUS*) and either the pear polygalacturonase inhibiting protein gene (*PGIP*) or the green fluorescent protein gene (*GFP*) were produced *via* somatic embryogenesis. Various media and culture conditions were tested in order to develop an efficient transformation method. Best results were obtained when embryogenic callus was initiated from anthers cultured on PIV medium and maintained in PT medium. Embryogenic lines of the rootstocks Saint George, 110 Richter and Freedom and from inflorescence primordia of Chardonnay and 110 Richter were also established using the same media. Inoculation with 10^9 cells·ml^{-1} *Agrobacterium* resulted in a higher number of selected calli than cultures inoculated with 10^7 or 10^8 cells·ml^{-1}. Plants were regenerated in a modified WP medium from up to 46 % of the selected callus. Approximately 80 % of the lines expressed *GUS* and either *PGIP* or *GFP* but a low correlation was found between β-glucuronidase and polygalacturonase inhibiting protein activities.

K e y   w o r d s : Grape, somatic embryogenesis, *Agrobacterium*, GUS, Saint George, 110 R, Freedom.

A b b r e v i a t i o n s : NAA, naphthaleneacetic acid; BA, bencylaminopurine; WP, Woody Plant; NOA, 2-naphthoxyacetic acid.

Introduction

Direct genetic modification induced by the introduction of single genes offers the unique opportunity to direct specific changes in existing grape cultivars, to make them more resistant to diseases and pests or to modify their fruit composition. It also offers the unprecedented opportunity to study how grape genes work (MEREDITH and REISCH 1996).

MULLINS *et al.* 1990, working with *Vitis rupestris*, were the first to obtain transgenic grapevines; followed by LE GALL *et al.* (1994), MARTINELLI and MANDOLINO (1994), KRASTANOVA *et al.* (1995), MAURO *et al.* (1995), PERL *et al.* (1996), FRANKS *et al.* (1998) and HANSON *et al.* (1999), who were able to transform wild *Vitis* species, interspecific hybrids and both table and wine varieties of *V. vinifera*. These authors used a wide variety of transformation and regeneration protocols, with some common factors, *e.g.* the use of embryogenic cultures as starting material for transformation and culture initiation from immature anthers.

Genetic transformation of grapes *via* somatic embryogenesis is a complex process that involves induction of somatic embryogenesis, maintenance of the cultures, transformation, selection, and regeneration of plants. The large number of transformable cultivars and rootstocks increases the complexity because genotype differences are observed at all culture steps. But despite these difficulties, several grape cultivars and rootstocks have been transformed successfully with genes that may improve plant health and fruit quality (VIVIER and PRETORIUS 2000).

In this paper we report the transformation of *V. vinifera* L. cvs Thompson Seedless and Chardonnay with either the pear polygalacturonase inhibiting protein encoding gene (*PGIP*) or the green fluorescent protein encoding gene (*GFP*). Our interest in the pear polygalacturonase inhibiting protein (PGIP) resides in the proposed role of PGIPs as components of the general plant defense mechanisms against pathogens (DE LORENZO *et al.* 2001); while the green fluorescent protein (GFP) is used as a tool to evaluate the role of signal sequences for the secretion of transgene products and the characterization of different promoters.

In this report, our primary emphasis is on the development of an efficient protocol for the transformation of these cultivars. Successful transformation *via Agrobacterium tumefaciens* of the cultivars discussed here has been reported previously (MAURO *et al.* 1995, SCORZA *et al.* 1996, FRANKS *et al.* 1998, IOCCO *et al.* 2001, LI *et al.* 2001). However, it is difficult to compare different protocols, particularly the overall efficiency of the system and the number of independent lines produced, because data are shown in various formats and/or cover partial aspects of the technique. The purpose of this paper is to present our results with a detailed description of each step so they can be easily contrasted and used as reference, especially by new groups working in the field.

Material and Methods

I n i t i a t i o n   a n d   m a i n t e n a n c e   o f   e m b r y o g e n i c   c a l l u s   f r o m   d i f f e r e n t   c u l t i v a r s : Inflorescences of *V. vinifera* L. cvs Chardonnay and Thompson Seedless and the rootstock *V. rupestris* Saint George were harvested from April to June 1999 and chilled at 4 °C for up to 20 d. After surface sterilization of the flowers (1.2 % solution of sodium hypochlorite for 20 min), green translucent anthers were excised and cultured on Petri dishes containing NB medium (LE GALL *et al.* 1994) or PIV medium

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(FRANKS et al. 1998). Cultures were incubated in the dark at 24 °C. The same experiment was repeated in 2000 and 2001. The rootstock 110 Richter (110 R) was also included in 1999 and 2001 and the rootstock Freedom in 2001.

Canes of Chardonnay were collected in February 2000 and October 2001. Canes of 110 R were also collected in October 2001. One node segments were surface-sterilized as described above and inflorescence primordia were dissected from buds under the microscope and cultured on PIV medium.

The pre-embryogenic calli that developed on NB medium were sub-cultured monthly on NB or ER (EMERSHAD and RAMMING 1994) media while those placed on PIV medium were sub-cultured monthly either on GS1CA (FRANKS et al. 1998) or PT media (HANSON et al. 1999).

Transformation: Four different transformation vectors (Fig. 1), all in *Agrobacterium tumefaciens* strain EHA 101, were used for transformation. Overnight cultures of the bacteria in LB medium (SAMBROOK et al. 1989, but with 5 g·l^{-1} NaCl) were diluted to 10^7, 10^8 or 10^9 cells·ml^{-1} and supplemented with acetosyringone to a final concentration of 20 µM. Embryogenic calli were placed on a sterile glass fiber filter (GFF) overlaid on co-cultivation medium (Tab. 1). Cultures were inoculated as described by HANSON et al. (1999). The *Agrobacterium* culture was poured over the callus and excess was blotted with sterile Whatman filter paper after 5 min. The callus on GFF was then transferred onto fresh co-cultivation medium. After 48 h in the dark, the callus pieces were sub-divided into small clumps, about 2 mm in diameter, and cultured on selection medium (Tab. 1). Putatively transformed callus sectors that developed from different clumps were considered to arise from independent transformation events and were transferred and maintained as independent lines on WP medium (LLOYD and MCCOWN 1981) supplemented with 600 mg·l^{-1} casein hydrolysate, 100 mg·l^{-1} glutamine, 100 mg·l^{-1} asparagine, 100 mg·l^{-1} arginine, 0.5 µM NAA and 2 µM BA, for embryo differentiation and germination (YORK MOY 1999, pers. comm.). Plants regenerated from putatively transformed embryos were multiplied through single node cuttings on half strength MS medium (MURASHIGE and SKOOG 1962) supplemented with 0.05 µM NAA and transferred to the greenhouse.

Plant analysis: β-glucuronidase (GUS) activity was assayed histochemically in leaves and petioles of *in vitro* plants according to JEFFERSON (1987). Fluorometric GUS analysis was performed using the substrate 4-methyl umbelliferyl glucuronide (MUG). Three hundred mg of leaf tissue was homogenized in 2 ml protein extraction buffer

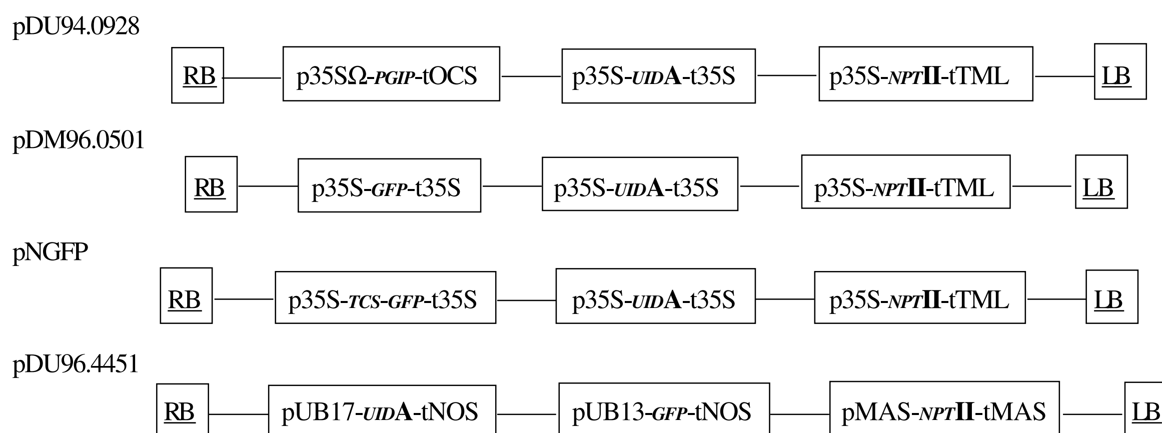

Fig. 1: Schematic representation of the binary plasmids pDU94.0928, pDM96.0501, pNGFP and pDU96.4451, used in this study. Abbreviations: p35S: CaMV 35S promoter; t35S: CaMV 35S terminator; Ω: TMV U1 Ω enhancer; tOCS: octopine synthase terminator; *NPT*II: neomycin phosphotransferase; tMAS: manopine synthase terminator; *PGIP:* pear PGIP gene; *GFP:* synthetic sGFP-TYG gene (MAXIMOVA et al. 1998); *uid*A: β-glucuronidase (GUS) gene; *TCS-GFP:* contains the amino terminal of a ribosome inactivating protein from *T. kirilowii;* pUBI3: ubiquitin 3 promoter; pUBI7: ubiquitin 7 promoter; pMAS: manopine synthase promoter; tTML: tumor morphology large 3' terminator; RB: right border; LB: left border.

Table 1

Composition of the media used for co-cultivation and selection

Previous culture conditions	Co-cultivation medium	Selection medium
PT	PT but lacking activated charcoal and supplemented with 4 µM picloram, 2.3 µM TDZ and 100 µM acetosyringone	PT supplemented with 100 µg·ml^{-1} kanamycin and 300 µg·ml^{-1} cefotaxime
NB	½ MS supplemented with 100 µM acetosyringone	NB supplemented with 100 µg·ml^{-1} kanamycin and 300 µg·ml^{-1} cefotaxime
GS1CA	GS1CA but lacking activated charcoal and hormones; supplemented with 100 µM acetosyringone	GS1CA supplemented with 100 µg·ml^{-1} kanamycin and 300 µg·ml^{-1} cefotaxime

consisting of 50 mM sodium phosphate buffer (pH 7), 10 mM EDTA, 0.1 % triton X-100, 0.1 % sodium lauryl sarcosine and 10 mM 2-mercaptoethanol. Aliquots of the extract were incubated with the MUG substrate. Fluorescence was quantitated using a Hoefer Scientific Instrument fluorometer as described by JEFFERSON (1987).

PGIP activity was determined by the inhibition of endo-PG activity from culture filtrates of *Botrytis cinerea* using the radial diffusion assay in agarose (TAYLOR and SECOR 1988). Samples were prepared by homogenizing 300 mg finely crushed leaves in 2 ml of 0.1 M sodium acetate buffer (pH 5) with 1M NaCl followed by centrifugation at 14,000 rpm for 5 min. GFP fluorescence was visualized with a Nikon fluorescence microscope equipped with illumination from a 100 W mercury lamp passed through a Nikon filter set with a 465 ± 495 nm bandpass excitation filter, a 505 nm dichroic mirror and a 515 ± 555 nm bandpass barrier filter.

Data were statistically analyzed with a one-way analysis of variance (ANOVA) using SAS 6.1 (SAS Institute, Cary, NC) at a significance level of $p < 0.05$. Mean values were compared using the Tukey Test when statistically significant differences were detected.

Results

Anthers produced both pre-embryogenic and non-embryogenic callus on NB and PIV media (Tab. 2). Pre-embryogenic callus was composed of cells arrested at a very early stage of development, with no globular or heart shaped embryos evident. This callus was distinguished by its yellow to light green color and compact structure. In contrast, non-embryogenic callus proliferated to form larger and friable white or transparent cell masses that typically turned brown after some weeks in culture. Tab. 2 shows that total callus formation was very variable from year to year and was not correlated with the rate of embryogenic callus production. In some cases, a high percentage of anthers developed callus but did not produce embryogenic tissue, *e.g.* Cabernet Sauvignon on NB in 1999 or Thompson Seedless on NB in 2000. Across all three years, callus growing on PIV medium had higher embryogenic capacity than that growing on NB medium. Pre-embryogenic calli that developed on PIV medium were smaller and darker than calli growing on NB medium, which were easily recognized by their yellowish cellular aggregates. PIV calli also appeared later, 4 to 7 months after culture initiation. The percentage of anthers that produced embryogenic callus on PIV medium in 1999 was low, *e.g.* no embryogenic callus was obtained from 110 R. For this reason, inflorescence primordia were also used as explants for callus induction in winter 2000 and fall 2001. Interestingly, they developed the same type of embryogenic callus and at similar rates as anthers (Fig. 2, Tab. 3).

Embryogenic calli that developed on NB and PIV medium were maintained on NB and PT media respectively and subcultured monthly. Because some sectors of the callus differentiated to globular or torpedo stages, only callus with pre-embryogenic appearance was retained. Fig. 3 shows that

Table 2

Embryogenic callus growth from immature anthers on NB or PIV media over three consecutive years (R, 110R; SG, Saint George; CH, Chardonnay, TS, Thompson Seedless, CS, Cabernet sauvignon and Fd, Freedom)

	NB			PIV		
	Number of flowers*	% of flowers producing callus	% of flowers producing embryogenic callus	Number of flowers	% of flowers producing callus	% of flowers producing embryogenic callus
1999						
R	480	11.5	0.0	510	24.7	0.0
SG	660	36.5	1.4	300	9.3	3.0
CH	1320	14.8	0.5	1320	4.0	4.0
TS	1050	91.3	0.8	1050	7.7	7.7
CS	1050	88.3	0.0	1110	8.9	0.2
2000						
SG	420	78.1	2.6	449	57.0	5.6
CH	527	71.7	2.5	521	7.9	7.7
TS	540	88.0	0.0	591	73.9	13.9
2001						
SG	494	31.4	7.9	358	18.2	10.1
Fd	482	51.9	7.3	450	59.6	4.0
R	401	14.2	1.0	396	15.2	12.6
CH	429	23.8	8.4	559	16.5	10.9
TS	352	57.4	2.6	450	9.1	8.4

* Anthers from a single flower were excised under the scope and cultured together, consequently each group of anthers was considered a flower.

Table 3

Embryogenic callus growth from inflorescence primordia on PIV media (CH, Chardonnay and R, 110 R)

	Number of inflorescences	% of inflorescences producing callus	% of inflorescences producing enbryogenic callus
CH-Winter 2000	67	55.2	14.9
CH-Fall 2001	65	100	15.4
R-Fall 2001	108	22.2	6.5

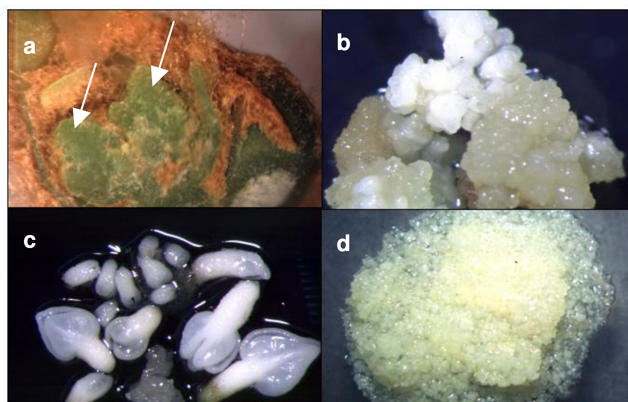

Fig. 2: Embryogenesis induction from inflorescence primordia. Dissected bud with arrows pointing at inflorescence primordia (**a**), pre-embryogenic callus of Chardonnay from inflorescence primordia cultured on PIV medium (**b**); embryos of Chardonnay that developed from callus produced by inflorescence primordia (**c**); pre-embryogenic callus obtained from anthers of Chardonnay and maintained in PT medium (**d**).

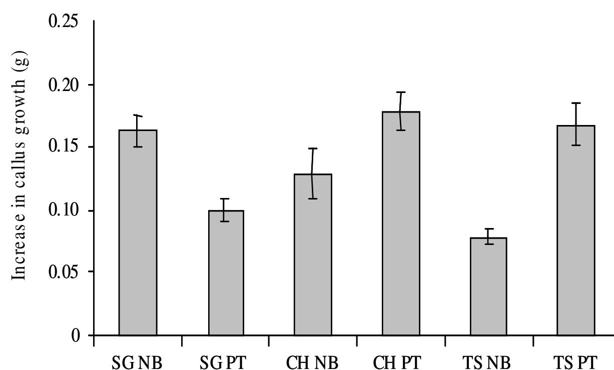

Fig. 3: Growth of embryogenic callus, expressed as increase in fresh weight, after one month of culture in PT or NB medium. Initial weight of the calli was 15 mg. Data presented are means of 10 replicates ± standard error (SG: Saint George; CH: Chardonnay; TS: Thompson Seedless).

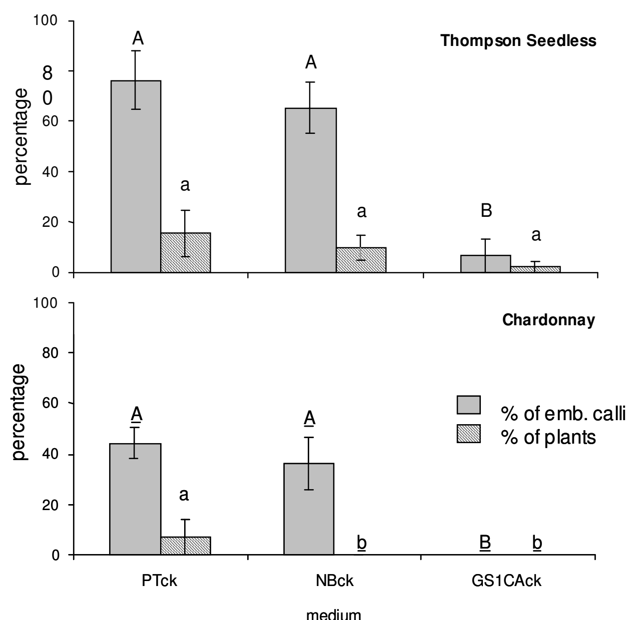

Fig. 4: Percentage of calli and plants growing in PT, NB or GS1CA selection medium containing 100 µg·ml^{-1} kanamycin and 300 µg·ml^{-1} cefotaxime. Calli were inoculated with 10^8 cells·ml^{-1} *Agrobacterium tumefaciens* carrying construct 35SΩ-*PGIP*. When many plants regenerated from a single callus, only one plant was selected. Data presented were obtained from a single experiment and are means of 5-8 replicates ± standard error. Mean values were compared with Tukey Test when statistical differences were detected with ANOVA using SAS 6.1. Means with the same letter are not significantly different.

growth of Chardonnay and Thompson Seedless callus was higher on PT medium, while Saint George callus grew better on NB medium. Attempts to maintain pre-embryogenic callus on ER or GS1CA media was unsuccessful because of gradual depletion of pre-embryogenic calli as a result of embryo differentiation. Pre-embryogenic calli growing on NB or PT medium were successfully transformed with the *PGIP* and *GFP* constructs. For both Chardonnay and Thompson Seedless, the number of calli that grew on selection medium and the number of plants obtained was higher for PT medium compared to NB medium, but the difference between the media was significant only for plants of Chardonnay (Fig. 4). The number of calli selected on GS1CA was significantly lower in Thompson Seedless and zero for Chardonnay.

In an experiment that focused on evaluating the concentration of the *Agrobacterium* suspension during the inoculation process, the inoculations conducted with a cell density of 10^9 cells/ml *Agrobacterium* resulted in a higher number of selected calli compared to those carried out with a cell density of 10^7 and 10^8 cells·ml^{-1} (Fig. 5). Similar results were obtained for both Chardonnay and Thompson Seedless although the percentage of callus that developed in Chardonnay at 10^9 cells·ml^{-1} was lower than the observed for Thompson Seedless. More than half of the inoculated callus pieces continued growing on selection medium but

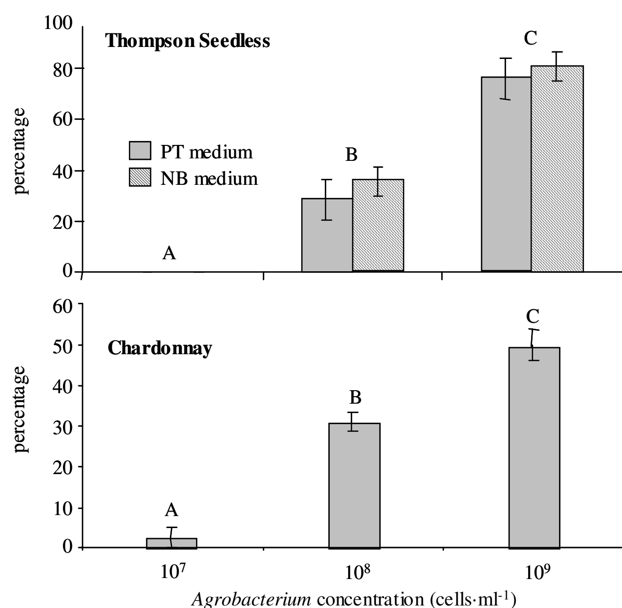

Fig. 5: Percentage of callus pieces growing on selection medium 3 months after inoculation with 10^7, 10^8, 10^9 cells·ml^{-1} of *Agrobacterium tumefaciens* carrying construct 35SΩ-*PGIP*. Data presented are means of 7 replicates ± standard error.

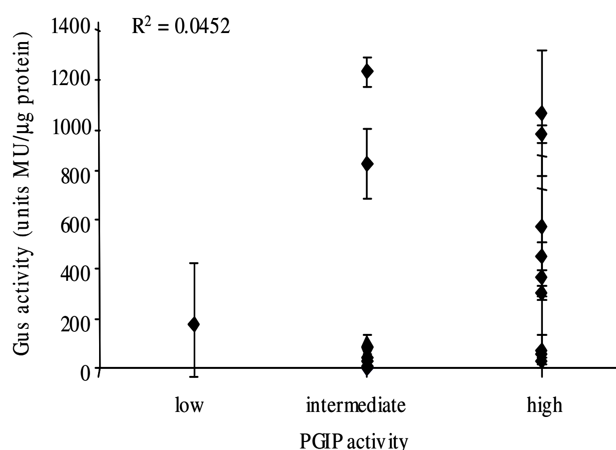

Fig. 6: GUS and PGIP activity measured in leaves of plants transformed with the *PGIP* and *GUS*. Nineteen plants expressing both genes were randomly selected. Leaves were collected from greenhouse plants and divided in two; one half of the blade was analyzed for PGIP activity and the other half for GUS activity. Plants with PGIP activity were divided in three categories according to the level of PG inhibition at 0, 2x and 10x extract dilutions. Lines that only showed activity in non diluted leaf extract were included in the low activity group, while lines that showed activity in 2x and 10x diluted leaf extract were included in the intermediate and high activity groups respectively. Data are means of 2 replicates. Standard deviations are represented by error bars.

the rates of plantlet regeneration from selected callus were considerably lower (Tab. 4), ranging from 16 to 46 % in Thompson Seedless and 0 to 34 % in Chardonnay, depending on the construct used. Most of the selected plants were transgenic (Tab. 4), with approx. 80 % of the plants expressing both genes, *e.g. GUS* and *PGIP* or *GUS* and *GFP*.

When GUS and PGIP activities were measured in randomly selected transgenic lines expressing both *PGIP* and *GUS*, a poor correlation was found. As shown in Fig. 6, some transgenic plants with intermediate or high level of PGIP activity displayed low GUS activity. Plants were also regenerated from callus inoculated with the *GFP* constructs. A strong fluorescence was visible in embryos and plants transformed with the 35S-*GFP* construct. In the transgenic

Table 4

Transformation of the grapevine cultivars Thompson Seedless, Chardonnay and Saint George with different constructs. Data obtained from 5 transformation experiments with callus selected on PT and NB media have been pooled for each of the three cultivars. The number of regenerated plants represents the number of selected calli that produced at least one plant. When many plants regenerated from a single callus, only one plant was selected and further multiplied; the rest were discarded. Numbers in parentheses are percentages of selected calli per total number of inoculated calli and calli producing plants per total number of inoculated calli

Construct	Number of inoculated calli	Number of selected calli (3-4 months after inoc.)	Number of calli producing plants	Number of plants with GUS activity	Number of plants with PGIP or GFP activity	Number of plants with GUS and PGIP or GFP activity
Thompson Seedless						
p35SΩ-*PGIP*	397	262 (66)	43 (11)	37	33	31
p35S-*GFP*	162	119 (73)	55 (34)	44	45	42
pUBI3-*GFP*	178	109 (61)	31 (17)	31	26	26
p35S-*TCS-GFP*	398	260 (65)	92 (23)	79	76	74
Chardonnay						
p35SΩ-*PGIP*	182	102 (56)	17 (9)	14	14	12
p35S-*GFP*	81	50 (62)	17 (21)	15	14	14
pUBI3-*GFP*	87	36 (41)	0 (0)			
p35S-*TCS-GFP*	178	100 (56)	13 (7)	12	11	11
Saint George						
p35SΩ-*PGIP*	6	6 (100)	1 (17)	1	1	1
p35S-*GFP*	7	7 (100)	1 (14)	1	1	1
pUBI3-*GFP*	8	7 (87)	1 (12)	1	1	1

plants, *GFP* expression was observed in leaves, roots and stem sections as expected for a transgene driven by the constitutive CaMV 35S promoter. All the leaves exhibited a uniform expression pattern except 6 lines of Thompson Seedless, which lacked of fluorescence in vascular tissue or displayed areas with different fluorescence intensity. Results obtained with the different GFP contructs will be the subject of a separate paper.

Based on all the results obtained in this study, a schematic flow chart is presented that outlines the main steps in the transformation procedure (Fig. 7).

Discussion

In this paper we report the successful introduction of the pear PGIP and GFP encoding genes into Chardonnay and Thompson Seedless grapevine cultivars following a protocol that involves 6 steps. Pre-embryogenic callus was initiated from anthers on PIV medium or NB media. FRANKS *et al.* (1998) and IOCCO *et al.* (2001) have used PIV medium for the callus initiation of several wine grape cultivars, including Cabernet Sauvignon, Chardonnay and Thompson Seedless. We found that this medium is also effective for rootstocks Saint George, 110 R and Freedom and when using flower primordia of Chardonnay and 110 R as starting material instead of anthers as was previously reported. Efforts to maintain the callus in GS1CA medium were unsuccessful. IOCCO *et al.* (2001) and TORREGROSA *et al.* (2002) also reported that the long-term maintenance on GS1CA medium was difficult because the majority of the callus differentiates into globular or torpedo structures. Nevertheless, we were able to maintain the pre-embryogenic cultures that developed on PIV medium by using PT medium for a year. After approximately a year of continuous subculture on PT, the calli lose their capacity to profilerate in culture and become necrotic. For this reason, and also because it is unclear whether prolonged culture of embryogenic cells would harbor spontaneous mutations or other genetic abnormalities (GRAY and MEREDITH 1992), new cultures were initiated every year. Interestingly, it was possible to apply the same scheme using inflorescence primordia as the initial explant. Bud dissection is laborious but considering the brief period the anthers become available each year, inflorescence primordia

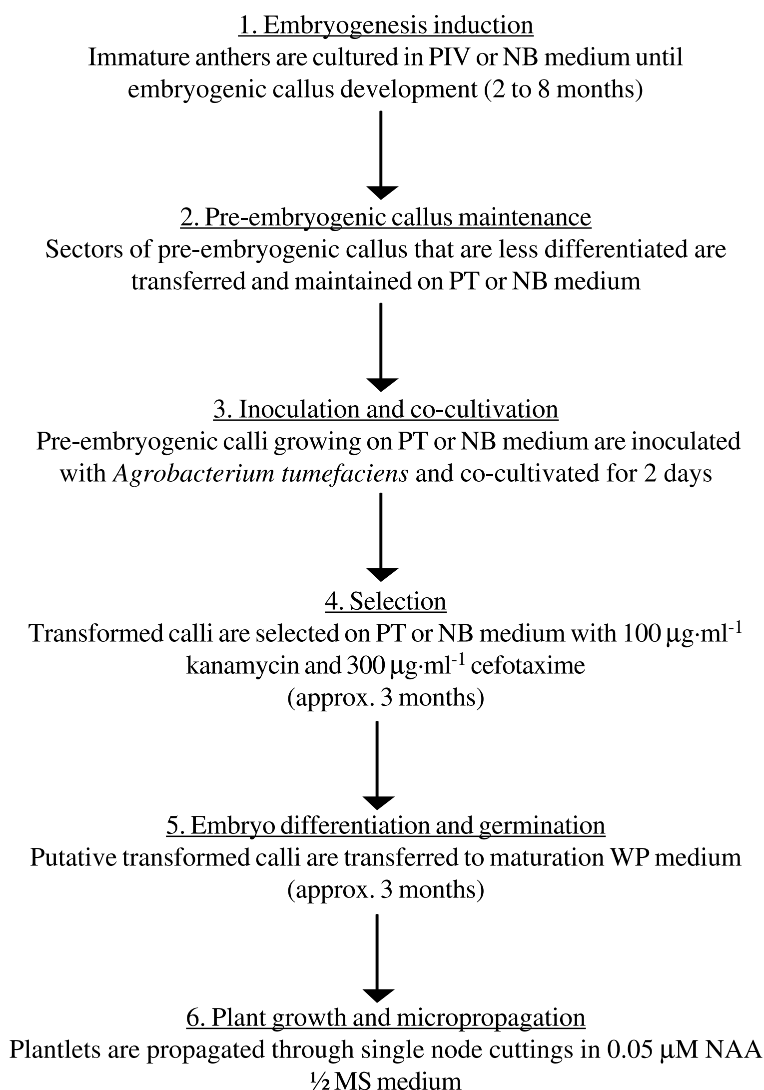

Fig. 7: Flow chart showing the most effective transformation procedure.

culture might constitute a valuable alternative. The embryogenic capacity of this explant confirms the significant potential as initial explants of reproductive organs, since ovaries and ovules have also been previously reported (GOLLES *et al.* 2000, KIKKERT *et al.* 2000, YAMAMOTO *et al.* 2000). Embryogenesis from anthers was also induced on NB medium but it was observed to be less efficient and the pre-embryogenic callus that emerged was slightly more differentiated. However the same medium was found to be appropriate for culture maintenance; callus could be multiplied for more than a year and, at least for Chardonnay, cultures growing on NB medium could be easily multiplied on liquid GM + NOA medium (MAURO *et al.* 1995, data not shown).

Pre-embryogenic callus growing on PT medium proved to be suitable for genetic transformation. It has been emphasized that embryogenic lines consisting of pre-embryogenic masses are preferred for transformation due to their high transformation and selection efficiencies (VIVIER and PRETORIUS 2000). PERL and ESHDAT (1998) have also remarked that transformation efficiency is very high when lines composed of fine cells, arrested in a very early pre-embryogenic state are co-cultivated with *Agrobacterium*. Callus growing on PT medium, and to a lesser extent on NB medium, responded to these characteristics, with a relatively high percentage of the inoculated callus groups growing on selection medium with kanamycin and differentiating into embryos. However, many of them germinated abnormally, showing enlarged hypocotyls and giant, malformed cotyledons. The abnormal development of somatic embryos has been attributed to abnormal apical meristem development (GOEBEL TOURAND *et al.* 1993) and precocious germination (FAURE *et al.* 1998), a process that involves continuous and simultaneous expression of embryogenic and post-embryogenic development programs, which has also been observed in grape immature zygotic embryos cultured *in vitro*. Several strategies have been proposed to avoid abnormal germination that include the daily sub-culturing of embryos onto fresh medium (COUTOS-THEVENOT *et al.* 1992), chilling (RAJASEKARAN *et al.* 1982) , dehydration (GRAY 1989) and section of cotyledons and hypocotyls (IOCCO *et al.* 2001), but none of these treatments has provided an effective solution and more research is needed to improve the efficiency of this step. As an alternative, organogenesis induction of transformed embryos should not be discarded.

The majority of the regenerated plants were found to be transgenic and expressed the transgenes in the entire plant as expected for transgenes driven by the constitutive CaMV 35S promoter but protein activity varied among independent transgenic plant lines. The low correlation between the activity of PGIP and GUS genes residing within the same construct-DNA, indicates that plant selection based on GUS activity alone can lead to plants with high PGIP activity being discarded. Lack of correlation between two transgenes has been reported several times and attributed to transgene silencing (HALPIN *et al.* 2001). In addition, post-translational glycosylation of PGIP may influence its inhibitor activity (POWELL *et al.* 2000) and may differ among independent transgenic lines. Transgenic plants expressing *GFP* and *PGIP* have been multiplied and transferred to the greenhouse for further analysis of transgene expression.

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