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Comparing 17-β-estradiol supply strategies for applying the XVE-*Cre/loxP* system in grape gene transfer (*Vitis vinifera* L.)

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Summary

Assays for enhancing the performance of 17-β-estradiol induction in the XVE-*Cre/loxP* system were performed on two transgenic 'Brachetto' plants obtained with the pX6-pKcpGVA construct, which is derived from the chemical-inducible pX6 vector carrying the neomycin phosphotransferase (*nptII*) gene and the XVE-*Cre/loxP* sequence. The 17-β-estradiol supply is expected to induce *Cre* recombinase expression resulting in *nptII* gene removal. We compared different hormone supply strategies during shoot organogenesis from meristematic proliferative tissue (MPT) or from the cut surface between leaf and petiole (SOLP) or during micropropagation from bud (MB). The effectiveness of the estradiol induction was evaluated on different tissues of the regenerated plantlets by means of *nptII* copy number quantification with Real time PCR. Results showed that the *Cre/loxP* inducible system functions effectively – however with different efficiencies- in both root and leaf tissues, and that micropropagation from buds combined with constant wetting with 17-β-estradiol is the most efficient and reproducible strategy for effective *in vivo* hormone induction.

K e y w o r d s : *V. vinifera,* gene transfer, site-specific DNA excision, marker-free, 17-β-estradiol, Real-time PCR.

A b b r e v i a t i o n s : BA: 6-benzyladenide; CN: Copy number; CNm: mean Copy number; GVA: grapevine virus A; IBA: indole-3-butyric acid; LB: Lennox (1955) medium; MPT: meristematic proliferative tissue; MS: Murashige and Skoog (1962) medium; MB: micropropagated bud; NAA: naphthaleneacetic acid; NN: Nitsch and Nitsch (1969) medium; *nptII:* neomycin phosphotransferase II; PVP: Polyvinylpyrrolidone; SOLP: shoot organogenesis from the cut surface between leaf and petiole.

Introduction

One of the main drawbacks of the technology for gene transfer is the transfer of vector sequences and marker genes into the plant genomes together with the genes of interest. Being the use of marker genes, especially where antibiotics resistance is concerned, a focal point in the debate on GMO safety (European Parliament and Council 2001, European Food Safety Authority 2004), various marker-free techniques for gene transfer, *i.e.* co-transformation, transposition, intrachromosomal and site-specific recombination (Miki and McHugh 2004, Puchta 2003) have been exploited in several plant species. Among these, site-specific recombination has been mostly applied in the production of crops (Ow 2007) whilst the use of inducible promoters to regulate the recombinase gene has also made it possible to apply this technique to perennial woody plants and vegetatively propagated species (Gidoni *et al.* 2008).

Chemically (Zuo *et al.* 2001) and heat-shock inducible (Kumar *et al.* 2009) promoters have mainly been exploited. The XVE system based on 17-β-estradiol induction is one of the most suitable strategies since the inability of plant steroid to act as inducer and the non-volatility of estradiol prevent inadvertent gene activation (Zuo *et al.* 2006).

In the *Vitis* genus, resistance to kanamycin conferred by neomycin phosphotransferase (NPTII) is the most widely applied selection strategy for gene transfer (Vidal *et al.* 2010). The use of alternative traits, such as the positive selection system based on D-xylose ketol-isomerase (Kieffer *et al.* 2004) or phosphomannose isomerase (PMI) (Vaccari and Martinelli 2009) turned out to be unsuitable. The co-transformation system associated with a combination of positive and negative selection has been successfully employed in 'Thompson Seedless' (Dutt *et al.* 2008) but being this method dependent on an efficient regeneration, it might not be extended to other *Vitis* genotypes.

Previous experience has shown the inducible site-specific DNA excision strategy based on the XVE system and 17-β-estradiol supply (Zuo *et al*. 2001) to be a promising method for *nptII* marker gene removal in the *Vitis* genus (Martinelli *et al.* 2009). Efficiency, however, needs to be improved (Dalla Costa *et al.* 2009), as has also been reported for other plant species, where hormone supply during various plant developmental stages has resulted in ineffective or limited induction (Zuo *et al*. 2001, Guo *et al*. 2003, Sreekala *et al*. 2005, Zhang *et al*. 2006).

Given the importance of an effective inducible system in grape for application in functional genomics as well as for undesired exogene removal, in this paper we present the first in-depth study aimed at optimizing the use of the XVE-*Cre/loxP* system in the *Vitis* genus. We concentrated on different 17-β-estradiol supply strategies in various plant morphogenetic systems.

Material and Methods

P l a n t m a t e r i a l , c o n s t r u c t s a n d g e n e t r a n s f e r : Gene transfer was mediated by *Agrobacterium tumefaciens* LBA 4404 carrying the pX6-pK-

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cpGVA construct (Turturo *et al.* 2003, see Fig. 1) which is derived from the chemical-inducible C*re/loxP* pX6 vector (accession no. AF330636) carrying the neomycin phosphotransferase (*nptII*) gene for kanamycin selection and the *Cre* recombinase gene whose expression is regulated by 17-β-estradiol (Zuo *et al.* 2001). In this vector, the GFP gene was replaced with a conserved coat protein sequence (200 bp) of the grapevine virus A (GVA) (Galiakparova *et al.* 2003) in sense and antisense orientations separated by the *pdk* intron (741 bp) for the expression of a hairpin RNA (Fig. 1). *Agrobacterium* co-cultures with embryogenic calli of 'Brachetto', obtained as in Martinelli *et al.* (2001), were performed in GS1CA medium as described in Vaccari and Martinelli (2009). Then, calli were moved onto the same fresh medium added with cefotaxime 300 mg·L^{-1} and after three weeks sub-cultured monthly on GS1CA to which kanamycin and cefotaxime were added in increasing and decreasing concentrations respectively (from 50 mg·L^{-1} to 150 mg·L^{-1} of kanamycin and from 300 mg·L^{-1} to 50 mg·L^{-1} of cefotaxime). Embryos at the torpedo stage were planted on germination medium (Martinelli *et al.* 2001) and the first emerging shoot from each embryo was cut and micropropagated on NN (Nitsch and Nitsch 1969) medium. Germination and micropropagation were induced on antibiotic-free media renewed monthly, at 24 ± 1 °C with a 16 h photoperiod (70 µmol·m^{-2}·s^{-1} cool white lights). Plantlets produced from individual somatic embryos were recorded as distinct plants and used as mother-plants for the further micropropagations.

Induction of nptII removal with 17-β-estradiol: Inductions with 17-β-estradiol for *nptII* removal were carried out on two genetically modified 'Brachetto' plants (named plant 1 and plant 2) converted from two distinct somatic embryos. For the destructive assay, 3 whole leaves, 3 cut-up leaves and the roots were excised from 2 plantlets propagated from plants 1 and 2 (Fig. 3) and incubated in liquid NN medium added with estradiol. For the assay based on shoot organogenesis from meristematic proliferative tissue (MPT) (Fig. 5, A and B), 10 explants of MPT (2 mm length x 2 mm thick) were obtained from 10 nodal sections of plants 1 and 2, according to Cadavid-Labrada *et al.* (2009), modified using NN medium containing 4.5 µM 6-benzyladenide (BA) and 5 µM indole-3-butyric acid (IBA). After estradiol induction in the same liquid medium, explants were moved to the same solidified medium, deprived of this inducer. Merging shoots were dissected and transferred onto solid NN medium for further elongation and rooting. For the tests based on shoot organogenesis from the cut surface between leaf and petiole (SOLP), according to Martinelli *et al.* (1996) (Fig. 5, C and D), 15 buds of plants 1 and 2 were placed on the same medium as MPT. Fifteen leaves from the emerging shoots of each plant were dissected for estradiol induction in liquid NN medium and transferred onto hormone-free half-strength MS solid medium (Murashige and Skoog 1962) containing 1 µM BA and 0.16 µM naphthaleneacetic acid (NAA). The shoots regenerated from the cut region between leaf lamina and petiole were dissected and moved to solid NN medium for elongation and rooting. In all these assays, the liquid induction phase with 17-β-estradiol was carried out by placing the explants for 48 h in 125 ml flasks containing 50 ml of liquid medium to which 17-β-estradiol was added to a final concentration of 20 µM and shaken continuously at 90 rpm. As for the induction assays performed during micropropagation from bud (Fig. 5, E and F), 8 nodes of plants 1 and 2 were planted on solid NN medium containing 20 µM 17-β-estradiol. During the first two weeks of culture each bud was wet daily with 200 µl of liquid NN medium containing 20 µM 17-β-estradiol and silwet L-77 (Lehle Seed) 0.005 %, and was then moved to the same fresh medium. All media were sterilized for 20 min in an autoclave at 121 °C and 1 atm. For solid media, 9 g·l^{-1} agarose was used. Estradiol powder (Sigma-Aldrich) was dissolved in absolute ethanol, stored at 4 °C and added to media after autoclaving. Cultures were maintained in a climate-room at 25 °C and a 16h photoperiod (70 µmol m^{-2}s^{-1} cool white light) in 9 cm diameter plastic Petri dishes, or - for plant micropropagation - in Magenta boxes.

Molecular assays: Genomic DNA was extracted from the plant tissues, according to Doyle and Doyle (1990) modified by the addition of 1 % PVP to the extraction buffer. Southern Blot assays for checking the presence of *nptII* exogene were performed as in Dalla Costa *et al.* (2009) on genomic DNA extracted from 6 transgenic plantlets randomly chosen from 21 plants regenerated from single somatic embryos using a specific probe amplified by PCR. The probe primers, designed with Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi), were: forward, 5′-GATGGATTGCACGCAGGTTC-3′, and reverse, 5′-GGAGCGGCGATACCGTAAAG-3′. The probe was digoxigenin-labeled with the PCR Dig Probe Synthesis Kit (Roche Diagnostics, Switzerland). The end-point PCR amplification with the P1 and P2 primers (see Figure 1) was performed on 100 ng of genomic DNA extracted from the leaves and roots used in the destructive assays. Reactions were carried out with the Thermocycler (Tgradient, Biometra) in a final volume of 25 µL containing GoTaq Green master mix 1X (Promega) and 0.5 µM of each primer designed by the Primer3 software (P1-forward, 5′-AAACTGAAGGCGGGAAACGA-3′, and P2-reverse, 5′-GGCTTTCGCCAACACCAACT-3′). The PCR thermal protocol consisted of an initial denaturing step of 2 min at 95 °C followed by 35 cycles of denaturation, annealing and extension of 30 s at 95 °C, 40 s at 60 °C and 60 s at 72 °C respectively, with a final extension of 5 min at 72 °C. The PCR products (10 µL) were electrophoresed as in Dalla Costa *et al.* (2009). The fragment amplified on root genomic DNA was extracted from the agarose gel using the NucleoSpin Extract II (Machenery-Nagel), and cloned into the pGEM-T vector (Promega) with T4 DNA ligase (Pomega) at a 1:1 vector/insert molar ratio during overnight incubation at 4 °C. The plasmids were transferred to *E. coli* strain JM109 according to the Promega technical manual, purified from the selected bacterial colonies with the QIAprep Spin Miniprep Kit (Qiagen) and sequenced with ABI 3730xl (Applied Biosystem) using the universal primers T7 and SP6 that bind to the vector sequence. Real-time PCR amplification of the *nptII* exogene

and the *chi* endogene were carried out in 96-well reaction plates using the iCycler iQ Thermocycler (Biorad) with the duplo target plasmid p-*nptII*/*chi* as standard calibrator according to Dalla Costa *et al*. (2009), on genomic DNA extracted from leaves and roots for the destructive assay and from different regions of the plants for the non-destructive inductions. Nuclease-free water was the negative control. Quantification of *nptII* in transgenic grapevines was calculated as exposed in Dalla Costa *et al.* (2009) with the following formula: (*nptII* gene copy number/*chi* endogene copy number) × 2, where the *nptII* and the *chi* copy numbers were calculated by the iCycler iQ optical System Software version 3.0a (Biorad) as mean values of the two replicate quantification cycles (Cq) on the basis of the standard curves obtained.

Statistical analysis: STATISTICA software version 8 (StatSoft) was used. The effectiveness of 17-β-estradiol induction was evaluated with a parametric two-way ANOVA (Snedecor and Chocran 1980) for both the destructive assays on leaves and roots and for the experiments comparing the three morphogenic systems. Subsequent to the ANOVA, significant differences between the *nptII* mean CN values of the controls and the treated samples of plants 1 and 2 – the latter considered independently - were assessed using multiple comparison post-hoc tests, *i.e.* the Newman-Keuls test (Keuls 1952) for the destructive assay (Tab. 1) and the Dunnet test (Dunnet and Tamhane 1992) for the other experiments (Tab. 2). The latter test was used to compare a group of treated biological replicates (N) with a control plant for each analyzed tissue, the statistical output being expressed as the number of treated plantlets among N replicates which differed significantly from the control.

Results and Discussion

In previous groundwork on *V. vinifera* 'Chardonnay' and 'Brachetto' transgenic plants obtained with the pX6 vector carrying the *nptII* and the *gfp* marker genes (Martinelli *et al.* 2009), we assessed the effectiveness of the chemically induced *Cre*/*loxP* system in removing *nptII* marker gene. Different concentrations and exposure times of 17-β-estradiol were evaluated on embryogenic callus proliferation and node micropropagation. Successful induction was checked by visualizing the expression of GFP fluorescence in putative transgenic cultures with a fluorescence stereomicroscope. No difference was found between the various estradiol concentrations (2, 5, 10 and 20 μM) and a 24 h application period was found to be effective.

In a further study (Dalla Costa *et al.* 2009), we extended our method for quantifying *nptII* removal. Estradiol was supplied to buds from transgenic 'Brachetto' plants produced with the pX6-pKcpGVA construct (Turturo *et al.* 2003). Solid *versus* liquid supply strategies combined with different concentrations (10 or 20 μM) of this hormone were compared on buds induced to elongate into plantlets. Separate examination of the different plant regions revealed significant levels of *nptII* removal only in the root samples.

These experiments showed that adoption of the induced site-specific DNA excision technique for successfully removing a marker exogene needs to be improved in grape. In other species, constraints are also reported since ineffective DNA excision and/or differing induction efficiencies have been obtained in different parts of the plant (Brand *et al.* 2006, Guo *et al*. 2003, Sreekala *et al.* 2005, Zhang *et al.* 2006, Zuo *et al.* 2001 and 2006).

In the present work, several strategies for improving 17-β-estradiol induction were thoroughly assessed in grape. First of all, we tested the response of different plant tissues to the hormone supply during a destructive assay. We then evaluated the best diffusion conditions within the cell tissues *in vivo* during plant morphogenesis and development. Indeed, both our previous experience and the above-mentioned literature seem to indicate the need to enhance induction in the cells where morphogenic processes take place. We expected to recover plantlets from these tissues free of the undesired exogenous sequence. This was the rationale behind a set of experiments comparing dif-

Table 1

Real-time PCR quantification of *nptII* marker gene removal following 17-β-estradiol induction during the destructive assay on whole and cut-up leaves, and roots. The p-*nptII*/*chi* duplo target plasmid was used for building the standard curve and exogene copy number values were calculated with the following formula: (nptII copy number/chi copy number) x 2, according to Dalla Costa *et al.* (2009). *nptII* CNm = *nptII* mean copy number from 6 measurements obtained by analyzing two biological replicates during three Real-time PCR sessions; F value = calculated Fisher values; P value = probability of no differences between quantification measures; * = significantly different from controls (non-induced samples) according to the Newman Kleus test

		whole leaves CN_m	cut-up leaves CN_m	roots CN_m
Plant 1	control (17-β-estradiol 0 μM)	0.49	0.49	0.52
	induced (17-β-estradiol 20 μM)	0.35*	0.39*	0.37*
Plant 2	control (7-β-estradiol 0 μM)	0.45	0.51	0.52
	induced (17-β-estradiol 20 μM)	0.31*	0.33*	0.36*

Two-Way ANOVA

	Line 1		Line 2	
Source of variation	F value	P value	F value	P value
17-β-estradiol concentration	35.75	< 0.001	69.72	< 0.001
Explant type	0.46	0.63	2.70	0.083

Table 2

Efficiencies of *nptII* marker gene removal quantified in different regions of plantlets regenerated from three different morphogenic systems following 17-β-estradiol induction. Real-time PCR quantifications were performed on DNA extracted from plantlets recovered from meristematic proliferative tissue (MPT), shoot organogenesis from the leaf/petiole cut surface (SOLP) and micropropagation of buds (BM) after induction with 20 μM 17-β-estradiol. The p-*nptII/chi* duplo target plasmid was used as calibrator. For both plants 1 and 2 and each treatment the *nptII* mean copy number (CNm) was calculated on different plant regions of treated plantlets (N), with 2 technical replicates assessed for each sample, during two independent Real-time PCR sessions. DNA was extracted from a transgenic plantlet without hormone supply as a control for both plants 1 and 2. AL = leaves of the apical bud; AI + BI = apical and basal internodes; BL = leaves of the basal bud; R = roots; D = Dunnet test to assess the number of treated plantlets out of N replicates with significantly different *nptII* CNm from the control for each plant region evaluated (e.g. 1:3 = 1 out of 3 treated plantlets differing significantly from its respective control); P values = probability of no differences between CNm measures. *** = highly significant difference

Plant	Plantlet region	Control		Induced MPT			Induced SOLP			Induced MB		
		N	CN_m	N	CN_m	D	N	CN_m	D	N	CN_m	D
1	AL	1	0.54	3	0.45	1:3	4	0.37	3:4	3	0.49	0:3
	A1 + B1		0.55		0.49	0:3		0.46	1:4		0.37	3:3
	BL		0.54		0.52	0:3		0.43	1:4		0.22	3:3
	R		0.57		0.46	1:3		0.33	2:4		0.07	3:3
2	AL	1	0.57	4	0.48	0:4	5	0.55	0:5	3	0.49	0:3
	A1 + B1		0.57		0.50	0:4		0.55	0:5		0.39	2:3
	BL		0.56		0.52	0:4		0.55	0:5		0.30	3:3
	R		0.48		0.47	0:4		0.13	4:5		0.06	3:3

	Two-Way ANOVA					
	MPT (P values)		SOLP (P values)		MB (P values)	
Source of variation	Plant 1	Plant 2	Plant 1	Plant 2	Plant 1	Plant 2
Plants (treated + control)	< 0.001***	0.15	< 0.001***	< 0.001***	< 0.001***	< 0.001***
Tissues	0.22	0.22	< 0.001***	< 0.001***	< 0.001***	< 0.001***

ferent morphogenic systems for plantlet recovery. After assessing several hormone supply strategies combined with organogenesis during a consistent number of experiments (data not shown), we selected the more promising ones to set up appropriate biological and technical replicates for an accurate statistical analysis.

Two transgenic 'Brachetto' plants (named plant 1 and plant 2) obtained with the pX6-pKcpGVA construct (Turturo *et al.* 2003, Fig. 1) were used. Southern Blot assays detected the presence of one copy of *nptII* (Fig. 2, lanes 1 and 2) in both plants, while *nptII* mean copy numbers quantified with Real time PCR on the genomic DNA from the leaves were 0.55 and 0.54 respectively for plants 1 and 2 (Dalla Costa *et al.* 2009).

In an initial assay, root and leaf tissues were compared (Fig. 3). End-point PCR performed with primers designed for recognizing the G10-90 promoter and a sequence of the GVA hairpin respectively (primers P1 and P2 in Fig. 1) amplified a fragment of 400 pb for both plant 1 (Fig. 4) and plant 2 (data not shown), proving that the DNA cassette flanked by *loxP* sites was successfully removed in each induced tissue. As expected, this sequence was not amplified in the respective controls (Fig. 4, lanes 4, 5, 6).

Moreover, sequencing of the 400 bp fragment amplified in the induced roots of plant 1 (Fig. 4, line 3) showed that the amplicon contained the G10-90 promoter and one *LoxP* site linked to the selected fragment of the coat protein sequence of the GVA virus, revealing the occurrence of a highly precise site specific recombination (Fig. 5).

A further quantitative analysis was performed on the same samples, according to Dalla Costa *et al.* (2009), in order to evaluate the efficiency of *loxP* site cassette removal. Given that *nptII* is located in the region flanked by the *loxP* sites, this analysis was based on copy number quantification of this exogene. Two biological replicates of the controls and the induced samples were analyzed during three Real time PCR sessions (Tab. 1). Significant rates of *nptII* copy number decrease were detected in the induced tissues of both plants, as verified by an ANOVA where P values related to hormone concentration were lower than 0.1 %, and by a multiple comparison Newman-Keuls test. Moreover, ANOVA revealed no significant differences between explant types (whole or cut-up leaves, or roots), the P values being higher than 0.63 for plant 1 and 0.083 for plant 2).

These results showed that the C*re*/*loxP* inducible system can function properly in the two different tissue types (roots and leaves), suggesting that the varying degrees of efficiency found in the different plant regions when buds were induced in the preliminary work (Dalla Costa *et al.* 2009) might be due to inadequate diffusion of 17-β-estradiol in the grape tissues during plant growth.

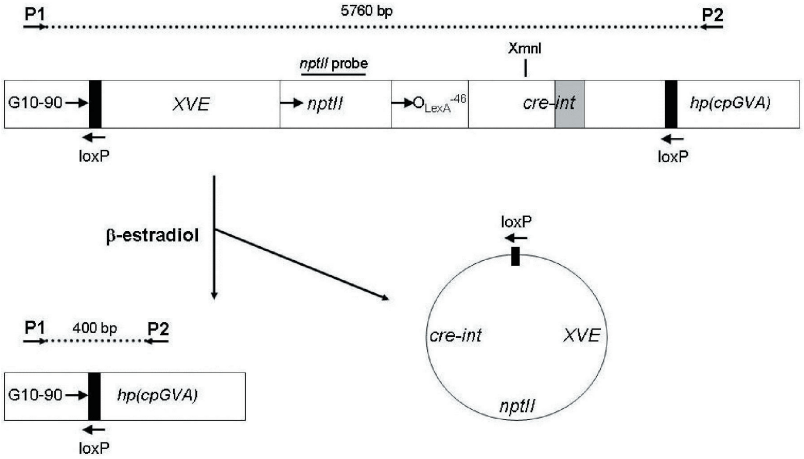

Fig. 1: Schematic representation of the pX6-pKcpGVA construct according to Turturo *et al.* 2003, obtained from the pX6 plasmid (Zuo *et al.* 2001). The representation illustrates the XmnI restriction enzyme binding site, the sequence recognized by the *nptII* probe for the Southern Blot and the binding sites for the primers P1-P2 respectively on the G10-90 promoter and on the conserved coat protein sequence of the GVA virus in sense orientation. Following an effective 17-β-estradiol induction, Cre recombinase cuts the DNA at each loxP sites producing excision of the loxP-flanked cassette and adjoining of the G10-90 promoter to the hp(cpGVA) sequence. As a result, the expected length of the P1-P2 amplification fragment is reduced from a 5760 bp to a 400 bp.

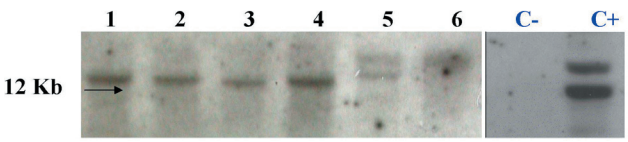

Fig. 2: Southern Blot analysis showing the presence of the nptII marker gene in genetically modified plantlets of V. vinifera 'Brachetto' obtained with the pX6-pKcpGVA construct. DNA was extracted from six genetically modified plantlets randomly chosen out of 21 plants regenerated from single somatic embryos (lanes 1 - 6), from a control wild type plantlet (C-) and from a tobacco transgenic line (C+) and digested with the restriction enzyme XmnI to check the presence of the nptII exogenous sequence.

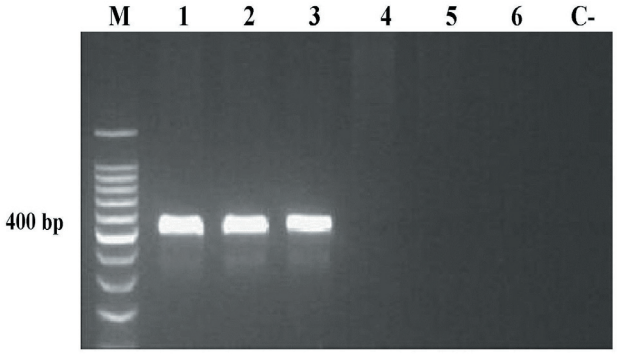

Fig. 4: End-point PCR assay on DNA from whole and cut-up leaves and roots to check *nptII* removal in the destructive assays with 17-β-estradiol induction. Lanes: M = molecular marker; 1, 2, 3 = induced samples, *i.e.* explants of leaves, cut-up leaves and roots induced with 17-β-estradiol; 4, 5, 6 = control samples, *i.e.* explants of leaves, cut-up leaves and roots without induction with 17-β-estradiol; C- = nuclease-free water as negative control. Primers P1 and P2 were used as shown in Fig. 1.

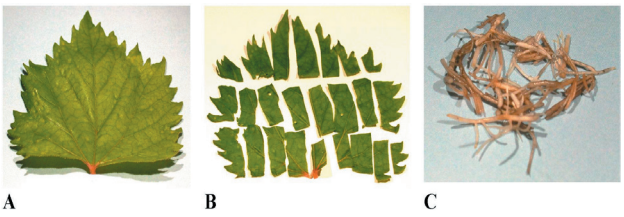

Fig. 3: Explants used for evaluating the effectiveness of 17-β-estradiol induction on nptII removal during the destructive assays. Whole (**A**) and cut-up leaves (**B**) and roots (**C**) from plants 1 and 2 were incubated in the dark for 48 h at 25 °C in liquid NN (Nitsch and Nitsch 1962) medium containing 20 µM 17-β-estradiol.

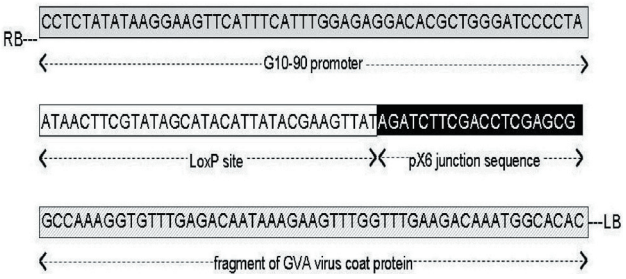

Fig. 5: Evaluation of Cre-mediated site-specific recombination by nucleotide sequencing of line 1 DNA after removal of the loxP cassette. The fragment amplified with P1 and P2 primers (see Fig. 1) on DNA from the roots of plant 1 induced during the destructive assay was cloned in pGEM-T vector and sequenced with the ABI 3730xl using the primer T7. Forward and reverse sequencings were carried out on the ABI 3730xl (Applied Biosystem) using primer T7 and SP6 (binding in the pGEM-T vector sequence) respectively. For the G10-90 promoter and the hairpin of the GVA virus coat protein, only the regions adjacent to the recombined loxP site are shown. RB = right border of T-DNA; LB = left border of T-DNA.

In the assays aimed at assessing different morphogenic systems for plantlet recovery, the hormone was supplied during shoot organogenesis from meristematic proliferative tissue (MPT) or from the cut surface between leaf and petiole (SOLP) or during micropropagation from bud (MB) (Fig. 6). Induction effect was evaluated on regenerated plantlets - with roots, leaves of the basal bud, leaves of the apical bud and apical and basal internodes analyzed

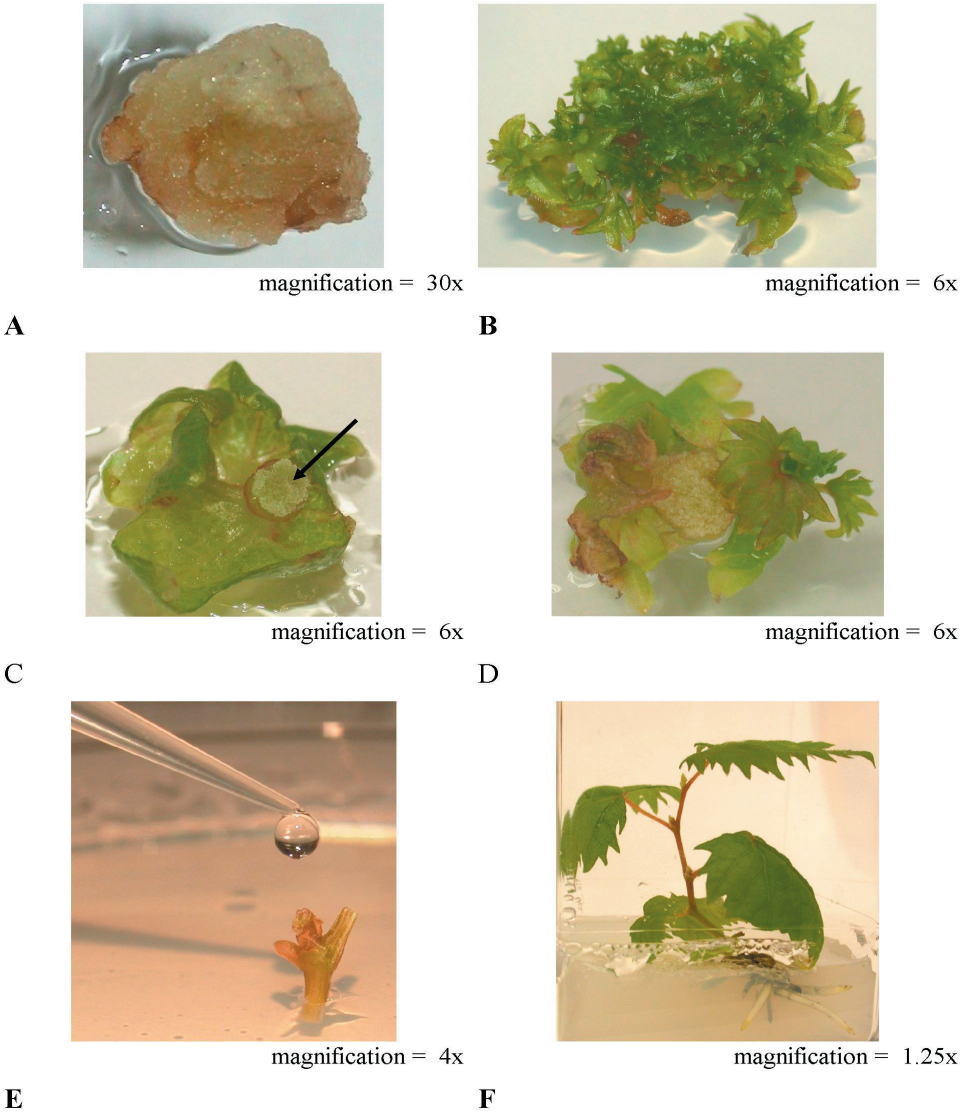

Fig. 6: Morphogenic systems for plantlet recovery used during the 17-β-estradiol induction experiments. The hormone was supplied during shoot organogenesis from meristematic proliferative tissue (MPT) (**A** and **B**) or from the cut surface between leaf and petiole (SOLP) (arrow, **C** and **D**) or during the micropropagation from bud (MB) (**E** and **F**).

separately - by means of *nptII* copy number quantification in two sessions of Real time PCR for each sample and data assessing with an ANOVA and a Dunnet test (Tab. 2).

In the assay carried out on meristematic proliferative tissue (Fig. 6 a and b), the ANOVA (P values for MPT) and the Dunnet test (induced MPT, column D) showed that *nptII* copy numbers quantified in the roots and the apical region differed from controls in 1 out of the 3 replicates (1:3) of plant 1. As for plant 2, no significant exogene removal was quantified in any of the evaluated plant regions of any of the replicates (0:4). These results show that induction is not reproducible in the replicates of plants 1 and 2 and between plants.

Regarding the strategy based on shoot regeneration from the cut surface between leaf and petiole (Fig. 6, c and d), after induction, leaves were placed on solid medium and 4 and 5 plantlets were regenerated from plants 1 and 2 respectively. ANOVA revealed significant differences between the plants (treated replicates *vs.* controls) and between the tissues of both plants (P values for SOLP). In particular, the Dunnet test (induced SOLP, column D) revealed significant *nptII* removal in the roots (2:4), basal leaves (1:4), internodes (1:4) and apical leaves (3:4) of the induced replicates of plant 1, but only in the roots of plant 2 (4:5). These results show that even though exogene removal in the various plant regions is better with the SOLP induction strategy than with MPT, it doesn't seem to be reproducible, as indicated by the differences obtained between the two plants.

The last of this set of trials was designed to improve the previous groundwork carried out on micropropagated buds (Dalla Costa *et al.* 2009, Martinelli *et al.* 2009): in addition to the presence of the hormone in the solid culture medium, buds were also wet constantly for two weeks with liquid medium containing the inducer and the surfactant Silwet L77. The ANOVA revealed significant differences between the plants (treated replicates and control) and between the tissues of both plants (P values for MB). In addition, the Dunnet test revealed significant *nptII* removal in the roots, basal leaves and apical and basal internodes of all the replicates (3:3) in both plants, the only exception being the apical and basal internodes of plant 2 (2:3).

These results show that induction of MB is effective and reproducible in these 3 regions in both the plants and in the replicates, whilst in the apical leaves a significant removal seems to be difficult to obtain (0:3 for both plants). Moreover, data on the efficiency of *nptII* excision (CN_m) show that removal was almost complete (88 %) in the roots of both plants (0.07 and 0.06) and reached 60 % and 47 % respectively in the basal leaves of plants 1 and 2 (0.22 and 0.30). These percentage efficiencies were calculated by comparing the CNm values of each treated sample versus the respective control, considering this latter as 100 %, *i.e.* 100 % - (*nptII* CN_m of the treated sample / *nptII* CN_m of the control) %. As for the other two treatments, CN_m values were higher, the only exception being the plant 2 roots in the SOLP assay (0.13).

In conclusion, our results show that micropropagation from buds combined with constant wetting with 17-β-estradiol is the most efficient and reproducible strategy for effective *in vivo* hormone induction. Exogene removal, however, was almost complete only in the roots, probably because the root apical meristem was more accessible to the hormone. Inaccessibility of this inducer to progenitor cells has been assumed in *Arabidopsis* (Zuo *et al.* 2001). In our experiments, this would also indicate the need for a continuous hormone supply during plant elongation. On the other hand, if properly targeted, our strategy would be useful where induction has to be obtained in specific plant regions, such as for studying the expression of genes in different tissues. Moreover, bud propagation offers several advantages over organogenesis being this the routine method for plant maintenance following transgenic plantlet production. Finally, plant recovery from buds is the favored system for preserving genotype identity during propagation (Torregrosa *et al.* 2001) given that somaclonal variation is an outcome - usually undesirable - of regeneration (Larkin and Scowcroft 1981).

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