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Elaboration of a Reliable Strategy Based on Real-Time PCR To Characterize Genetically Modified Plantlets and To Evaluate the Efficiency of a Marker Gene Removal in Grape (*Vitis* spp.)

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We have developed an effective strategy based on real-time PCR assay for the molecular characterization of genetically modified grape and to quantify the efficiency of a marker gene removal. This research has been implemented in *Vitis vinifera* cv. Brachetto plantlets where exogenes were inserted during cocultures of embryogenic calli with *Agrobacterium tumefaciens* carrying the chemically inducible site-specific *cre/loxP* pX6 vector where the expression of the *cre* recombinase is regulated by 17-β-estradiol. The neomycin phosphotransferase gene (*nptII*) for the kanamycin resistance trait was inserted as part of the gene transfer protocol, and this exogene was employed as a case study for carrying out our research. The 9-*cis*-epoxycarotenoid dioxygenase (*nced2*) and chalcone isomerase (*chi*) genes coding for two enzymes, involved respectively in abscisic acid and flavonoid biosynthesis, proved to be valuable reference endogenes for real-time PCR assays. Two types of duplo-target plasmids were exploited for building the standard curves: in one type (p-*nptII/nced2*) the *nptII* sequence is linked to the *nced2* sequence; in the other (p-*nptII/chi*) it is linked to the *chi*. These calibrators were intended to simulate an ideal genetically modified plant carrying a homozygous single-copy exogene insertion. The repeatability test confirmed the suitability of both plasmid calibrators. Foreign gene stability can be monitored during long-term plant preservation, and the method proved to be suitable for quantifying the efficiency of *nptII* gene removal induced by 17-β-estradiol.

KEYWORDS: *Vitis vinifera*; genetically modified grape; real-time PCR; duplo-target plasmid; nine-cis-epoxycarotenoid dioxygenase2 gene (*nced2*); chalcone isomerase gene (*chi*); neomycin phosphotransferase gene (*nptII*); *cre/loxP*

INTRODUCTION

Vitis vinifera is an economically important fruit crop grown for the production of wine, juice, table grape, and raisins as reported by the Organisation Internationale de la Vigne et du Vin (*1*). In the *Vitis* genus, marker genes and agronomically important traits have been transferred into wild species, interspecific hybrids and rootstocks, as well as into various *V. vinifera* cultivars (*2, 3*).

Recently, characterization of genes involved in main pathways (*4, 5*) and advances in grape genome sequencing (*6, 7*) have been obtained in various laboratories. This line of research requires a reliable technology for transgenic plant production for studying in planta the function of genes involved in important biologic processes.

In the overall process of establishing a genetically modified plant, molecular characterization is crucial and requires verification of transgene integration in the host genome and certain related phenomena.

Above all, foreign DNA insertion into the plant genome is random and frequently produces multiple copy number integration in one or more chromosome locations (*8*). While this would be useful in overexpression analysis (*9*), multiple copies have, on the other hand, been related to unstable expression and silencing of the transgene (*10*−*12*). Conversely, single-copy insertions have proved to be stable even after several subsequent breeding generations, and are therefore preferred in various applications (*13*).

For these reasons, copy number needs to be evaluated not only when transgene expression is assessed in planta for advanced biological studies, where a precise molecular characterization of the plant is necessary, but also for plant commercialization. Moreover, exogene quantification is mandatory for deliberate in vivo release, according to European regulation (*14*). Consequently, a reliable method for transgene copy number assessment is essential.

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Southern blot is the most commonly applied assay for DNA quantification. Recently, however, real-time PCR has also been shown to provide a suitable analysis, and is often preferred due to certain technical advantages, such as rapidity, low quantity DNA requirement, high repeatability and reproducibility, and the possibility of applying statistical tests to the quantitative results (*9*).

So far, real-time PCR has been successfully employed to analyze transgenic maize (*15*−*17*), tomato (*18*), soybean (*19*), rice (*20, 21*), rape (*8, 22*), and wheat (*23*).

As for grape, transgenic plant evaluation based on real-time PCR needs to be further developed since there are no reports in the literature of effective methods based on this assay specifically adopted for the *Vitis* genus. Moreover, besides exogene quantification, this assay seems to be an interesting tool for assessing specific aspects of the gene transfer technology, among them the efficiency of a marker gene removal.

As in other plants, in grape gene transfer, the *nptII* gene conferring resistance to the antibiotic kanamycin is mainly associated with the gene of interest in order to effectively discriminate between the cells that have inserted the foreign gene and those that have not (*3, 24, 25*). The latter have to be removed from the cultures as the *nptII* gene enables them to survive in a medium to which has been added the otherwise toxic antibiotic. Given that the exclusive function of the marker genes is to aid the gene transfer process, their presence in transgenic plants may be undesirable. Furthermore, the use of antibiotic resistance is becoming one of the more controversial and less socially acceptable issues of transgenic plant production, and is subjected to restrictions (*14*).

To eliminate the *nptII* gene after the insertion of the target gene in grapevine, we exploited the self-excision strategy based on the site-specific *cre/loxP* pX6 vector where the expression of the *cre* recombinase is regulated by 17-β-estradiol. The hormone is specifically responsible for precise recombination and DNA excision between two directly repeated *LoxP* recognition sites flanking the selectable marker gene (*26*).

In the present research, we define an effective real-time PCR method for estimating a transgene copy number in grape and for assessing its stability during long-term in vitro preservation. Our work has been developed in *Vitis vinifera* cv. Brachetto plantlets where exogenes have been inserted via *Agrobacterium*-mediated gene transfer during cocultures of embryogenic calli.

In our previous experiments, we investigated certain critical parameters of the real-time PCR method in the analysis of DNA of grape origin, such as that of wine and must (*27*), and the results reported in the present paper are based on a thorough examination of certain crucial aspects of this technique. First, we identified the most suitable endogenous reference genes, which must conform to certain basic requirements, such as species-specificity, low intraspecific variability, and presence in a single or a low copy number in the genome (prEN ISO 2426 and 21569). Accordingly, the *nced2* (9-*cis*-epoxycarotenoid dioxygenase) (*27, 28*) and *chi* (chalcone isomerase) (*29, 30*) genes coding for two enzymes involved respectively in abscisic acid and flavonoids biosynthesis were assessed.

Moreover, we developed specific duplo-target plasmids to be applied as reliable calibrators in the quantification, in light of recent literature in which multiple-target plasmids have been described as promising tools for foreign DNA detection analysis (*31*−*33*).

The real-time PCR method presented here seems to be a valuable tool for foreign gene analysis in grapevine.

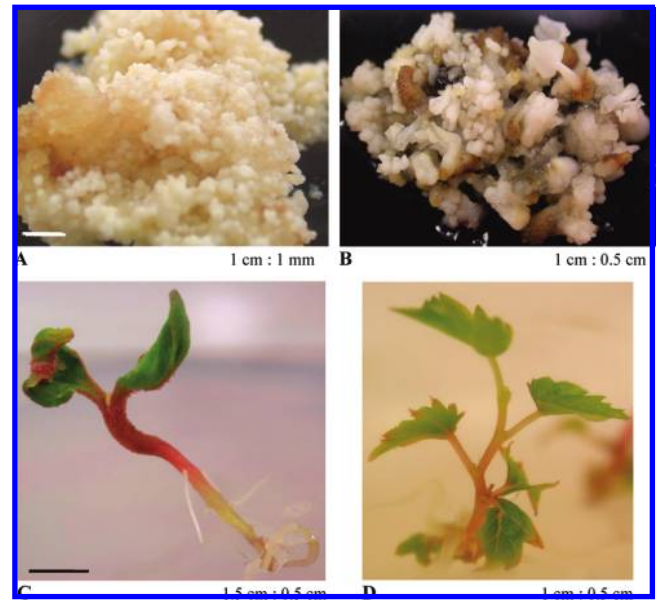

Figure 1. Crucial steps along the path from callus to plantlet of *V. vinifera* cv. Brachetto according to ref *35*: (**A**) Highly morphogenic embryogenic callus showing predominance of pro-embryos, globular and torpedo structures. A well-established embryogenic callus can indefinitely preserve morphogenic potential through secondary somatic embryogenesis on a suitable propagation medium. (**B**) Embryogenic callus after 6 months on the differentiation medium, showing an asynchronous morphogenic development, with the predominance of individualized and mature (arrow) somatic embryos. (**C**) Well-shaped, well-polarized, isolated somatic embryo with root and shoot axes, a hypocotyl and two cotyledons obtained after 2 months on germination medium. (**D**) Young plantlet regenerated from a mature somatic embryo after 1 month on a germination medium and induced to elongation.

We also adopted this method as a suitable application for quantifying the efficiency of *nptII* marker gene removal. Since this aspect of the gene transfer technique is the focus of much attention in biotechnology, our research would open up interesting perspectives with respect to optimizing gene transfer technology in grapevine.

MATERIALS AND METHODS

Plant Material and Constructs for the Gene Transfer. This study was performed on 9 randomly chosen genetically modified plantlets of *Vitis vinifera* cv. Brachetto obtained in our laboratory (**Figure 1**). Gene transfer was performed during cocultures of grape embryogenic calli with *Agrobacterium tumefaciens*, according to ref *34*. Cefotaxime was added to the culture medium after a two-day coculture to prevent further *Agrobacterium* contamination, in an initial concentration of 300 mg/L which was gradually reduced, during the following subcultures, to 50 mg/L. Embryogenic callus subcultures, embryo differentiation, plantlet regeneration and micropropagation were performed as described in ref *35*. Plants 1 and 2 were obtained after cocultures of *Agrobacterium* strain LBA4404 carrying the chemical-inducible site-specific *cre/loxP* pX6 vector (*26*), where GFP gene was replaced with a conserved coat protein sequence of the grapevine virus A (GVA) in sense and antisense orientation, coding for a hairpin RNA (pX6-pKcpGVA (*36*)). In the construct, expression of the *cre* recombinase is regulated by 17-β-estradiol. The remaining 7 plants were obtained employing *Agrobacterium* strain EHA105 carrying the pPLT7000 construct with the *vhb* gene for the *Vitreoscella stercoraria* hemoglobin (*37*). Both the above-described constructs also carried the neomycin phosphotransferase (*nptII*) gene for kanamycin selection. This antibiotic was added to the medium one month after the *Agrobacterium* cocultures, in an initial concentration of 50 mg/L gradually increasing to 150 mg/L during the embryogenic callus subcultures. Finally, embryo conversion into plantlets and micropropagation were induced on an antibiotic-free

Table 1. Differences between *nced2* Ct and *chi* Ct (ΔCt) Obtained from Real-Time PCR Amplifications of DNA Extracted from Various *V. vinifera* cvs. (Chardonnay, Traminer, Cabernet Sauvignon, Riesling, Moscato giallo, Merlot, Marzemino, Pinot Noir) and Rootstocks (Kober 5BB and SO4) from the In-Field Collection of Our Institute and Extracted from the Genetically Modified *Vitis vinifera* cv. Brachetto Plant Obtained with the pX6-pKcpGVA Construct (Referred to as Sample 1)[a]

genotype	ΔCt
Chardonnay	0.60
Traminer	0.75
Cabernet Sauvignon	0.60
Riesling	0.65
Moscato giallo	0.55
Merlot	0.40
Marzemino	0.65
Pinot Noir	0.70
Kober 5BB	0.55
SO4	0.60
Brachetto	0.60
mean ΔCt	0.60
RSDr (%)	15.00

[a] The *nced2* Ct and *chi* Ct values are the means of two replicates.

medium. Plants converted by single embryos were recorded as individual mother-plants of the distinct clones obtained by further micropropagation (**Figure 1**).

Induction of *nptII* Removal with 17-*β*-Estradiol. A genetically modified plant produced with the pX6-pKcpGVA construct (plant sample 1 in **Table 2**) was micropropagated, and 60 buds were used for the induction assays with the *β*-estradiol (Sigma-Aldrich). Six different treatments, resulting from two different supply strategies (solid versus liquid) combined with three hormone concentrations, were evaluated by either adding the *β*-estradiol to the solid media or pouring on the buds 3 drops of the hormone at final concentrations of 0 (control), 10 or 20 *μ*M. In each treatment, 12 buds were induced, with induction repeated twice every 15 days, producing shoots for the real-time PCR assays.

Southern Blot. The 9-*cis*-epoxycarotenoid dioxygenase (*nced2*, accession no. AM457017), the chalcone isomerase (*chi*, accession no. AM430986), and the *nptII* (accession no. AF330636) genes were analyzed. For each gene, a specific probe was amplified by PCR using suitable primers, designed by the Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi), as follows: forward, 5′-TTAGCCCAAACCCAACTCTCCT-3′, and reverse, 5′-ACTGCGGTGGCTGAGTTATCTG-3′, for *nced2*; forward, 5′-AAGATGTCTCCAGTGCCGTCAG-3′, and reverse, 5′-GGAAAATCTCGCCAAAATCCAG-3′, for *chi*; forward, 5′-GATGGATTGCACGCAGGTTC-3′, and reverse, 5′-GGAGCGGCGATACCGTAAAG-3′, for *nptII*. Probes were digoxigenin-labeled with the PCR Dig Probe Synthesis Kit by Roche Diagnostics, Switzerland. Ten micrograms of genomic DNA was extracted from grape leaves using the Doyle and Doyle method (*38*), modified by the addition of 1% PVP to the extraction buffer and digested with the appropriate restriction endonuclease, i.e. *Sac*I (Sigma) or *Xba*I (Sigma) for *nced2*, *Xba*I (Sigma) for *chi*, and *Xmn*I (Promega) for *nptII*, according to the manufacturers' instructions. Digestion mixture was precipitated, electrophoresed on a 0.9% agarose gel in 0.5× TBE, and capillary-blotted in 10× SSC (i.e., 1.5 M NaCl, 150 mM sodium citrate) onto a positively charged nylon membrane (Roche Diagnostics, Switzerland). Prehybridization was carried out for 3 h at 68 °C in the hybridization buffer (Roche Diagnostics, Switzerland) where the probe was added for further overnight hybridization at 68 °C. The membrane was washed with a solution of 2× SSC, 0.1% SDS. Dig-chemiluminescent detection was performed according to the Roche Diagostics protocol, and the autoradiograph was developed after a 4 h exposure.

Quantitative Real-Time PCR. The real-time PCR amplification of the two endogenes *nced2* and *chi* and of the exogene *nptII* was performed in 96-well reaction plates on the iCycler iQ Thermocycler (Biorad), in a 25 *μ*L final volume containing 1X Platinum Quantitative PCR SuperMix-UDG (Invitrogen), 100 ng of genomic DNA, 0.3 *μ*M primers (Sigma) and a 0.2 *μ*M specific Taqman probe (Sigma). The latter were designed by the Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) as follows: forward, 5′-CGGAGCAACCTGTCTGTCACTC-3′, reverse, 5′- CGCCGTTTCGCAGATAAACTC-3′, and probe, FAM-5′-CTTCCTGTCTCCGGCACCAT-3′-TAMRA for *nced2*; forward, 5′-GAGGCTGGGGATGAGAAAATTG-3′, reverse, 5′-CCCATCTCTCCTTCAACCACCT-3′, and probe, FAM-5′-AAGCTGAGAAGGTTGCTCCGGT-3′-TAMRA for *chi*; forward, 5′-CTTGCCGAATATCATGGTGGAA-3′, reverse, 5′-GGTAGCCAACGCTATGTCCTGA-3′, and probe, FAM-5′-TTCTGGATTCATCGACTGTGGC-3′-TAMRA for *nptII*. The thermal protocol used was as follows: UDG PCR decontamination for 2 min at 50 °C and for 2 min at 95 °C, followed by 50 cycles of denaturation and annealing/extension of 15 s at 95 °C and of 1 min at 60 °C respectively. Fluorescence signals were detected during the annealing/extension phase. The standard curves were built by means of 4 decreasing concentrations for each calibration plasmid in a serial dilution of 1:5 (1,000,000; 200,000; 40,000; 8,000 plasmid molecules). Nuclease-free water was used as negative control. Plasmids and plant samples were analyzed in duplicate. For each genomic DNA sample, the copy numbers of *nptII* and of the endogenes (*nced2* or *chi*) were calculated by the iCycler iQ optical System Software, version 3.0a (Biorad), as mean values of the two replicate threshold cycles (Ct) on the basis of the standard curves obtained. Estimation of the *nptII* insertion copies in transgenic grapevines was calculated with the following formula: (*nptII* gene copy number/endogene copy number) × 2.

For the melting curve analysis, 100 ng of genomic DNA was amplified in a 25 *μ*L final volume containing 0.625 U of Ampli*Taq* Gold (Applera), Gold buffer 1X (Applera), 200 *μ*M of each dNTP, 4 mM of $MgCl_2$, 0.4 *μ*L of Sybr Green I 10X (Cambrex) and 0.5 *μ*M primers as reported above. The thermal program consisted of a first step of 2 min at 50 °C and a second step of 10 min at 95 °C followed by 45 cycles of denaturation and annealing/extension of 15 s at 95 °C and of 1 min at 60 °C respectively. After amplification, melting analysis was performed by heating the reaction mixture from 55 to 95 °C at a rate of 0.5 °C/10 s.

Duplo-Target Plasmid Calibrators. For the real-time PCR assays, two different duplo-target plasmids were prepared to be used as standard calibrators. Each plasmid carried a duplo-target amplicon consisting of the *nptII* sequence linked in one case to the grape endogene sequence *nced2*, in the other case to *chi*. These amplicons were built by two subsequent PCR reaction steps. In the first PCR step, selected sequences of the *nptII* gene and of each endogene were separately amplified in a final volume of 50 *μ*L, containing 1.25 U of Ampli*Taq* Gold (Applied Biosystems), 200 *μ*M of each dNTP, 2 mM of $MgCl_2$, 100 ng of DNA and 0.3 *μ*M of the specific primers. Primer sets were designed as described in the previous section, with the following modifications (in bold): forward, 5′-CTTGCCGAATATCATGGTGGAA-3′, and reverse, 5′-**AATGCGTCGAGCTCAACTGT**GGTAGCCAACGCTATGTCCTGA-3′, for *nptII*; forward, 5′-**ACAGTTGAGCTCGACGCATT**CGGAGCAACCTGTCTGTCACTC-3′, and reverse, 5′-CGCCGTTTCGCAGATAAACTC-3′, for *nced2*; forward, 5′-**ACAGTTGAGCTCGACGCATT**GAGGCTGGGGATGAGAAAATTG-3′, and reverse, 5′-CCCATCTCTCCTTCAACCACCT-3′, for *chi*. The extension sequences designed according to ref *39* to allow the hybridization of the complementary ends during the second PCR step are reported in bold. Amplifications were performed on the PCR Thermocycler (Tgradient, Biometra) with the following thermal protocol: a first denaturing step of 9 min at 95 °C followed by 45 cycles of denaturation, annealing and extension of 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C respectively, with a final extension of 5 min at 72 °C. PCR products were electrophoresed at a constant voltage (100 V) with a loading buffer (Promega) and Sybr Gold 10X (Molecular Probes) on a 2% agarose gel (Sigma) that was scanned by Gel Doc 2000 (Biorad). In the second PCR reaction step, the *nptII* amplified fragment was linked to one or the other of the two endogene sequences. Regarding the *nptII*−*nced2* duplo-target amplicon, 1 *μ*L of each of the *nptII* and the *nced2* amplified fragments obtained in the first PCR step was mixed into a 50 *μ*L final volume containing 1.25 units of Ampli*Taq* Gold (Applied Biosystems), 200 *μ*M of each dNTP, 2 mM of $MgCl_2$ and 0.3 *μ*M of each primer, i.e. forward, 5′-CTTGCCGAATATCATGGTGGAA-3′, and reverse, 5′-CGCCGTTTCGCAGATAAACTC-3′. The same method was used

Table 2. Repeatability of Quantification of p-*nptII/nced2* and p-*nptII/chi* Duplo-Target Plasmid Calibrators[a]

plant sample	calibrator	*n*	mean CN	CI 95%	RSDr %	"*t*"
1	p-*nptII/nced2*	8	0.50	±0.055	13	1.45
	p-*nptII/chi*	6	0.55	±0.084	15	
4	p-*nptII/nced2*	6	0.97	±0.052	5	0.11
	p-*nptII/chi*	6	0.96	±0.130	13	
6	p-*nptII/nced2*	5	0.21	±0.044	17	0.51
	p-*nptII/chi*	6	0.20	±0.041	19	

[a] Mean quantification values of exogene copy number (mean CN), respective confidence intervals (CI) at 95%, and relative repeatibility standard deviations (RSDr %) were evaluated on the DNA extracted from 3 samples of genetically modified *Vitis vinifera* cv. Brachetto plants obtained with the pX6-pKcpGVA vector (sample 1) and with the pPLT7000 construct (samples 6 and 4), and analyzed in replicates (*n*). Student's test "*t*" values tabulated for 10 degrees of freedom at 95% = 2.228.

for building the *nptII−chi* duplo-target amplicon, except for the reverse primer 5′-CCCATCTCTCCTTCAACCACCT-3′. The thermal protocol was the same as that used for the first step amplifications. Aliquots of the PCR products were run on a 2% agarose gel, as described above, and quantified in comparison with a standard quantity of Lambda DNA (New England Biolabs-Celbio). The two duplo-target amplicons were cloned into the pGEM-T Easy vector (Promega) with the T4 DNA ligase (Promega) at a 1:1 vector/insert molar ratio, during overnight incubation at 4 °C, and transferred to *Escherichia coli* strain JM109, according to the Promega technical manual. The plasmids were extracted from the selected colonies, purified with the QIAprep Spin Miniprep Kit (Qiagen) and eluted in 100 μL nuclease-free water (Promega). Plasmid concentrations were measured at 260 nm with the BioPhotometer (Eppendorf). Their respective molarities in the solutions were calculated according to the nomogram for double-stranded DNA (*40*). The inserted fragments were checked by sequencing (CRIBI, Padova). Plasmid solutions were diluted in a TE buffer (pH 8.0) at a concentration of 10^8 copies/μL, subdivided into 100 μL aliquots and stored at −20 °C.

Statistical Analysis. The quantification performances of the two duplo-target plasmid calibrators (p-*nptII/nced2* and p-*nptII/chi*) used in the real-time PCR were compared using Student's "*t*" test (*41*) (**Table 2**). An analysis of variance (ANOVA) with a hierarchical design (*42*) was used to compare the mean copy numbers of the *nptII* exogene quantified with real-time PCR in different regions of the plantlets (**Table 4**). In the experiments based on β-estradiol induction, the effects of the different hormone concentrations (0, 10 or 20 μ M) and of their supply strategies (solid versus liquid) on *nptII* removal were evaluated with a nonparametric Friedman test (*43*) and with a parametric two-way ANOVA analysis (*44*); the latter analysis was also used for verifying the potential interaction of these effects (**Table 5**). Subsequently, the effects of the two hormone concentrations (10 or 20 μM) were compared using a least significant difference (LSD) test (*42*) (**Table 5**). Finally, the effects of hormone induction on different tissues of the plant for each supply strategy were analyzed and compared using the nonparametric Kruskal−Wallis test (*45*) and the Bonferroni−Dunn multiple procedure (*46*) (**Table 6**).

RESULTS AND DISCUSSION

In this study, we report a real-time PCR method developed for detailed molecular characterization of genetically modified grape. We focused in particular on exogene copy number quantification and removal of the *nptII* marker gene.

The analysis was carried out on 9 plant samples of *Vitis vinifera* cv. Brachetto which were regenerated from individual somatic embryos produced from embryogenic calli after *Agrobacterium*-mediated gene transfer. Two constructs, one harboring a sequence of the grapevine virus A (GVA) (*36*) (samples 1 and 2), the other a sequence of the *Vitreoscella* hemoglobin gene (*37*) (samples 3−9), were employed for producing the genetically modified plants. In addition, the neomycin phosphotransferase (*nptII*) gene for the kanamycin resistance trait is present in both constructs as part of the gene transfer protocol. The strategy described here is based on this exogene as a case study both for exogene quantification and for marker gene removal evaluation.

Table 3. Quantification of *nptII* Exogene Copy Number Using Southern Blot (CN_{SB}) and Real-Time PCR (CN_{Rt}) Assays Performed with p-*nptII/nced2* and p-*nptII/chi* Duplo-Target Plasmid Calibrators[a]

		p-*nptII/nced2*		p-*nptII/chi*	
plant sample	CN_{SB}	*n*	mean CN_{Rt}	*n*	mean CN_{Rt}
1	1	8	0.50	6	0.55
2	1	3	0.53	2	0.53
3	2	2	0.83	2	0.99
4	2	6	0.97	6	0.96
5	2	2	0.97	2	1.0
6	1	5	0.21	6	0.20
7	1	2	0.19	4	0.17
8	2	2	0.83	4	0.67
9	2	3	0.63	2	0.65

[a] Nine randomly chosen genetically modified plantlets of *Vitis vinifera* cv. Brachetto obtained with the pX6-pKcpGVA (samples 1 and 2) or the pPLT7000 constructs (samples 3−9) were analyzed. Relative Southern blot assay is presented in **Figure 3**. Exogene copy numbers obtained with real-time PCR analysis were calculated with the following formula: (exogene copy number/reference gene copy number) × 2. CN_{Rt} for both plasmids are the mean values of repeated measures (*n*).

Table 4. Evaluation of *nptII* Gene Stability in 3 Different Regions (Leaves of the Apical Bud "L"; a Mixture of Tissues Consisting of the Apical Bud and the Two Superior Internodes "M"; and Roots "R") of 3 Plantlets Micropropagated from the Genetically Modified Plant Sample 1[a]

	plant 1		plant 2		plant 3	
region	*n*	mean CN	*n*	mean CN	*n*	mean CN
L	3	0.57	3	0.52	3	0.52
M	3	0.56	3	0.52	3	0.64
R	3	0.56	3	0.57	3	0.50

Analysis of Variance (Fischer Test)

source of variance	F value	P value	d.f.
between plants	0.29	0.75	2(n); 6(d)
between tissues, within plant	1.11	0.39	6(n); 18(d)

[a] The *nptII* mean copy number (CN) of repeated measures (*n*) was calculated with the calibrator p-*nptII/chi* and analysis of variance with a hierarchical design was carried out. F value = calculated Fischer values; P value = probability of assessing no differences between quantification measures; d.f. = degrees of freedom for the variances used at the numerators (n) and at the denominators (d) to calculate the F values, according to ref *42*.

For the real-time PCR assay, the "relative quantification" method (*9*) was applied. This is based on two standard curves, for the foreign and the reference endogenous genes respectively, built with a known standard calibrator. The curves provide the exogene and reference endogene copy numbers of an unknown DNA sample.

A preliminary detailed investigation was carried out in order to define crucial parameters of real-time PCR analysis in grape, including selection of the most suitable reference endogenes, the real-time amplification system and the calibration system, as detailed and discussed below.

Selection of Suitable Endogenous Reference Genes. A major aspect of the real-time PCR assay for accurately analyzing genetically modified grape is the selection of a suitable endogenous reference gene. Endogenes must conform to certain critical requirements, such as species-specificity, low intraspe-

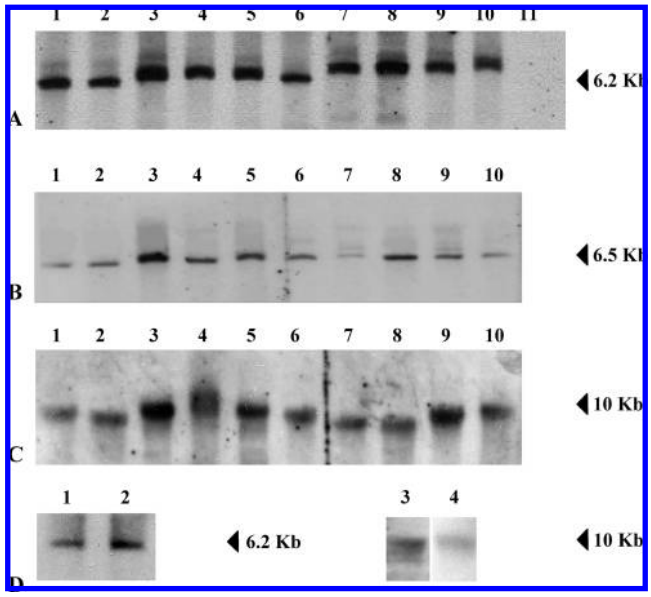

Figure 2. Southern blot analysis for quantification of the copy number presence of the endogenous genes for the 9-*cis*-epoxycarotenoid dioxygenase (*nced2*) and for the chalcone, isomerase (*chi*) in different *V. vinifera* cultivars (samples 1−7), various grape rootstocks (samples 8−10), and the *nced1* amplicon: results obtained for *nced2* endogene after DNA digestion with *Sac*I (**A**) and with *Xba*I (**B**) restriction enzymes, and for *chi* endogene after DNA digestion with *Xba*I restriction enzyme (**C**). Lanes: 1 = Chardonnay; 2 = Traminer; 3 = Pinot Noir; 4**A** = Marzemino, 4**B** = Cabernet Sauvignon, 4**C** = Lambrusco f.f.; 5 = Imperatrice; 6 = Superior seedless; 7 = Regina; 8 = 140 Ruggeri; 9 = SO4; 10 = Kober 5BB; 11**A** = *nced1* amplicon. In (**D**) are reported the analysis of the *nced2* (samples 1, 2) and *chi* (samples 3, 4) endogenes performed on the DNA extracted from genetically modified Brachetto plants, obtained with the pX6-pKcpGVA. Lanes: 1 and 2 = probe for *nced2*, digestion with *Sac*I restriction enzyme; 3 and 4 = probe for *chi*, digestion with *Xba*I restriction enzyme.

cific variability, and presence in a single or a low copy number in the genome (prEN ISO 2426 and 21569). Accordingly, on the basis of Data Bank analyses and our preliminary experiment (*27*), two genes were identified as promising candidates, i.e. the gene for the 9-*cis*-epoxycarotenoid dioxygenase (*nced2*) (*28*) and the gene for the chalcone isomerase (*chi*) (*29*, *30*).

An initial GenBank search provided the mRNA sequences of both genes (AY337614.1 for *nced2* and X75963 for *chi*), while a *Blastn* search (www.ncbi.nlm.nih.gov/BLAST/) yielded the gene sequences based on recently released data from the grape genome for *V. vinifera* Pinot Noir (*7*) and obtained from a highly homozygous *V. vinifera* genotype (*6*). Comparison of mRNA with their corresponding DNA sequences showed, for *nced2*, absence of introns, alignment with a single contig (AM457017.1) and a shared identity of 76% with the isoform *nced1*. Furthermore, *chi* was found to have 4 exons and 3 introns, that is, a gene structure similar to the homologous barley, rice and maize genes where *chi* is present in 1, 1, and 3 copies respectively (*47*). The above-mentioned data from grape genome sequencing also proved a single copy presence for the *chi* gene in grape.

In addition, Southern blot analysis performed on 7 different *Vitis vinifera* renowned cultivars of grapevine (Chardonnay, Traminer, Pinot Noir, Marzemino, Cabernet Sauvignon, Lambrusco f.f., Brachetto), 3 table-grape cultivars (Imperatrice, Superior seedless, Regina), and 3 rootstocks (140 Ruggeri, SO4, Kober 5BB) (**Figure 2**) verified decisively the copy number of these genes in the *Vitis* genus. In our analysis, *nced2* proved to be present in a single copy in each genotype assessed. Moreover, the high specificity of the *nced2* probe was verified by additionally blotting the *nced1* amplicon which, as expected, turned out to be undetected (**Figure 2A**, lane 11). Furthermore, the *chi* gene also proved to be a single copy gene. This result confirmed the findings previously reported by ref *29* for the cv. Lambrusco f.f. where the same restriction enzyme was employed.

Real-Time Amplification Systems. In order to select the most suitable primer and probe sets for real-time PCR amplification of the *nced2* and *chi* endogenes, many different DNA sequences were designed using the Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and carefully checked on serially diluted DNA extracted from a *V. vinifera* cv. Chardonnay plant (data not shown). The selection of these sets (described in Material and Methods) was based on the best amplification profiles, standard curves and melting curves obtained. The standard curves, in fact, presented R^2 (0.999 for both *nced2* and *chi*) and slope values (−3.529 for *nced2* and −3.562 for *chi*), both within the ranges recommended by ENGL method performance requirements ($R^2 \geq 0.98$ and $-3.1 \geq$ slope ≥ -3.6) (*48*). In addition, single and sharp melting peaks were obtained (data not shown), proving the presence of a unique type of allele for the amplified genes in the genome.

The performances of the two endogenes were compared on the basis of their amplification curves (Ct values). Ct values of *nced2* curves are slightly higher than those of *chi* curves, giving ΔCt values close to 0.5. This minor divergence may be attributed to differing performances of the specific primer and probe systems rather than to dissimilar gene copy numbers, as found with Southern blot assays.

These results were also verified on several grape genotypes (*V. vinifera* cvs Chardonnay, Traminer, Cabernet Sauvignon, Riesling, Moscato giallo, Merlot, Marzemino, Pinot Noir, Brachetto, and the rootstocks Kober 5BB and SO4), where the amplification performances of the two genes were tested and the differences between *nced2* Ct and *chi* Ct ($\Delta Ct = Ct_{nced2} - Ct_{chi}$) were calculated (**Table 1**). The ΔCt mean value was 0.60, and the repeatability standard deviation (RSDr %) was 15%, i.e. a value that proves an ordinary variability among these genotypes, according to the principal biometry manuals (*42*).

Finally, regarding the *nptII* exogene, the suitable primer and probe set designed with the Primer3 software was assessed and verified on the DNA extracted from a *V. vinifera* cv. Brachetto plant, known to be genetically modified for this foreign gene.

Calibration System. To build the real-time PCR standard curves, suitable calibrators are required, i.e. DNA at a known percentage of exogene extracted from properly genetically modified matrices. Certified reference materials are available for the principal genetically modified plants and transgenic events released on the market (Institute for Reference Materials and Measurements, IRMM, Belgium), while for other species reference materials have to be prepared by the experimenter. According to the literature, the calibrator is usually DNA extracted from a genetically modified plant with a known foreign gene copy number, as verified with Southern blot analysis (*15*, *17*, *49*). This strategy, however, is hindered by the occurrence of possible exogene chimerical events associated with the gene transfer technique, leading to a mixture of cells with differing copy number insertions, most frequently deletions, that Southern blot assay is not capable of identifying. For this reason, the reliability of such calibrators employed in real-time PCR assay is questionable.

Table 5. Evaluation of *nptII* Gene Removal Produced by β-Estradiol Supplied to the Buds with Two Different Strategies (Liquid versus Solid) and at Three Different Concentrations (0, 10, 20 μM) in the Whole Plantlets[a]

	β-estradiol 0 μM		β-estradiol 10 μM		β-estradiol 20 μM	
supply strategy	*N*	mean CN	*N*	mean CN	*N*	mean CN
β-estradiol in liquid	6	0.50	6	0.30	6	0.26
β-estradiol in solid	6	0.50	6	0.28	6	0.28

Friedman Test

source of variation	χ^2_F value	P value
β-estradiol concentration	23.58	<0.001***
β-estradiol supply	0.077	0.05

Two-Way ANOVA

source of variation	F value	P value
β-estradiol concentration	61.60	<0.001***
β-estradiol supply strategy	0.024	0.05
interaction	0.388	0.05

LSD Test

	mean CN absolute differences		
hormone supply strategy			LSD value
liquid	0 μM − 10 μM	0.20*	
	0 μM − 20 μM	0.24*	
	10 μM − 20 μM	0.04	
solid	0 μM − 10 μM	0.22*	0.046
	0 μM − 20 μM	0.22*	
	10 μM − 20 μM	0.00	

[a] The *nptII* mean copy number (CN) was calculated on 6 shoots (*N*) with the calibrator p-*nptII/chi*, and two analyses of variance, respectively nonparametric (Friedman test) and parametric (two-way ANOVA), were carried out. χ^2_F value = calculated Friedman value; P value = probability of assessing no differences between quantification measures; F value = calculated Fisher values; LSD value = calculated least significant difference value at a 95% probability level for a pairwise comparison of different β-estradiol concentrations; * = significant value; *** = highly significant value.

Recently, the use of multiple-target plasmids is finding increasing favor in foreign DNA detection analysis in food and feed (*32*, *33*). Moreover, our previous experiments with soybean and maize (*31*) have shown that duplo-target plasmids as standard curve calibrators are valuable alternatives to genomic DNA of certified standards, as they offer the possibility of being fully sequenced, provide highly reproducible analyses and accurate performances, and are cheap and stable in the long term.

Therefore, in order to elaborate a suitable method for analyzing genetically modified grape, we developed ad hoc duplo-target plasmids, carrying the sequences of both reference endogenous and exogenous genes, which simulates the DNA extracted from an ideal genetically modified plant carrying a homozygous single-copy exogene insertion. In the present research, two duplo-target plasmids were developed, p-*nptII/nced2* and p-*nptII/chi*, both of which contain a sequence of the gene for kanamycin resistance linked to one or the other of the two chosen endogen sequences.

To discover which was the more reliable calibrator, we compared the real-time PCR quantification performances obtained with the two plasmid systems. Our results showed both duplo-target plasmids to be suitable calibrators.

First, standard curves of the *nced2* and *chi* endogenes were both found to be optimal since the correlation coefficients (R^2) (respectively 0.999 and 1.000) and amplification efficiencies related to slopes (respectively −3.408 and −3.404) fall within the ranges recommended by ENGL method performance requirements ($R^2 \geq 0.98$ and $-3.1 \geq$ slope ≥ -3.6) (*48*). Moreover, comparison of these standard curves with those obtained for genomic DNA amplifications showed equivalent values for the R^2 and slope parameters. As for the *nptII* exogene, the values obtained with the p-*nptII/nced2* and p-*nptII/chi* calibrators were 0.999 for R^2 in both cases and −3.240 and −3.391 respectively for slopes, while for genomic DNA the values were 0.998 for R^2 and −3.471 for slopes.

Ct values obtained with the duplo-target plasmid amplifications showed higher *nced2* values compared with *nptII*, while identical values were obtained for *chi* and *nptII*. As already pointed out (in Real-Time Amplification Systems), Ct values obtained with genomic DNA amplifications were higher for *nced2* than for *chi*. We consider these results to be due to similar amplification performances of the two endogenes in the plasmidic and genomic systems, thus proving their equivalence, and to show the suitability of the duplo-plasmids as calibrators for analyzing genomic DNA in real-time PCR. The differences in the Ct values between the two endogenes would not, therefore, affect copy number quantification.

Finally, the repeatability, or precision performance, of the quantifications provided by the two plasmid calibrators for determining the copy number of the *nptII* exogene was evaluated on DNA extracted from 3 samples of genetically modified *Vitis vinifera* cv. Brachetto plants obtained either with the pX6-pKcpGVA (*36*) (sample 1) or with the pPLT7000 construct (*37*) (samples 4 and 6), both carrying the kanamycin resistance trait, as reported in **Table 2**. For each plant, at least 5 quantification assays were performed. Mean quantification values of *nptII* exogene copy number (mean CN), respective confidence intervals (CI) at 95%, and relative repeatability standard deviations or coefficient of variation (RSDr %) were calculated employing both duplo-target plasmid calibrators. The RSDr % values obtained were well below 25%, that is the acceptance criterion suggested by ENGL method performance requirements (*48*). This fact proves the high precision of both methods. In addition, the degree of similarity between the exogene copy number mean values quantified with the two plasmid calibrators was evaluated with Student's "*t*" test, which showed no significant differences. It is worth stressing that this latter result could attest to the quantification accuracy of both methods. The levels of precision and accuracy obtained proved the equivalence of the two systems assessed.

Quantification of Exogene Copy Number by Southern Blot and Real-Time PCR Assays. Southern blot and real-time PCR assays were carried out in order to analyze the *nptII* exogene copy number on the DNA extracted from 9 randomly chosen genetically modified plantlets of *Vitis vinifera* cv. Brachetto obtained with the pX6-pKcpGVA (*36*) (samples 1 and 2) or with the pPLT7000 construct (*37*) (samples 3−9). The data obtained using the two methods were compared.

Among the 9 plants assessed, Southern blot analysis (**Figure 3**) showed a single-copy insertion for *nptII* in samples 1, 2, 6 and 7, and a two-copy number integration in the others.

Regarding the real-time PCR, exogene copy number was quantified using the relative quantification method (*49*) and application of the following formula: exogene copy number insertion = (exogene copy number/reference gene copy number) × 2. In the formula, the ratio between the two genes has to be

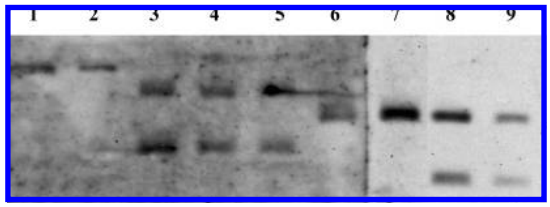

Figure 3. Southern blot analysis for quantification of copy number integration of the *nptII* exogene assessed on genomic DNA extracted from 9 genetically modified *V. vinifera* cv. Brachetto plants. DNA was digested with *Xmn*I restriction enzyme. Lanes: 1 and 2 = gene transfer obtained with the pX6-pKcpGVA vector; 3−9 = gene transfer obtained with the pPLT7000 construct.

Table 6. Evaluation of *nptII* Removal Produced by β-Estradiol Supplied to the Buds with Two Different Strategies (Liquid versus Solid) in 5 Different Regions of the Plantlets (the Leaves of the Apical Node "L_A"; the Two Apical Nodes and Internodes "IN_A"; the Leaves of the Basal Node "L_B"; the Two Basal Nodes and Internodes "IN_B" and the Roots "R")[a]

supply strategy	region	*N*	mean CN	KW value	P value	CD
β-estradiol in liquid	L_A	9	0.47	16.40	<0.001***	b
	IN_A	9	0.43			b
	L_B	9	0.47			b
	IN_B	9	0.45			b
	R	6	0.01			a
β-estradiol in solid	L_A	9	0.54	25.88	<0.001***	b
	IN_A	6	0.52			b
	L_B	9	0.45			b
	IN_B	9	0.43			b
	R	9	0.13			a

[a] The *nptII* mean copy number (CN) was calculated with the calibrator p-*nptII*/*chi*, and a nonparametric analysis of variance (Kruskal−Wallis test) was carried out. *N* = number of measures obtained from 3 plantlets analyzed in 3 independent replicates. Three independent replicates of 2 plantlets from the "R" and the "IN_A" samples induced with β-estradiol, both in liquid and in solid, were analyzed. KW value = calculated Kruskal−Wallis values; P value = probability of assessing no differences between quantification measures; *** = highly significant value; CD = multiple comparisons through the evaluation of the critical difference with the Bonferroni−Dunn procedure ("a" refers to a result different from "b").

doubled since a single-copy exogene insertion in a diploid T_0 plant is expected to have exogene hemizygosity and endogene homozygosity (*8*).

The two duplo-target plasmid quantifications were found to be very similar in almost all the samples analyzed, providing a variable range of exogene copy numbers among the different plant samples (**Table 3**).

We should point out that inconsistencies in copy number quantifications obtained with real-time PCR and Southern blot assays were found. We would exclude possible imprecision of the real-time PCR assay, as discussed above. First of all, our results are based on two different systems, both providing the same results (Student's "*t*" test, **Table 2**) and proving high precision (RSDr % values well below 25%, **Table 2**), while the calibrators applied are sequenced, thus reliable, plasmids. Moreover, the relative quantification method is widely used in transgenic plant analysis and is considered reliable for GMO detection (*32*). Finally, these results remain constant in further analyses performed on different parts of the plants (**Table 4**) as discussed below.

Comparison of our results with the few studies reported in the literature shows that similar mismatches between real-time PCR and Southern blot assays have already been found in tomato (*18*) and maize (*17*) during quantification assays based on real-time PCR and the relative quantification method, and in maize (*15, 16*) and tobacco (*49*) when real-time PCR and different quantification methods were applied. The levels of copy number integration and the reliability of the assays have been taken into account in interpreting these incongruities, and a combination of both methods has been suggested for precise exogene estimation (*9*).

We believe that the observed differences in quantification between the two techniques may be attributed to the different type of information that each method provides. In fact, for a given target exogene, Southern blot assay estimates the uppermost quantifiable integration pattern present in the DNA extracted from the transgenic cells of a sampled plant tissues, while real-time PCR based on a relative standard curve provides quantification of the total number of both foreign and endogenous genes present in the cell pool and exogene copy number is the results of their ratio. This ratio could be influenced by the possible presence of cell mixture with different integration patterns of exogene integration due to exogene instability or chimerical integration (*50*). The latter case may be the result of possible foreign gene deletions during the several morphogenic steps leading from embryogenic callus to plantlet regeneration. Moreover, this may be the outcome of an ineffective selection of the antibiotic (*51*). With Southern blot analysis, on the other hand, the genetically modified cells can give positive results, concealing the possible presence of nontransgenic ones.

We performed the molecular characterization on genetically modified plantlets germinated from single somatic embryos, putatively carrying the kanamycin-resistance trait, regenerated from embryogenic calli selected on medium to which this antibiotic had been added (**Figure 1**). Even though somatic embryos are described as being of single-cell origin (*52*), participation of more cells in the morphogenesis cannot be excluded (*53*). This may have given rise to regenerants where transgenic cells coexist with wild-type cells, and may explain the values of the mean CNrt detected (**Table 3**). As a consequence, the ratio between the copy number values obtained with real-time PCR (CNRt) and with Southern blot (CN_{SB}) analyses would show the extent of transgenic cell presence in the plant tissues. In our study, this ratio is recurrently close to 1/2, with the exception of samples 6, 7 and 9, where it is close to 1/5, 1/5 and 1/3 respectively, suggesting the presence of more wild type cells than transgenic cells. Further meticulous micropropagation in the presence of kanamycin may be a suitable solution for removing nontransgenic cells from the plantlets.

Moreover, the combination of Southern blot and real-time PCR assays also provides precious information for characterizing the origin of the regenerants. In fact, when a highly proliferating embryogenic callus (**Figure 1**, **A** and **B**) is employed for gene transfer, secondary embryogenesis from a single somatic embryo may regenerate clones of the same exogene integration event. In our research, for instance, among 9 plantlets investigated, the presence of the 4 integration sets detected with Southern blot (**Figure 3**) (i.e., samples 1 and 2; samples 3, 4, and 5; samples 6 and 7; and samples 8 and 9) is confirmed with real-time PCR copy number quantifications (**Table 3**), with the exception of samples 8 and 9. For these latter, in fact, while the same restriction patterns were obtained with Southern blot, different values were obtained with real-time PCR.

Finally, the effectiveness of our method for assessing the stability of a foreign gene was performed on sample 1, i.e. a *V. vinifera* cv. Brachetto plant where *nptII* exogene was transferred with the pX6-pKcpGVA (*36*). The mother-plant was micropropagated for one year in the absence of kanamycin, then a plantlet was randomly chosen from the clone produced and three buds were separated for originating three distinct plant samples.

After 50 days in the kanamycin-free elongation medium, DNA was extracted from three different parts of the plants by setting apart, respectively, three expanded leaves of the apical bud (L), a mixture of tissues consisting of the apical bud and the two superior internodes (M), and the roots (R). Exogene copy number was quantified with real-time PCR assay using the p-*nptII*/*chi* plasmid calibrator.

Table 4 reports the mean copy number values of three measures for the three distinct regions of each plant. All the values were very similar to those obtained from plant sample 1 (0.55 ± 0.084, **Table 2**), and fall into the confidence interval of the mean copy number calculated ($0.47 < 0.55 < 0.64$). Furthermore, analysis of variance with a hierarchical design gave Fischer values which definitely prove no significant differences between the three plants nor between the different regions. This test confirms the reliability of our approach to characterizing genetically modified grape and to following exogene stability during long-term plant preservation.

Evaluation of the Efficiency of *nptII* Marker Gene Removal with 17-β-Estradiol Induction. Since there is nothing in the literature reporting successful production of marker-free transgenic grape, our research is a first attempt to apply the marker gene removal technique in *Vitis*. The technique exploited is the chemical-regulated, site-specific DNA excision (*26*), which could be a promising strategy in this fruitcrop. In fact, the insertion of the marker gene would allow an effective selection to be made between cells that have inserted the foreign gene and those that have not. Subsequent induction of the Cre/*loxP* system would bring about removal of the undesired genetic material that was inserted during the gene transfer process.

In our experience, induction with β-estradiol is one of the most critical factors of the overall process. Concentration, supply strategies and application time of the hormone have to be carefully evaluated in order to carry out efficient marker gene removal from the plant tissues.

In this paper, the real-time PCR assay we have elaborated seems to be a valuable tool for estimating the efficiency of *nptII* gene excision induced by β-estradiol. Hormone induction was performed on the buds of the genetically modified plant, produced with the pX6-pKcpGVA construct, which contained 0.55 ± 0.084 copy number of the *nptII* gene (plant sample 1, **Table 2**). After one-month induction, the buds elongated into 2 cm plantlets with 1 node, 2 leaflets and the root. The 12 plantlets obtained from each induction treatment (two supply strategies $\times$ 3 hormone concentrations) were divided into two groups.

From the first group, 6 plantlets were chosen for DNA extraction from the whole plant including the root. Real-time PCR assays were performed in order to assess the amount of *nptII* gene present in the samples after β-estradiol induction. The results are reported in **Table 5**, which shows data from both the nonparametric and parametric analyses according to ref *54*.

Comparison of the inductions with 0 (control), 10 and 20 μM of hormone on the basis of a nonparametric analysis of variance (Friedman test) shows significant differences among the mean copy numbers (CN) (P value <0.001), while no significant differences were found between the two supply strategies (P value >0.05). These findings were also confirmed by a further parametric analysis (two-way ANOVA). This test, moreover, was able to exclude a possible interaction between the β-estradiol concentration and the β-estradiol supply strategy (P value >0.05).

Regarding the different hormone concentrations, a pairwise comparison was performed with the least significant difference (LSD) test. In both liquid and solid supply strategies, the only significant differences observed were between controls and hormone-induced plantlets, while no significant differences were found between the two concentrations of β-estradiol (10 and 20 μM), the absolute differences between their mean CNs being always lower than the LSD values. Moreover, the two supply strategies (solid versus liquid phase) did not differ significantly (P value >0.05).

The plantlets of the second group were grown for another 45 days (**Figure 1D**) before molecular analysis was carried out in 5 different regions, i.e. the leaves of the apical node, the two apical nodes and internodes, the leaves of the basal node, the two basal nodes and internodes, and the roots (**Table 6**). The analysis of variance shows significant differences among the 5 tissues for both hormone supply strategies (P values <0.001***). According to the Bonferroni−Dunn procedure (CD), these differences are to be attributed to the root region, where exogene removal was obtained at the highest level (roots showed the lowest *nptII* mean copy numbers, 0.01 and 0.13). Moreover, when the hormone was supplied to the solid medium, the *nptII* copy numbers in the various regions increased progressively from the root to the apical parts of the plantlets, suggesting that exogene removal tends to a gradient.

Conclusions. In conclusion, the real-time PCR strategy we developed for characterizing genetically modified grape was shown to be effective, and two endogenous genes were identified as suitable candidates for real-time PCR quantification in grape. Preliminary research (*27*, *55*) had already shown the *nced2* gene to be suitable. And the present study has fully confirmed its reliability on the basis of new meticulous experiments and more advanced methods. First of all, the recently released genome data (*6*, *7*) allowed us a better choice of primer and probe systems. In addition, its presence in single-copy number was checked on a much larger sample of grape genotypes. Finally, specific duplo-target plasmids—instead of linear amplicons—were assessed. The suitability of a second endogene, i.e. *chi*, was also tested and proved.

Both duplo-target plasmids developed for the two genes in this study were shown to be suitable standard calibrators for simulating an ideal transgenic plant, and this approach is a decidedly innovative strategy not previously applied in the *Vitis* genus. Both methods proved to offer highly precise results and accuracy as discussed above.

Additionally, this assay was shown to be appropriate for evaluating the efficiency of a marker gene removal based on the self-excision strategy. We believe that the latter point is a highly relevant and original aspect of the present study.

Our research was developed on grape; however, the assay elaborated here may also be suitable for characterizing a wide range of crops, after adaptation to the specific characteristics of the selected plant system, in particular the choice of species-specific endogene.

ABBREVIATIONS USED

Chi, chalcone isomerase; CN, copy number; Ct, threshold cycle; ΔCt, Cts difference; *nced2*, 9-*cis*-epoxycarotenoid dioxygenase; *nptII*, neomycin phosphotransferase; PVP, polyvinil pirrolidon; R^2, correlation coefficient; RSDr, repeatability standard deviation; SDS, sodium dodecyl sulfate; SSC, sodium chloride−sodium citrate; TBE, Tris borate EDTA; TE, Tris-Cl EDTA; UDG, uracil-DNA glycosylase.

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