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Biolistic transformation of grapevine using minimal gene cassette technology

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Abstract The use of minimal gene cassettes (MCs), which are linear DNA fragments (promoter + open reading frame + terminator) lacking the vector backbone sequence, was compared to the traditional use of whole circular plasmids (CPs) for transformation of grapevine. Embryogenic cell suspensions of 'Chardonnay' (*Vitis vinifera* L.) were transformed via particle co-bombardment using two nonlinked genes in either MCs or CPs. One construct contained the *npt*-II selectable marker and the second construct contained the *MSI99* antimicrobial peptide gene. A total of five lines each from MC and CP treatments that showed positive signals by PCR for both the *npt*-II and *MSI99* genes were selected. Southern blot analyses revealed up to five integration events in the DNA treatments. Transcription levels determined by semi-quantitative RT-PCR varied among transgenic lines. No significant differences were found in transgene transcription between lines from MC and CP transformation. The correlation between *npt*-II and *MSI99* transcription levels was positive ($P<0.05$), however, no correlation between the transcription level and the number of integration events was observed. Transgenic lines presented a similar phenotype in leaf morphology and plant vigor compared to non-transgenic lines. Moreover, transgenic lines from both MC and CP DNA treatments produced fruit as did the non-transgenic lines in the third year of growth in the greenhouse. Our data confirm the effectiveness of the minimal cassette technology for genetic transformation of grapevine cultivars.

Keywords Integration events · Magainin genes · Minimal cassette · Transgene-transcription · *Vitis vinifera*

Abbreviations CP(s): circular plasmid(s) · MC(s): minimal cassette(s) · *MSI99*: synthetic analog of magainin-2 gene · RT-PCR: reverse transcription-polymerase chain reaction

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Introduction

Grapevine (*Vitis* sp.) genetic transformation is now routinely achieved by both *Agrobacterium*- and biolistic- mediated DNA delivery systems (Colova-Tsolova et al. 2001; Kikkert et al. 2001). With both methods, all transgenes such as reporter genes, selectable markers and genes of agronomic interest have been transferred to regenerable embryogenic tissues via the construction of circular recombinant plasmids. Whole plasmids are convenient carriers, however, undesirable backbone sequences of the vectors were found integrated in the genome of transgenic plants developed with both *Agrobacterium* (Cluster et al. 1994; Kononov et al. 1997; DeBuck et al. 2000) and biolistic (Christou 1997; Kohli et al. 1999) systems. Sequences of bacterial origin in the plasmid backbone such as the origin of replication and AT-rich sequences were reported to have negative effects on transformation, such as promotion of transgene rearrangements and illegitimate recombination (Muller et al. 1999) as well as methylation of the transgene and an inability to bind eukaryotic nuclear proteins leading to gene silencing (Jakowitsch et al. 1999). Furthermore, the persistence of these unwanted bacterial sequences in addition to selectable bacteria genes in commercial transgenic plants is a biosafety concern.

For *Agrobacterium*-mediated gene transfer, circular plasmids are needed for transformation. Moreover, some sequences in the Ti plasmid backbone, such as *vir* genes, in both cointegrating and binary vector systems, are needed

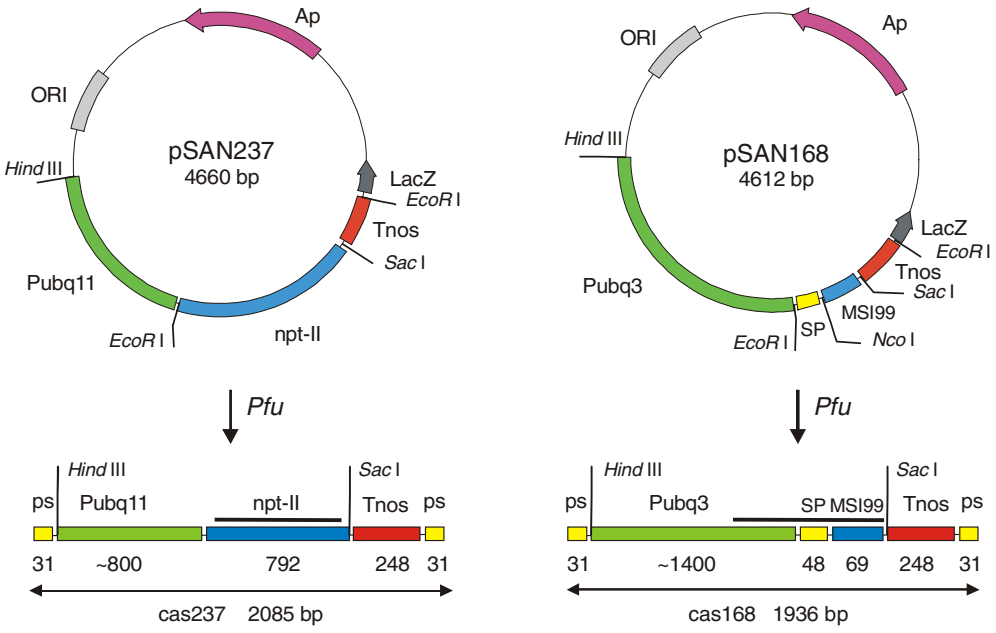

Fig. 1 Schematic representation of DNA constructs used for biolistic cotransformation. Minimal gene cassettes (*below*) were developed by PCR (*Pfu*) amplification from circular plasmids (*above*). Diagrams of the constructs are not to scale. Elements of the cassettes are as follows: *Pubq3* and *Pubq11* are *A. thaliana* ubiquitin promoters; *Tnos* is the *A. tumefaciens* T-DNA nopaline synthase terminator; *npt*-II is the *E. coli* neomycin phosphotransferase II gene; SP is a signal peptide from pea vicilin protein; *MSI99* is a synthetic derivative of the natural magainin 2 gene and PS is an upstream and downstream cassette protection sequence. The numbers in the figures correspond to the sequence size in base pairs (bp). The total size of the cassettes includes multicloning sites (not shown). *Hind*III and *Sac*I correspond to unique restrictions sites on the transformation vectors. ORI and Ap correspond to the plasmid origin of replication and a selective ampicillin gene, respectively. Bars above cassettes correspond to the probe size

to facilitate the transfer of T-DNA into the plant cell (for review, see Gruber and Crosby 1993; Zupan et al. 2000). In contrast, backbone sequences have no effect on the biolistic delivery of foreign genes into plant cells (Klein et al. 1987). However, backbone sequences in circular and linear plasmids may stabilize and protect transgenes from degradation by cellular nucleases after bombardment, during the cell repair and integration process (Hunold et al. 1994).

Minimal gene cassettes which contain only a promoter, open reading frame and terminator (lacking vector backbone sequences) were first reported by Fu and coworkers (2000) for transformation of rice. Recent reports using the same model plant (Breitler et al. 2002; Loc et al. 2002) and also potato (Romano et al. 2003) demonstrated the potential of this minimal cassette technology in the transformation of herbaceous plants. Previously, higher biolistic transformation efficiencies were reported with linearized plasmids, both double- and single-stranded forms, compared to circular plasmids (Uzé et al. 1999). Here we focused on a comparison of transgenic grapevines arising from minimal cassettes (MCs) technology with those derived from the standard procedure that uses circular plasmids (CPs). In each case, two constructs were used; one construct contained the *npt-II* selectable gene and the second construct carried a magainin antimicrobial gene, both driven by *Arabidopsis* constitutive promoters. Magainins (Zasloff 1987) are short antimicrobial peptides with demonstrated broad-spectrum in vitro antimicrobial activity (Kristyanne et al. 1997) including activity against grapevine bacterial pathogens (Li and Gray 2003).

In this work we report the successful co-transformation, regeneration and transcription of both *npt*-II and magainin transgenes in 'Chardonnay' (*Vitis vinifera* L.), using minimal gene cassette technology. Transgenic plants were grown in the greenhouse and produced fruit in their third growing season.

Material and methods

Gene constructs

Two circular pUC19-based plasmids, pSAN168 and pSAN237, were used as the templates to develop the linear minimal gene cassettes, cas168 and cas237 (Fig. 1). pSAN168 (4612 bp) carries a signal peptide (SP) from pea vicilin protein fused to a synthetic *MSI99* gene, a derivative of the natural magainin-2 gene (DeGray et al. 2001), under control of the *Arabidopsis* ubiquitin-3 promoter (Pubq3) and the *Agrobacterium* T-DNA nopaline synthase (nos) terminator (Tnos). pSAN237 (4660 bp) harbors the selectable marker neomycin phosphotransferase II (*npt-II*) gene under control of the *Arabidopsis* ubiquitin-11 promoter (Pubq11) and Tnos. Minimal gene expression cassettes were amplified from the source plasmids with the *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA) according to the manufacturer's instructions using specific primers designed to protect cassette borders and containing restriction sites for cloning. Each primer has 44 bases (b): 13 b from the original cassette, 11 b from the vector and

Table 1 Specific primers used in the study

Primer[a]	DNA fragment	Size (bp)	Sequence 5′ to 3′[b]
Cassette design (amplification and protection of minimal gene cassettes)			
SAN168-for			TCC **TTA ATT AA**T ATA AGA AGC ACT ATA GGG AAA GCT TCG GAT TT
	Cas168	1936	
SAN168-rev			GTT **GGC GCG CC**T ATA AGA AGC CAG TGA ATT CCC GAT CTA GTA AC
SAN237-for			GTT **GGC GCG CC**T ATA AGA AGC ACT ATA GGG AAA GCT TCT TGC CT
	Cas237	2085	
SAN237-rev			TCC **TTA ATT AA**T ATA AGA AGC CAG TGA ATT CCC GAT CTA GTA AC
PCR and hybridization (probe design) analyses			
5′-npt-II			CAA GAT GGA TTG CAC GCA GG
	npt-II	779	
3′-npt-II			TTG AGC AGT TCT TCC GCT ATC
Pubq3-for			CAC GTA TCA TAT GAT TCC TTC C
	MSI99 cassette	400	
MSI99-rev			GAG CTC GTT AGG AGT TCA GG
RT-PCR analysis (transcription level)			
SP-for			ATG CTT CTC GCT ATT GCC
	SP + MSI99	117	
MSI99-rev2			TTA GGA GTT CAG GAT CTT CAC
EF1-α-for			ATT GTG GTC ATT GGY CAY GT
	EF1-α cDNA	700	
EF1-α-rev			CCT ATC TTG TAV ACA TCC TG

[a]Abbreviations for and rev indicate forward and reverse primer, respectively

[b]Bold nucleotides correspond to *Pac*I (TTAATTAA) and *Asc*I (GGCGCGCC) restriction sites designed for minimal cassette cloning. Underlined nucleotides correspond to *Hind*III (AAGCTT) and *EcoR*I (GAATTC) restriction sites from the original gene cassette in the circular plasmid.

20 b extra containing either *Pac*I or *Asc*I restriction sites (Table 1) . Primer design was checked with the Lasergene software (DNAstar, Madison, WI). Thus, each linear cassette comprised an ubiquitin promoter, a coding region, plus the Tnos and was flanked both upstream and downstream by a 31-bp protection sequence (Fig. 1). PCR-amplified cas168 and cas237 cassettes were double digested with *Pac*I and *Asc*I (NE BioLabs, Beverly, MA) and the fragments ligated in pNEB193 (NE BioLabs) to yield pNEBcas168 and pNEBcas237, respectively. Both pSAN- and pNEB-derived plasmids were cloned in *Escherichia coli* strain DH10b. Plasmids were isolated from bacteria using a plasmid purification kit (QIAGEN, Valencia, CA), sequenced (Cornell facilities) and finally pNEB-derived plasmids were double digested with *Pac*I and *Asc*I. Cassettes were separated from the plasmid backbones on 0.8% ultrapure agarose (GibcoBRL, Grand Island, NY) gels, purified from the agarose using the QIAEXII gel extraction kit (QIAGEN) and diluted in TE buffer (1.0 mM Tris and 0.1 mM Na_2EDTA, pH 8.0). DNA was quantified with a fluorometer and concentration was adjusted to 0.5 and 1.0 μg/μl for MCs and CPs, respectively.

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Plant material and transformation

Embryogenic cultures of cv. ‘Chardonnay’ were initiated from anthers and maintained on semi-solid medium approximately 2 years before establishing cell suspensions. The suspensions used (line 98Cd18) were 1-year old prior to transformation. Embryogenic cell suspensions were prepared for transformation as described by Vidal et al. (2003). Biolistic transformation was carried out by microprojectile co-bombardment following the procedure described in Kikkert et al. (2004) and using a prototype of the commercially available PDS-1000/He biolistic device (BioRad, Hercules, CA). The competency of the grape embryogenic cells was assessed by the β-glucuronidase (GUS) assay after bombardment with the vector pBI426, which contained a *gus*/*npt*-II gene fusion (Hébert et al. 1993). Cobombardment was made with a molar ratio of 1:1 for the unlinked linear minimal cassette [cas168 (Pubq3::SP::MSI99::Tnos) to cas237 (Pubq11::*npt*-II::Tnos)] or circular plasmid [pSAN168 to pSAN237]. Post-bombardment cell handling, embryo germination, plant regeneration and propagation were performed as described elsewhere (Vidal et al. 2003) with the exception that a constant concentration of 15 mg/l kanamycin [in hormone-free half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) with MS vitamins] was used for the selection of candidate embryos.

Molecular analyses

Integration of foreign DNA into the plant genome was determined by polymerase chain reaction (PCR) and

Table 2 Molecular analyses and phenotypic observation on transgenic and non-transgenic lines

Lines from different DNA treatments	Southern blots/*Hind*III digestion[a]		RT-PCR/Ratio[c]		Phenotype of greenhouse-grown plants		
	MSI99 probe[b]	*npt*-II probe	MSI99/EF	*npt*-II/*EF*	Leaf morphology[d]	Main shoot length (cm)[e]	No. of clusters produced[f]
Circular plasmids							
P1	2	1	7.7	nd	Normal	101 ± 33	2
P2	1	1	0.0	nd	nd	nd	nd
P3	4	3	3.1	nd	Normal	146 ± 10	5
P4	1	1	4.7	nd	nd	nd	nd
P5	3*	5	14.0	nd	Abnormal	49 ± 03*	0
Minimal cassettes							
C1	4*	1	0.6	2.8	Normal	153 ± 05	0
C2	2	2	5.4	3.5	Normal	103 ± 07	3
C3	0	0	0.0	0.1	Normal	128 ± 13	4
C4	1	1	9.3	4.2	Abnormal	114 ± 03	0
C5	0	1	0.9	1.6	nd	nd	nd
Non-transformants							
NT1	0	0	0.0	0.0	Normal	116 ± 09	7
NT2	0	0	0.0	0.0	Normal	114 ± 16	4

nd no data

*$P<0.05$

[a]Number of integration sites in genomic DNA containing either the *MSI99* or npt-*II* transgene fragment

[b]Presence of a 1.5 kb pairs diagnostic fragment, corresponding to integration of Ubiquitin 3 promoter::signal peptide::*MSI99* coding region cassette fragment after *Hind*III + *Sac*I double digestion, is indicated with an asterisk

[c]Transcription level determined as ratio between *MSI99* or *npt*-II gene and elongation factor (*EF*) gene transcription

[d]A leaf was considered normal with five lobes and a half open petiole sinus

[e]Average ± standard error of shoot length. Asterisk indicates a significant difference

[f]Number of clusters from total of three plants per line

Southern blot analyses. Total genomic DNA was extracted from leaves of transgenic and nontransgenic plants using the method described by Lodhi et al. (1994). DNA extraction was done from both in vitro- and greenhouse-grown plants. PCR amplification, Southern blots and digoxigenin probes for hybridization analyses were made as described by Vidal et al. (2003). Reverse transcription-polymerase chain reaction (RT-PCR) amplification of RNA was used to detect the presence of SP::MSI99 (117 b) and npt-II (779 b) mRNAs and determine gene expression. Total RNA was isolated from leaves of in vitro grown plants as described by Renault et al. (2000). Semi-quantitative RT-PCR analysis and hybridization of RT-PCR products were performed as described elsewhere (Vidal et al. 2006). The constitutive alpha subunit of the translation elongation factor 1 (*EF1*-α) gene was used as a control for quantification. Primers used for specific amplification of either DNA or cDNA as well as for generation of probes for hybridization analyses are shown in Table 1. All molecular analyses were performed at least twice. The GUS histochemical assay was performed as described by Hébert et al. (1993). The transcription level between MC and CP treatments, as well as correlation (Pearson moment product) between the number of integration events and transcription levels was statistically analyzed using SigmaStat software (SPSS Inc., Chicago, IL).

Plant morphology

Tissue culture lines that were positive in the molecular analyses and grew well in vitro were acclimated and grown in a greenhouse as described by Vidal et al. (2006). A total of seven transgenic and two nontransformed control lines were selected (Table 2). Three vegetatively propagated plants per line were analyzed. The plants were allowed to go dormant during the winter months. During the third growing season, visual observation of plants was based on several recognized descriptors for grapevine (IPGRI 1997) such as the number of lobes per leaf (O-068), the shape of the petiolar sinus (O-079) and the length of shoot internodes (O-353). In addition, the length of the primary shoot was measured and the number of inflorescences per line was counted between 2 and 3 months after bud break in greenhouse-grown plants.

Results

Plant regeneration

Embryogenic cell suspensions of ‘Chardonnay’ were used for biolistic transformation with three different DNA treatments in two separate experiments. In the first experi-

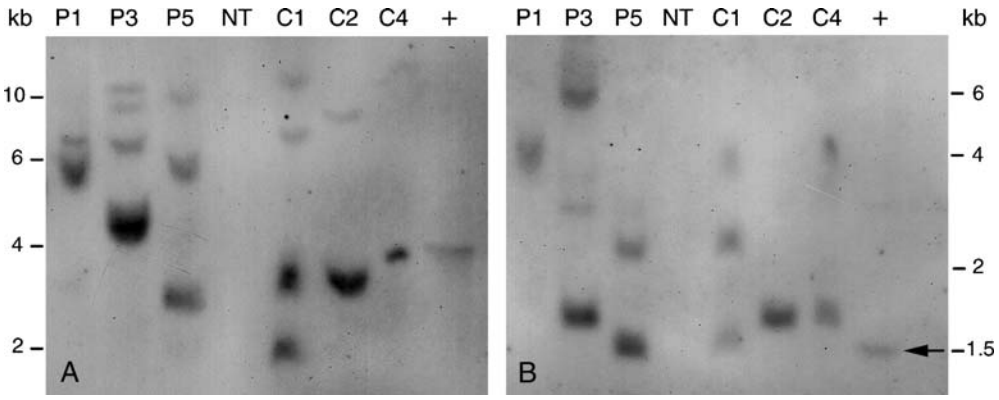

Fig. 2 Southern blots of genomic DNA from MSI99-transgenic 'Chardonnay' lines bombarded with either circular plasmids (P) or minimal cassettes (C) and a nontransformed (NT) plant. **a** DNA digested to completion with *Hind*III, which cuts once in the transgene contructs. **b** DNA digested with *Hind*III plus *Sac*I which cut once each in the transgene constructs and liberate a 1.5 kb diagnostic fragment indicated by an *arrow* (←). Digested DNA was blotted onto a membrane and hybridized with a digoxigenin-specific probe of the PCR-amplified MSI99 cassette. As DNA positive controls (+), pSAN168 digested with either *Hind*III or *Hind*III plus *Sac*I was used. Pictures were scanned from hybridization films

ment, cell suspensions were co-bombarded with two CPs (pSAN168 and pSAN237). In the second experiment, cell suspensions were co-bombarded with two MCs (cas168 and cas237) lacking plasmid backbone sequences (Fig. 1). In addition, separate aliquots of cells were also bombarded with pBI426 containing the *gus* (*uidA*) reporter gene. The competency of the embryogenic cells for transformation was evaluated by transient GUS expression. Two days after bombardment, an average of 6431 ± 733 blue spots per plate was obtained from nine bombarded plates from both experiments, indicating successful transient transformation and good potential for stable transformation.

Germinated embryos were selected on 15 mg/l kanamycin. A total of 57 embryos from CP co-transformation and 40 embryos from MC cotransformation at 90 and 105 days after bombardment, respectively, were harvested. All embryos emerged from different places on the filter paper support containing the embryogenic cells; therefore they were treated as independent transgenic lines. In this work, one year after the bombardment, 28 plants out of 57 embryos (approximately 50%) from CP co-transformation and 16 plants out of 40 embryos (approximately 42%) from MC co-transformation were regenerated.

Transgene integration

An initial screening by PCR on healthy-appearing in vitro plants was used to detect the presence of both *npt*-II selectable and *MSI99* nonselectable transgenes. PCR analysis of genomic DNA showed 5 lines (31%) from MC and 10 lines (35%) from CP co-transformation with positive bands of the expected size for both *npt-II* (779 bp) and *MSI99* (400 bp) gene fragments, as well as for plasmid controls (pSAN168 or pSAN237), whereas no amplification was detected from DNA of nontransformed plants and a pUC19 negative control. These positive signals were confirmed by dot blot hybridization for all lines except line C3 from MC transformation, indicative of a possible chimeric status for this line (data not shown). In addition to the five PCR-positive lines transformed with the MCs, a representative group of five PCR-positive lines transformed with CPs were selected and vegetatively propagated for further analyses (Table 2).

The integration patterns of the gene constructs incorporated into the genomic DNA after cotransformation with either MCs or CPs was studied by Southern blots. Hybridization was performed with probes corresponding to cassette fragments containing the *MSI99* or the *npt*-II genes (Fig. 1). DNA from selected lines was digested with *Hind*III to determine the number of integration events. *Hind*III cuts only once in each of the four DNA constructs (Fig. 1) and the other cut is elsewhere in the plant genome. Single digestion with *Hind*III revealed multiple integration events for both MC and CP cotransformations (Table 2). A representative Southern blot from this experiment probed with the MSI99 fragment is shown in Fig. 2a. The number of integration events ranged from 1 to 4 and 5 sites for *MSI99* and *npt*-II genes, respectively (Table 2). A double digestion with *Hind*III and *Sac*I was carried out to determine the partial integrity of the MSI99 cassette (Fig. 1). The expected diagnostic fragment ($\sim$ 1.5 kb) containing the *MSI99* gene (Pubq3::SP::MSI99) was observed using the MSI99 probe in one out of three lines cotransformed with MCs and also in one out of three lines cotransformed with CPs (Fig. 2b).

Gene transcription

To estimate transgene expression, semi-quantitative RT-PCR of total RNA was chosen over the standard Northern blot technique to more readily detect the small size (117 b) of the expected mRNA transcript (SP + MSI99). This transcript was detected in four out of five lines from both MC and CP cotransformations, while no signals were detected in the nontransgenic control (Fig. 3). All five lines from MC transformation as well as all lines from CP transformation (data non shown) were positive for transcription of the *npt*-II gene (Fig. 3b). Amplification

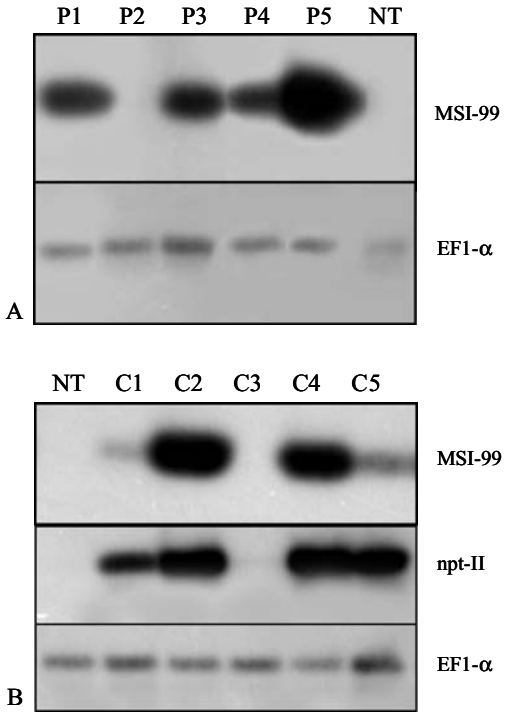

Fig. 3 Semi-quantitative RT-PCR analyses of MSI99-transgenic 'Chardonnay' lines. Transcription of exogenous [*MSI99* (117 bp) and *npt*-II (779 bp)] and endogenous [*EF1-α* (700 bp)] genes in lines obtained from **A** circular plasmid (P) and **B** minimal cassette (C) DNA treatments as well as from nontransformed (NT) plants was analyzed. Differences among transcription levels of transgenic lines were estimated after 20 cycles of PCR on equivalent total cDNA amounts. Hybridization signals are from digoxigenin-specific probes as described in Vidal et al. (2006)

of the constitutive *EF1-α* gene indicated that only cDNA from the EF1-α transcript was amplified as only a 700-bp fragment was detected by hybridization with the EF1-α probe, lacking the plant intron (200 bp) in the endogenous gene (900 bp) sequence. Comparative amplification of RT-PCR products at 20 cycles within both MCs and CPs treatments showed lines with different levels of transcription for both *MSI99* and *npt*-II transgenes (Table 2). However, no significant differences in *MSI99* transgene transcription between lines from MC and CP treatments were found.

Though only five lines each from MC and CP transformation were analyzed, we checked the correlation between the transcription levels and the number of integration events and found no significant relationship. For example, among plants obtained from the MC transformation, the Southern blots showed four and two integration sites for the MSI99 fragment in lines C1 and C2, respectively, but the transcription level was much higher in line C2 than line C1 (Fig. 3b). However, the correlation coefficient between MSI99 and *npt*-II transcription levels within lines from the MC treatment was positive ($R = 0.882$, $P < 0.05$). Although the MSI99 transcription was detected in eight transgenic lines, the exact size of the diagnostic fragment (1.5 kb) was found in two lines (Table 2). Additional bands of higher molecular weight (Fig. 2b) could indicate rearrangements of the cassette fragment or loss of a restriction site, probably *Hind*III, during the integration process.

Plant phenotype and grape productivity

A total of three CP and four MC transgenic lines as well as two nontransgenic lines were simultaneously acclimated and grown in the greenhouse for three seasons. During the third growing season, transgenic and nontransgenic lines were examined for their morphology and productivity (Table 2). Leaf morphology was similar in both nontransgenic lines as well as two lines from CP treatment and three lines from MC treatment with five lobes and half open petiole sinus, considered normal and typical of 'Chardonnay' plants regenerated from embryogenic cultures. However, lines P5 and C4 from CP and MC treatments, respectively, presented abnormal morphology compared to control plants. Line P5 had small leaves with 3 to 5 lobes and shorter internodes. Line C4 also had three to five lobes per leaf, but with deeper sinuses. Red leaf veins were present in all lines but the extent of coloration ranged from the base of the vein to the full length of the vein. The mean shoot length for all lines were measured 2 months after the bud break was 114 cm. One-way ANOVA showed no significant differences among lines ($P < 0.05$) except for line P5 which showed a significant reduction in growth ($P = 0.004$) compared with nontransgenic controls. The fertility of transgenic and nontransgenic plants was based on flowering and cluster production. Two lines each from the CP and MC treatments as well as both nontransgenic lines produced two to seven flower clusters that resulted in berry set and fruit ripening (Table 2).

Discussion

The first report on the use of minimal cassettes for plant transformation (Fu et al. 2000) appeared during the course of our ongoing project to transfer antimicrobial peptide genes into grapevines. So we sought to investigate the potential of using minimal gene cassettes instead of traditional circular plasmid DNA for co-transformation of a woody plant such as grapevine. Transformation was performed with two unlinked genes in separate constructs. One construct carried the *npt*-II marker gene and the second construct harbored an *MSI99* antimicrobial gene. We focused mainly on *MSI99* gene integration and transcription because this was the gene of interest in our program, while the *npt*-II gene was used only as a selective marker. A lower percentage of plant regeneration and lines positive for the *MSI99* gene from both MC and CP co-transformations was obtained in this study compared to previous results in our lab (Vidal et al. 2003), possibly due to the use of aging embryogenic cultures for transformation. A more rigorous study should be carried out with fresh embryogenic cell suspensions as well as with a larger sample size to compare transformation efficiency between CP and MC treatments. Also, we isolated minimal cassettes from agarose gels using

silica kits, however, better cassette purification by electroelution from the gel or the use of affinity columns (Breitler et al. 2002) could improve transformation with MC.

It was suggested that the plasmid backbone plays a significant role in the integration process by providing both recombination hot spots and regions of homology to promote homologous recombination (Kohli et al. 1998). Fu and coworkers (2000) observed in transgenic rice a simpler integration pattern and lower copy number with MC compared to CP technology. Similar trends were also described by Loc et al. (2002) in a study carried out with only four transgenic rice plants obtained from different MC treatments. On the other hand, Breitler et al. (2002) with transgenic rice and Romano et al. (2003) with transgenic potato using larger sample sizes did not find any differences in the number of integration events and copy numbers between MCs and CPs. Our results with grapevine point to no significant differences in the number of integrations between MCs and CPs, although the sample size of our experiment does not allow for a rigorous comparison. The expected diagnostic fragment containing the *MSI99* gene was only observed in one line from each MCs and CPs treatment (Table 2). During the transgene integration process it is possible to lose some DNA sequences including one or both restriction sites placed at 5′ and 3′ ends of the cassette (Fig. 1), but still the cassette could be functional for DNA transcription and expression. To our knowledge this is the first report of successful use of minimal cassette technology for transformation of a woody plant.

In our study, most transgenic lines from both MC and CP treatments expressed the transgenes, as determined by RT-PCR. However, different levels of transcription were found among lines within both DNA treatments (Table 2). Lines C3 and C5 were positive by PCR for the MSI99 gene but negative by Southern blot. This result could be due to a chimeric status for these two lines. This experiment was a cotransformation with two separate constructs followed by selection on kanamycin medium. The expression of the *npt*-II gene is indispensable for the selection of embryos but not the expression of the *MSI99* gene, which is in agreement with the result for lines C3 and C5 (Table 2). Fu et al. (2000) reported up to 30% lower transgene expression or complete transgene silencing in transformants from CPs compared to those from MCs. Loc et al. (2002) reported the absence of transgene silencing and higher levels of transgene expression in lines from MC compared to those from CP. Breitler et al. (2002), in a comparative study of gene expression between plants from MCs and CPs, reported a loss of expression of the *yfp* gene over the course of plant development on whole plasmid-derived plants. They pointed out that integration of backbone sequences of bacterial origin could favor the recognition of foreign DNA and subsequent silencing of the integrated genes. Romano et al. (2003) reported that the co-expression of two integrated transgenes delivered as separated constructs was 75–80% with both MCs and CPs technology. They found no correlation between the number of integration sites and GUS activity in transgenic plants from CPs, but no data from MCs were presented. In previous experiments carried out in our lab using CPs for transformation, between 12 and 55% of transgenic plants showed either low levels of transgene transcription or complete transgene silencing (Vidal et al. 2006). In this work, no significant differences in MSI99 transcription level between MC and CP treatments were found, suggesting that the minimal upstream and downstream cassette protection could be enough to prevent cassette erosion by host nucleases after bombardment. The lack of *MSI99* gene expression in line P2, according to RT-PCR, could be due to transgene silencing. Preliminary ELISA tests were also carried out to detect *MSI99* transgene expression using a specific antibody designed and raised by Sigma-Genosys (The Woodlands, TX). However, we were unable to detect the peptide consistently in leaf tissue from both in vitro- and greenhouse-grown plants (data not shown). A similar difficulty was mentioned by other authors reporting transformation of plants using the *MSI99* gene (Li et al. 2001).

Integration and transcription of both *npt*-II and *MSI99* transgenes were not shown to affect the phenotype of greenhouse-grown ‘Chardonnay’ plants. In a visual observation of several parameters of leaf morphology and shoot vigor, most transgenic plants showed a phenotype common to nontransgenic plants. Only two plants, one each from MC and CP treatments, presented a different phenotype compared to nontransgenic control plants. ‘Chardonnay’ plants produced from embryogenic culture have lobed leaves. This leaf phenotype is different from the description of mature leaves of ‘Chardonnay’ used in ampelography for the identification of grapevine cultivars (Galet 1990). The same feature was reported in transgenic plants of cv. ‘Sultana’ (*Vitis vinifera* L.) that were regenerated from embryogenic tissues (Franks et al. 1998). This phenomena was considered a reversion to juvenility and that the switch to a mature phenotype would occur when the plants begin to fruit. However, in our experiment with ‘Chardonnay’ when both transgenic and nontransgenic plants set fruit for the first time in the greenhouse, leaves were still lobed. As early as 4 years after the bombardment of embryogenic cells, both transgenic and nontransgenic plants produced bunches that ripened during the third year of growth in the greenhouse.

Successful transgene integration and expression was found with both MC and CP treatments. The donor construct DNAs integrated as complete or incomplete units in the recipient genome DNA (Kohli et al. 1998; Chen et al. 1998). Therefore, the absence of backbone sequence in minimal cassette constructs, should help to avoid potential pitfalls associated with the vector sequence such as rearrangements and gene silencing. In contrast, the use of minimal cassettes could lead to erosion of DNA at cassette ends during the integration process with the subsequent loss of diagnostic fragments and eventually loss of transgene expression. MCs as designed in this work performed similarly to CPs in terms of integration and transcription and the short flanking regions seem sufficient in protecting the cassette. In general, this and previous reports on the minimal cassette strategy point out the potential utility of this ‘clean DNA’ technology in the future for plant cell transformation as is common practice for animal cell transformation.

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