Transgenic Research (2006) 15:69–82
DOI 10.1007/s11248-005-4423-5

Evaluation of transgenic 'Chardonnay' (*Vitis vinifera*) containing magainin genes for resistance to crown gall and powdery mildew

José R. Vidal[1,4,*], Julie R. Kikkert[1], Mickael A. Malnoy[2], Patricia G. Wallace[1], John Barnard[3] & Bruce I. Reisch[1]

[1]*Department of Horticultural Sciences, New York State Agricultural Experiment Station, Cornell University, Geneva, NY, 14456, USA*
[2]*Department of Plant Pathology, New York State Agricultural Experiment Station, Cornell University, Geneva, NY, 14456, USA*
[3]*Computer Center, New York State Agricultural Experiment Station, Cornell University, Geneva, NY, 14456, USA*
[4]*Departamento de Biotecnología, ETS Ingenieros Agrónomos, Universidad Politécnica de Madrid, 28040, Madrid, Spain*

Received 5 April 2005; accepted 25 October 2005

Key words: *Agrobacterium vitis*, disease resistance, grapevine transformation, magainin genes, ubiquitin promoter, *Uncinula necator*

Abstract

Magainins, short peptides with broad-spectrum antimicrobial activity *in vitro*, were assayed for their ability to confer resistance to pathogens in transgenic grapevines. Embryogenic cell suspensions of 'Chardonnay' (*Vitis vinifera* L.) were co-transformed by microprojectile bombardment with a plasmid carrying the *npt-II* gene and a second plasmid harboring either a natural magainin-2 (*mag2)* or a synthetic derivative (*MSI99*) gene. Magainin genes and the marker gene were driven by *Arabidopsis* ubiquitin-3 and ubiquitin-11 promoters, respectively. A total of 10 mag2 and 9 MSI99 regenerated lines were studied by Southern blot hybridization, which showed 1–6 transgene integration events into the plant genome. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) revealed a variable range in transcription levels among mag2 and MSI99 lines. A positive correlation between number of integration events and transcription level was observed ($p < 0.05$). Plants were acclimated and challenged in the greenhouse with either *Agrobacterium vitis* strains (bacterial crown gall pathogen) at 10^8 cfu/ml or *Uncinula necator* (fungal powdery mildew pathogen) at 10^5 conidia/ml for evaluation of disease resistance. A total of 6 mag2 and 5 MSI99 lines expressing the antimicrobial genes exhibited significant reductions of crown gall symptoms as compared to non-transformed controls. However, only two mag2 lines showed measurable symptom reductions in response to *U. necator*, but not strong resistance. Our results suggest that the expression of magainin-type genes in grapevines may be more effective against bacteria than fungi. Additional strategies to enhance transgene expression and the spectrum of resistance to grape diseases are suggested.

Abbreviations: AMP – antimicrobial peptide; *mag2* – natural magainin-2 gene; *MSI99* – synthetic analog of magainin-2 gene; RT-PCR – reverse transcription-polymerase chain reaction

*Author for correspondence
E-mail: joseramon.vidal@upm.es

Introduction

Plant biotechnology offers the possibility to improve agricultural traits such as disease resistance of economically important crops without modifying cultivar identity. Most commercial grape cultivars (*Vitis vinifera* L.) are very susceptible to bacterial and fungal diseases, which cause important economical losses worldwide. Bacteria such as *Agrobacterium tumefaciens*, biovar 3 (*A. vitis*), which causes crown gall disease, and fungi such as *Uncinula necator*, which causes powdery mildew disease, are major grapevine pathogens (Pearson & Goheen, 1988). Grapes have been genetically transformed using both *Agrobacterium*-mediated and biolistic bombardment technologies for the purpose of increasing pathogen resistance and stress tolerance (Colova et al., 2001; Kikkert et al., 2001). A long-term objective of the grape industry is to increase cultivar resistance to plant pathogens resulting in the reduced use of labor and fungicides with benefits for winegrowers, consumers and the environment.

Different strategies and genes have been used in genetic engineering to enhance resistance to major plants pathogens (Mourgues et al., 1998; Punja, 2001). One strategy has been the use of antimicrobial peptides (AMPs). These are natural defensive compounds found in many organisms, ranging from bacteria to humans and plants, that protect the host from invading pathogens. Among these compounds are the magainins (Zasloff, 1987), isolated from the skin of the African clawed frog, *Xenopus laevis*. Magainins and their analogs are small (21 to 26 amino acids) cationic peptides, with α-helical structure and demonstrated broad-spectrum antimicrobial activity that inhibits the growth *in vitro* of bacteria and fungi (Kristyanne et al., 1997; Alan & Earle, 2002) including major grapevine pathogens such as *A. tumefaciens* (Li & Gray, 2003). Magainins have a strong affinity for microbial membranes due to a high concentration of anionic phospholipids in the outer leaflet of their bilayer, and very low toxicity to animal and plant cells due in part to the presence of cholesterol or other sterols in the host membranes (Maloy & Kari, 1995; Kristyanne et al., 1997). Magainins disturb membrane function either by forming ion channels or by depolarizing the membrane leading to leakage of metabolites and cell death (Bechinger, 1997). The selective activity of magainins and their synthetic derivatives on microbial membranes and their simple amino acid sequence make them potential candidates for genetically engineering disease-resistant plants (Powell et al., 1995). Recently, transgenic plants that expressed high levels of magainin peptides exhibited significant levels of resistance to a broad range of both fungi and bacteria, including pathogens causing botrytis and powdery mildew diseases (Smith et al., 2001). The expression of synthetic magainins, such as *Myp30* (Li et al., 2001) and *MSI99* via either the chloroplast genome (DeGray et al., 2001) or the nuclear genome (Chakrabarti et al., 2003) in transgenic plants, enhanced resistance to bacterial and fungal pathogens.

In this paper, we report in 'Chardonnay' (*Vitis vinifera*) stable transformation and expression of either a natural magainin-2 (*mag2*) or a synthetic derivative (*MSI99*) gene under control of the *Arabidopsis* ubiquitin-3 promoter. Some transgenic lines exhibited enhanced resistance to crown gall and powdery mildew diseases in greenhouse tests.

Material and methods

Plasmids and transformation

Three circular pUC19-based plasmids were used for transformation of grapevine by microprojectile co-bombardment (Figure 1a). pSAN237 harbors the selectable marker neomycin phosphotransferase II (*npt-II*) gene under control of the *Arabidopsis* ubiquitin-11 promoter (Pubq11) and the *Agrobacterium* T-DNA nopaline synthase (nos) terminator (Tnos); pSAN167 carries a signal peptide (SP) from pea vicilin protein (Pang et al., 1992) fused to a natural magainin-2 (*mag2*) gene under control of the *Arabidopsis* ubiquitin-3 promoter (Pubq3) and Tnos; pSAN168 harbors the same SP fused to the synthetic *MSI99* gene, a derivative of *mag2* (Figure 1b), under control of Pubq3 and Tnos. Embryogenic cell suspensions of 'Chardonnay' were co-bombarded with pSAN237 (*npt-II*) and a second vector, either pSAN167 (*mag2*) or pSAN168 (*MSI99*). Transformation, embryo selection and plant regeneration were performed as described by Vidal et al. (2003).

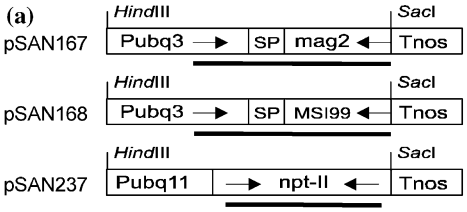

Figure 1. Transformation vectors and peptide sequences. (a) Schematic representation of the gene cassettes in the pUC19 vector used for biolistic co-transformation. Diagrams of the cassettes are not to scale. Elements of the cassettes are as follows: *Pubq3* and *Pubq11* are *A. thaliana* ubiquitin promoters; *Tnos* is the *A. tumefaciens* T-DNA nopaline synthase terminator; *npt-II* (792-bp) is the *E. coli* neomycin phosphotransferase II gene; SP (48-bp) is a signal peptide from pea vicilin protein; *mag2* (72-bp) is a natural magainin 2 gene from *Xenopus laevis* and *MSI99* (69-bp) is a synthetic derivative. *Hin*dIII and *Sac*I correspond to unique restrictions sites on the transformation vectors. Arrows on the cassettes indicate primer annealing sites. Bars correspond to the probe size. (b): Amino acid (aa) sequence of mag2 (23 aa) and MSI99 (22 aa) peptides. The amino acids in natural mag2 (+4 charged) are above and those substituted in synthetic MSI99 (+6 charged) are below. The carboxyl group (COOH) in mag2 was replaced with an amide group (NH2) in MSI99.

Plant material

Based on kanamycin selection, positive signals from PCR and/or dot blot hybridization and good plant growth *in vitro*, 10 mag2 and 9 MSI99 transgenic lines plus two non-transformed controls were selected for molecular characterization and later disease resistance evaluation in the greenhouse. Rooted in vitro lines were transferred to 500 ml pots with sterile Cornell Mix (Boodley & Sheldrake, 1982) in which one-half of the vermiculite was replaced with perlite. Plants were grown in a growth chamber at 100% relative humidity at 23 ± 1°C with a 14:10-h light:dark photoperiod for one month. Relative humidity was slowly lowered during the second month to 85% for acclimatization. Three month old plants were transferred to one-gallon pots, fertilized with Osmocote PLUS® (Scotts, Marysville, OH), moved to the greenhouse and grown at 25 ± 2°C with natural light for later evaluation of disease resistance. Plants in the greenhouse were systemically treated for insect control (Marathon® 1%, Olympic, Mainland, PA). After the first-year evaluation, plants were moved to a cold frame for dormancy induction and then into storage at 4°C for 3 months. Potted plants were then pruned, fertilized and grown again in the greenhouse as described above for a second-year evaluation of disease resistance.

PCR and Southern blot analyses

Integration of foreign DNA into the plant genome was determined by polymerase chain reaction (PCR) and Southern blot analyses. DNA isolation from leaves of both tissue culture- and greenhouse-grown plants as well as PCR analyses were performed as previously described (Vidal et al., 2003). In order to detect the presence of the transgenes, a 750-bp fragment of the *npt-II* marker gene was amplified with specific primers [forward 5′-CAAGATGGATTGCACGCAGG-3′ & reverse 5′-TTGAGCAGTTCTTCCGCTATC-3′, Tm 60°C]. Similarly, for *mag2* and *MSI99* antimicrobial genes, primers were designed to amplify a ~400-bp region spanning from 1105-bp within the Pubq3 promoter [5′-CACGTATCATATGATTCCTTCC-3′ forward primer] to the 3′ end of the AMP genes [5′-TTAACTGTTCATGATCTCACC-3′ and 5′-GAGCTCGTTAGGAGTTCAGG-3′ reverse primers (annealing at 58°C) for *mag2* and *MSI99* fragments, respectively].

Genomic DNA (5 μg) from both tissue culture- and greenhouse-grown plants was digested to completion with either *Hin*dIII to determine number of integration events or *Hin*dIII plus *Sac*I (Promega, Madison, WI) to detect integration of the complete Pubq3 + SP + AMP cassette fragment (Figure 1). DNA fragments were electrophoresed in a 0.8% agarose gel, blotted to a neutral nylon membrane (Magnagraph, Osmonics, Minnetonka, MN) by capillary transfer and hybridized with a digoxigenin-labeled probe. Fragments for probes corresponding to *mag2*, *MSI99* and *npt-II* genes were amplified from the original constructs, pSAN167, pSAN168 and pSAN237, respectively, using specific primers as shown above (Figure 1a). The PCR products were excised from the gel, purified and randomly labeled using the DIG DNA labeling kit (Roche, Indianapolis, IN) according to the manufacturer's procedure. Hybridization and chemiluminescence detection was performed as described by Vidal et al. (2000). X-ray films (BioMax light, Kodak, Rochester, NY) were exposed to the blots from 1 to 10 h.

Semi-quantitative RT-PCR analysis

Reverse transcription-polymerase chain reaction (RT-PCR) amplification of RNA provides an easy means to detect the presence of small transcripts and gene expression. Total RNA was isolated from leaves of in vitro plants as described by Renault et al. (2000) with some modifications and scaled to 2 ml microcentrifuge tubes. Two hundred mg of leaves were ground twice in liquid nitrogen to a fine powder, transferred to a tube with 1 ml of extraction buffer [200 mM Tris–Cl pH 8.5, 300 mM LiCl, 10 mM EDTA, 1.5% SDS (w/v) and 2% PVPP (w/v)] and 0.8 ml of chloroform, mixed by vortexing, and centrifuged at 13,000 rpm for 10 min. The supernatant was extracted twice with phenol:chloroform:isoamylalcohol [25:24:1 (v/v/v)]. Polysaccharides were precipitated with 0.5 volume of 100% ethanol and centrifuged at 13,000 rpm for 15 min. Soluble nucleic acids were precipitated with 1/20 volume of 3 M sodium acetate and 2 volumes of 100% ethanol after incubation at −20°C for 4 h. The pellet was resuspended in diethyl pyrocarbonate (DEPC)-treated water, and for precipitation of RNA, 5 M LiCl was added 1:1 (v/v), incubated overnight on ice and centrifuged at 13,000 rpm for 30 min. RNA was resuspended in 50 µl DEPC-treated water and quantified with a spectrophotometer (BioMate™, Thermo Spectronic, Rochester, NY).

First strand cDNAs were synthesized from 1 µg of total RNA in a 30 µl reaction volume using an oligo dT primer and MMLV reverse transcriptase following the Promega protocol and application guide. Amplification of cDNA from SP + mag2 and SP + MSI99 transcripts via first strand cDNA was performed using specific primers for 5′-end-SP (forward primer 5′-ATGCTTCTCGCTATTGCC-3′) and 3′-end-mag2 (reverse primer 5′-TTAACTGTTCATGATCTCACC-3′, annealing at 52°C) or 3′-end-MSI99 (reverse primer 5′-TTAGGAGTTCAGGATCTTCAC-3′, annealing at 52°C) genes. Preliminary amplification was done in a 25 µl reaction volume containing 1 µl cDNA, 200 µM of each dNTP, 0.5 µM of each specific primer, 1.0 unit of *Taq* DNA polymerase (Sigma, St. Louis, MO) and 1× specific buffer. The reaction was cycled 35 times at 94°C for 1 min, 52°C for 1 min and 72°C for 1 min with an initial denaturation step at 95°C for 2 min and a final elongation step at 72°C for 10 min.

As a control for quantification, in first reactions, a cDNA fragment (700 bp) from the alpha subunit of the translation elongation factor 1 (EF1-α) was amplified with degenerate primers (forward primer 5′-ATTGTGGTCATTG-GYCAYGT-3′ and reverse primer 5′-CCTATCT-TGTAVACATCCTG-3′, annealing at 54°C), which were designed from the conserved coding region of the gene sequences. A test for linearity of the cDNA amplification among samples was performed with different amounts of RT-PCR substrate (cDNA) as described by Malnoy et al. (2003) following the PCR steps described above. In a second reaction, comparative amplification RT-PCR of mag2 and MSI99 cDNA was carried out with an equivalent RT reaction product and the reaction was cycled only 20 times. Twenty µl of PCR product were resolved by electrophoresis on 1.5% agarose gels and products blotted on a neutral nylon membrane (Magnagraph). For SP + mag2 and SP + MSI99 transcripts, construct-specific probes were used as described for Southern blots. For the EF1-α transcript, a specific probe was generated with forward and reverse degenerate primers from cDNA and DIG labeled as described above. Specificity of the probes was confirmed by hybridization. Hybridization and detection were carried out as described for Southern blots. The intensity of the hybridization signals was quantified by direct scanning of the films with a Kodak Image Station 440 (Kodak, Boston, MA) and the ratio of mag2/EF1-α and MSI99/ EF1-α was calculated.

Resistance to crown gall disease and data analysis

Transgenic and non-transformed lines were assayed for crown gall resistance (Table 1). Four vegetatively-propagated plants per line were evaluated for resistance to *A. tumefaciens* strain (C58) and two strains of *A. vitis* (TM4 and CG450) (Otten et al., 1996) in two consecutive years. *Agrobacterium* strains were cultured on solid potato dextrose agar (PDA) medium at 28 ± 0.1°C for 24–48 h before the inoculation. Plants for first-year screening (after acclimatization from tissue culture) and second-year screening (after cold storage from greenhouse culture) were grown in the greenhouse until each had more than 20 nodes. Each plant was inoculated at 20 different sites (5 sites per strain plus 5 sites for an

Table 1. Molecular and disease resistance data from AMP-transgenic 'Chardonnay' lines and non-transgenic controls assayed in greenhouse trials for resistance to crown gall and powdery mildew diseases.

Line [a]	Southern blot	RT-PCR	Disease resistance		
	Integration [b]	AMP/EF1-α [c]	Crown gall [d]		Powdery mildew [e]
	*Hin*dIII	ratio	TM4	CG450	2003
mag2-2	2	1.56	−2.61 ± 0.62**	−1.61 ± 0.61*	41.52 ± 3.20
mag2-3	2	2.48	−5.06 ± 0.67**	−6.79 ± 1.19**	39.17 ± 7.03
mag2-5	3	0.10	nt	nt	42.04 ± 1.77
mag2-9	4	1.83	−2.61 ± 0.62**	−1.24 ± 0.61*	35.15 ± 2.56*
mag2-12	0	0.04	nt	nt	43.37 ± 3.38
mag2-13	3*	0.19	−1.05 ± 0.57	−0.44 ± 0.61	42.81 ± 3.97
mag2-16	1*	0.00	−1.40 ± 0.58*	−1.51 ± 0.61*	35.50 ± 0.68*
mag2-17	4*	4.77	−1.25 ± 0.57*	−0.85 ± 0.61	45.04 ± 2.14
mag2-19	1	0.00	−0.38 ± 0.57	0.67 ± 0.60	37.96 ± 2.73
mag2-20	2*	1.45	−1.94 ± 0.59*	−0.74 ± 0.61	40.81 ± 1.75
NT81	0	0.00	0	0	47.33 ± 1.52
MSI99-3	4*	6.71	−0.15 ± 0.57	−0.93 ± 0.61	45.74 ± 2.14
MSI99-4	0	0.00	−0.72 ± 0.57	−0.44 ± 0.61	43.70 ± 2.98
MSI99-8	3	11.71	−1.49 ± 0.58*	−1.10 ± 0.61	39.85 ± 0.46
MSI99-10	3	1.77	0.56 ± 0.57	0.32 ± 0.60	46.11 ± 2.57
MSI99-15	2*	6.50	−2.26 ± 0.60**	−0.86 ± 0.61	38.74 ± 0.95
MSI99-16	3*	6.65	0.12 ± 0.57	0.07 ± 0.60	43.59 ± 1.48
MSI99-32	3*	7.68	−2.40 ± 0.60**	−1.53 ± 0.61*	43.37 ± 5.32
MSI99-34	4*	3.08	−1.68 ± 0.58*	0.06 ± 0.60	37.67 ± 3.51
MSI99-37	4*	13.95	−1.84 ± 0.58*	−1.61 ± 0.61*	nt
NT82	0	0.00	0	0	49.83 ± 2.47

[a] Correspond to PCR positive transgenic antimicrobial peptide (AMP), mag2 or MSI99, or non-transformed (NT) lines.
[b] No. of AMP gene integrations in genomic DNA after digestion with *Hin*dIII. Full integrity of the promoter plus the AMP gene (indicated by an asterisk corresponding to a 1.5 kb fragment) was determined by double digestion of genomic DNA with *Hin*dIII and *Sac*I (Figure 1a).
[c] Transcription level of AMP gene as compared with the alpha subunit of translation elongation factor 1 (*EF1-a*) gene, estimated on equivalent total cDNA amount at 20 cycles of RT-PCR. The line mag2-16 showed a weak hybridization signal when amplified for 35 cycles.
[d] Estimate (log of odds) ± standard error of the gene-expression effect on gall size in transgenic lines as compared with non-transformed control (*$p < 0.05$; **$p < 0.001$) inoculated with *A. vitis* strains TM4 or CG450. Data correspond to 2nd year. 'nt' indicates not tested.
[e] Percentage ± standard error of powdery mildew disease based on 27 leaves (9 leaves per plant, 3 plants per line) 14 days after inoculation with *U. necator*. Significant reduction of disease symptoms at 14 days after inoculation (*$p < 0.05$). Data correspond to 2nd year.

H_20-inoculated negative control). Each inoculation site was on the stem, opposite each leaf, right below the node. To inoculate, a needle (0.6 mm diameter) was dipped in the fresh bacteria culture ($\sim 1 \times 10^8$ cfu/ml) and then inserted into the stem to cause an injury. Inoculated plants were grown in the greenhouse at 25 ± 2°C. Day length was ~14 h and no supplemental lighting was used. Susceptibility to *Agrobacterium* strains was evaluated 60 days after inoculation based on crown gall size which was evaluated visually following a disease index (DI): DI = 0, no gall symptoms; DI = 1, small gall size; DI = 2, medium gall size; DI = 3, large gall size and DI = 4, very large gall size. Plant responses (gall size) to *Agrobacterium* strains were recorded on this five-category ordinal scale (0–4). Data were analyzed using a proportional odds model with the program Genstat 5 (VSN International Ltd, Hemel Hempstead, UK).

A group of AMP lines were assayed to test *in vitro* peptide activity in transgenic plants against strain TM4 and compared to a non-transgenic line. Explant preparation (green shoots), inoculation *in vitro* and bacterial growth on plant tissue

was done as described by Herlache et al. (2001). The TM4 culture was grown for 48 h at 28°C on PDA and suspended to $OD_{600} = 0.1$ in sterile distilled water. The suspension was diluted 100-fold and 2 µl ($\sim 2 \times 10^3$ cfu/ml) were placed on the cut end of a shoot section that was placed vertically into water agar. Whole explants were individually macerated 24 h post inoculation with a plastic pestle driven by a drill for 30 sec in 1 ml sterile water in an Eppendorf tube. Suspensions were serially diluted and plated on PDA. TM4 colonies were counted after 48 h of incubation at 28°C. A total of 10 explants per line were assayed and each line was evaluated at least 3 times. Analyses of contrasts were carried out to test for significant differences among lines using the SAS program (SAS Institute, Cary, NC).

Resistance to powdery mildew disease and data analysis

Transgenic and non-transformed lines were assayed for powdery mildew resistance (Table 1). Three plants per line were evaluated for resistance to *U. necator* in two consecutive years. In the first year, plants were rooted and acclimated 4-months prior to inoculation, while in the second year growth followed a period of dormancy. The leaf surface of actively growing shoots (30–50 cm long containing five young leaves plus the apex) was sprayed with *U. necator* inoculum at 1×10^5 conidia per ml using a Preval sprayer (Precision Valve Corp., Yonkers, NY) as described by Gadoury et al. (2003). Inoculated grapevines were watered every two days and grown for 14 days in the greenhouse at 25 ± 2°C during the day and at 18 ± 2°C during the night with a day length of $\sim$14 h. Susceptibility to powdery mildew infection was recorded at 7 and 14 days after inoculation based on the number of infected leaves (up to nine leaves per plant) and the percentage of leaf area infected, which was estimated by visual observation of the leaf surface. Severity of disease for the whole plant was evaluated and then calculated for each line. Plant resistance to *U. necator* was analyzed using analysis of variance. Transgenic lines and a non-transformed control were compared by least significant difference test controlling for the false discovery rate. The statistical analysis was made using the software R (Ihaka & Gentleman, 1996).

Results

Transgene integration and integrity

PCR and Southern blot analyses were carried out with DNA samples from both *in vitro*- and greenhouse-grown plants. PCR products of genomic DNA were of the expected size for the AMP-containing fragments (400-bp) in both mag2 and MSI99 lines as well as for plasmid controls (pSAN167 or pSAN168), whereas no amplification was detected from DNA of non-transformed plants, as well as pSAN237 and pUC19 negative controls. Similarly, a $\sim$750-bp fragment representing the region within the *npt-II* gene was amplified from both mag2 and MSI99 lines (data not shown). This band was also amplified from the pSAN237 positive control. No PCR products were detected from a non-transgenic plant, and pSAN168 and pUC19 negative controls.

Band patterns from Southern blot were similar for each plant line regardless of sample origin. Genomic DNA was digested with *Hin*dIII to confirm the integration of foreign genes and determine the number of integration events. For all constructs, one *Hin*dIII restriction site occurs at the 5′ end of the Pubq promoter (Figure 1a). Other sites should occur elsewhere in the plant genome. *Hin*dIII digested genomic DNA of all mag2 and MSI99 lines showed hybridization signals when probed with the *npt-II* fragment, as did the pSAN237 positive control, but the non-transformed 'Chardonnay' negative control did not (data not shown). Hybridization signals appeared in 9 of 10 mag2 and 8 of 9 MSI99 lines probed with mag2 and MSI99 sequences, respectively (Figure 2a, b). No hybridization signals were detected among non-transformed negative controls. Hybridization signals indicated multiple integration events, which ranged from 1 to 6 for the *npt-II* gene and from 1 to 4 for both mag2 and MSI99 genes (Table 1). Band patterns were different among all lines, indicating independent transformation events. The integrity of the Pubq3 + SP + AMP cassette fragment in transgenic lines was determined using *Hin*dIII and *Sac*I enzymes in a double digestion, which should result in a 1.5-kb fragment (Figure 1a). Out of 19 transgenic lines, 4 mag2 and 6 MSI99 lines showed a hybridization signal of the expected size for the cassette fragment with mag2 and MSI99 probes,

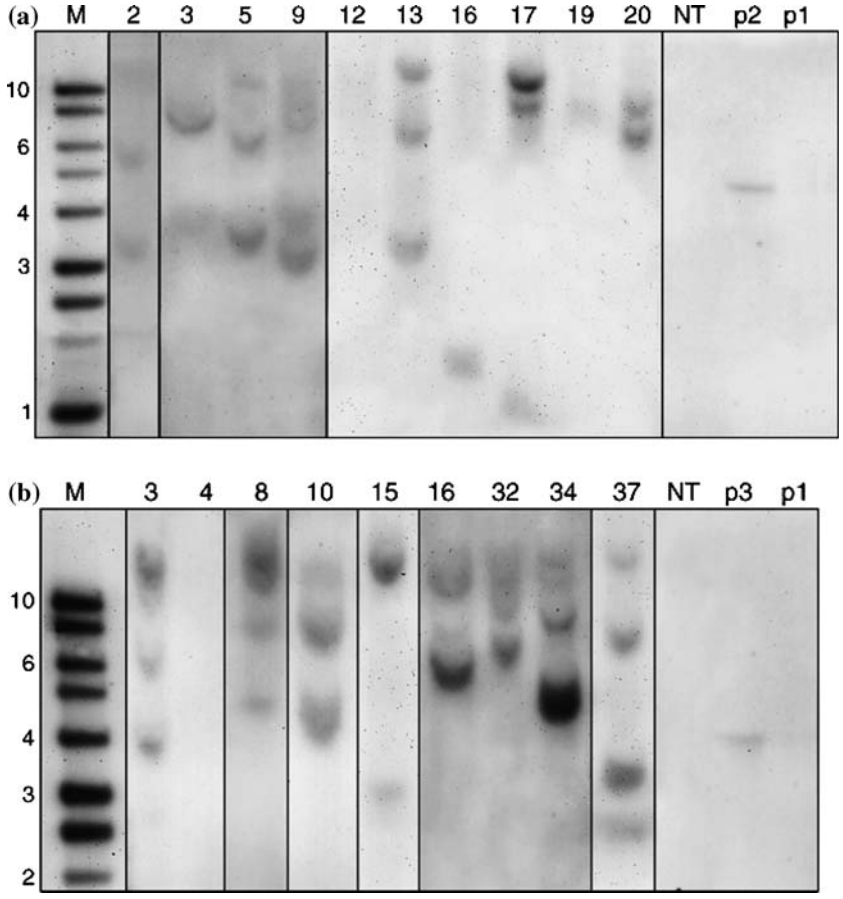

Figure 2. Southern hybridization analyses of mag2 (a) and MSI99 (b) transgenic and non-transformed (NT) 'Chardonnay' lines. Genomic DNA from greenhouse plants was digested to completion with *Hin*dIII, which cut the plasmids once (Figure 1a), and hybridized with digoxigenin-labeled probes for the *mag2* (a) or the *MSI99* (b) fragment. Plasmid controls: p1 = pSAN237, p2 = pSAN167, p3 = pSAN168. M is a 1 kb DNA ladder (Promega), in kilo bases (kb) on the left, used as a molecular size standard. Pictures were assembled from different films using Adobe Photoshop.

respectively (Table 1), along with other bands of different sizes (data not shown).

Gene transcription and expression

Because of the small size of the transcripts [SP + mag2 (120-b) and SP + MSI99 (117-b) mRNAs] RT-PCR of total RNA was chosen over the standard Northern blot technique to more readily detect the gene transcripts and evaluate the expression. The expected size of the cDNA, 120-bp for mag2 cDNA and 117-bp for MSI99 cDNA, was obtained for most of the Southern blot positive lines, however no signals were detected for the non-transgenic control. All 10 mag2 lines were positive for transcription of the *npt-II* gene, providing a proof of function for the ubq11 promoter in grapes, but only 8 lines were positive for *mag2* gene transcription after 20 cycles of RT-PCR (Figure 3a). However, mag2-16 also showed a weak hybridization signal when DNA blotting was done after 35 cycles of RT-PCR (data not shown). For the MSI99 lines, 8 of 9 lines were positive for the transcription of the *MSI99* gene, confirming that the ubq3 promoter functions in grapes (Figure 3b). The *EF1-α* gene (900-bp) includes a 200-bp intron. We confirmed that our RT-PCR data was based only on plant mRNA because hybridization with the EF1-α probe detected only a 700-bp fragment. Comparative amplification of RT-PCR products at 20 cycles in both mag2 and MSI99 lines showed a wide range of transcription levels among the lines (Table 1).

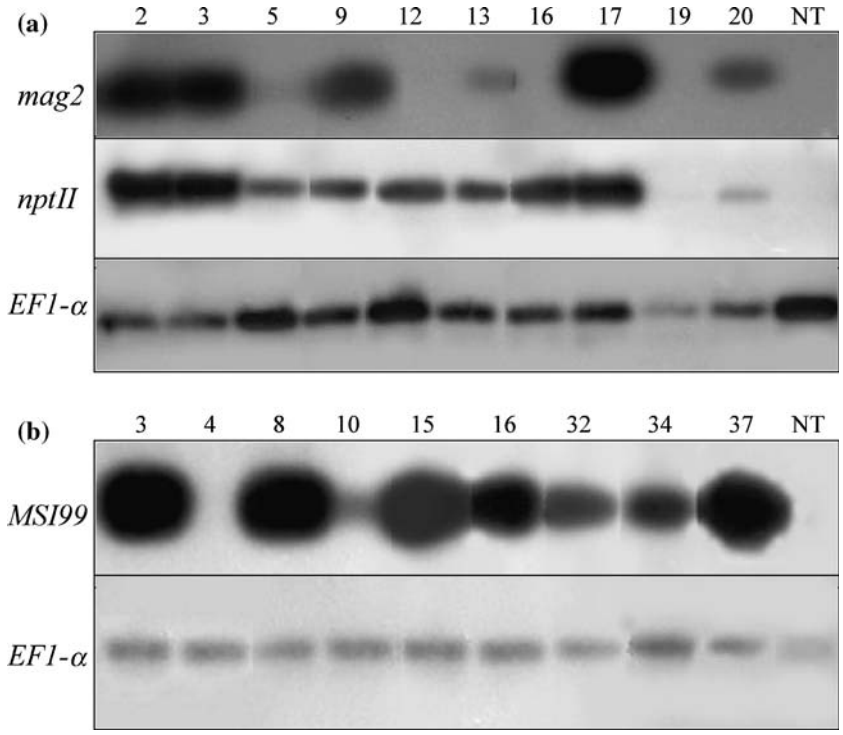

Figure 3. Semi-quantitative RT-PCR analyses of transgenic 'Chardonnay' lines. Comparative transcription of *mag2*, *npt-II* and *EF1-α* genes in mag2 lines (a) and *MSI99* and *EF1-α* genes in MSI99 lines (b) using leaves of in vitro transgenic and non-transformed (NT) plants. Differences among transcription levels of transgenic lines were estimated after specific PCR (20 cycles) on equivalent total cDNA amounts. Hybridization signals are from digoxigenin-labeled probes.

Test for resistance to Agrobacterium vitis

As expected, water-inoculated sites of transgenic and non-transgenic plants were free of crown gall symptoms. However, plants inoculated with the C58 tumorigenic strain developed small galls (DI = 1) sporadically. Sites inoculated with strains TM4 or CG450 showed variable gall sizes within and between plants for each line. All transgenic lines showed crown gall symptoms but most had smaller galls than did the non-transgenic controls. In general, gall size in the second year was larger than the first year due to better plant growth. In the second year, out of 8 transgenic lines evaluated that carried the natural *mag2* gene, 6 lines inoculated with the TM4 strain and 4 lines inoculated with the CG450 strain, showed statistically significant reductions of symptoms as compared with the non-transgenic control (Figure 4a). Similarly, out of 9 lines evaluated harboring the synthetic *MSI99* gene, 5 lines inoculated with the TM4 strain and 2 lines inoculated with the CG450 strain showed statistically significant reductions of symptoms as compared with the non-transformed control (Figure 4b). Most of the transgenic lines that were significantly resistant during the second year (Table 1) were also significantly resistant in the preliminary experiment one year earlier (data not shown).

Antimicrobial activity of the AMP produced by 4 transgenic lines (mag2-9, mag2-16, MSI99-15, MSI99-32) was evaluated on a TM4 bacterial population over time. Bacteria grew on grape explants at similar rates for all lines, however bacteria population means at 24-h post-inoculation were lower in all transgenic lines as compared to a non-transgenic (NT) control. The single degree of freedom contrast between transgenic and non-transgenic means was significant ($p = 0.04$). Pairwise comparisons between line means indicated that the only significant differences were between NT [$32 \pm 3 \times 10^4$ cfu/ml; mean ± standard error] and mag2-16 [$24 \pm 2 \times 10^4$ cfu/ml] ($p = 0.032$) and MSI99-32 [$23 \pm 2 \times 10^4$ cfu/ml] ($p = 0.024$) at 24 h post-inoculation.

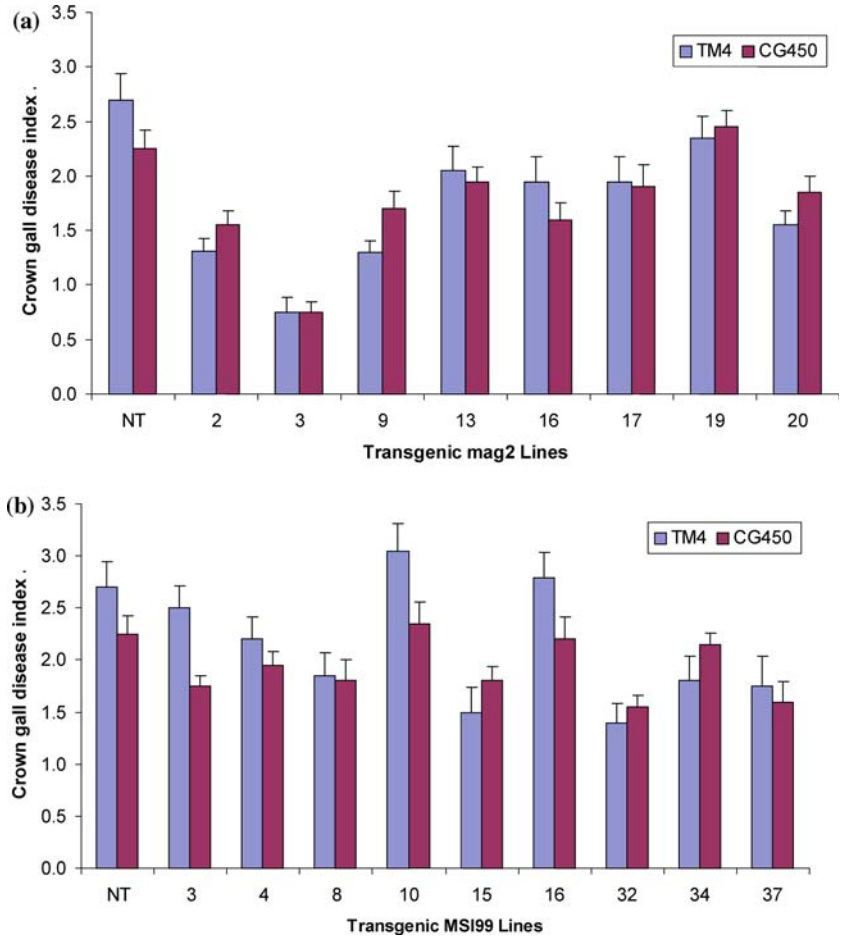

Figure 4. Test of transgenic 'Chardonnay' vines for resistance to *Agrobacterium vitis* strains (TM4 or CG450). Average of crown gall disease index in mag2 (a) and MSI99 (b) lines as compared to non-transformed (NT) control at 60 days after inoculation. Mean values per line were determined from 20 inoculation sites per strain (5 sites per plant, 4 plants per line). Vertical bars are standard errors of the means.

Test for resistance to Uncinula necator

All three plants for each line were similar in size at the time of inoculation and grew vigorously thereafter with the exception of the mag2-3 line, which grew more slowly. Percentage of powdery mildew infection on the leaf surface after inoculation was used to measure the extent of resistance of the transgenic plants. Severity was recorded at one week on 4 leaves (the initially inoculated leaves) and at two weeks on 9 leaves (4 initially inoculated leaves plus 5 younger leaves infected naturally via secondary inoculum). All transgenic and non-transgenic plants were heavily infected after inoculation, but measurable symptom reductions were observed in several transformed lines. The severity of infection was higher in the second year than in the first (lower growth). The difference between the first and second year was statistically significant ($p < 0.001$). In the first year, there was no significant difference among transgenic lines and non-transgenic controls with both *mag2* and *MSI99* genes (data not shown). In the second year, severity of disease at one week after inoculation (based on 12 leaves: 4 leaves per plant, 3 plants per line) did not show significant differences between transgenic lines and non-transgenic controls. However, severity of disease at two weeks after inoculation (based on 27 leaves: 9 leaves per plant, 3 plants per line), showed two mag2 lines (9 and 16) with reduced symptoms that differed significantly from the NT81 control [$p = 0.013$ and $p = 0.039$, respectively] (Table 1).

Discussion

The expression of AMPs in transgenic plants has not always resulted in enhanced resistance to plant pathogens. The lack of improved resistance could be due to peptide degradation by host proteases (Hightower et al., 1994) as well as peptide localization in plant tissues. To improve their antimicrobial activity, many natural peptides have been modified to develop synthetic derivatives with more potent antimicrobial activity (Powell et al., 1995). To reach the target pathogens early in the infection process, plant expression vectors were constructed with signal peptides to target the peptide into the cytoplasm and the extracellular spaces (Pang et al., 1992). Recently, several natural and synthetic peptides from the defensin, cecropin and magainin families were successful expressed in transgenic banana, potato and tobacco resulting in resistance to plant pathogens *in vitro* and in planta (Cary et al., 2000; Gao et al., 2000; Osusky et al., 2000; Chakrabarti et al., 2003). In a previous work, we reported the transformation and regeneration of 'Chardonnay' vines (a woody plant) containing either mag2 (natural magainin) or MSI99 (synthetic derivative) genes, which were selected while still *in vitro* mainly by PCR (Vidal et al., 2003). Here, we report in some of those grapevine lines stable integration and expression of either mag2 or MSI99 genes, both fused in frame to a signal peptide, and compare their antimicrobial effect against a bacterial and a fungal pathogen.

All transgenic 'Chardonnay' lines analyzed were independent transformants, as none shared the same hybridization pattern. This shows the advantage of using embryogenic cell suspensions for grape transformation versus traditionally used embryogenic callus or somatic embryos in which regenerated grapes were produced from unique transformation events (Franks et al., 1998; Iocco et al., 2001). The number of transgene integrations into different regions of the genome was 1–6, similar to what was found in previous reports in grapes using *Agrobacterium*-mediated transformation (Yamamoto, 2000; Iocco et al., 2001). However, the average of integrations obtained here via biolistics (2.5 for AMPs and 2.6 for *npt-II*) is higher than the number obtained from *Agrobacterium*-mediated transformation, in agreement with the results from other bombardment experiments (Dai et al., 2001). Analysis of Southern blot and RT-PCR data showed positive correlation between the number of integrations and the level of transcription ($R = 0.527$, $df = 18$, $p < 0.05$). MSI99-34 and MSI99-37 lines both have four integrations of the transgene but different levels of transcription (Table 1) that could be due to a position effect of the transgenes into the host genome (Chakraborty et al., 2000). Most mag2 and MSI99 lines positive by Southern analysis were also positive by RT-PCR analysis at 20 cycles. The line mag2-16 had one complete insert but scored positive by RT-PCR only at 35 cycles. This line was also positive by PCR, suggestive of transcriptional gene silencing. However this line showed resistance to both bacterial and fungal pathogens. Another line, mag2-12, was negative by Southern analysis but positive by RT-PCR. This line was also positive by PCR suggesting a possible chimeral status.

Transgenic grapevines with enhanced resistance to fungal pathogens have been reported. The expression of a rice chitinase gene in *V. vinifera* 'Neo Muscat' conferred resistance to powdery mildew and anthracnose diseases (Yamamoto et al., 2000). Transgenic 110 Richter rootstock (interspecific hybrid) expressing a eutypine reductase gene from *Vigna radiata* had enhanced resistance to Eutypa dieback disease (Legrand et al., 2003). The expression of a fungal endochitinase gene in 'Chardonnay' plants showed symptom reductions to powdery mildew and Botrytis bunch rot (Reisch et al., 2003). Here, we report the expression of magainin-type genes, which confer in preliminary studies significant resistance to the bacterial disease, crown gall, and moderate symptom reduction to the fungal disease, powdery mildew. All magainin lines significantly resistant to the CG450 strain were also significantly resistant to the TM4 strain. There were additional lines with significant resistance to the TM4 strain (Table 1). One plausible explanation is that the TM4 strain is more sensitive to the peptides than is CG450. This hypothesis is supported by an *in vitro* antimicrobial assay of the effects of the mag2 peptide (0.05–2.0 μM) on growth of these two strains. In two separate experiments carried out with a microbiology growth reader, it was shown that strain TM4 was much more sensitive to mag2 than was CG450 (Rosenfield, Kikkert, Reisch, Vidal, personal communication). Peptide-producing plants showed

reductions in gall size, but none showed a gall-free response. It could be that magainin production in the plant is reducing the bacterial population, in agreement with a previous antimicrobial assay *in vitro* with pure peptide (Li & Gray, 2003), and that this is reflected in reduced gall size. To test this hypothesis we studied the antimicrobial activity in planta. Two transgenic lines, mag2-16 and MSI99-32, significantly reduced the bacterial population in tests *in vitro* compared to non-transgenic lines. In contrast with the effect on bacteria, the resistance of our magainin plants to the fungus *U. necator* was rather limited. Only two lines, mag2-9 and mag2-16, showed a significant reduction of powdery mildew at two weeks after inoculation. This result suggests to some extent that the resistance of these transgenic lines to powdery mildew in the field could be greater since the inoculum present in early natural infections is less concentrated than was the inoculum used in our experiment. Field trials are underway and evaluation for powdery mildew resistance in natural conditions will ultimately require several years.

Tolerance to bacterial strains was associated with a high level of mRNA transcription (Table 1). Line mag2-16 was an exception, having significant tolerance to both TM4 and CG450 strains, but only showing a low level of mRNA transcription. However, this line harbors a complete copy of the gene cassette, composed of the promoter and the coding sequence. We evaluated mRNA transcription from *in vitro* grown plants. One possible explanation of transgene silencing in line mag2-16 could be transgene methylation in tissue culture plants. Franks et al. (1998) reported methylation of T-DNA in a transgenic grapevine, which had low glucuronidase (GUS) expression in tissue culture leaves. The authors suggested a decrease of transgene methylation when the transgenic line was transferred from tissue culture to a glasshouse, since higher GUS expression was later observed. Another explanation of the missing mRNA in line mag2-16 could be rapid mRNA turnover (Mitchell & Tollervey, 2000). Malnoy et al. (2003) reported in some transgenic pears high levels of expression of lactoferrin protein from weak transcription of the lactoferrin gene. In addition, somaclonal variation due to *in vitro* culture of embryogenic tissues associated with genetic transformation (Torregrosa et al., 2001) could help explain the resistance exhibited by the line mag2-16.

Expression of the synthetic *MSI99* gene in transgenic plants enhanced, to some extent, disease resistance to several fungal pathogens in tobacco (*Sclerotinia sclerotiorum*, *Alternaria alternate* and *Botrytis cinerea*) and banana (*Fusarium oxysporum* f.sp. *cubense* and *Mycosphaerella musicola*) (Chakrabarti et al., 2003) as well as resistance in tomato to *Pseudomonas syringae* pv *tomato* (Alan et al., 2004). Expression of the MSI99 peptide in tobacco via the chloroplast genome inhibited growth of the bacterial pathogen, *P. syringae* pv *tabaci*, as well as fungal pathogens (DeGray et al., 2001). Another engineered magainin analog, Myp30, resulted in significantly increased resistance in tobacco to the oomycete *Peronospora tabacina* and the bacterium *Erwinia carotovora* subsp. *carotovora* (Li et al., 2001). Cationic and hydrophobic AMPs, including magainins, are selective for prokaryotic membranes (bacteria) rather than eukaryotic ones (animals and plants) due to the predominantly negatively charged phospholipids in the outer leaflet of the prokaryotic membranes (for review see Zasloff 2002). The basis for the different susceptibilities of bacterial and fungal species against particular peptides remains unexplained (Zasloff, 2002). Recently, Gallucci et al. (2003) reported that ergosterol in fungal membranes may be a possible target of magainin 2 for channel formation. Fungi have eukaryotic membranes and could be more resistant than bacteria to the action of AMPs, an observation that is consistent with our study. In addition to the overall charge of the peptide, its efficiency, power and spectrum is related to other features such as size, sequence, structure, hydrophobicity, and amphipathic capacity (Tossi et al., 2000). MSI99 was derived from mag2 by modifying its amino acid sequence to enhance its lytic abilities (Figure 1b). However, in our study, which is supported by 2-years of greenhouse experiments, we found that the mag2 peptide is slightly more effective than is MSI99 in their activity against *A. vitis* (bacterial pathogen) and *U. necator* (fungal pathogen) when expressed in transgenic 'Chardonnay' plants. In addition, we found that both peptides when expressed in transgenic grapes are more active against *A. vitis* strains (prokaryotic membrane) than *U. necator* (eukaryotic membrane), which is in agreement to

the reported affinity of AMPs to negatively charged prokaryotic membranes (Maloy & Kari, 1995; Zasloff 2002).

Arabidopsis thaliana ubiquitin promoters have been successfully assayed for the constitutive expression of open reading frames in dicot plants (Norris et al., 1993). A comparison among different constitutive and inducible promoters revealed an adequate strength for the ubiquitin promoter but with a lower expression level than the CaMV-35S promoter (Holtorf et al., 1995). We used *Arabidopsis* ubiquitin promoters for the constitutive expression of *npt-II* and AMP genes in grapevine. Use of the CaMV-35S promoter may help to increase the expression of AMP genes and therefore to increase the resistance to plant diseases. Positive hybridization signals by Southern blot and RT-PCR analyses as well as the phenotypic resistance exhibited in some transgenic lines provide proof that the *Arabidopsis* ubiquitin-3 and -11 promoters are functional in *V. vinifera*. To our knowledge, this is the first report concerning the functioning of the *Arabidopsis* ubiquitin promoters in grapes. In addition, an antibody specific for both mag2 and MSI99 peptides was designed and raised by Sigma-Genosys (The Woodlands, TX). Using a protocol for detection of small peptides (Li et al., 2001), mag2 and MSI99 could be detected by ELISA at low levels in fresh leaf extracts of a number of lines, in agreement with the RT-PCR results. However, we were unable to detect these peptides (~2.4-kDa) consistently in both *in vitro* and greenhouse-grown plants (data not shown), suggesting the difficulty to detect these very small peptides by this technique. Similar difficulty was mentioned by Li et al. (2001) for MSI99 detection in transgenic tobacco plants.

Magainin genes may be promising AMPs for genetic engineering of grapevines for disease resistance. The action of AMPs is concentration dependent (Biggin & Sansom, 1999) and varies with the membrane composition of the targeted pathogen (Gallucci et al., 2003). Different strategies could be followed to improve their antimicrobial activity. In addition to modeling more active peptides, transgene expression under control of stronger and/or tissue specific promoters, as well as the expression via the chloroplast genome, are promising approaches. Also, the synergistic effect via a multiple gene strategy, by linking a magainin gene with another gene encoding a protein with a different mode of action, may allow a broader antimicrobial protection of grapevine cultivars.

Acknowledgments

The authors thank Thomas Burr (Cornell University) for providing the *A. vitis* strains and David Gadoury (Cornell University.) for providing the *U. necator* inoculum, and both for critically reviewing this manuscript and helpful discussions. We also gratefully acknowledge Claire-Lise Rosenfield for running the antimicrobial assay *in vitro* with the Bioscreen reader and Steve Luce for excellent technical assistance. The magainin constructs were kindly provided by John Sanford (FMS Foundation) and Franzine Smith (Sanford Scientific Inc.). This research was partially supported by grants from the American Vineyard Foundation, the USDA Viticulture Consortium-East, and the New York Wine and Grape Foundation. J.R. Vidal was supported in part by Kaplan Funds and a postdoctoral grant from the Spanish Ministry of Education and Science.

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