SUMMARY

Phomopsis viticola (Sacc.) Sacc. is the phytopathogenic fungus causing a severe disease of grapevine known as Phomopsis cane and leaf spot. Protoplasts from mycelium of P. viticola were successfully transformed either with plasmids carrying the bacterial hph gene, conferring resistance to hygromycin B (pAN7-1, pOHT, pOHT-AMA1), or the Bmlr gene from Neurospora crassa, causing resistance to benzimidazole fungicides (pBT6). Up to more than 300 transformants per µg of plasmid DNA were obtained with the hph marker gene. The highest effectiveness was obtained with pOHT, whereas pBT6 yielded around 25 transformants per µg of plasmid DNA. Southern blot analysis showed the occurrence of multiple integration events in the fungal genome of all tested plasmids. Experiments of co-transformation with pOHT and pBT6 were successful and about 70% of transformants were resistant to both hygromycin B and benomyl. The “Instant Gene Bank” technique and mutagenesis through Restriction Enzyme Mediated Integration (REMI) were attempted. As reported for other phytopathogenic fungi, the REMI technique proved to be a powerful method for obtaining mutant strains with variation in phenotypic traits.

Key words: Genetic transformation, phytopathogenic fungi, Phomopsis cane and leaf spot, grapevine.

INTRODUCTION

Phomopsis viticola (Sacc.) Sacc., one of the species in the genus Phomopsis and Diaporthe infecting grapevines (Kuo and Leu, 1998; Merrin et al., 1995; Mostert et al., 2001; Scheper et al., 2000; Uecker and Kuo, 1992), is the causal agent of a disease called Phomopsis cane and leaf spot. This disease occurs in all grape-growing areas of the world, where it causes decline and yield losses of susceptible cultivars.

Despite of its economical importance, P. viticola has never been studied at the genetic level. The fungus seems to be prevalently mitosporic, although perithecia have been found in Australia (Scheper et al., 1997, 2000). Recent observations showed this fungal species includes isolates that can be clustered in vegetative compatibility groups (VCGs) (Pollastro et al., 1998, 2002).

Improved knowledge of the genetics of this fungus could be important for clarifying its biology and epidemiology, and would provide more solid bases for improving protection strategies.

Genetic transformation is a powerful tool for investigating fungi and their interactions with the host plant. The low level of homology required for integration in the fungal genome allows the use of heterologous genes that have often been employed for molecular studies of genetically uncharacterised but economically important fungi (Goosen et al., 1992).

Transformation techniques requires selectable markers, like dominant or semi-dominant genes responsible for antibiotic resistance, e.g., Sb ble or hph, conferring resistance to phleomycin or hygromycin B, respectively (Gritz and Davies, 1983; Kaster et al., 1983; Jain et al., 1992; Santos et al., 1996), or for fungicide resistance, like, for instance, Bmlr from Neurospora crassa Shear et Dodge, that confers resistance to benzimidazoles (Orbach et al., 1986).

The effectiveness of genetic transformation is generally low (1-25 transformants µg⁻¹ plasmid DNA) when heterologous genes are used (Fincham, 1989; Hynes, 1986). A method for enhancing the effectiveness of transformation involves no-integrative autonomously replicating plasmids carrying DNA sequences with a replication origin, as the 2-µm plasmid (Beggs, 1978) and chromosomal ARSs (Autonomous Replication Sequences) of Saccharomyces cerevisiae Meyen ex Hansen (Stinchcomb et al., 1979). ARS-like sequences have rarely been observed in filamentous fungi (Huang et al., 1989; Santos et al., 1996; Suarez and Eslava, 1988). AMA1 sequence (Autonomous Maintenance in Aspergillus) of Aspergillus nidulans Eidam being an important example (Gems et al., 1991; Aleksenko and Clutterbuck, 1996, 1997).

Autonomously replicating plasmids carrying the AMA1 sequence have been successfully used in a simple gene-cloning strategy, known as “Instant Gene Bank”,

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based on co-transformation of linearized plasmids and genomic DNA fragments obtained with an appropriate restriction enzyme. Plasmids and useful flanking genomic sequences can be readily recovered from transformants (Gems et al., 1994; Verdoes et al., 1994).

Restriction enzyme-mediated integration (REMI) is a technique favouring single-copy plasmid integration. It is a useful tool for mutagenesis and gene cloning because single-copy insertions make it easy to rescue transforming plasmid and flanking sequences (Kahmann and Basse, 1999; Maier and Schafer, 1999).

The present paper reports the results of experiments for the genetic transformation of *P. viticola* with plasmids carrying the *bph* gene, conferring resistance to hygromycin (pAN7-1, pOHT and pOHT-AMA1), or the *Bml* gene, conferring resistance to benomyl (pBT6). Plasmids pOHT and pBT6 were also used for co-transformation experiments.

**MATERIALS AND METHODS**

**Fungal strains and media.** The wild-type strain PV3 of *P. viticola*, belonging to VCG III, one of prevalent VCGs in Apulia (Southern Italy), was used in most transformation experiments. Other strains, selected as representatives of other common VCGs, i.e. PV2 (VCG II), PV5 (VCG IV), PV26 (VCG V), and PV62 (VCG VI), were used to verify and confirm the results obtained with strain PV3.

Genomic DNA of the benzimidazole-resistant mutant PV3 was used with the “Instant Gene Bank” technique. The mutant was obtained by selection on a benomyl-amended medium (1 μg ml⁻¹) of UV-irradiated (30 mJ; 60% lethality) conidia of strain PV15-1 (VCG III).

*P. viticola* isolates were grown on potato dextrose agar (PDA), i.e. infusion of 200 g l⁻¹ peeled and sliced potatoes kept at 60°C for 1 h, 20 g l⁻¹ dextrose, 20 g l⁻¹ technical agar Oxoid n. 3, pH 6.5. Potato dextrose broth (PDB), i.e. infusion of 200 g l⁻¹ peeled and sliced potatoes kept at 60°C for 1 h, 20 g l⁻¹ dextrose, 20 g l⁻¹ technical agar Oxoid n. 3, pH 6.5. Potato dextrose broth (PDB), i.e. PDA with no agar, was used as a liquid medium to grow the fungus in shaken cultures.

The SHV medium, a modified SH medium (0.6 M sucrose, 5 mM HEPES, 1 mM (NH₄)₂HPO₄, 6 or 10 g l⁻¹ bacto-agar Difco, pH 5.3) (Shirane and Hatto, 1986) amended with 0.2 mg l⁻¹ biotin and 1 mg l⁻¹ thiamine, was used for reversion of fungal protoplasts.

Putative transformants were grown and maintained on PDA or on the basal medium described by Correll et al. (1987) [BM; 30 g l⁻¹ sucrose, 1 g l⁻¹ KH₂PO₄, 0.5 g l⁻¹ MgSO₄·7H₂O, 0.5 g l⁻¹ KCl, 10 mg l⁻¹ FeSO₄·7H₂O, 2 ml l⁻¹ of micronutrient solution, 20 g l⁻¹ technical agar Oxoid n. 3, pH 6.5; micronutrient solution: 5 g citric acid, 5 g ZnSO₄·7H₂O, 4.75 g FeSO₄·7H₂O, 1 g Fe(NH₄)₂(SO₄)₆·6H₂O, 250 mg CuSO₄·5H₂O, 50 mg MnSO₄·H₂O, 50 mg H₂BO₃, 50 mg Na₂MoO₄·2H₂O, 95 ml l⁻¹ water] supplemented with 0.2 mg l⁻¹ biotin and 1 mg l⁻¹ thiamine (BMS) and amended with 25 μg ml⁻¹ hygromycin B or 1 μg ml⁻¹ benomyl. Filter-sterilised vitamins and selective compounds were added to autoclaved media cooled down at about 40°C.

**Plasmids.** The plasmids pAN7-1 (Punt et al., 1987) and pOHT-T contain the *Escherichia coli* hygromycin B phosphotransferase gene (*bph*) conferring hygromycin resistance. The *bph* gene is under the control of different promoters from *A. nidulans*: gpd in pAN7-1 and *OliC* in pOHT. The plasmid pOHT-AMA1 is derived from pOHT by insertion of the *AMA1* sequence of *A. nidulans* (Gems et al., 1991). The plasmid pBT6 contains the *N. crassa Bmh* gene coding for β-tubulin and conferring resistance to benzimidazole fungicides (Orbach et al., 1986).

The strains DH5α or JM101 (for pBT6) of *E. coli* were used for the maintenance and propagation of plasmids.

Plasmid DNA was extracted using standard procedures (Sambrook et al., 1989) and purified using the Prep-A-Gene DNA purification kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions.

**Preparation of protoplasts.** Fungal mycelium was grown in PDB in shaken culture (150 rpm) for 36-48 h, harvested by filtration through a double layer of Miracloth (Calbiochem, La Jolla, CA, USA) washed three times with 0.6 M MgSO₄ and suspended (10 ml per g of fresh weight) in 1.2 M MgSO₄ containing 5 mg ml⁻¹ Novozyme 234 (InterSpex Products, Inc., Foster City, CA, USA). The suspension was kept 1-2 h at 30°C under mild shaking (70 rpm) until releasing of a sufficient number of protoplasts. Mycelium residues were then removed by filtration on one layer of Miracloth. Two ml 0.6 M KCl were gently layered on the top of the protoplast suspension. The discontinuous gradient was centrifuged at 3,500 rpm for 15 min at 4°C, and purified protoplasts were collected from the interface between the two phases. Protoplasts were washed 10 ml 0.6 M KCl, 50 mM CaCl₂, pelleted by centrifugation at 1300 rpm for 10 min at 4°C, and suspended in 0.6 M KCl, 50 mM CaCl₂ at the concentration of 10⁷ protoplasts in 50 μl.

**Genetic transformation.** Ten μg plasmid DNA were dissolved in 7-10 μl of ultrapure water and the volume brought to 50 μl with 0.6 M KCl, 50 mM CaCl₂, 2.5 μl 50 mM spermidine (Sigma-Aldrich, Milan, Italy). The solution was added to an equal volume of protoplast suspension.

After 10 min on ice, 50 μl 25% PEG 3350 (Sigma-Aldrich, Milan, Italy) in 50 mM CaCl₂, 10 mM Tris-HCl, pH 7.5 were added to the mixture and kept 20 min on ice. A further incubation for 10 min on ice was done after adding 250 μl of the same PEG solution. The reaction was stopped with the addition of 700 μl 0.6 M KCl, 50 mM CaCl₂.

In co-transformation experiments, 10 μg of each pOHT and pBT6 were added together to the protoplast
suspension. In the case of the “Instant Gene Bank” technique, 10 or 50 μg of KpnI-linearised pOHT-AMA1 and genomic DNA of the PV33 strain digested with the same enzyme were added to the protoplast suspension. HindIII-linearized pOHT was used for the REMI technique; the same restriction enzyme (10 or 50 U) was added to protoplast suspension in absence or presence of the appropriate buffer (10 mM Tris-HCl, 100 mM NaCl, 5 mM MgCl2, 1 mM 2-mercaptoethanol, pH 8.0; Buffer B; Roche Diagnostics, Milan, Italy).

Protoplast reversion and selection of transformants. Following transformation, 500 μl of protoplast suspension were dispersed into 60 ml SHV medium containing 6 g l⁻¹ agar cooled to 45°C after autoclaving. The medium was immediately poured in aliquots of 10 ml in Petri dishes (bottom-agar). After 15-16 h (5-6 h in the last experiments) at 25±1°C, the bottom-agar was overlaid with 6 ml SHV medium containing 10 g l⁻¹ agar and the selective compound (top-agar): 25 μg ml⁻¹ hygromycin B or 1 μg ml⁻¹ benomyl, i.e. concentrations inhibiting the growth of wild-type sensitive strains, according to the marker gene used.

Colonies developed within 5-10 days at 25±1°C were counted and numerous putative transformants were individually transferred onto BMS (initial tests) or PDA on non-selective medium (PDA); the response to the concentrations used for selection.

Amended with hygromycin B or benomyl, at the same concentrations as above, hygromycin B or benomyl at the above concentrations. Through growth tests on PDA amended with hygromycin B or benomyl at the above concentrations.

Suitable colonies were transferred into 2-ml Eppendorf microcentrifuge tubes and powdered with micropestles under liquid nitrogen. The mycelium was added with 600 μl of lysis buffer [100 mM Tris-Cl, pH 8.0; 1.4 M NaCl; 20 mM EDTA, pH 8.0; 2% CTAB (w/v); 0.2% 2-mercaptoethanol (v/v)] kept at 75°C for 30 min before usage. The suspension, frozen in liquid nitrogen and thawed at 75°C for three times, was kept at 75°C for 1 h. Afterwards, 600 μl chloroform were added and, after a centrifugation at 12,000 rpm for 15 min, the aqueous phase was transferred into a new tube, added with 600 μl isopropanol and maintained 2 h at −20°C. DNA was pelleted at 12,000 rpm for 15 min. The pellet was washed with 70% ethanol, centrifuged at 12,000 rpm for 5 min, and the supernatant discarded. DNA was dried under vacuum, then dissolved in 200 μl TE (10 mM Trizma base; 1 mM EDTA; pH 8.0).

Genomic DNA (25 μg) was digested with restriction enzymes recognizing only one or no site in the plasmid used for transformation: HindIII, EcoRV and XhoI for pOHT; HindIII and EcoRV for pAN7-1; EcoRV for pBT6. Restriction reactions were carried out according to manufacturer’s instructions (Roche Diagnostics, Milan, Italy).

Aliquots of restricted DNA, or undigested DNA from pOHT-AMA1 transformants, were mixed with 2 μl of loading buffer (Sambrook et al., 1989), loaded on 0.8% agarose gel (Bio-Rad Laboratories, Hercules, CA, USA), run in 0.5xTBE buffer (45 mM tris-borate; 1 mM EDTA, pH 8.0) at 110 V for 1 h (Sub-Cell Electrophoresis Cell; Bio-Rad Laboratories, Hercules, CA, USA), and transferred onto positively charged nylon membrane (Roche Diagnostics, Milan, Italy) by capillary blotting (Sambrook et al., 1989). Membranes were exposed to UV radiations (150 mJ) in a GS Gene Linker chamber (Bio-Rad Laboratories, Hercules, CA, USA) for DNA crosslinking.

An EcoRI-EcoRI fragment (about 1 kb) containing the hph gene and the trpC terminator from pOHT was used as a probe for transformants obtained with pOHT or pAN7-1. Both KpnI-linearized pOHT-AMA1 and its HindIII-EcoRI III portion containing the complete AMA1 sequence were used as probes for transformants obtained with that plasmid. Whole plasmid pBT6 linearized with BglII was used as a probe for benomyl-resistant transformants. Plasmid fragments was eluted from agarose gel by using the Qiax II Gel Extraction Kit (Qiagen, Milan, Italy), following the manufacturer’s instructions.

Probes were labelled with digoxigenin using a commercial kit (DIG-High Prime; Roche Diagnostics, Milan, Italy) based on the random primed labelling method. Labelled probes were purified through chromatography on Sephadex G-50 (Sigma-Aldrich, Milan, Italy) columns.

A commercial kit (DIG Luminescent Detection Kit; Roche Diagnostics, Milan, Italy) was used according to the supplier’s instructions for hybridisations. These were carried out in a Hybridiser HB-2D (Techne, Cambridge, UK) at 65°C for 14 h in the “standard hybridization buffer” containing 50 ng ml⁻¹ labelled probe. Stringency washes were in 0.1xSSC, 0.1% SDS at 65°C. Autoradiography films (X-ray film, Fuji Photo Film G.M.B.H., Dusseldorf, Germany) were exposed to the membrane in hypercassette with intensifying screen (RPN1643, 24 x 30 cm, Amersham Biosciences Italia, Milan, Italy).

RESULTS

Transformation with pAN7-1 and pOHT. The hph gene conferring resistance to hygromycin B carried by pAN7-1 and pOHT was used as dominant selectable
marker for transforming *P. viticola*. The plasmids were used with protoplasts of the PV3 strain either in circular form or after linearization with *Hind*III.

With both plasmids, colonies appeared on the selective hygromycin B-amended medium after 2-4 days of incubation. Fast-growing and slow-growing colonies were obtained, hereafter denoted “large” and “small” colonies (Fig. 1).

The efficiency of transformation with pAN7-1 was satisfactory (5-10 putative transformants per µg of plasmid DNA), but it was definitively higher with pOHT. Unexpectedly, circular form of pOHT yielded a higher efficiency (107-330 putative transformants µg⁻¹ plasmid DNA) than the linear plasmid (100 putative transformants per µg⁻¹ plasmid DNA) (Table 1).

Transformation experiments were extended to other strains representative of four different VCGs to evaluate the reliability of the procedure. Transformation rates varied with single strains from 78 to 330 putative transformants µg⁻¹ plasmid DNA (Table 1).

Representative hygromycin B-resistant colonies were transferred onto fresh selective medium (BMS or PDA amended with 25 µg ml⁻¹ hygromycin B). The majority of “large” colonies confirmed their resistance to the antibiotic. “Small” colonies did not yield uniform results in repeated experiments, because a variable proportion of them was normally sensitive to selectable markers. These colonies were interpreted as being “abortive” transformants (Table 1).

A sample of putative transformants obtained with pOHT, 10 “large” and 10 “small” colonies, were grown on BMS as such or amended with 20 or 100 µg ml⁻¹ hygromycin B to ascertain if the two typologies of colonies had different levels of resistance. Isolates deriving from “small” colonies grew slower than either the original strains PV3 or the isolates derived from “large” colonies in a statistically significant manner (Fig. 2). On hygromycin B-amended media, colony growth of wild type strain PV3 was inhibited, while the growth of “large” and “small” colonies was very similar, with no statistically significant differences, at both the tested concentrations of the antibiotic (Fig. 2).

The stability of resistant phenotypes was evaluated on samples of 25 transformants (including 5 from “small” colonies) obtained with pAN7-1 and 195 (75 from “small” colonies) obtained with pOHT. Putative transformants were transferred three times on no-selective medium, and each generation was tested for antibiotic resistance. All transformants from pAN7-1 and 94% of those derived from pOHT maintained their
Table 1. Examples of results obtained in transformation experiments carried out with plasmids containing the hph gene as selectable marker.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Fungal strain</th>
<th>VCG</th>
<th>Plasmid</th>
<th>N. colonies on the selective medium</th>
<th>N. putative transformants</th>
<th>N. tested colonies</th>
<th>N. resistant colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Large</td>
<td>Small</td>
<td>Total</td>
<td>µg pDNA</td>
</tr>
<tr>
<td>A</td>
<td>PV3 III</td>
<td>pAN7-1</td>
<td>pAN7-1 linearized</td>
<td>20</td>
<td>7</td>
<td>27</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pOHT</td>
<td>pOHT linearized</td>
<td>21</td>
<td>22</td>
<td>43</td>
<td>10</td>
</tr>
<tr>
<td>B</td>
<td>PV3 III</td>
<td>pOHT</td>
<td>128</td>
<td>408</td>
<td>536</td>
<td>107</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td>PV3 III</td>
<td>pOHT</td>
<td>38</td>
<td>1611</td>
<td>1649</td>
<td>330</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PV2 II</td>
<td>pOHT</td>
<td>151</td>
<td>979</td>
<td>1130</td>
<td>226</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PV5 IV</td>
<td>pOHT</td>
<td>242</td>
<td>598</td>
<td>840</td>
<td>168</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PV26 V</td>
<td>pOHT</td>
<td>53</td>
<td>339</td>
<td>392</td>
<td>78</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PV62 I</td>
<td>pOHT-AMA1</td>
<td>43</td>
<td>489</td>
<td>532</td>
<td>106</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td>PV3 III</td>
<td>pOHT-AMA1</td>
<td>53</td>
<td>1383</td>
<td>1436</td>
<td>287</td>
<td>48</td>
</tr>
<tr>
<td>REMI</td>
<td>PV3 III</td>
<td>pOHT linearized</td>
<td>238</td>
<td>111</td>
<td>349</td>
<td>70</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pOHT linearized+10U HindIII</td>
<td>311</td>
<td>253</td>
<td>564</td>
<td>113</td>
<td>152</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pOHT linearized+10U HindIII+buffer</td>
<td>362</td>
<td>412</td>
<td>774</td>
<td>155</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pOHT linearized+50U HindIII</td>
<td>58</td>
<td>1478</td>
<td>1536</td>
<td>307</td>
<td>190</td>
</tr>
<tr>
<td></td>
<td></td>
<td>POHT linearized+50U HindIII+buffer</td>
<td>483</td>
<td>824</td>
<td>1307</td>
<td>261</td>
<td>147</td>
</tr>
</tbody>
</table>

a Starting from 5·10⁶ protoplasts used in the transformation procedure and distributed into six Petri dishes.

b Resistance was evaluated by growing putative transformants on BMS or PDA amended with 25 µg ml⁻¹ hygromycin B.

c No tested resistance phenotype.
resistance phenotype up to the third generation with no differences between those deriving from “large” and “small” colonies.

Genomic DNA of 15 transformants obtained with pAN7-1 was digested with HindIII or EcoRV and used in Southern hybridisation with a probe containing the bpb gene and the trpC terminator. All transformants, but not wild type strain PV3, yielded positive results. Based on the number of hybridisation bands observed with genomic DNA digested with HindIII, which cuts pAN7-1 in a single site, it was concluded that from 2 to 5 integration events had taken place in most transformants, with the exception of L3, L5 and L20 (Fig. 3). Genomic DNA digested with EcoRV enzyme, which has not restriction sites in pAN7-1, gave often only one band of high molecular size (more than 8.4 kbp), but additional bands were seen in several transformants.

Genomic DNA of 16 transformants obtained with pOHT, 8 from “large” and 8 from “small” colonies, was digested with HindIII, XhoI or EcoRV. Multiple integrative events were distinguishable for DNA of strains L23, L24, L25 and L30 digested with HindIII, S26 and S43 digested with XhoI. For some of other transformants only single bands were seen, but the intensity of the signal suggested the presence of more than one copy of plasmid due to multiple integrative events (Fig. 3).

Fig. 3. Example of Southern analysis of DNA from wild-type strain, PV3, and transformants of P. viticola obtained with pAN7-1 (above) or pOHT (below) giving “large” (L) or “small” (S) colonies. DNA was digested with HindIII and fractioned in 0.8% agarose after digestion. Gel blots were hybridised with a digoxigenin-labelled 1 kb sequence containing most part of the bpb gene and the trpC terminator.

Transformation with pBT6. P. viticola colonies transformed with the Bm1 gene of N. crassa were observed on the selective medium after 5-8 days of incubation. They had a variable growth, but unlike those engineered with the bpb gene, no different types of colonies were distinguishable.

The efficiency of transformation with pBT6 was satisfactory (20-25 putative transformants per µg of plasmid DNA) but lower than that given by pOHT.

A sample of 65 resistant colonies was transferred on fresh PDA amended with 1 µg ml⁻¹ benomyl; 30 of them were normally sensitive to the fungicide and were classified as “abortive” transformants.

Thirty-five transformants were grown on PDA amended with benomyl at concentration inhibiting the growth of wild-type strains (1 or 10 µg ml⁻¹). All transformants grew slowly at the lowest concentration and were inhibited at the highest concentration of the fungicide. The transformants were transferred for three consecutive times on non-selective medium for testing the stability of their resistant phenotypes. Most of the colonies (31) retained resistance till the third generation.

Southern hybridisation was carried out with genomic DNA of 12 transformants digested with EcoRV, an enzyme that has not cutting sites in pBT6, using the plasmid linearized with BgIII as a probe. Under high stringency conditions, a common band (about 3.5 kbp in size) was seen in all isolates, PV3 included. Likely, it represents the homologue β-tubulin gene of P. viticola. DNA from 7 transformants gave two additional bands while others, like B122, gave a highly fluorescent band, suggesting integration of multiple copies of the plasmid (Fig. 4).

Transformation with pOHT-AMA1. In experiments with the pOHT-AMA1, plasmid containing the AMA1 sequence from A. nidulans, two well distinguishable kinds of colonies with different growth were obtained, as observed when the bpb gene was used as a selectable marker. Transformation rate was 287 putative transformants per µg of plasmid DNA, which is very similar to that obtained with pOHT (Table 1). Hence, unlike A. nidulans, the AMA1 sequence did not improve significantly transformation efficiency in P. viticola.

Only 1 out of 46 tested transformants lost resistance to hygromycin B after three generations on non-selective medium. This is in contrast with mitotic instability reported for other fungal species transformed with AMA1-containing plasmids (Gems et al., 1991; Verdoes et al., 1994).

Undigested genomic DNA of 17 transformants was used in Southern assay using either the whole plasmid

Fig. 4. Southern analysis of DNA from the wild-type strain, PV3, and transformants of P. viticola obtained with pBT6. DNA was digested with EcoRV and fractioned in 0.8% agarose after digestion. Gel blots were hybridised with digoxigenin-labelled pBT6 linearized with BgIII.
or the AMA1 sequence as probes. In both assays, all isolates showed hybridisation with one band of high molecular weight (more than 23,000 kpb), while bands expected for non-integrated plasmids were not detected (Fig. 5).

Notwithstanding the observed integration of pOHT-AMA1, “Instant Gene Bank” was attempted through co-transformation with pOHT-AMA1 and genomic DNA from the benzimidazole-resistant mutant PV33 digested with KpnI, that were used at 1:1 or 1:5 ratio. Colonies resistant to benomyl did not appear on selective medium even after long incubation.

Co-transformation with pOHT and pBT6. Plasmids pBT6 and pOHT were used in experiments of co-transformation. Transformants were selected on media amended with hygromycin B or benomyl. The efficiency of transformation was comparable to that obtained with the use of single plasmids. In particular, 321 and 24 putative transformant colonies per μg of plasmid DNA where obtained, respectively, when hygromycin B or benomyl were used for selection (Table 2).

Table 2. Efficiency of transformation in co-transformation experiments with the plasmids pOHT and pBT6.

<table>
<thead>
<tr>
<th>Selective compound</th>
<th>N. colonies on the selective medium</th>
<th>N. tested colonies</th>
<th>N. resistant colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Large</td>
<td>Small</td>
<td>Total</td>
</tr>
<tr>
<td>Hygromycin B</td>
<td>44</td>
<td>1560</td>
<td>1604</td>
</tr>
<tr>
<td>Benomyl</td>
<td>-</td>
<td>-</td>
<td>122</td>
</tr>
</tbody>
</table>

a Starting from 5·10⁶ protoplasts used in the transformation procedure and distributed into six Petri dishes.

b Resistance was evaluated by growing putative transformants on PDA amended with 25 μg ml⁻¹ hygromycin B or 1 μg ml⁻¹ benomyl.

Table 3. Resistance to benomyl and hygromycin B of colonies obtained by co-transformation experiments.

<table>
<thead>
<tr>
<th>Selective compound</th>
<th>N. tested colonies</th>
<th>N. resistant colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hygromycin B</td>
<td>hygromycin B+ benomyl</td>
</tr>
<tr>
<td>Hygromycin B</td>
<td>46</td>
<td>16</td>
</tr>
<tr>
<td>Benomyl</td>
<td>83</td>
<td>73</td>
</tr>
</tbody>
</table>

Some of the transformants were tested for resistance to both selective compounds. Among 46 isolates selected on hygromycin B, 63% were resistant only to the antibiotic and 35% were also resistant to benomyl. Among 83 isolates selected on benomyl, 8% was resistant only to the fungicide and 88% were also resistant to hygromycin B. Unexpectedly, a few colonies were resistant only to the compound that was not used for selection, likely because of the instability of the selected resistance character. As a whole, about 70% of 129 tested isolates were transformed with both plasmids and showed double resistance to hygromycin B and benomyl (Table 3).

Application of REMI technique. The REMI technique was tested by using pOHT linearized with HindIII and by adding the same enzyme to the transformation mixture at two different concentrations (10 or 50 U) in presence or not of the appropriate buffer.

The number of transformants was generally higher when the enzyme was included in the reaction mixture than when only linearized pOHT was used. Particularly, the best transformation efficiency was obtained with 50 U HindIII (260-307 transformants per μg of DNA); this condition also reduced markedly the proportion of “small” colonies on the selective medium. In addition, “abortive” transformants were never observed (Table 1). More than 500 transformants were obtained with the REMI technique, which often showed morphological variability in comparison to the parental strain PV3.

DISCUSSION

The transformation procedure described yielded stable transformants of *P. viticola* with four different plasmids containing as selectable marker the bacterial gene
bph, responsible of resistance to hygromycin B, or the Bm1 gene of N. crassa, responsible for resistance to benzimidazole fungicides. All plasmids transformed P. viticola successfully. The efficiency of transformation was 5-10 transformants per µg of DNA with pAN7-1 and 20 times higher with pOHT and pOHT-AMA1. pBT6 yielded 20-25 transformants per µg of DNA. The efficiency of transformation, although variable, was comparable or even higher than that reported for other fungal species (Fincham, 1989; Hynes, 1986).

With reference to the bph gene marker, the different results obtained with pOHT and pAN7-1 can be ascribed to different activities of promoters; thus, in P. viticola the gpd promoter carried by pOHT is more effective than the gpd promoter carried by pAN7-1. The effectiveness of pOHT in transforming P. viticola, which was confirmed with four distinct strains representing different VCGs of the fungus, was higher than that reported for other fungi (Cullen et al., 1987; Hamada et al., 1994; Huang et al., 1989; Punt et al., 1987). Though the efficiency of transformation induced by pOHT was always high, it was variable in different experiments, likely due to physiological differences among protoplasts that are a well-known cause of variation in the uptake of foreign DNA (Hamada et al., 1994).

The use of the bph gene in transformation of P. viticola always originated two kinds of colonies with different growth on the selective medium. The slow-growing colonies are generally believed abortive transformants (Cullen et al., 1987; Hamada et al., 1994; Huang et al., 1989; Punt et al., 1987). In some of experiments, however, a variable proportion of slow-growing colonies showed stable resistant phenotypes, displaying a resistance level similar to that of fast-growing colonies but with a reduced vigour on non-selective medium. This suggests that slow-growing colonies include abortive transformants but perhaps also transformants in which the plasmid integrated in genomic loci involved in colony growth.

Most of tested transformants showed a stable resistance to hygromycin B or benzimidazole fungicides even when they were grown for three generations in absence of selective pressure. Molecular analysis showed that multiple integration events in the fungal genome occurred very frequently for all the tested plasmids. This kind of integration, indicated as “II type”, is indeed very frequent when heterologous genes are used in transforming filamentous fungi (Fincham, 1989; Goosen et al., 1992; Hynes, 1986). In transformation experiments with pOHT, the number of plasmid copies integrated into the fungal genome was similar for transformants with different growth, which supports the above hypothesis on the origin of slow-growing colonies obtained using the bph gene as a selectable marker.

When competent protoplasts of P. viticola were exposed to both plasmids pOHT and pBT6, they were co-transformed with a high frequency: 70% of strains selected on benomyl- or hygromycin B-amended medium showed double resistance to both compounds. High frequency of co-transformation may be a very useful tool for transformation with no selectable markers, avoiding the need of construction of particular vectors, and can be applied in gene cloning techniques, such as the “Instant Gene Bank” by using auto-replicative plasmids (Aleksenko and Clutterbuck, 1997; Gems et al., 1994; Verdoes et al., 1994).

The “Instant Gene Bank” technique was tested with P. viticola using the plasmid pOHT-AMA1. Unfortunately, the plasmid did not yield the expected results. The efficiency of transformation was not increased as compared to the original pOHT and the resistant phenotypes of transformants were stable even in absence of selective pressure, in contrast with what generally reported for auto-replicating plasmids (Gems et al., 1991; Huang et al., 1989; Suárez and Eslava, 1988). Southern analysis carried out with both the whole plasmid and the AMA1 sequence definitively showed that the plasmid was integrated in the genome of all examined transformants and auto-replicating plasmids were not detected. Hence, the AMA1 sequence seems no effective in allowing plasmid auto-replication in P. viticola.

When pOHT-AMA1 and genomic DNA of a benomyl-resistant mutant of P. viticola were used for co-transforming a strain normally sensitive to the fungicide, no resistant colonies were obtained. This corroborated the hypothesis that the AMA1 sequence does not have the same function observed in A. nidulans.

REMI has been successfully applied for detecting and characterising genes responsible for pathogenicity in several fungi, such as Alternaria alternata (Fr.:Fr.) von Keissler (Kodama et al., 1998; Tsuge et al., 1998; Tanaka et al., 1999), Cochliobolus heterostrophus Drechsler (Lu et al., 1994), Colletotrichum magnus S.F. Jenkins et Winstead (Redman et al., 1999), Gibberella fujikuroi (Sawada) Ito et K. Kimura (Linnemannstöns et al., 1999), Glomerella graminicola (Ces.) G.W. Wils. (Epstein et al., 1998), Magnaporthe grisea Barr. (Sweigard et al., 1998; Balhadere et al., 1999), Mycosphaerella zeae-maydis Mukunya et Boothroyd (Yun et al., 1998), and Ustilago maydis (DC.) Corda (Bölk er et al., 1995). This technique confirmed its efficiency also with P. viticola when linearized pOHT was used in presence of the appropriate restriction enzyme. Numerous transformants were obtained and their preliminary characterisation showed variation in morphological traits. A deeper characterization of transformants is in progress to evaluate the possible existence of variation in pathogenicity or vegetative compatibility.

ACKNOWLEDGEMENTS

Research granted by the Italian Minister of Agricultural and Forestry Politics, project “Genetic variability of fungal pathogens and selection for resistance of grapevine to biotic stress” (Coordinated Research “As-
sisted selection for resistance to biotic stress and qualitative improvement of fruit trees”, National Programme on Plant Biotechnology); by the University of Bari, project “Epidemiology and genetics of phytopathogenic microorganisms”; and by CEGBA (Centro di Eccellenza in Genomica Comparata in Campo Biomedico e Agrario), Research Line n. 7.

The plasmids pAN7-1 and pBT6 were obtained from Fungal Genetics Stock Center (Department of microbiology, university of Kansas Medical Center, Kansas City, USA). The plasmids pOHT and pOHT-AMA1 were kindly gifted by M. Ward (GENENCOR International, San Francisco, USA) and G. Del Sorbo (Department of Fruit-culture, Botany and Plant Pathology - Section of Plant Pathology, University of Naples “Federico II”, Italy), respectively.

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Genetic transformation of Phomopsis viticola

Journal of Plant Pathology (2003), 85 (1), 43-52


