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Transformation of fungal grapevine trunk disease pathogens with the green fluorescent protein gene

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Summary. *Eutypa lata* and *Phaeomoniella chlamydospora* are important fungal grapevine trunk disease pathogens. Studies of their epidemiology and disease progression are hampered by the slow growth rates of the fungi and a lack of molecular tools. A protoplast-based transformation system was developed using hygromycin resistance as a positive selectable marker. The system was used to introduce a green fluorescent protein reporter gene that was expressed in the hyphae of both species. The reporter strains will be useful tools for further epidemiological studies. Furthermore the transformation system will allow for genetic dissection of candidate pathogenicity or virulence genes by targeted replacement or disruption, in order to discern the role of these genes in the disease process.

Key words: *Eutypa, Phaeomoniella*, dieback, Petri disease.

Introduction

Grapevine trunk diseases such as eutypa dieback and Petri disease (young vine dieback) are found wherever grapevines are grown (Gubler *et al.*, 2004). A key epidemiological question is how the pathogens causing these diseases invade and colonise vines. The dieback pathogen *Eutypa lata* (Pers.:Fr) Tul. invades via pruning wounds, probably by ascospores that are drawn a few millimetres into cut xylem vessels. The success of inoculations depends on the spore load, the time of year, the age of the wood inoculated, and the variety of grapevine. Several years may elapse before symptoms and the anamorph appear, while the teleomorph is formed even later (Carter, 1991). The fungus *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & Mugnai) Crous & W. Gams has been implicated in Petri disease. In addition to invading through pruning wounds it may also be present in grapevines propagated from infected mother stock and can possibly be the result of stress-induced stimulation of an asymptomatic latent infection (Gubler *et al.*, 2004). Once inside the plant tissues growth of each of these pathogens is certainly slow, although the actual rate of growth is not known (Creaser and Wicks, 2000).

In recent years substantial advances have been made in methods to detect fungal pathogens or their toxins in grapevine wood. For example PCR assays have been developed to identify pathogens in infected vines (Lecomte *et al.*, 2000; Tegli *et al.*, 2000) and phytotoxin metabolite profiles have been

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studied (Sparapano *et al.*, 2000; Molyneux *et al.*, 2002). However, although these methods are useful diagnostic tools and are epidemiologically informative, they involve destructive sampling and do not give a clear picture of the distribution and spread of fungal mycelia within the woody tissue.

A new method to observe the growth of fungal plant pathogens in their plant hosts involves the green fluorescent protein (*gfp*) gene, derived from the jellyfish *Aequorea victoria* (Lee *et al.*, 2002). Fungi that have been transformed with, and express, the *gfp* gene can be clearly visualised by fluorescence imaging (Lorang *et al.*, 2001). Infection patterns and host colonisation can be tracked in detail, as shown in barley plants infected with *gfp*-containing *Fusarium graminearum* (Skadsen and Hohn, 2004).

The purpose of the present investigation was to develop a transformation system for the trunk disease pathogens *E. lata* and *P. chlamydospora* so that GFP-tagged strains can be developed and used to study infection and colonisation in detail. A transformation system will also enable the powerful tool of reverse genetics to be used with these fungi so as to make targeted gene replacements and to directly test whether specific toxins are required for pathogenicity.

Materials and methods

Cultures and plasmids

Eutypa lata strain E10-10 was isolated from a Cabernet Sauvignon vine at Erindale Vineyard, Hawkes Bay, New Zealand on 18 November 1988. The *P. chlamydospora* strain was a New Zealand vineyard isolate provided by Hayley Ridgway, Lincoln University, Canterbury, New Zealand.

The plasmid pAN7-1 contained the *Escherichia coli* hygromycin B phosphotransferase (*hph*) gene fused to the *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) gene promoter and the *A. nidulans trpC* terminator (Punt and van den Hondel, 1992). Plasmid pCT74 contained an SGFP variant of the *gfp* gene from the jellyfish *A. victoria* fused to the *Phytophthora tritici-repens ToxA* promoter, along with a hygromycin resistance cassette (Lorang *et al.*, 2001). The plasmid pBCH-*gfp* (used only with *E. lata*) contained a 2.27 kb *Not*I fragment from pFAT-*gfp* (Fitzgerald *et al.*, 2003) that contained an SGFP variant of the *gfp* gene driven by the *Aspergillus nidulans gpdA* promoter, placed into the *Not*I site of pBC-hygro, a plasmid that contained the *hph* gene (Silar, 1995). Plasmid DNA was purified using a QIAGEN Plasmid Mega kit (Qiagen, Hilden, Germany).

Culturing

Eutypa lata and *P. chlamydospora* were grown on potato dextrose agar (PDA) at 22°C. For protoplast isolation or for DNA extraction, mycelial plugs were inoculated onto autoclaved cellophane discs (Waugh Rubber Bands, Wellington, New Zealand) on PDA plates. Transformants were purified and subcultured by transferring single hyphae from the colony margin onto fresh medium.

Protoplast isolation and transformation

Cellophane discs with mycelium grown at 22°C for 5 days (*E. lata*) or 15 days (*P. chlamydospora*), were removed from the surface of four plates and placed in 20 ml of OM buffer (1.4 M $MgSO_4$, 10 mM Na_2HPO_4, pH 5.8) containing 5 mg ml^{-1} Glucanex (Chemcolour Industries, Auckland, New Zealand) and incubated at 37°C with gentle shaking (80 rpm) for 3 h. Protoplasts were separated from mycelial debris by filtration through sterile miracloth (Calbiochem Corporation, La Jolla, CA, USA), placed in Corex centrifuge tubes in 5-ml aliquots and overlaid with 2 ml of ST buffer (1 M sorbitol, 100 mM Tris-HCl pH 8.0). After centrifugation at 1085 *g* for 5 min at 20°C, protoplasts were collected from the interface and washed twice with 5 ml of STC buffer (1.2 M sorbitol, 50 mM $CaCl_2$, 50 mM Tris-HCl pH 8.0) then re-centrifuged as before. Protoplasts were re-suspended in STC buffer at a concentration of $0.8–2.0\times10^7$ ml^{-1}.

The transformation procedure was based on that of Punt and van den Hondel (1992). Plasmid DNA (5 mg of pAN7-1, pCT74 or pBCH-*gfp*) was added to a protoplast suspension containing approximately $1.2–3.0\times10^6$ protoplasts in 150 μl STC buffer. After incubation at 20°C for 20 min, sterile 40% polyethylene glycol (PEG) 6000 solution in STC buffer was added in three successive steps of 250, 250 and 850 ml, with thorough mixing between additions. After a further 20 min at 20°C the mixture was diluted with 5 μl STC buffer and the protoplasts collected by 10 min centrifugation at 1085 *g* and 20°C. The protoplast pellet was re-suspended in 500 ml STC buffer and 100 μl aliquots spread

onto plates containing 20 ml of PDA osmotically stabilised with 0.8 M sucrose. For selection of transformants, after incubation for 24 h at 20°C, the plates were overlaid with 5 ml of PDA top agar containing sufficient hygromycin B to give a final overall concentration of 100 μg ml^{-1}.

DNA extraction and molecular analysis of transformants

Mycelium was harvested from 2–3 cellophane-PDA plate cultures after 10 days (*E. lata*) or 21 days (*P. chlamydospora*) at 22°C and freeze-dried overnight. DNA was extracted using one of two methods. For PCR-quality DNA a rapid method using cetyltrimethylammonium bromide (CTAB) based on that of Doyle and Doyle (1987) was used. Freeze-dried mycelium (30–50 mg) was ground in liquid nitrogen with a mortar and pestle and added to 600 μl CTAB buffer containing 2% (w:v) CTAB, 1% (w:v) PVP40, 1.4 M NaCl, 20 mM EDTA, 0.1 M Tris HCl (pH 8.0) together with 2 μl RNAse (20 mg ml^{-1}). After mixing and incubating at 65°C for 30–45 min and then cooling, 600 μl chloroform was added, mixed, and left to stand at room temperature for 2 min. Following a brief centrifugation (1 min at 15,000 *g*) the upper (aqueous) phase was transferred to a clean tube and 600 μl isopropanol was added and gently mixed by inversion to precipitate the DNA. Using only gentle pulse centrifugation (max 30 sec at 15,110 *g*) to settle the DNA pellet, three successive washes were carried out with 70% ethanol. Finally the pellet was air-dried and the DNA re-suspended in 100 μl TE buffer (10 mM Tris pH 8.0, 1 mM EDTA). DNA of higher purity for Southern blotting was extracted using the method of Al-Samarrai and Schmid (2000) which removed polysaccharides with a salt precipitation step, except that an additional chloroform step was included after the second precipitation step and that lithium chloride was added to a final concentration of 0.1 M immediately prior to ethanol precipitation to increase the yield of the DNA.

The presence of the *hph* and *gfp* genes in the transformants was tested by PCR amplification with primers 5'hph2672 (5'-ATCTTAGCCAGACGAGCG-3') and 3'hph3032 (5'-GTCTGCTGCTCCATACAAGC-3') or GFP1 (5'-GGAACTGTTCACTGGCGTGG-3') and GFP2 (5'-AGGATGGATCCGTGCAGCTG-3') respectively. The expected sizes of the PCR products were 360 bp and 520 bp respectively. Amplification reactions (50 μl) contained 1×PCR buffer (Roche Molecular Biochemicals, Mannheim, Germany), 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM each primer, 2 U *Taq* DNA polymerase (Roche) and 50 ng genomic DNA. Cycling conditions were: 2 min at 94°C; 35 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 60 sec; 72°C for 7 min.

Restriction digests and gel electrophoresis of fungal DNA were according to standard methods (Sambrook *et al.*, 1989). Approximately 2.5 μg of genomic DNA, from transformants or the untransformed control (*E. lata* E10-10), was digested with *Eco*RI, *Cla*I or *Hin*dIII, electrophoresed on a 1% TBE agarose gel along with 100 pg linearised pAN7-1 and pCT74 plasmid DNA and transferred to nylon membranes for hybridisation. The *hph* and *gfp* gene probes were PCR products obtained using pAN7-1 or pCT74 templates and the primers shown above, purified with a QIAquick PCR purification kit (Qiagen) and labelled with Digoxigenin (DIG)-11-dUTP using a High Prime DNA labeling kit (Roche). Hybridisation was at 42°C overnight in DIG Easy Hyb buffer (Roche). Chemiluminescent detection of DIG-labelled probes was according to the manufacturer's instructions.

Microscopy

Mycelium was mounted in 75% glycerol and examined by fluorescence microscopy using excitation filter BP 460–490 nm and barrier filter BA510-550 nm. Actively growing mycelium was used for microscopy and the untransformed wild type strains were used as negative controls.

Results and discussion

Protoplast yields and transformation frequency

Approximately 4–10×10^6 protoplasts were generally obtained from mycelium grown on four cellophane discs for each of the species. Protoplast regeneration on stabilised non-selective media after PEG treatment was generally between 0.2 and 0.5% of the numbers estimated by haemocytometer counts.

Growth of mycelia and regeneration of protoplasts of untransformed *E. lata* and *P. chlamydospora* was completely inhibited by 100 μg ml^{-1} hygromycin B, whilst transformants were able to

grow. The transformation frequencies were low with an average (n = 4) of 1 or 4 stable transformants per μg transforming plasmid DNA for *E. lata* and *P. chlamydospora* respectively, for each of the vectors used. Stable transformants were those that retained hygromycin resistance after a period of growth on non-selective media. Although low, these transformation frequencies were similar to those reported for some other phytopathogenic fungi (Blakemore *et al.*, 1989; Hamada *et al.*, 1994). With the vector pBCH-*gfp*, slow-growing 'abortive' *E. lata* transformants were also seen on the selective transformation plates that were not hygromycin resistant when subcultured onto fresh plates. These are believed to show only transient expression of a non-integrated form of the *hph* gene and are a common occurrence in fungal transformations (Goosen *et al.*, 1992).

Molecular characterisation of transformants

Several stable, hygromycin-resistant transformants were selected for further analysis. The transformants and wild-type controls were grown for DNA extraction. The yield of DNA from the two extraction methods used was generally 0.2 μg mg^{-1} dry weight of mycelium, although DNA extracted using the rapid CTAB method was not of sufficient purity for restriction endonuclease digestion. Using genomic DNA from purified transformants and wild-type untransformed controls, PCR amplification confirmed the presence of both the *hph* and *gfp* gene in most transformants, although in a few cases (e.g. *E. lata* transformant S4) only the *hph* gene was present (results for *E. lata* pCT74 transformants are shown in Figure 1). These results were verified by Southern hy-

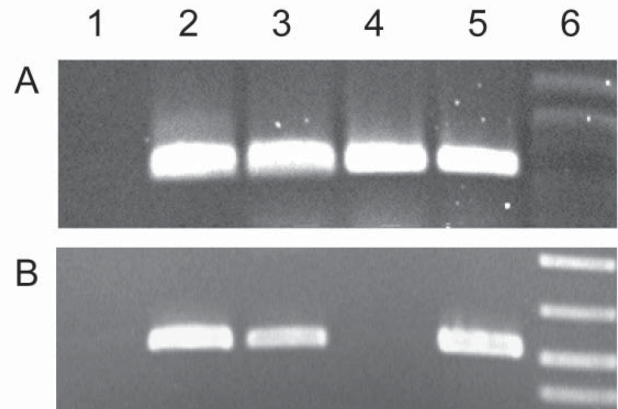

Fig. 1. PCR amplification of pCT74 transformants with hygromycin B phosphotransferase (*hph*) primers (A) or green fluorescent protein (*gfp*) primers (B) and templates of genomic DNA from wild-type *Eutypa lata* E10-10 (lane 1), pCT74 control plasmid (lane 2), *E. lata* pCT74 transformants S6, S4, S1 (lanes 3–5 respectively). Lane 6 contains a size marker ladder (Invitrogen 1 kb+). Sizes of PCR products are 360 bp in A and 520 bp in B.

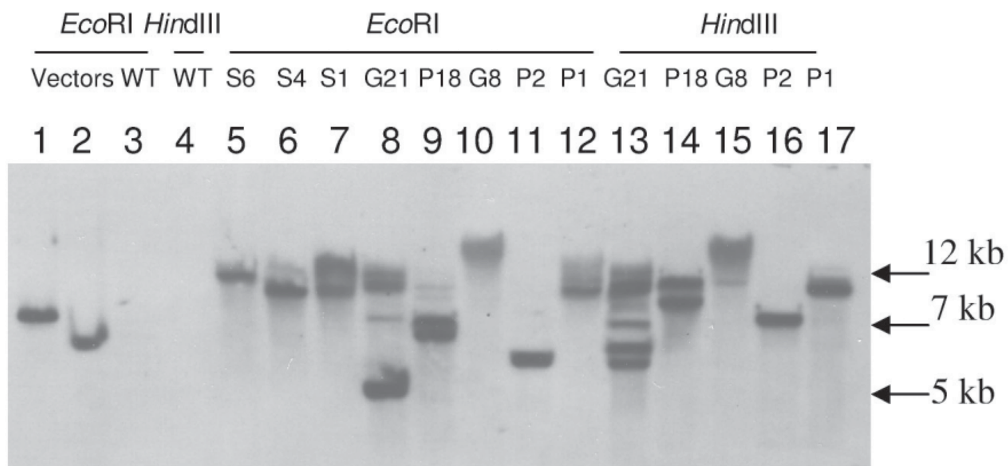

Fig. 2. Southern blot hybridised with a DIG-labelled hygromycin B phosphotransferase (*hph*) gene fragment. Lane 1, pBCH linearised with *Eco*RI; lane 2, pAN7-1 linearised with *Eco*RI; lanes 3 and 4, genomic DNA from wild-type *Eutypa lata* E10-10 digested with *Eco*RI and *Hin*dII respectively; lanes 5–17, genomic DNA from transformants digested with *Eco*RI (lanes 5–12) or *Hin*dIII (lanes 13–17). Transformants obtained using pCT74 (S1, S4, S6), pBCH-*gfp* (G8, G21) or pAN7-1 (P1, P2, P18).

bridisation. Figure 2 shows *hph* gene hybridisation results for *E. lata* transformed with plasmid pAN7-1 (transformants P1, P2, P18) and the *gfp*-containing plasmids pBCH-*gfp* (G8, G21) and pCT74 (S1, S4, S6). The results suggest that the plasmids integrated into the genome at random sites (different-sized hybridising bands in each transformant), and often in multiple copies (e.g. P18, G21). Similar results (not shown) were seen with *P. chlamydospora*. Both random and multi-site integration are common phenomena in the transformation of plant pathogenic filamentous fungi (Oliver *et al.*, 1987; Rikkerink *et al.*, 1994; Bradshaw *et al.*, 1997).

The presence of the *gfp* gene in *E. lata* transformants S1, S6, G8 and G21 and in *P. chlamydospora* transformants P1 and P7 was verified by Southern blotting (data for transformants S1, S6, P1 and P7 are shown in Fig. 3). The presence of *hph* and lack of *gfp* in transformant S4 suggested that either only a part of the pCT74 vector, containing the *hph* gene, integrated into the genome, or the entire vector integrated but the *gfp* gene was lost during subsequent purification steps under hygromycin selection. A single copy of the *gfp* gene appeared to be present in *E. lata* S6 and *P. chlamydospora* P1, but *P. chlamydospora* P7 had multiple copies.

GFP expression of transformants

The *gfp* gene was expressed in *E. lata* pCT74 transformants S1 and S6 (S6 is shown in Figure 4A) but not in pCT74 transformant S4 or in pBCH-*gfp* transformants G8 and G21 (data not shown). The lack of expression in the latter could be due to the use of a different promoter (*A. nidulans gpdA* in pBCH-*gfp* and *P. tritici-repens ToxA* in pCT74) or to a mammalian codon bias of the *gfp* gene in pBCH-*gfp* that was originally derived from pGreen Lantern (Invitrogen) (Fitzgerald *et al.*, 2003). The

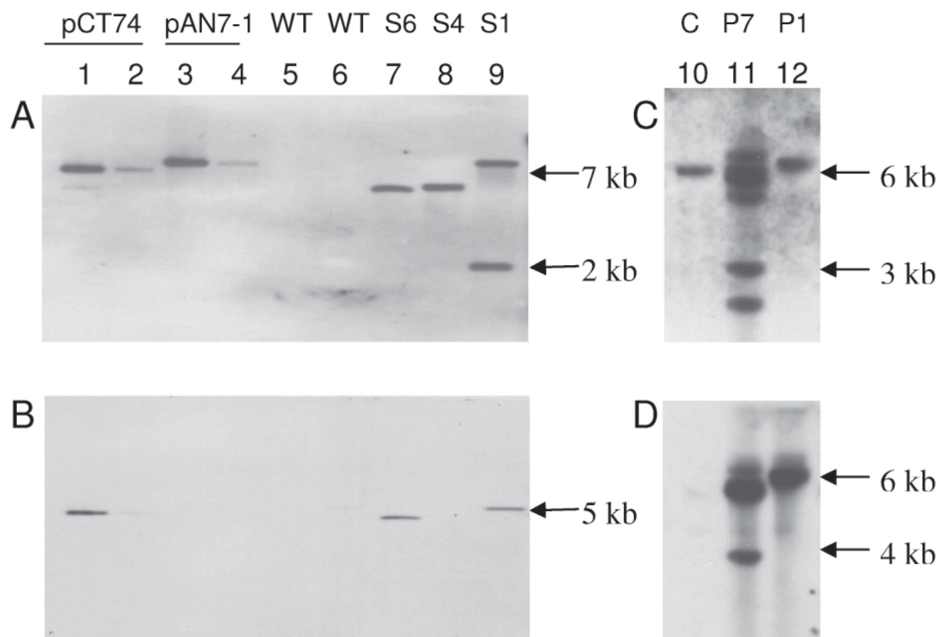

Fig. 3. Southern blot hybridised with DIG-labelled hygromycin B phosphotransferase (*hph*) (A, C) and green fluorescent protein (*gfp*) (B, D) gene fragments. Lanes 1–4, 100 pg (lanes 1, 3) or 10 pg (lanes 2, 4) of vector controls linearised with *Cla*I (pCT74) or *Hin*dIII (pAN7-1); lanes 5 and 6, genomic DNA from wild-type *Eutypa lata* E10-10 digested with *Cla*I and *Hin*dII respectively; lanes 7–9, genomic DNA from pCT74 *E. lata* transformants S1, S4 and S6 digested with *Cla*I; lane 10, genomic DNA from control (pAN7-1) transformant (C) of *Phaeomoniella chlamydospora*; lanes 11–12, genomic DNA from pCT74 *P. chlamydospora* transformants P7 and P1 digested with *Cla*I.

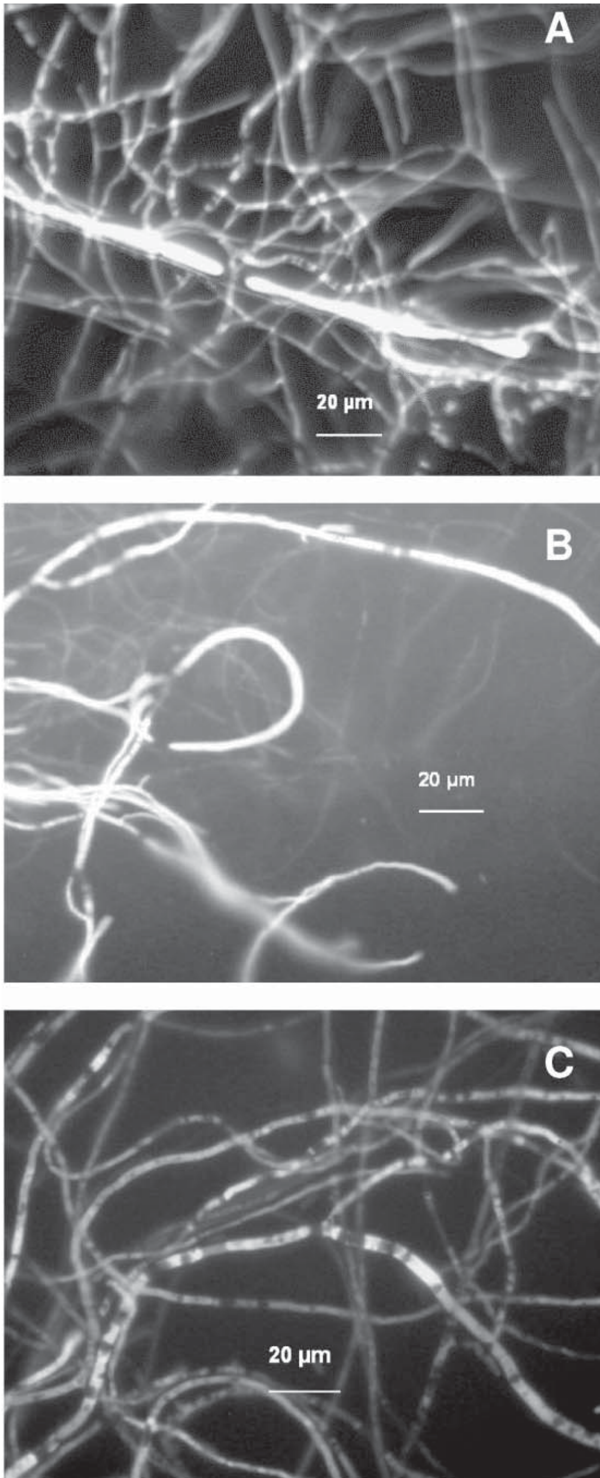

Fig. 4. Green fluorescent protein fluorescence of hyphae of pCT74 transformants of *Eutypa lata* S6 (A), and of *Phaeomoniella chlamydospora* P7 showing actively growing hyphal tips (B) and older vacuolated hyphae (C).

gfp gene was also expressed in four *P. chlamydospora* pCT74 transformants tested, including P1 and P7**.** Isolate P7, which has multiple copies of the *gfp* gene, fluoresced brightly. Actively growing hyphal tips showed uniform fluorescence (Fig. 4B) whilst older hyphae contained patches devoid of fluorescence that appeared to be associated with vacuoles (Fig. 4C). Colonies of isolate P1, which had a single copy of the *gfp* gene only, had a background glow from dense areas of hyphae but most individual hyphae had little or no detectable fluorescence.

The ability to transform these fungi and the availability of *gfp*-expressing strains will enhance studies of the epidemiology and lifecycle of these pathogens. For example, strains of *E. lata* and *P. chlamydospora* expressing the *gfp* gene can be used to track the growth of these fungi through grapevine wood. Unlike PCR approaches, GFP analysis can give a complete three-dimensional picture of where the fungus is present within the plant. It will be possible to accurately track the rate of growth and to determine the distribution of hyphae in the host tissues. It will also enable these hyphae to be distinguished from hyphae of any biological control agent applied to the same tissues.

Another application of the transformation system will be to discern which genes have a role in pathogenicity or virulence to grapevines. One area where this will be of particular interest is that of toxin biosynthesis. A number of toxins have been implicated with roles in the disease process (Smith *et al*., 2003) and when genes for the biosynthesis of these toxins are available it will be possible to make targeted gene replacements and to test directly whether these specific toxins are required for pathogenicity.

Conclusions

A protoplast-based transformation system using hygromycin selection was developed for the grapevine trunk pathogens *E. lata* and *P. chlamydospora* and was used to transform these fungi to express the heterologous green fluorescent protein (*gfp*) gene. The ability to transform *E. lata* and *P. chlamydospora* and the availability of *gfp*-expressing strains will enhance studies of the epidemiology and lifecycle of this pathogen.

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