Introduction

Unlike many plant pathogens, Botrytis cinerea is prominent year round and causes infection under a broad range of environmental conditions (Williamson et al., 2007). To reduce grey mould damage caused by the fungus, a number of control measures have been developed but these often exhibit a limited period of efficiency. As a result, researchers have focused upon the identification and characterization of novel gene targets. To identify such targets, recent advances in reporter system technologies, such as enhanced green fluorescent protein (eGFP), may allow for better visualization of physiological processes occurring within the fungus and between the pathogen and its host.

GFP has been employed in numerous biological studies because of its ability to form internal chromophores without substrates and its ease of visualization using non-destructive methods in living cells (Heim et al., 1994; Prasher et al., 1992). Modifications to optimize the structural composition of GFP have enabled efficient expression in a number of eukaryotes (Chiu et al., 1996; Cubitt et al., 1995; Yang et al., 1996; Zhang et al., 1996), including filamentous fungi (Lorang et al., 2001). The so-called enhanced GFP (eGFP) has been found to be 35 times brighter than the unal-
tered A. victoria GFP. Next, modifications to DsRed have led to the development of monomeric red fluorescent protein (mRFP), a highly stable reporter protein with advantages similar to those observed when using eGFP (Matz et al., 1999). The spectral properties of eGFP and mRFP are such that both proteins can be used simultaneously without interference.

Unlike eGFP or mRFP, LUC and GUS require external substrates before visualization of activity. Luciferase catalyzes the oxidation of luciferin thereby emitting light. LUC has been utilized in in vivo cell imaging and tracking and gene expression studies (de Wet et al., 1985; Doyle et al., 2004; Welsh and Kay, 2005). The GUS reporter gene requires the hydrolysis of glucuronides before pigmentation can be observed. GUS-based systems have been utilized to detect protein activity and developmental analysis without background interference (Bevan et al., 1989; Bonner et al., 1984; Finnegan et al., 1989; Masson and Fedoroff, 1989). One of the most useful applications of the GUS is as a reporter of promoter activity in promoter-GUS fusion experiments.

Prior to this report, B. cinerea expression vectors did not allow for easy manipulation; vectors did not contain a multiple cloning site (MCS) which could be easily modified. Instead, researchers were forced to generate expression vectors de novo for each transformation procedure. In this report we evaluate two vector systems, pOT and pLOB, for the expression of various reporter proteins, including eGFP, mRFP, LUC and GUS. We observed successful transformation of each reporter gene through molecular and protein expression studies. Of the four reporter proteins, eGFP and GUS reporter fusions have been successfully transformed and depicted in B. cinerea (Guimaraes et al., 2004; van Kan et al., 1997). Overall, eGFP, mRFP, GUS and LUC activity demonstrate the efficiency of the pOT vector system, which can be utilized in manipulating B. cinerea and other related ascomycetes.

Materials and Methods

Fungal strains and growth conditions. Experimental analyses and transformation was performed on Botrytis cinerea haploid strain B05.10 (Buttnier et al., 1994). The fungus was grown on malt agar containing 5% (w/v) malt extract and 1.8% (w/v) agar. Plates were then incubated at 20°C under near-UV light to induce sporulation. Conidia were harvested 7–14 days post inoculation in 10 ml water containing 0.05% (v/v) Tween 80 and collected by centrifugation at 3,000 × g for 5 min. For DNA extraction, 5% (w/v) malt extract broth was inoculated with 5 × 10⁸ spores and incubated at 20°C and 180 rpm for 24 h. Fungal mass was harvested by centrifugation and lyophilized.

Transformation of B. cinerea. Transformants were produced using methodology described by Hamada (1994) with modifications described by van Kan et al. (1997). Protoplasts were obtained by incubating 5 × 10⁸ spores in 100 ml of 1% (w/v) ME at 20°C at 180 rpm for 24 h. The mycelia were harvested and washed with KC solution (0.6 M KCl, 50 mM CaCl₂) by centrifugation at 3,000 × g for 5 min. The mycelium was incubated with β-1,3-glucanase (5 mg ml⁻¹ in KC solution) (InterSpex Products, Inc., USA) at 140 rpm and 25°C for 1.5–2 h.

For genetical insertion, 10 μg of transformation vector and 1 μg of hygromycin resistance vector were diluted in 95 μl KC solution. The solution was incubated on ice for 5 min before 5 μl of 5 mM spermidine was added and incubated on ice for an additional 5 min. One hundred microliter fresh PEG solution [25% (w/v) PEG 3350 in 10 mM Tris-HCl (pH 7.4) and 50 mM CaCl₂] was added and incubated at room temperature for 20 min. The total transformation mixture was combined with 200 ml of SH agar before being poured into 20 petri dishes. After 24 h incubation at 20°C, an equal volume of SH agar containing 50 μg ml⁻¹ hygromycin B was added to developing cultures. Emerging colonies were transferred to MEA plates supplemented with 100 μg ml⁻¹ hygromycin B to select against heterokaryon. Colonies were also transferred to MEA plates without hygromycin B. Monospore colonies were obtained by harvesting, diluting and plating on MEA plates containing 100 μg ml⁻¹ hygromycin B. Transformants were further purified by three rounds of subculturing in the presence of hygromycin to ensure they were monokaryotic.

Standard nucleic acid techniques. Fungal genomic DNA was isolated as described (Schouten et al., 2002). PCR analysis was performed as described by Saiki et al. (1988) using Reddy Mix (Bioline, UK). Primers used during PCR analysis are listed in Table 1. Purified products (Wizard Gel Extraction Kit, Promega Corp., UK) were cloned using the PCR 2.1 TOPO Cloning Kit (Invitrogen). Ligation strategies were designed using Clone Manager Suite (Science & Educational Software, USA). Sequence analysis and alignment
Improved expression vectors for *Botrytis cinerea* were aided by BLAST analysis at NCBI (Altschul et al., 1990).

**Protein concentration determination.** Protein concentration was estimated by Bradford assay (Bradford, 1976). Bovine serum albumin (BSA) dilutions, from 0 to 1 mg ml\(^{-1}\), were utilized to determine standard protein concentration. Measurements were recorded in OD units in triplicate (Benchmark Microplate Reader, Bio-Rad Laboratories Inc., UK).

**eGFP and mRFP visualization.** eGFP and mRFP transformants were grown on MEA for 3 days at room temperature before analyzing reporter protein activity. Developing mycelium was collected by lightly scraping a culture with a sterile plastic pipette tip before being submerged into 50 \(\mu\)l water. Ten microliters of the mycelium suspension was then transferred to a microscope slide with glass cover slip. eGFP and mRFP were visualized in situ using epifluorescence. Specifically, eGFP activity was depicted with a Leica filtercube I3 (Leica Microsystems GmbH, Germany), while mRFP activity was assessed with a Leica TCS-NT confocal laser scanning microscope attached to a Leica DM IRBE inverted epifluorescence microscope and recorded with Leica Confocal Software.

**GUS assay.** GUS transformants were grown on malt extract agar for 3 days at room temperature before analyzing reporter protein activity. GUS activity was histochemically visualized using 0.01% (w/v) X-Gluc (5-bromo-4-chloro-3-indoyl-\(\beta\)-glucuronide acid, CHA salt) (Nettleship, 2002) first dissolved in N,N'-dimethylformamide and adjusted to 100 mM sodium phosphate buffer (pH 7.0) and 0.05% (v/v) Tween 20 and inoculated overnight at 37°C with freshly grown fungal plugs. GUS-positive transformants developed a blue coloration that was recorded.

GUS expression was quantified by a fluorometric assay using MUG (4-methylumbelliferyl-\(\beta\)-D-glucuronide) as substrate (Gallagher, 1992). Fungal tissue (300 mg) was homogenized with 500 \(\mu\)l GUS extraction buffer [50 mM phosphate buffer (pH 7.0), 10 mM EDTA, 0.1% triton X-100, 0.1% sarcosyl and 10 mM \(\beta\)-mercaptoethanol]. One hundred microliters of supernatant was removed after centrifugation at 13,000 \(\times\) g for 5 min and mixed with 400 \(\mu\)l GUS extraction buffer containing 1 mM MUG. Samples were incubated at 37°C before 100 \(\mu\)l aliquots were removed at 0 min, 30 min and 60 min. Aliquots were mixed with 100 \(\mu\)l GUS reaction stop solution (200 mM Na2CO3) and added to fluorometer plate wells in triplicate before being measured and recorded at an excitation and emission wavelength of 365 nm and 455 nm, respectively, on a fluorometer (Fluorolite 1000, Dynatech Laboratories Ltd., USA) in relative fluorometric units (RFUs).

**LUC assay.** Luciferase assay was adopted from Turner et al. (1994). Fungal tissue (300 mg) was homogenized with mortar and pestle. Ground material was mixed vigorously with 500 \(\mu\)l 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM DTT. After centrifugation at 13,000 \(\times\) g for 5 min, 50 \(\mu\)l of the supernatant was transferred to a bioluminescence photometer reaction cuvette and assayed with 100 \(\mu\)l ATP buffer [500 mM HEPES (pH 7.8), 1 M MgCl2, 20 mM ATP in 100 mM phosphate buffer (pH 7.5)] and 100 \(\mu\)l 0.5 mM D-luciferin (Sigma-Aldrich Inc., UK) in 100 mM phosphate buffer (pH 7.5). Luciferase activity was recorded in relative light units per microgram protein.

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**Table 1.** Primer list and sequences utilized for *Botrytis cinerea* vector construction and transformation determination.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence 5′-3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSPHI MRFP F</td>
<td>GGTCATGAAATGGCCTCTCCGAGG</td>
</tr>
<tr>
<td>BAMHI MRFP R</td>
<td>GGGGATCCCTAGCAGCGGTGAG</td>
</tr>
<tr>
<td>HYG BSPHI F</td>
<td>GGTCATGAAATGGCCTCTCCGAGG</td>
</tr>
<tr>
<td>HYG BGLII R</td>
<td>GGAGATCTCAATTCTTGGCCCTGACGTGTC</td>
</tr>
<tr>
<td>OLIC2</td>
<td>ATTAACGCGGAGACGTATT</td>
</tr>
<tr>
<td>OLIC F</td>
<td>CCCCATGATGATTGATGATGAT</td>
</tr>
<tr>
<td>OLIC25</td>
<td>CCCCATGATGATTGATGATGAT</td>
</tr>
<tr>
<td>OLIC28</td>
<td>GGCCGCGGTCACGTGACGACGCTT</td>
</tr>
<tr>
<td>OLIC R</td>
<td>GGCCGCGGTCACGTGACGACGCTT</td>
</tr>
<tr>
<td>TRPC</td>
<td>GCACCTTTGCTTGGAC</td>
</tr>
<tr>
<td>TUBA F</td>
<td>CTATAATGTACATCCGATCTCCAAC</td>
</tr>
<tr>
<td>TUBA R</td>
<td>TTTCGGAGACTGATGTTGAC</td>
</tr>
<tr>
<td>TUB2</td>
<td>GGAGCTTAGCAGGATGAAAC</td>
</tr>
</tbody>
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(RLU \( \mu g^{-1} \) protein) by a luminometer (Lumat LB 9507, EG & G Berthold, Germany) and determined in triplicate.

Results

Vector system construction

pLOB: The pLOB vector system is based on a pre-established hygromycin resistance cassette (van Kan et al., 1997), pLOB1 (Genbank Accession No. AJ 439603). Because pLOB1 derived expression vectors did not contain a multiple cloning site (MCS) which could be easily modified, they have been redesigned to allow for easy manipulation. Specifically, the new system, pLOB, involves the co-transformation of the pLOB-HYG hygromycin resistance cassette with an expression vector, pLOB-MCS, containing a multiple cloning site between the \( A. \) nidulans oliC promoter and pLOB1 terminator region (Fig. 1A). Formation of the pLOB-MCS toolkit required the modification of the pMCS004 plasmid (Heneghan et al., 2007), comprising a 120 bp MCS flanked by an \( Agaricus \) bisporus gpdII promoter and an \( A. \) nidulans trpC terminator. The pMCS004 plasmid was digested with Sac II and Nco I, to excise the gpdII promoter, and ligated with a fragment containing the \( A. \) nidulans oliC promoter, amplified from pLOB1 with primer pair OLIC25 and OLIC28 (Table 1). The \( A. \) nidulans trpC terminator was excised with BamHI and Xho I digests and ligated with the pLOB1 terminator region, amplified by PCR with primers TUBA F and TUBA R (Table 1). The hygromycin resistance cassette, pLOB-HYG, a derivative of pLOB-MCS, was constructed by amplifying the hygromycin resistance gene (\( \textit{hph} \)) from pLOB1 and ligated into the pOT-MCS backbone as previously described above (Fig. 1B). Since both vector systems, pLOB and pOT, differ only by terminator sequence, they will be referred to herein pLOB(\( \text{t-trpC} \)) and pOT(\( \text{t-trpC} \)). Expression and resistance vectors derived from both systems will also be labeled according to the terminator region. For instance, pLOB-MCS and pOT-MCS expression cassettes will be referred to as pLOB-MCS(\( \text{t-trpC} \)) and pOT-MCS(\( \text{t-trpC} \)), while resistance cassettes pLOB-HYG and pOT-HYG are designated pLOB-HYG(\( \text{t-trpC} \)) and pOT-HYG(\( \text{t-trpC} \)) respectively.

Formation of reporter gene constructs

Reporter genes, eGFP (732 bp; Ma et al., 2001), mRFP (690 bp; Campbell et al., 2002), GUS (1,824 bp; Amey et al., 2002), and LUC (1,785 bp; Burns et al., 2005), were isolated and ligated into pLOB-MCS(\( \text{t-trpC} \)) and pOT-MCS(\( \text{t-trpC} \)) expression vectors as follows. Digestion with Nco I and BamHI I of the pMCS004 based vectors pGP004, pGUS004 and pLUC004 that each contain the \( B. \) cinerea gpdII promoter, the \( Aspergillus \) nidulans trpC terminator and the reporter genes eGFP on a \( Nco \text{-BamHI} \) fragment yielded eGFP, GUS and LUC fragments, flanked by Nco I and BamHI I restriction sites. The mRFP coding sequence was amplified by PCR from plasmid pPJ-1 (provided by Peter Joyce by permission of Dr. Roger Y. Tsien, University of Bristol, UK) with primers BspHI mRFP F and BamHI mRFP R (Table 1) that introduce BspHI I and BamHI I restriction sites to allow directional cloning. Fragments were independently ligated into the pLOB-MCS(\( \text{t-trpC} \)) and pOT-MCS(\( \text{t-trpC} \)) backbones digested with Nco I and BamHI I. Resulting pLOB-MCS(\( \text{t-trpC} \)) and pOT-MCS(\( \text{t-trpC} \)) expression vectors will be referred herein as pLOB-eGFP(\( \text{t-trpC} \)), pLOB-mRFP(\( \text{t-trpC} \)), pLOB-GUS(\( \text{t-trpC} \)) and pLOB-LUC(\( \text{t-trpC} \)), whereas pOT-MCS(\( \text{t-trpC} \)) based vectors will be designated pOT-eGFP(\( \text{t-trpC} \)), pOT-mRFP(\( \text{t-trpC} \)), pOT-GUS(\( \text{t-trpC} \)) and pOT-LUC(\( \text{t-trpC} \)).

Evaluation of pLOB(\( \text{t-trpA} \)) and pOT(\( \text{t-trpC} \))

\( B. \) cinerea was co-transformed with the hygromycin resistance cassette, pLOB-HYG(\( \text{t-trpA} \)), and eGFP, mRFP, GUS and LUC expression vectors derived from pLOB-MCS(\( \text{t-trpA} \)). The fungus was also co-transformed with pOT-HYG(\( \text{t-trpC} \)) and eGFP, mRFP, GUS and LUC expression vectors derived from pOT-MCS(\( \text{t-trpC} \)). Both systems yielded similar numbers of hygromycin resistant transformants (Table 2). The pLOB(\( \text{t-trpA} \)) based transfor-
Fig. 1. pLOB_{tubA} and pOT_{trpC} vector systems.

(A) pLOB_{tubA} regulatory sequences are the *A. nidulans* oliC promoter and what is reported to be the *B. cinerea* tubA terminator. pLOB-MCS_{t-tubA} contains a 120 bp multiple cloning site (MCS), while pLOB-HYG_{t-tubA} contains the hygromycin resistance gene (*hph*). (B) pOT_{trpC} regulatory sequences are the *A. nidulans* oliC promoter and *A. nidulans* trpC terminator. pOT-MCS_{t-trpC} contains a MCS, while pOT-HYG_{t-trpC} contains the *hph*. Fragment sizes are found above each segment, while compete vector sizes and names are shown at the end of each plasmid.
mants, however, were found to be more variable in growth rate and experienced irregular growth patterns (results not shown).

Hygromycin B resistant colonies were screened for transgene incorporation by studying reporter activity. eGFP (Fig. 2, A and B) and mRFP (Fig. 2, G and H) expression was detected by microscopy, while GUS activity was demonstrated histochemically (Fig. 2, D and E) and fluorometrically (Fig. 3A) and LUC expression was determined and quantified by luminometer (Fig. 3B). Although initial analysis indicated that both systems proved effective in expressing eGFP, mRFP, GUS and LUC, it was noted that in a number of eGFP transformants, not all cells displayed green fluorescence. The highest proportion of reporter protein expressing transformants was obtained with eGFP constructs (30–100% of recovered transformants), as compared to the other reporter systems. For mRFP constructs specifically, only two transformants expressed the reporter to detectable levels (Table 2). Transformants expressing reporter proteins were subjected to PCR analysis to confirm the integration of the entire expression construct. Primers sets OLIC2 & TUB2 and OLIC2 & TRPC (Table 1) were employed to determine successful integration of the entire reporter gene in pLOB(t-tubA) and pOT(t-trpC) based transformants, respectively (results not presented). Co-transformation frequencies were higher in pOT(t-trpC) based transformants; overall, 41.2% of pOT(t-trpC) based and 20.4% of pLOB(t-tubA) based transformants tested positive for reporter protein expression and all contained an intact reporter construct as confirmed by PCR (Table 2). Transformation frequencies (per μg expression vector) were also higher for pOT(t-trpC) based transformants (1.7 transformants per μg expression vector) in comparison to pLOB(t-tubA) based transformants (0.65 transformants per μg expression vector). Reporter gene expression was found to be stable throughout several rounds of subculturing.

Discussion

We present the development of two vector systems, pLOB(t-tubA) and pOT(t-trpC), for molecular manipulation in *B. cinerea*. Prior to this report, *B. cinerea* expression vectors did not allow for easy manipulation, in particular because they did not contain a multiple cloning site (MCS) which could be easily exploited for transgene expression. Also, promoter and terminator elements were not easily interchangeable. As a result, researchers were forced to generate expression vectors de novo for each transformation procedure. Both vector systems were evaluated with a range of exogenous genes, including eGFP, mRFP, GUS and LUC, whose protein activity can be easily visualized and quantified. Reporter gene technology in fungi has proven useful in studying plant-pathogen interactions (Lorang et al., 2001), but its use in *Botrytis cinerea* has thus far been limited. Whilst eGFP and GUS reporter fusions have been successfully expressed in *B. cinerea* (Guimaraes et al., 2004; Rolland et al., 2003; van Kan et al., 1997), expression levels were weak. In these previous studies, eGFP and GUS transformants were generated through the transformation of pLOB1 based vectors [regulated by the *Aspergillus nidulans olIC* promoter and a region reported to be the *B. cinerea tubA* termi-
Improved expression vectors for \textit{Botrytis cinerea} [Genbank Accession No. AJ439603].

The pLOB\textsubscript{(t\textsubscript{tubA})} system (controlled by the \textit{A. nidulans} oli\textit{C} promoter and what was reported to be the \textit{B. cinerea} tub\textit{A} terminator region) consists of the pLOB-HYG\textsubscript{(t\textsubscript{tubA})} hygromycin resistance cassette and the pLOB-MCS\textsubscript{(t\textsubscript{tubA})} expression vector, while the pOT\textsubscript{(t\textsubscript{trpC})} vector system (controlled by the \textit{A. nidulans} oli\textit{C} promoter and \textit{trpC} terminator elements) consists of the pOT-HYG\textsubscript{(t\textsubscript{trpC})} resistance cassette and pOT-MCS\textsubscript{(t\textsubscript{trpC})} expression vector. Both vector systems, pLOB\textsubscript{(t\textsubscript{tubA})} and pOT\textsubscript{(t\textsubscript{trpC})}, were tested for efficient expression in \textit{Botrytis cinerea} with eGFP, mRFP, GUS and LUC. Successful transformation and expression of each reporter gene was confirmed through molecular and protein

![Fig. 2. Examples of eGFP (A and B), GUS (D and E) and mRFP (G and H) expressing pLOB\textsubscript{(t\textsubscript{tubA})} transformants. Wild type controls were also tested for eGFP (C), GUS (F) and mRFP (I) expression. GFP (at ×63 objective) and mRFP (at ×40 objective) was microscopically recorded while GUS expression was observed histochemically. Samples pictured include eGFP 1 (A), eGFP 155 (B), GUS 166 (D), GUS 163 (G), mRFP 1 (G) and mRFP 6 (H).]
expression studies. However, many pLOB (t-
tubA) transformants exhibited abnormal growth rates and pat-
terns, an occurrence that may be resultant of transcript
instability (Judelson et al., 1992; Lemke and Peng,
2004; Wang et al., 1988; Yost et al., 1990). When com-
paring the percentage of reporter gene expressing co-
tranformants, a greater percentage of pOT (t-trpC)
based transformants were found to express the introduced
reporter gene activity in comparison to the wild type (WT, white bars). 
pLOB (t-tubA) and pOT (t-trpC) based transformants are colored grey
and black, correspondingly.

expression studies. However, many pLOB (t-tubA) transformants exhibited abnormal growth rates and patterns, an occurrence that may be resultant of transcript instability (Juddelson et al., 1992; Lemke and Peng, 2004; Wang et al., 1988; Yost et al., 1990). When comparing the percentage of reporter gene expressing cotranformants, a greater percentage of pOT (t-trpC) based transformants were found to express the introduced reporter gene. Also a greater proportion of transformants exhibited detectable levels of eGFP in comparison to LUC, GUS and mRFP reporter systems. These results correspond to observations in Clonostachys ro-
sea, where 35% of recovered transformants demonstrated high levels of GUS activity, while almost 100% of colonies tested expressed eGFP activity (van West et al., 1999). In this case, however, it was observed that GUS expression was not detectable in transformants with a single vector integration site. A number of the B. cinerea transformants that we analyzed displayed heterogeneous eGFP expression only in a proportion of cells. Similar observations were reported in Phytophthora palmivora (van West et al., 1999). Of the four marker proteins, eGFP and GUS reporter fusions have previously been successfully transformed and expressed in B. cinerea (Guimaraes et al., 2004; Rolland et al., 2003; van Kan et al., 1997). This is, however, the first report of LUC and mRFP expression in B. cinerea, which may prove advantageous for further molecular and protein investigations.

On the whole, the expression levels of eGFP in Botrytis cinerea, under the control of either pOT (t-trpC) or pLOB (t-tubA) based systems, are considered low in comparison to the ascomycetes Sordaria macrospora and Acremonium chrysogenum (Pöggeler et al., 2003). Since the A. nidulans oliC promoter and A. nidulans trpC terminator were also used to regulate reporter gene expression in these two fungi, similar levels of eGFP expression were expected in B. cinerea. As a result, further vector optimization studies are required to attain greater levels of reporter protein expression. For instance, one may consider the utilization of a vector that contains an intron sequence in the 5’-UTR, which is believed to provide a greater degree of tran-
script stability and thereby increases the heterologous protein production level (Burns et al., 2005). Even so, the pOT vector system may be used as a tool to improve ease of transgene expression and increase previously observed gene silencing frequencies in B. cinerea and other ascomycetes (Patel et al., 2008).

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References


