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## Transformation of *Botrytis cinerea* with a Green Fluorescent Protein (GFP) Gene for the Study of Host-pathogen Interactions

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**Abstract:** *Botrytis cinerea* is a plant pathogenic fungus that causes diseases generally known as gray mold in a wide variety of agriculturally important crops. To effectively reduce gray mold diseases and minimize synthetic chemical use on fruits pre- and postharvest, it is necessary to have an understanding on latent infections and the behaviours of the pathogen under various treatments. We described here a procedure for *B. cinerea* transformation based on particle bombardment. Utilising a co-transformation system, we successfully introduced a Green Fluorescent Protein (GFP) reporter gene and a hygromycin B resistant (Hy<sup>R</sup>) selectable marker into the fungal conidia. Within the five Hy<sup>R</sup> positive colonies, one isolate BC-2b that displayed strong green fluorescence under a fluorescent compound microscope confirmed the GFP gene insertion by direct PCR. Morphological observation, cultural evaluation and pathogenicity tests on flower petals and fruits of strawberry revealed that the GFP transformant of *B. cinerea* maintained the characteristics of the wild type isolate and was able to express the GFP gene in hyphae and conidia of the fungus both *in vitro* and *in vivo*. This proved that the transformant could be a powerful tool for our future studies on the interactions between the pathogen and its fruit hosts.

**Key words:** *Botrytis cinerea*, Green Fluorescent Protein (GFP), host-pathogen interactions, particle bombardment, strawberry, transformation

### INTRODUCTION

*Botryotinia fuckeliana* (de Bary) Whetz, a teleomorph of *Botrytis cinerea* (Pers.; Fr.) is a plant pathogenic fungus that causes diseases generally known as gray mold in a wide variety of agriculturally important crops, including various fruits, such as apple, grape, raspberry and strawberry, all over the world. Infection by *B. cinerea* not only can greatly reduce fruit yield and quality but also significantly shorten shelf life of harvested fruits. It is a great challenge to control gray mold because the pathogen can attack crops at any stage of growth and can infect all plant part (Elad and Evensen, 1995). The fungus is considered one of the most interesting fungal pathogens since it can live pathogenically, but also survive saprophytically (Rosslenbroich and Stuebler, 2000).

For the development of effective measures to control gray mold on fruits, an understanding of the interactions between *B. cinerea* and its fruit hosts under different pre- and post-harvest conditions or treatments is critically needed; an isolate that can be traced in its host tissue and other ecological niches is necessary for this understanding. The particular constitution of the nuclear system in *Botrytis* makes it difficult to obtain mutants to serve this purpose (Büttemer *et al.*, 1994), but fungal

transformation using a vital marker may provide an effective solution. A Green Fluorescent Protein (GFP) has been employed as a visual marker or reporter gene in a number of fungi (Aboul-Soud *et al.*, 2004; Balint-Kurti *et al.*, 2001; Inglis *et al.*, 1999, 2000; Rohel *et al.*, 2001; Spellig *et al.*, 1996). An isolate of *B. cinerea* with expression of GFP gene would be a very useful tool for monitoring the responses of the fungal pathogen to the dynamic changes of its fruit hosts and to the potential physical, chemical and biological treatments during both growing and storing periods. The GFP isolate may also be used as a tool to study interactions between biocontrol agents and plant pathogens (Zeilinger *et al.*, 1999; Lu *et al.*, 2004).

The aim of this research was to introduce a GFP gene to a wild-type *B. cinerea* by using a particle bombardment transformation technique and to obtain a traceable isolate that is morphologically stable and pathologically similar to the wild-type, thus can be used for future *in vivo* studies.

### MATERIALS AND METHODS

**Sources of fungus and plant:** The wild-type isolate of *B. cinerea* was originally isolated from a strawberry (*Fragaria × ananassa*) fruit with typical gray mold

symptoms and stored on Potato Dextrose Agar (PDA) slant at 4°C before use. Conidia were obtained by culturing the fungus on PDA plate at 22°C for a week. Strawberry was used for testing fungal pathogenicity: flowers were collected from Guelph, Ontario and fruit was California products purchased locally.

**Transformation vectors:** GFP gene (*gfp*) was recovered by PCR from plasmid yEGFP3 (Cormack *et al.*, 1997) with a forward primer 5' - GTCATGAGCAAAGGTGAAGAATTATTCCTGG- 3' and reverse primer 5' - CGGATCCTTATTTGTACAATTCATCCATACC- 3', which introduced a *RcaI* site (TCATGA) at ATG start codon and a *BamHI* site (GGATCC) at TTA stop codon of the GFP gene. The resulting 718 bp PCR fragment was ligated in pBluescript SK+ digested with *EcoRV*. To insert the *gfp* to a transformation vector PAN2-4 (Punt *et al.*, 1997), the vector was digested with *NcoI* and *BamHI* before the *gfp* with *RcaI* and *BamHI* hanging ends were ligated to the vector. A transformation vector containing *Hy<sup>R</sup>* (a hygromycin B resistant gene) and pMOcosX (Orbach, 1994) was co-transformed to aid the selections.

#### Transformation and selection

**Bombardment:** Conidial suspension (200 µL) of *B. cinerea* at  $5 \times 10^6$  conidia/mL was spread on a thin layer PDA with 1.5 M sorbitol, 8 mL in a standard Petri dish (100×15 mm). M17 tungsten particles (1.1 µm, Bio-Rad Laboratories, Inc., Hercules, CA) were used as microprojectiles. The plasmids PAN2-4GFP and pMOcosX were precipitated onto tungsten particles using a DNA-coating protocol modified from Sanford *et al.* (1993). Aliquots of 4 µL (approximately 0.8 µg DNA precipitated onto 1 mg tungsten) were applied to Kapton flying discs. One disc was shot to each plate by a helium-driven gene gun (PDS He-1000 Biolistic Gun, Dupont). The distance between the helium source and the flying disc was 1.0 cm and the distance between the particle launch site and the target cells was 9.0 cm. The helium pressure used to accelerate the flying disc was 1,000 psi.

**Selection:** The bombarded plates were overlaid with 16 mL of PDA amended with 300 µg of hygromycin B/mL and incubated at 22°C. Single colonies grown out of the medium were transferred onto fresh PDA plates amended with 100-400 µg mL<sup>-1</sup> hygromycin B mL<sup>-1</sup>. Colonies developed on the hygromycin B medium were reselected for presence of green fluorescence under a fluorescent compound microscope equipped with a chroma filter (ex 470 em 525 nm).

**Stabilization:** Colonies with green fluorescence were considered transformants, which were subcultured 4 times on PDA with hygromycin B before being used for single conidial re-isolation. Conidia produced by the transformants were washed off from the culture plates and used for a conidial suspension of  $1 \times 10^4$  conidia mL<sup>-1</sup>, which (100 µL) was evenly distributed on each PDA plate with hygromycin B. Single colonies formed on the plates were checked for the presence of green fluorescence and the ones with strong fluorescence were chosen for a second single conidial re-isolation. The selected conidial colonies were further stabilized by up to 6 sub-culturing on hygromycin B containing PDA.

#### Characterization of the transformants

**Detection of GFP gene in the transformants:** Putative transformants were screened by PCR using primers specific for GFP gene and the *gpd* promoter (P1876: CGGTGTATGAAACCGGAAAG; G177: GACTAAGGTTGGCCATGGAA) and the expected amplified DNA fragment of 611 bp was analyzed by electrophoresis.

**Morphological characterization:** Stable transformants (single conidial isolates) with strong GFP expression and the wild-type isolate were cultured on PDA, four plates per isolate, at 22°C for comparison in growth rate and sporulation. The colony diameters were measured every other day until the plate was fully covered and sporulation were evaluated by estimating percent area of the culture colony covered with conidia.

#### Pathogenicity on strawberry and fluorescence *in vivo*

**Evaluation with strawberry fruits:** Fruits were surface disinfested using 0.5% sodium hypochlorite and rinsed with sterile distilled water. The treated fruits were inoculated with 10 µL of a conidial suspension of *B. cinerea* at  $1 \times 10^5$  conidia/mL at a wound site (2 mm in diameter and 2 mm in depth) made with a sterile pointer. The inoculated fruits, 12 fruits per *isolate*, were incubated in a plastic container (32×19×12 cm), which was placed in a growth chamber at 22°C with 80% RH. The disease development was observed daily and type of the symptoms and size of the lesion on each fruit was recorded.

**Evaluation with strawberry flower petals:** Individual petals were disinfested similarly as the fruit and inoculated by placing a drop of a conidial suspension of *B. cinerea* on the petal surface. Amount of mycelial growth and level of sporulation were visually determined.

**Observation of *in vivo* presence of green fluorescent pathogen:** Aerial mycelia and conidia were removed from diseased part of both fruits and petals to make a microscope specimen slide, which was observed under a fluorescent microscope. Diseased fruit and petal tissues were also sectioned and observed under the fluorescent microscope for appearance of green fluorescent pathogen within the tissues.

## RESULTS

**Transformation:** GFP gene (*gfp*) recovered from plasmid yEGFP3 (Cormack *et al.*, 1997) was inserted to the transformation vector PAN2-4 (Punt *et al.*, 1997). The *gfp* was fused to a region of downstream of the *Aspergillus nidulans* *gpd* promoter and of upstream of the *A. nidulans* *trpC* terminator (Fig. 1). The fusion was confirmed by sequencing.

After co-transformation of the *gfp* vector with the vector containing Hy<sup>R</sup>, five hygromycin B resistant colonies (transformants) were obtained initially and the efficiency of the transformation was 1.0 transformants/ $\mu$ g pMOcosX plasmid DNA. Among these transformants, only one, 20%, displayed green fluorescence when observed under a fluorescent compound microscope. Direct PCR has confirmed that *gfp* gene has been transformed into this transformant (Fig. 2). Single conidia derived from the transformant also showed positive in the PCR screening (Fig. 2).

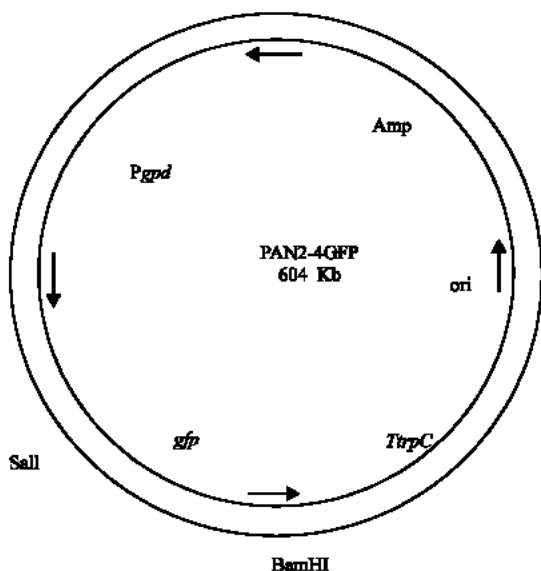


Fig. 1: GFP plasmid construction. GFP gene (*gfp*) was fused between the *A. nidulans* promoter *gpd* and the terminator *trpC*

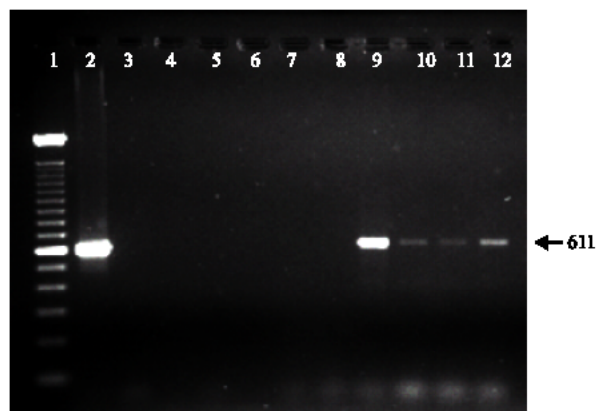


Fig. 2: Agarose gel showing banding for PCR-positive *Botrytis cinerea*, 1. DNA ladder; 2. PAN2-4GFP plasmid control; 3. H<sub>2</sub>O control; 4. *B. cinerea* wild-type; 5-9: Hy<sup>R</sup> resistant *B. cinerea* isolates; only isolate BC-2b (lane 9) is GFP positive; 10-12: single conidial isolates from isolate BC-2b

### Characterization of the transformants

**Stability of fluorescence:** Green fluorescence did not appear in all hyphal cells in the first and second subcultures of the transformants (Fig. 3a). The partially expressed isolates were sequentially subcultured for 4 times on media amended with hygromycin B before further selection was made by single-conidium isolations. A complete GFP expression was achieved through three sequential single-conidial isolations and serial subcultures up to an additional 6 times followed by the final single-conidial isolations (Fig. 3b and c); although fluorescence intensity varied sometimes in different observations.

**Growth and sporulation:** The original transformant (isolate BC-2b) and isolates derived from the transformant by single conidial isolations had similar growth rate of  $7.7 \pm 0.2$  mm/day to the wild-type isolate when cultured on PDA without hygromycin B. Also, isolate BC-2b and the single conidial cultures derived from it sporulated as well as the wild type isolate. Under microscope, hyphae and conidia of the transformants could not be distinguished from that of the wild type isolate (Fig. 3d).

### Pathogenicity on strawberry and fluorescence *in vivo*:

On strawberry fruit and flower petals, inoculation with isolate BC-2b resulted in typical gray mold symptoms, the same as that of the wild-type isolate (Fig. 4). There was no significant difference between the two isolates in lesion diameters ( $p = 0.05$ ), which ranged from 15 to 28 mm after

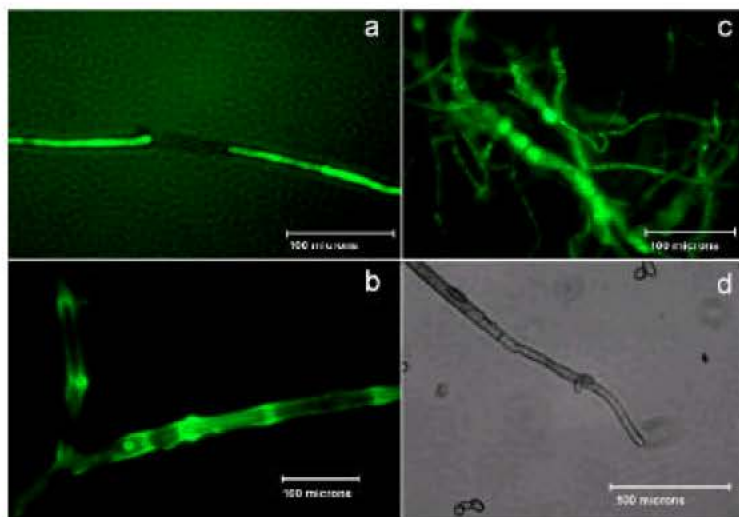


Fig. 3: The transformant BC-2b: The first subculture on PDA with  $100 \mu\text{g mL}^{-1}$  hygromycin B  $\text{mL}^{-1}$ , a portion of the hyphae shows green fluorescence (a). The third subculture on PDA with  $100 \mu\text{g mL}^{-1}$  hygromycin B  $\text{mL}^{-1}$  (b). The 5th subculture on PDA with  $100 \mu\text{g mL}^{-1}$  hygromycin B  $\text{mL}^{-1}$  (c). The wild type isolate shows no green fluorescence (d)



Fig. 4: Pathogenicity comparison of GFP transformant BC-2b (Right) with the wild-type isolate (Left) on strawberry. The strawberry fruits were surface disinfested, wounded and inoculated before incubation at  $22^\circ\text{C}$  for 5 days

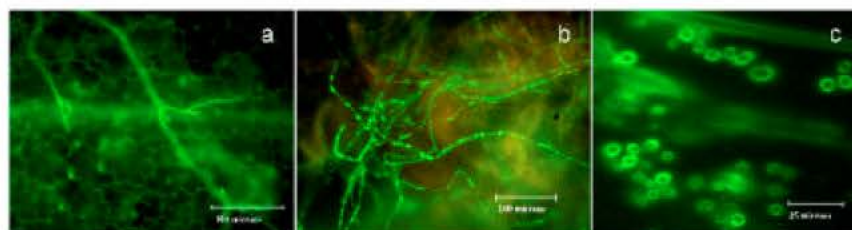


Fig. 5: Characterization of the transformant BC-2b on strawberry. The GFP transformant BC-2b in the tissue of strawberry flower petal 48 h post inoculation (a). The GFP transformant BC-2b in the tissue of strawberry fruit 96 h post inoculation (b). Conidia of the GFP transformant BC-2b produced on the inoculated strawberry fruit after one week incubation at  $22^\circ\text{C}$  (c)

5-days incubation at 22°C. When samples were taken from the diseased tissues of inoculated flower petals and fruits, fungal hyphae in the inoculated strawberry flower petals (Fig. 5a) and fruits (Fig. 5b) apparently showed green fluorescence under the fluorescent microscope. Conidia produced on the inoculated fruits also showed green fluorescence (Fig. 5c).

## DISCUSSION

GFP (*gfp*) gene was successfully transformed into *B. cinerea* and expressed in its hyphae and conidia both *in vitro* and *in vivo*; the transformants maintained the characteristics of the wild-type isolate not only in morphology but also in pathogenicity. GFP has often been used as a reporter gene in fungi to study gene expressions (Aboul-Soud *et al.*, 2004; Chen *et al.*, 2003; Dumas *et al.*, 1999; Lorang *et al.*, 2001) and as a visual marker in biocontrol agents for studying their hyperparasitism to fungal pathogens and in a few cases for the study of the interactions of plant-pathogens (Bottin *et al.*, 1999; Jansson, 2003; Lu *et al.*, 2004; Rohel *et al.*, 2001). The successful transformation of *B. cinerea* provides powerful tools for studying the interactions between *B. cinerea* and its fruit hosts. They are also very useful in determining the response of *B. cinerea* on/in plant surface and tissues to different pre- and postharvest treatments. All the information is essential for the development of a novel system to control gray mold in the field and during transportation and storage. Potentially, the transformants should also be able to be used in gray mold disease systems on other crops such as grapes, tomatoes and so on.

This research aimed to use GFP gene as a visual marker for the study of the interactions between plants and the pathogen, thus the stability and uniformity in characteristics such as pathogenicity, growth rate, sporulation, etc., of the transformants are especially important. Therefore, similarities in morphological and physiological characteristics were considered when transformants were selected and also later in the single conidial selections. All characteristics which may have effects on fungal infection and disease development have thoroughly been evaluated not only *in vitro* but also on strawberry leaves, flowers and as well as fruit. Although the selected transformants were relatively stable after passing several subcultures and were as pathogenic as the wild-type isolate on the detached plant tissues, careful comparison to wild-type isolates (controls) should be made once the transformants are being used under greenhouse and field conditions.

Particle bombardment with conidia, rather than electroporation of the protoplast was used for the transformation although the later seems to be more common in gene transformation in fungi. When aiming for gene expression, transformation with electroporation may have advantages such as high transformation rate and more likely single copy insertion over bombardment (Hamada *et al.*, 1994). However, beside that, protoplast generation is both time consuming and laborious; the processes of producing protoplasts often affect their physiological characteristics, such as colony morphology, growth rate and sporulation, giving rise to great variation among resulting fungal isolates (Boland and Smith, 1991). This variation will carry over to the transformation processes and make the selection of transformants, which need to possess same characteristics as the wild type in this case, more difficult. The results from our experiment on the transformation of *Monilinia fructicola* indicated electroporation process significantly affected fungal sporulation (Li *et al.*, unpublished observations).

To aid the selections, a transformation vector containing Hy<sup>R</sup> was co-transformed with *gfp* gene, which was fused to a region of downstream of the *Aspergillus nidulans gpd* promoter and of upstream of the *A. nidulans trpC* terminator. Although *gfp* has been used as a marker for selection (Bae and Knudsen, 2000), it is nearly impossible to visually select transformants because of the low transformation frequency in fungi. However, the introduction of Hy<sup>R</sup> gene may limit the use of the transformants because of the concern for the escape of drug-resistant gene. Co-transformation has provided a potential for further election of fungal isolate without the resistant gene. This is particularly important for this research because the aim of the transformation is to study the pathogen-plant interactions. Although the study may be conducted at the primary stage only in laboratory, extensive studies are necessary in some controlled areas, such as a greenhouse. In the later situation, an isolate with GFP but not Hy<sup>R</sup> may be needed.

The initial colonies showed only partial fluorescence; this might be due to the nucleus nature of this fungus. Through analysis of field isolates of *B. cinerea*, Bütternner *et al.* (1994) found that their DNA content per nucleus varied considerably and concluded that aneuploidy/polyploidy was a widespread phenomenon in this species. In their homokaryotic transformation, Hamada *et al.* (1997) showed that there is a Mendelian segregation of hygromycin B resistance in *B. cinerea*. From the results of serial conidial transfers, they suggested that either heterokaryotic transformants contained active and inactive nuclei or inactivation was the result of conidial transfer. This inactivation could be

maintained through meiosis and may explain the scarce recovery of hygromycin-resistant progeny; alternatively, chromosomal rearrangements might lead to non-expression of the hygromycin resistance gene. Further work is needed to understand the mechanism(s) of silencing in *Botrytis* (Hamada *et al.*, 1997). Since copies of integrated plasmid segregated in progeny with a 1:1 ratio, transforming DNA can be used in future to tag *B. cinerea* pathogenicity genes. The transformants will be used first to study the pre-harvest latent infections associated with post-harvest decays of strawberries.

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#### REFERENCES

- Aboul-Soud, M.A.M., B.W. Yun, L.A. Harriet and G.J. Loake, 2004. Transformation of *Fusarium oxysporum* by particle bombardment and characterisation of the resulting transformants expressing a GFP transgene. *Mycopathologia*, 158: 475-482.
- Bae, Y.S. and G.R. Knudsen, 2000. Cotransformation of *Trichoderma harzianum* with  $\beta$ -glucuronidase and green fluorescent protein genes provides a useful tool for monitoring fungal growth and activity in natural soils. *Applied Environ. Microbiol.*, 66: 810-815.
- Balint-Kurti, P.J., G.D. May and A.C.L. Churchill, 2001. Development of a transformation system for *Mycosphaerella* pathogens of banana: A tool for the study of host/pathogen interactions. *FEMS Microbiol. Lett.*, 195: 9-15.
- Boland, G.J. and E.A. Smith, 1991. Variation in culture morphology and virulence among protoplast-regenerated isolates of *Sclerotinia sclerotiorum*. *Phytopathology*, 81: 766-770.
- Bottin, A., L. Larche, F. Villalba, E. Gaulin, M.T. Esquerre-Tugaye and M. Rickauer, 1999. Green Fluorescent Protein (GFP) as gene expression reporter and vital marker for studying development and microbe-plant interaction in the tobacco pathogen *Phytophthora parasitica* var. *nicotianae*. *FEMS Microbiol. Lett.*, 176: 51-56.
- Bütternner, P., F. Koch, K. Voigt, T. Quidde, S. Risch, R. Blaich, B. Bütternner and P. Tudzynski, 1994. Variations in ploidy among isolates of *Botrytis cinerea*: Implications for genetic and molecular analyses. *Curr. Genet.*, 25: 445-450.
- Chen, N., T. Hsiang and P.H. Goodwin, 2003. Use of green fluorescent protein to quantify the growth of *Colletotrichum* during infection of tobacco. *J. Microbiol. Methods*, 53: 113-122.
- Cormack, B.P., G. Bertram, M. Egerton, N.A.R. Gow, S. Falkow and A.J.P. Brown, 1997. Yeast-enhanced green fluorescent protein (yEGFP): A reporter of gene expression in *Candida albicans*. *Microbiology*, 143: 303-311.
- Dumas, B., S. Centis, N. Sarrazin and M.T. Esquerre-Tugaye, 1999. Use of green fluorescent protein to detect expression of an endopolygalacturonase gene of *Colletotrichum lindemuthianum* during bean infection. *Applied Environ. Microbiol.*, 65: 1769-1771.
- Elad, Y. and K. Evensen, 1995. Physiological aspects of resistance to *Botrytis cinerea*. *Phytopathology*, 85: 637-643.
- Hamada, W., Ph. Reignault, G. Bompeix and M. Boccara, 1994. Transformation of *Botrytis cinerea* with the hygromycin B resistance gene, *hph*. *Curr. Genet.*, 26: 251-255.
- Hamada, W., M.C. Soulie, P. Malfatti, G. Bompeix and M. Boccara, 1997. Stability and modulated expression of a hygromycin resistance gene integrated in *Botrytis cinerea* transformants. *FEMS Microbiol. Lett.*, 154: 187-193.
- Inglis, P.W., P.R. Queiroz and M.C. Valadares-Inglis, 1999. Transformation with green fluorescent protein of *Trichoderma harzianum* 1051, a strain with biocontrol activity against *Crinipellis perniciosa*, the agent of witches' broom disease of cocoa. *J. Gen. Applied Microbiol.*, 45: 63-67.
- Inglis, P.W., F.J.L. Aragao, H. Frazao, B.P. Magalhaes and M.C. Valadares-Inglis, 2000. Biolistic co-transformation of *Metarhizium anisopliae* var. *acridum* strain CG423 with green fluorescent protein and resistance to glufosinate ammonium. *FEMS Microbiol. Lett.*, 191: 249-254.
- Jansson, J.K., 2003. Marker and reporter genes: Illuminating tools for environmental microbiologists. *Curr. Opin. Microbiol.*, 6: 310-316.
- Lorang, J.M., R.P. Tuori, J.P. Martinez, T.L. Sawyer, R.S. Redman, J.A. Rollins, T.J. Wolpert, K.B. Johnson, R.J. Rodriguez, M.B. Dickman and L.M. Ciuffetti, 2001. Green fluorescent protein is lighting up fungal biology. *Applied Environ. Microbiol.*, 67: 1987-1994.

- Lu, Z., R. Tombolini, S. Woo, S. Zeilinger, M. Lorito and J.K. Jansson, 2004. *In vivo* study of *Trichoderma*-pathogen-plant interactions, using constitutive and inducible green fluorescent protein reporter systems. *Applied Environ. Microbiol.*, 70: 3073-3081.
- Orbach, M.J., 1994. A cosmid with a Hy<sup>R</sup> marker for fungal library construction and screening. *Gene*, 150: 159-162.
- Punt, P.J., M.A. Dingemans, A. Kuyvenhoven, R.D. Soede, P.H. Pouwels and C.A. van der Hondel, 1997. Functional elements in the promoter region of the *Aspergillus nidulans* *gpdA* gene encoding glyceraldehydes-3-phosphate dehydrogenase. GenBank direct submission; Accession Z32750.
- Rohel, E.A., A.C. Payne, B.A. Fraaije and D.W. Hollomon, 2001. Exploring infection of wheat and carbohydrate metabolism in *Mycosphaerella graminicola* transformants with differentially regulated green fluorescent protein expression. *Mol. Plant Microb-Interact.*, 14: 156-163.
- Rosslénbroich, H.J. and D. Stuebler, 2000. *Botrytis cinerea*-history of chemical control and novel fungicides for its management. *Crop. Prot.*, 19: 557-561.
- Sanford, J.C., F.D. Smith and J.A. Russell, 1993. Optimizing the biolistic process for different biological applications. *Methods Enzymol.*, 217: 483-509.
- Spellig, T., A. Bottin and R. Kahmann, 1996. Green Fluorescent Protein (GFP) as a new vital marker in the phytopathogenic fungus *Ustilago maydis*. *Mol. Gen. Gene.*, 252: 503-509.
- Zeilinger, S., C. Galhaup, K. Payer, S.L. Woo, R.L. Mach, C. Fekete, M. Lorito and C.P. Kubicek, 1999. Chitinase gene expression during mycoparasitic interaction of *Trichoderma harzianum* with its host. *Fung. Genet. Biol.*, 26: 131-140.