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# Green Fluorescent Protein (GFP) as a Reporter Gene for the Plant Pathogenic Oomycete *Phytophthora ramorum*

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ABSTRACT. Transgenic *Phytophthora ramorum* strains that produce green fluorescent protein (GFP) constitutively were obtained after stable DNA integration using a polyethylene glycol and $CaCl_2$-based transformation protocol. Green fluorescent protein production was studied in developing colonies and in different propagules of the pathogen to evaluate its use in molecular and physiological studies. About 12% of the GFP transformants produced GFP to a level detectable by a confocal laser scanning microscope. Green fluorescent protein could be visualized in structures with vital protoplasm, such as hyphal tips and germinating cysts. In infection studies with *Rhododendron*, one of the GFP expressing strains showed aggressiveness equal to that of the corresponding non-labelled isolate. Thus, GFP could be used as a reporter gene in *P. ramorum*. Limitations of the technology are discussed.

**Key Words.** Chromists, DNA transformation, stramenopiles.

THE fungal-like oomycete *Phytophthora ramorum* is a severe pathogen on tree species and woody ornamentals in Europe and in North America (Werres et al. 2001) (http://www.suddenoakdeath.org; http://rapra.csl.gov.uk/). Since its first detection in the early 1990s in Germany and in the Netherlands the geographical distribution of *P. ramorum* and the host range has increased dramatically. Up to now more than 80 genera from several different plant families have been described as natural host plants. *Phytophthora ramorum* has been regulated as a quarantine organism (http://www.eppo.org/QUARANTINE/Alert_List/alert_list.htm).

In order to prevent the spread of the pathogen with asymptomatic plants, more detailed information on the infection process, on the tissue colonization, and on the latency period would be needed. These studies would require suitable tools for histological studies in plant tissue. The development of a reporter gene system could be such a helpful tool allowing monitoring of the development and spread of the pathogen in infected plant tissue.

In *Phytophthora* species, the successful application of molecular technologies like high-efficiency co-transformation has been demonstrated (Judelson 1993; van West et al. 1999a). Different vectors have been constructed for expressing sequences that incorporate a variety of promoters enabling expression over a range of levels (Judelson and Michelmore 1989, 1991). Selectable marker genes have been tested and applied (Bailey, Mena, and Herrera-Estrella 1991; Judelson, Tyler, and Michelmore 1991). The application of visible marker genes, such as beta-glucuronidase and green fluorescent protein (GFP), has also been reported (Bottin et al. 1999; Judelson and Michelmore 1991; Si-Ammour, Mauch-Mani, and Mauch 2003; van West et al. 1999a; Vijn and Govers 2003).

The main objective of the present study was to introduce the reporter gene *gfp* into isolates of *P. ramorum*. A $CaCl_2$- and polyethylene glycol (PEG)-based DNA transformation protocol was developed and detection levels of GFP-expressing transformants were evaluated.

## MATERIALS AND METHODS

***Phytophthora ramorum* isolates and culture conditions.** *Phytophthora ramorum* isolates (Table 1) were grown on 5% (w/v) carrot-piece agar (Werres et al. 2001). For zoospore production, 14-day-old agar cultures were flooded with 7 ml sterile water and then incubated at 4 °C for one-hour followed by 1-h incubation at room temperature.

**Transformation vector.** The transformation vector used in this study was p34GFN (Si-Ammour et al. 2003). This vector contains the selectable *nptII* marker and the visible marker *gfp* expressed under the control of the ham34 promoter and terminator sequences from *Bremia lactucae*.

**Transformation.** The four wild-type isolates of *P. ramorum* were used as recipients for transformation according to the protocol of Judelson et al. (1991) with minor modifications. All the steps were completed under aseptic conditions. For the production of protoplasts, 72-hour-old mycelium from a germinating zoospore suspension was used. Vegetable juice V8 (Campbell Soup Company, Camden, NJ) was added to the zoospore suspension at a final concentration of 10% (v/v). For protoplasts production, the mycelium was washed briefly in KC osmoticum (0.64 M KCl, 0.20 M $CaCl_2$) and then incubated at room temperature for 35 min with a solution of KC containing 5 mg/ml lysing enzymes from *Trichoderma harzanium* (L1412, Sigma, Steinheim, Germany) and 2 mg/ml cellulase (C8546, Sigma). The suspension of protoplasts recovered from the macerated mycelium was filtered through a 50-μm nylon mesh, pelleted at 700 g for 4 min, washed once with 10 ml KC, once with 10 ml KC/MT (0.64 M KCl, 0.20 M $CaCl_2$/1 M mannitol, 10 mM Tris/HCl pH 7.5), and re-suspended in MT (1 M mannitol, 10 mM Tris/HCl pH 7.5) medium at a concentration of $0.15–0.5 \times 10^7$ protoplasts/ml. A mixture of 20 μg vector DNA and 60 μg lipofectin (Invitrogen, Basel, Switzerland) was prepared, pre-incubated at room temperature, and added to the 1 ml protoplast suspension. A solution of 1 ml of 50% (w/v) PEG 3350 (Sigma) was added to the protoplast suspension and after a 5-min incubation, the mixture was diluted in 20 ml of 10% (v/v) V8 juice-1 M mannitol medium. After 24 h of

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Table **1.** *Phytophthora ramorum* wild-type isolates used for transformation.

| Isolate no | Host plant | Origin | Mating type | Lineage |
|---|---|---|---|---|
| BBA 9/95 | *Rhododendron* sp. | Germany | A1 | EU1[a] |
| BBA 26/02 | *Viburnum* sp. | Belgium | A2 | EU1[a] |
| BBA MSOD03-02[b] | *Rhododendron* sp. | Canada | A1 | EU1[c] |
| BBA PR01[d] | *Quercus agrifolia* | USA | A2 | NA1[a] |

[a]According to Ivors et al. (2004, 2006).
[b]Isolate was kindly supplied by Stéphan Brière (CFIA, Canada).
[c]Simone Prospero/Everett Hansen (pers. commun.).
[d]Isolate was kindly supplied by Kelly Ivors (North Carolina State University).

incubation at 18 °C in the dark, the protoplasts germinated and were transferred to V8 agar (100 ml/l Campbell V8 juice, 3 g/l $CaCO_3$, 15 g/l Agar, Becton Dickinson, Heidelberg, Germany) containing 20 µg/ml of geneticin antibiotic (G418, Invitrogen) for cultivation.

**Molecular analysis of gene integration.** Quantitative real-time PCR (qRT-PCR) was used to check the integration and expression of the *gfp* and *nptII* genes in the transformants. Primers targeting the *nptII* and the *gfp* gene were, respectively, KAN-F (5′-ATGATTGAACAAGATGGATTGCACGCAGG-3′) and KAN-R (5′-TCAGAAGAACTCGTCAAGAAGGCGATAGAAG-3′) and GFP-F (5′-ATGGGCAAGGGCGAGGAACTGTTCAC-3′) and GFP-R (5′-TCACTTGTAGAGTTCATCCATGCCATGCG-3′). Amplification mixtures (20 µl final vol.) were made using the Light Cycler Fast Start DNA Master SYBR Green I kit (Roche Diagnostics, Rotkreuz, Switzerland), to which 1 ng of template DNA and primers at a final concentration of 5 mM were added. Amplifications were carried out in capped capillary tubes in a Lightcycler 2.0 Real-time PCR system (Roche Diagnostics) using an initial denaturation at 95 °C for 10 min, followed by 45 cycles at 95 °C for 10 s, 60 °C for 5 s, and 72 °C for 15 s. A melting curve analysis was used to check qPCR reactions for primer–dimer artifacts, contamination, reaction specificity, and accurate quantification. Additionally qPCR products were checked by DNA sequencing. Amplicons were purified using a Minelute PCR Purification Kit (Qiagen, Hombrechtikon, Switzerland), according to the manufacturer's specifications. Quantity and quality were checked as described above for DNA extraction. Amplicons were sequenced directly in both sense and antisense directions. All samples were sequenced twice and a consensus sequence was created from the duplicates.

**Immunocytochemistry.** Green fluorescent protein expression was verified by immunocytochemical detection with Alexa Fluor®488 labelled rabbit anti-GFP antibodies (Invitrogen, Karlsruhe, Germany). Mycelium or germinated cysts of transformants and control strains were mounted on Polysine™ coated slides (Menzel, Bielefeld, Germany). Samples were washed twice in 1 × PBS (phosphate buffered saline) and blocked (3% [w/v] BSA, 0.1% [v/v] Tween 20, 1 × PBS) for 30 min at room temperature. Following the blocking step, samples were incubated for 3 h with the anti-GFP antibody (diluted to 1:200) and washed three times in 1 × PBST (PBS + 0.2% [v/v] Tween 20). Specimens were mounted in 40% (v/v) glycerine in 1 × PBS for microscopic observations.

**Microscopic analysis.** A confocal laser scanning microscope (CLSM) TCS SP2 (Leica Microsystems GmbH, Wetzlar, Germany) was used for microscopic observations. Green fluorescent protein and anti-GFP antibody excitation were achieved with a 488-nm argon krypton laser at power levels from 10–50%. Green fluorescent protein or the Alexa Fluor® 488 emissions were detected in a separate channel at wavelengths of 500–530 nm. Autofluorescence of *P. ramorum* was excited with a 488-nm argon krypton and a 633-nm neon helium laser and detected in a separate channel at wavelengths of 531–600 nm. Observations were performed using 0.8 20X and 1.4 63X oil immersion lenses. Green fluorescent protein expression of transgenic *P. ramorum* structures was confirmed by generating emission spectrum profiles (CLSM lambda scan, wavelengths: 500–580 nm, 14 steps). Images and lambda scans were analysed with the LCS 2.61 software (Leica Microsystems GmbH).

**Plant material and infection studies.** Detached leaves of *Rhododendron* cv. ''Cunningham's White'' were used for infection studies. Two wounded and two non-wounded leaves for each transformed and non-transformed strain were inoculated with a single drop (50 µl) of an aqueous zoospore suspension containing $\sim 3 \times 10^5$ zoospores per drop. The leaves were wounded by cutting the lower epidermis of the leaf with a blunt scalpel. Inoculated leaves were incubated in a moist chamber at 20 °C and a photoperiod of 16 h light. Pathogenicity of the strains was evaluated by observing necrosis development around the inoculation point within 12 d after inoculation.

## RESULTS

**Transformation of *Phytophthora ramorum* with *gfp* gene constructs.** The construct p34GFN (Si-Ammour et al. 2003), which was introduced into *P. ramorum* strains BBA 9/95, BBA 26/02, BBA MSOD03-02, and PR01, included two expression cassettes: one contained the selective marker gene neomycine phosphotransferase (*nptII*); and the other the visual reporter gene *gfp*. Both *nptII* and *gfp* were fused to promoter and terminator sequences of the *ham34* gene of *B. lactucae*. First analysis of gene integration was done with PCR (Fig. 1). The concomitant presence of the *nptII* and the *gfp* coding sequences was checked by qRT-PCR. All antibiotic-resistant transformants gave a positive PCR result. Cycle threshold values, defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level), varied considerably between transformants using equal amounts of genomic DNA from the different transformants, indicating that integration of the construct occurred at one site or at a multiplicity of sites. Besides tandem integrations, multiple single integrations of p34GFN were also detected in some of the transformants when the conserved tubulin housekeeping gene was used as an internal control to calculate transgene copy number (data not shown). In total, 40 putative *ham34-gfp* transformants were obtained. To test whether these putative transformants expressed the *ham34-gfp* construct, GFP production was analysed in sporangia and germinated cysts with a CLSM. Real-time reverse transcription PCR allowed us to confirm the expression of the *gfp* gene in these transformants.

**Microscopic analysis of expression of GFP.** Although the vegetative growth rates on selective agar medium of the 40 putative transformants indicated an expression of the transferred cassette, only five putative transformants (i.e. BBA 26/02_4, BBA 9/95_G1, BBA 9/95_G4, BBA 9/95_G6, BBA 9/95_G8) exhibited a detectable fluorescence that could be confirmed as GFP. With CLSM (lambda scan) it was possible to prove the *gfp* expression. The GFP emission maximum was determined at a wavelength between 510 and 520 nm (Fig. 2,3).

In these strains, *gfp* expression could be observed in all *P. ramorum* propagules studied: hyphae, chlamydospores, zoosporangia, and germinating zoospores (Fig. 4–9). The GFP expressed by the five transformants appeared to be very weak and showed very rapid fading when exposed to UV light or 488-nm argon laser light. Thus, the scanning time was very limited. The use of the anti-fading mountant solution Citiflour AF1 (Citifluor Ltd., London, UK) did not improve the bleaching of GFP. The detection of the weak and rapidly fading GFP could be improved using Alexa Fluor®488 labelled anti-GFP antibodies (Fig. 10–13).

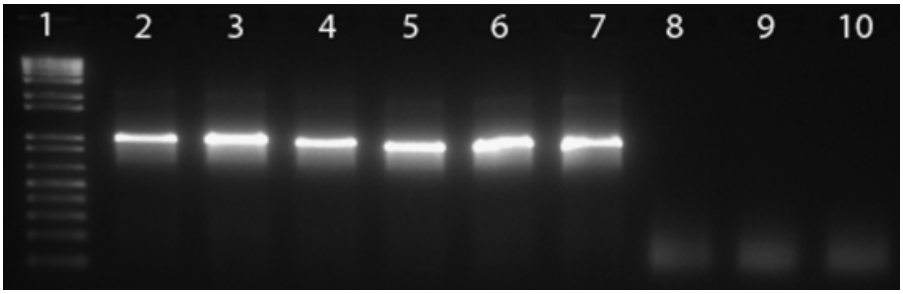


Fig. **1.** PCR detection of the *gfp* gene in the genome of the *Phytophthora ramorum* transformants. 1, 1 kb DNA ladder; 2–6, transformants BBA 9/95_G1, BBA 9/95_G4, BBA 9/95_G6, BBA 9/95_G8 and BBA 26/02_4; 7, Cloned *gfp* genes as positive control; 8, 9, wild-type strains BBA 9/95 and BBA 26/02 of *P. ramorum*; 10, PCR negative control.

Green fluorescent protein signals were mainly observed in germinating cysts and active growing hyphal tips from mycelium that contained active protoplasm (Fig. 4,5). Green fluorescent protein expression was also observable in the protoplasm of zoosporangia and chlamydospores (Fig. 6–9). Vacuolar structures in older hyphae and in zoosporangia (Fig. 7) did not show any GFP signals.

Green fluorescent protein antibody signals were obtained mainly from hyphae and zoosporangia (Fig. 10–13). Similarly to direct GFP observation with CLSM, antibody signals were found mainly on propagules with active protoplasm. No GFP antibody signal was observed in the chlamydospore plasm.

Older *P. ramorum* propagules expressed a very strong yellow autofluorescence (emission maximum: 550–560 nm) (Fig. 2,3, 5–9). This yellow autofluorescence could be observed in the wild type as well as in transformed strains, and was mainly present in walls of chlamydospores (Fig. 9) and zoosporangia (Fig. 6–8) and in the zoosporangium papillae (Fig. 6–8). This natural autofluorescence was resistant to photobleaching and interfered with the GFP signals. Consequently conventional epifluorescence microscopy did not provide a reliable differentiation of GFP fluorescence from this autofluorescence. Emission signals of GFP and the natural autofluorescence of *P. ramorum* could better be distinguished when detected with CLSM in separate channels, which were precisely adjusted to the emission of GFP (505–525 nm) and autofluorescence (530–600 nm), respectively. This situation was improved when the specimens were treated with the Alexa Fluor488® labelled antibodies (Fig. 10–13). The antibody signals were strong, stable, and easily distinguishable from the natural autofluorescence of *P. ramorum*.

**Vegetative growth rate of transformed and wild-type strains.** The majority of the transformed strains did not show a significant reduction of vegetative growth on non-selective medium. Four of the five strains with detectable GFP expression (i.e. BBA 9/95_G1, BBA 9/95_G4, BBA 9/95_G8) showed growth rates similar to the corresponding wild types: 1.8–3.5 mm/24 h (Table 2). Only BBA 9/95_G6 and BBA 26/02_4 grew significantly slower: 0.7 and 2.6 mm/24 h (Table 2). On selective medium (geneticin, 20 µg/ml), some transformed strains (BBA 9/95_G1, BBA 9/95_G2, BBA 9/95_G3) reached the edge of the Petri dish (9 cm diam.) within 18 days, while non-transformed wild-type isolates stopped growing when reaching a colony diameter of approximately 4 mm.

Independently from the wild type, a few of the transformed strains showed atypical colony morphology and distorted hyphae, absence of chlamydospores or a reduced production of zoosporangia, respectively, zoospores.

**Aggressiveness of transformed and wild-type strains.** Development of necrosis on detached leaves varied considerably between transformed strains. Among the five transformed strains with good GFP expression (BBA 26/02_4, BBA 9/95_G1, BBA 9/95_G4, BBA 9/95_G6, BBA 9/95_G8), only BBA 26/02_4 showed pathogenicity similar to the corresponding wild type (data not shown). In addition many of the less aggressive transgenic strains were unable to infect non-wounded leaves (data not shown).

## DISCUSSION

A protocol for the first successful genetic transformation of the oomycete *P. ramorum* has been developed. Using a PEG/$CaCl_2$-

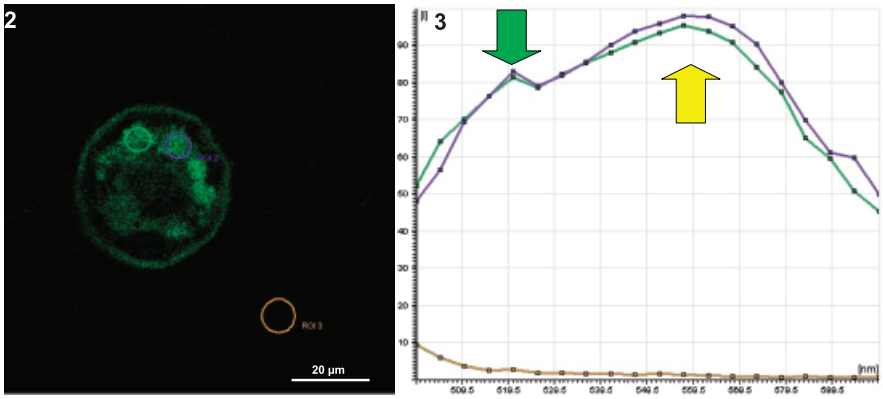


Fig. **2,3.** Emission spectra profiles of transgenic *Phytophthora ramorum* strain BBA 9/95_G6. **2.** Confocal laser scanning micrograph of a chlamydospore of transgenic *P. ramorum* strain BBA 9/95_G6. Areas selected for analysis of emission are highlighted in rings. **3.** Lambda scan showing weak green fluorescent protein emission peak at 515–520 nm (left arrow) and strong autofluorescence at 550 nm (right arrow), lower curve: background.

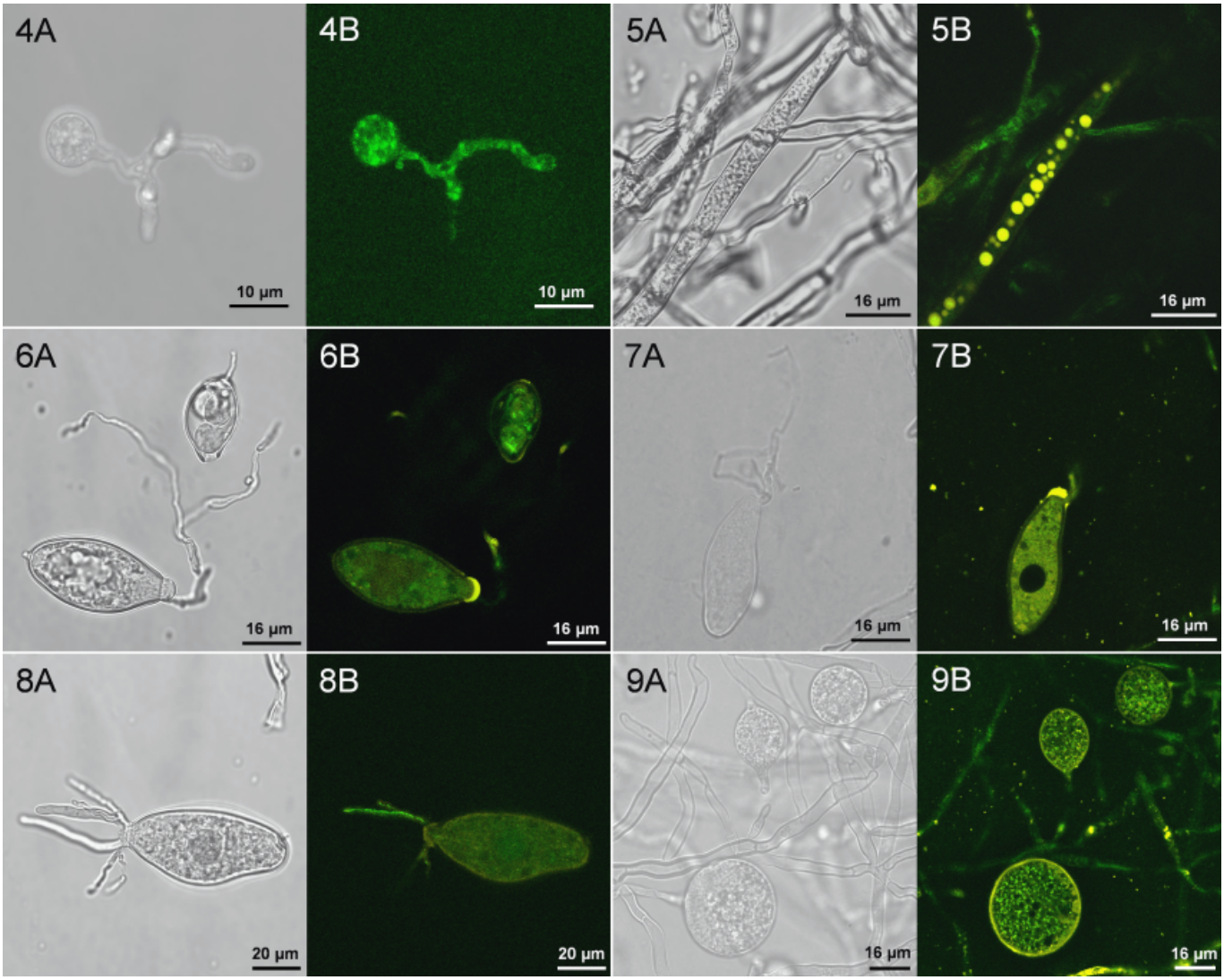


Fig. **4–9.** Confocal laser scanning micrographs (CLSM) indicating green fluorescent protein (GFP) expression of transgenic *Phytophthora ramorum* strains BBA26/02_4 (4, 6) and BBA9/95_G6 (5, 7, 8, 9). **4.** Germinating cyst. **5.** Hyphae grown in vitro. **6–8.** Zoosporangia releasing zoospores (6 above) and germinating. **9.** Chlamydospores grown in vitro. Image on left (**A**): transmission images; images on right (**B**): CLSM image with green coloured signals indicating GFP expression in protoplasm and yellow coloured signals showing natural autofluorescence in wall and papilla of zoosporangia and wall of chlamydospores of *P. ramorum*.

and liposome-mediated transformation protocol, we were able to integrate a cassette containing the genes *nptII* as selective marker and *gfp* as visible marker expressed under the control of the *ham34* promoter and terminator sequences of *B. latucae*. The expression of the transformed *gfp* gene could be confirmed by direct observation with CLSM and with GFP-specific antibodies.

Only about 12% of the transformed strains that were able to grow on selective medium showed a GFP expression detectable with CLSM or antibodies. However, PCR analysis proved that *nptII* and *gfp* sequences were also present in transformants without detectable GFP fluorescence. Transformation with other *Phytophthora* species resulted in a similar (13%; Bottin et al. 1999) or higher (85%; Si-Ammour et al. 2003) amount of GFP-fluorescent transformants. Reduced transgenic expression could be caused by many factors. A well-known important influencing factor would be the genotype of the recipient organism (Michielse et al. 2005; Pasternak et al. 1999). In the present study, the genotype could be the main reason for the low number of GFP-expressing transformants of *P. ramorum*. It might be that not all four wild-type isolates used exhibited a genetic background optimal for genetic transformation because under identical experimental conditions only transformants of two of the four wild-type isolates showed GFP expression after transformation. The influence of the genotype at species level on transformation efficiency was also observed by Si-Ammour et al. (2003) using the same transformation protocol for a single wild-type isolate of *Phytophthora brassicae* and *Phytophthora infestans*. Higher transformation efficiency could be obtained with *P. brassicae*.

As additional factors the genomic location, copy number, and arrangement of the integrated genes would certainly have a major influence on transgenic expression. In the present study, no results are available yet to reveal this. However, the results of qRT-PCR, which indicated tandem and multiple integrations of the vector p34GFN, could be the cause for post-transcriptional gene silencing of the transferred genes.

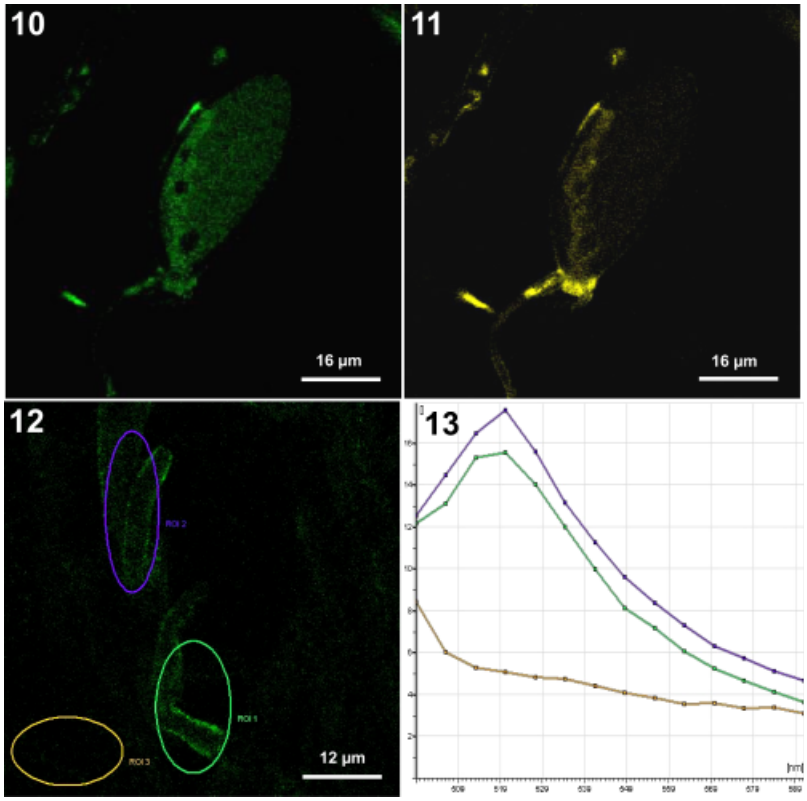


Fig. **10–13.** Immunocytochemical identification of green fluorescent protein (GFP) in transgenic *Phytophthora ramorum* strain BBA 9/95_G8 using Alexa Fluor 488-labelled anti-GFP antibodies. **10, 11.** Confocal laser scanning micrograph (CLSM) of Alexa Fluor 488-labelled zoosporangium. **10.** Channel for Alexa Fluor 488 detection (510–525 nm) **11.** Channel for detection of autofluorescence (530–525 nm). **12.** CLSM image of Alexa Fluor 488-labelled hyphae. Areas selected for Lambda scan are highlighted by encirculation. **13.** CLSM Lambda scan of Alexa Fluor 488-labelled hyphae showing Alexa Fluor 488-emission peak at 520 nm (upper curves), lower curve: background.

The processes of protoplasting and gene integration represent severe interventions in the life cycle of organisms. This could result in distorted hyphae and spores, as was observed in some of the transformants of *P. ramorum*. The altered metabolism of additional proteins due to transformation could be the cause for the reduced vegetative growth rate and the low pathogenicity in some of the transgenic strains. Reduced in vitro growth in transformed strains compared with the wild-type isolates was also reported by Bae and Knudsen (2000) and Si-Ammour et al. (2003).

Table **2.** Vegetative growth rate of transformed and wild-type strains.

| Isolate | *n* | Growth rate mm/24 h | |
|---|---|---|---|
| | | Mean | SD |
| BBA 9/95 (wild type) | 6 | 3.0[a] | 0.1 |
| BBA 9/95-G1 | 4 | 3.0[a] | 0.1 |
| BBA 9/95-G2 | 6 | 1.8[a] | 0.5 |
| BBA 9/95-G3 | 6 | 3.5[a] | 0.1 |
| BBA 9/95-G4 | 6 | 3.4[a] | 0.2 |
| BBA 9/95-6G | 6 | 0.7[a,*] | 0.1 |
| BBA 9/95-G8 | 6 | 2.5[a] | 0.2 |
| BBA 26/02 (wild type) | 6 | 3.1[b] | 0.1 |
| 26/02-4 | 6 | 2.6[b,**] | 0.0 |

[a]Normality test failed ($P = 0.001$).
*Data were significantly different from wild type (BBA 9/95) with $P < 0.05$ (ANOVA on ranks, Dunn's method).
[b]Normality test passed ($P = 0.05$).
**Data were significantly different from wild type (BBA 26/02) with $P < 0.001$ (ANOVA Dunnett's method).

The constitutive character of the *ham34* promoter was demonstrated by Judelson et al. (1991) with *P. infestans*. However, in the present study, GFP expression could not be observed in every tissue type. Green fluorescent protein fluorescence was detectable here only in the presence of active protoplasm (e.g. in hyphal tips, zooporangia, and chlamydospores) but not in the walls of zoosporangia and in the tissue of older hyphae, characterized by the presence of many vacuoles. Similar observations were made with *Phytophthora parasitica* var. *nicotianae* transformed with *hsp70* promoter regulated *gfp* (Bottin et al. 1999) and with *Phytophthora palmivora* transformed with genes regulated by *ham34* and *hsp70* promoters (van West et al. 1999b). The constitutive character of both promoters was previously shown (Judelson et al. 1991). Bottin et al. (1999) suggested a specific down-regulation of the *hsp70* promoter or cytoplasm quiesence as a cause for the absence of GFP expression in sporangiophores of *P. parasitica* var. *nicotianae*.

In all transformants of *P. ramorum*, direct observation of GFP fluorescence was influenced by low GFP expression and by high autofluorescence of these strains. Immunocytochemistry using anti-GFP antibodies proved to be a convenient method for detecting the weak and slow fading GFP. It allowed visualization of GFP in all structures of *P. ramorum* except chlamydospores. The absence of antibody signals in chlamydospores may be due to the impermeability of the thick spore wall for antibodies.

The first results of infection studies indicate that *gfp*-transformed strains could be used to study the infection process, latency, and tissue colonization of *P. ramorum* in plant tissue. However, for further studies, transformed isolates that show a higher expression of the marker gene *gfp* would be preferable.

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