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FEMS Microbiology Letters 178 (1999) 71–80

FEMS MICROBIOLOGY LETTERS

Green fluorescent protein (GFP) as a reporter gene for the plant pathogenic oomycete *Phytophthora palmivora*

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Received 1 June 1999; accepted 24 June 1999

Abstract

Transgenic *Phytophthora palmivora* strains that produce green fluorescent protein (GFP) or β-glucuronidase (GUS) constitutively were obtained after stable DNA integration using a polyethylene-glycol and $CaCl_2$-based transformation protocol. GFP and GUS production were monitored during several stages of the life cycle of *P. palmivora* to evaluate their use in molecular and physiological studies. 40% of the GFP transformants produced the GFP to a level detectable by a confocal laser scanning microscope, whereas 75% of the GUS transformants produced GUS. GFP could be visualised readily in swimming zoospores and other developmental stages of *P. palmivora* cells. For high magnification microscopic studies, GFP is better visualised and was superior to GUS. In contrast, for macroscopic examination, GUS was superior. Our findings indicate that both GFP and GUS can be used successfully as reporter genes in *P. palmivora*.

Keywords: β-Glucuronidase; Green fluorescent protein; Oomycete; *Phytophthora palmivora*

1. Introduction

The oomycete *Phytophthora palmivora* (Butler) is an ubiquitous plant pathogen with a wide host range. One of the major diseases caused by *P. palmivora* is black pod of cocoa. It is estimated that *P. palmivora* alone is responsible for annual losses of 20–30% of the world's cocoa crop and in certain regions, this figure may be as high as 90–95% [1]. An important developmental stage of *P. palmivora* is the ability to form swimming zoospores from sporangia in wet soils or in water films on the surfaces of plants. Sporangiogenesis and zoospore release occur rapidly, especially in tropical areas where frequent rainfall prevails and optimum growth temperatures (20–30°C) exist. Released zoospores swim towards non-infected plant tissue, encyst, produce a germ

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0378-1097/99/$20.00
PII: S0378-1097(99)00320-1

tube and subsequently infect the plant via appressoria. The tactic responses by zoospores to swim towards host (root) tissue has been subject of much research and debate [2–5]. From these studies, it can be concluded that during the motile and prepenetration phases, zoospores are mainly concerned with locating any plant (root) tissue, rather than the identification of the host species [3,4,6]. Therefore, it seems likely that general tactic responses such as non-specific chemotaxis, electrotaxis, autotaxis and induced encystment play important roles in locating plants.

We are interested in the molecular, cellular and physiological processes underlying the early events of pathogenesis, such as the release of zoospores, the tactic responses by the zoospores to host tissue and to other zoospores, encystment and penetration processes. We therefore wanted to develop a reporter gene system suitable for examining cell stages of the life cycle of *P. palmivora* with the final aim of helping to characterise the host-pathogen interactions. Since the development of DNA transformation procedures for *Phytophthora* species such as *Phytophthora infestans* [7], transformants expressing reporter genes have been generated for a variety of studies. Examples include the use of β-glucuronidase (GUS) as a reporter or marker gene for the analysis of promoter activity of the in planta induced gene *ipi*O [8], studies of sexual oospore formation [9] and the monitoring of in planta disease progression [10]. With this reporter gene system, temporal and spatial expression patterns could be characterised in detail. Although non-destructive GUS assays have been described and used successfully in plants [11], other, more recently developed systems exploiting luciferase (LUC) and green fluorescent protein (GFP) have many advantages [12,13]. In principle, these systems could be used to monitor expression during developmental processes in a single cell. Successful use of LUC as a reporter gene has been described for *P. infestans*, although only a small percentage of the transformants showed a detectable LUC activity [8].

Here, we analyse the suitability of GFP as a reporter gene in *P. palmivora*. A $CaCl_2$ and polyethylene-glycol-based DNA transformation protocol was developed and transformants expressing GFP were compared with transformants expressing GUS for usefulness in future studies. The advantages and disadvantages of both systems are discussed.

2. Materials and methods

2.1. Strains, culture conditions and developmental stages

A *P. palmivora* isolate (P6390) was kindly supplied by M.D. Coffey and grown under licence from the Scottish Ministry of Fisheries and Food (licence PH/2/1999). All transgenic isolates were in the P6390 background. Isolates were grown routinely on rye agar medium supplemented with 2% (w/v) sucrose (RS-medium) [14] in the dark at 18 or 28°C. Mycelia for isolation of DNA and RNA were obtained by growing *P. palmivora* in liquid RS-medium for 7 days at 28°C. To obtain sporangia, cultures were grown for 4 days in the dark and subsequently transferred into the light for 2–3 days. Sporangia were isolated according to the method described by van West et al. [8]. To obtain zoospores, mycelia were flooded with water, incubated at −20°C for 4 min and then at room temperature (18–21°C) for 1 h. Cysts were obtained by continuously shaking a zoospore suspension for 2 min. Cysts were allowed to germinate by incubation in water at room temperature for 1 h.

2.2. Plasmids for transformation of P. palmivora

Plasmid pHAMT35G, which contains the coding region of the GUS gene (*uid*A) of *Escherichia coli* fused to the *ham34* promoter and terminator of *Bremia lactucae* (Fig. 1), was kindly provided by H.S. Judelson. Plasmid pTH209 consisted of the *hsp70* promoter of *B. lactucae*, fused to the coding sequences of the neomycin phosphotransferase (*nptII*) gene and the *ham34* terminator (Fig. 1). pTH209 was also provided by H.S. Judelson. Our studies suggest that the promoters of *hsp70* and *ham34* are constitutive promoters in *P. palmivora*.

Plasmid pVW2, which contains a mammalian enhanced GFP open reading frame (GenBank U55763) fused to the *ham34* promoter and terminator of *B. lactucae*, was prepared as follows. A polymerase

chain reaction (PCR) was performed on pEGFP-C1 (Clontech Laboratories, Palo Alto, CA, USA) with primers PVW5 (5′-CCGATATCCATGGTGAG-CAAGGGCGAGGAGCTG-3′) and PVW6 (5′-GGCCCGGGTACCTACTTGTACAGCTCGTCC-ATGCCG-3′), resulting in the amplification of a 730-bp DNA fragment. This PCR fragment was digested with *Nco*I and *Kpn*I and cloned into *Nco*I-*Kpn*I-digested vector pHAMT35G, resulting in pVW2.

E. coli strain XL1blue (Stratagene, La Jolla, CA, USA) was used for general cloning experiments and was routinely grown at 37°C in Luria-Bertania medium [15]. Plasmid DNA was isolated and purified with Qiagen/Filter Plasmid Midi kit (Qiagen, Hilden, Germany). Non-linearised plasmids were used in transformations.

2.3. Transformation of P. palmivora

Stable transformation was conducted according to the protocol described by van West et al. [8] with some modifications. Modifications were: a sporangial suspension of *P. palmivora* strain P6390 was diluted with water to 10^5 sporangia per ml and mixed with two times concentrated amended Lima bean medium [16]. After growth for 18–24 h at 28°C, young mycelia were harvested by filtration. To generate protoplasts, young mycelia were treated as described by van West et al. [8]. The protoplast suspension was diluted to 1×10^6–5×10^6 protoplasts per ml mannitol/Tris buffer (1 M mannitol, 10 mM Tris (pH 7.5)) and 1 ml of the protoplast suspension was mixed gently with a pre-incubated lipofectin-DNA mixture. The lipofectin-DNA mixture was prepared by mixing 50 μl water, containing 30–45 μg of the plasmids, pHAMT35G or pVW2 and 15 μg of the selectable plasmid pTH209, with 60 μl of lipofectin reagent (Gibco BRL Life Technologies, Paisley, UK) in polystyrene tubes. This suspension was incubated for at least 15 min at room temperature. After the transformation and regeneration of the protoplasts [8], colonies appeared on selection plates containing 5 μg ml^{-1} geneticin within 3–6 days and were propagated on RS-medium containing 10 μg ml^{-1} geneticin at 28°C. A transformation efficiency of up to two transformants per 1 μg selection plasmid DNA was achieved.

2.4. Selection of ham34-gfp and ham34-gus transformants

Integration of the pHAMT35G construct in the *P. palmivora* genome was examined by Southern analysis. The *ham34-gus* transformants were tested in vitro for GUS activity, by transferring pieces of agar containing mycelia into the GUS staining solution (Section 2.5).

Integration of pVW2 was investigated by Southern analysis and GFP activity was determined by analysing sporangia and germinated cysts using a Bio-Rad MRC 1024 laser scanning confocal microscope (LSCM) system with a Nikon DIAPHOT 200 inverted microscope (Bio-Rad Laboratories, Hemel Hempstead, UK).

2.5. GUS assays

Zoospores, cysts, germinated cysts, sporangia and mycelia were vacuum-infiltrated for 10 min with a GUS staining solution (0.5 mg ml^{-1} 5-bromo-4-chloro-3-indoxyl-beta-D-glucuronide (Sigma, Poole, UK), 100 mM sodium phosphate (pH 7.0), 1% Triton X-100, 1% DMSO and 10 mM EDTA). Infiltrated material was kept in the staining solution for 2 h at 37°C. Subsequently, stained material was washed twice with 50% (v/v) glycerol to remove the staining solution and resuspended in 50% glycerol for long term storage.

2.6. Southern and Northern analysis

Genomic DNA of *P. palmivora* was isolated from mycelium as described by Raeder and Broda [17] with minor modifications. Total RNA from *P. palmivora* was isolated, blotted and hybridised as described previously [8].

DNA templates for probe synthesis were a 716-bp *Nco*I-*Kpn*I fragment of pVW2 containing the GFP coding sequence, a 796-bp *Hin*dIII fragment from pSTA31 [18] containing the *act*A coding sequence derived from *P. infestans* and a 1870-bp *Nco*I-*Kpn*I fragment of pHAMT35G [19] containing the coding region of the GUS gene. All DNA templates were gel-purified with the Qiaex II Agarose Gel Extraction kit (Qiagen, Hilden, Germany). Probes were radiolabelled with [α-^{32}P]dCTP using a Random Prim-

ers DNA Labeling System (Gibco BRL Life Technologies, Paisley, UK). To remove non-incorporated nucleotides, the Qiaquick Nucleotide Removal kit (Qiagen, Hilden, Germany) was used. Conditions for homologous hybridisations were as described by van West et al. [8]. Heterologous hybridisations (actin) were performed at 60°C and blots were washed at 60°C in 0.5×SSC (75 mM NaCl and 7.5 mM sodium citrate) and 0.5% sodium dodecyl sulfate.

2.7. Microscopy and preparation of biological material

Microscopic analysis of *ham34-gus* transformants was performed with a Nikon BH-2 microscope and photographs were taken using a Nikon 601 camera unit.

Cells of the *ham34-gfp* transformants were mounted with a cover slip in water containing the anti-fading agent *p*-phenylene diamine (0.5 μg μl^{-1}). Propidium iodide (0.1 μg μl^{-1}) was added in some cases as a counter stain for cells lacking intact membranes. Bio-Rad LaserSharp software (version 3.2) was used to control image acquisition. The following settings were used: laser excitation wavelength 488 nm, emission filter 585 nm, 10% laser power, iris 2.5, gain 1365, black level 4 and zoom 2.1. Images were obtained using a 60× (1.4 N.A.) lens with immersion oil. In general, images of cells were scanned 3–5 times and a Kalman filter was applied. Images of motile zoospores were scanned only once.

3. Results

3.1. Transformation of P. palmivora with GFP and GUS gene constructs

A construct containing the *ham34* promoter of the oomycete *B. lactucae* fused to the coding sequence of the GUS gene (*uid*A, encoding GUS), pHAMT35G, was introduced into a *P. palmivora* strain P6390 by co-transformation with pTH209. The latter construct contains the geneticin resistance gene (*nptII*) fused to the *hsp70* promoter of *B. lactucae*, as a selection marker (Fig. 1). Both the *ham34* and the *hsp70* promoters are constitutively active in *B. lactucae* and *P. infestans* [20]. In order to establish whether eight

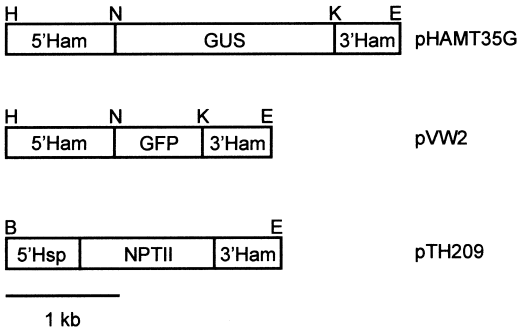

Fig. 1. Transformation constructs used in reporter gene studies. pHAMT35G contains the coding sequence of the *E. coli* GUS gene fused to the promoter (5′-Ham) and terminator (3′-Ham) of the *ham34* gene of *B. lactucae* [20]. For pVW2, the coding sequence of the mammalian codon optimised GFP gene was fused to the *ham34* promoter and terminator. pTH209 consists of the coding sequence of the *nptII* gene (NPTII) fused to the *hsp70* promoter (5′-Hsp) and *ham34* terminator. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; N, *Nco*I.

putative co-transformants (EY1p-EY8p) contained the *ham34-gus* construct, Southern blot analysis was performed. Genomic DNA was digested with restriction enzyme *Eco*RI, recognising a single restriction site in pHAMT35G, blotted on to a nylon membrane and hybridised with a probe specific for the GUS encoding sequence (Fig. 2A). This resulted in a strong hybridising band of the size of the linearised plasmid in most of the transformants, indicating that multiple integration of the construct occurred at one site or a multiplicity of sites (e.g. EY1p and EY4p in Fig. 2A). Interestingly, the hybridisation results showed that all putative co-transformants had the *ham34-gus* construct integrated in their genome, indicating a co-transformation efficiency of 100% (data not shown). Besides tandem integrations, also multiple single integrations of pHAMT35G were detected in some of the transformants (e.g. EY3p in Fig. 2A).

P. palmivora transformants expressing the GFP gene constitutively were obtained by co-transformation of strain P3609 with pVW2 and pTH209. Transformation vector pVW2 contains the *ham34* promoter and terminator fused to the coding sequence of a mammalian codon-optimised GFP gene (Fig. 1). In total, 64 putative *ham34-gfp* transformants (UY1p-UY64p) were obtained. To test whether these

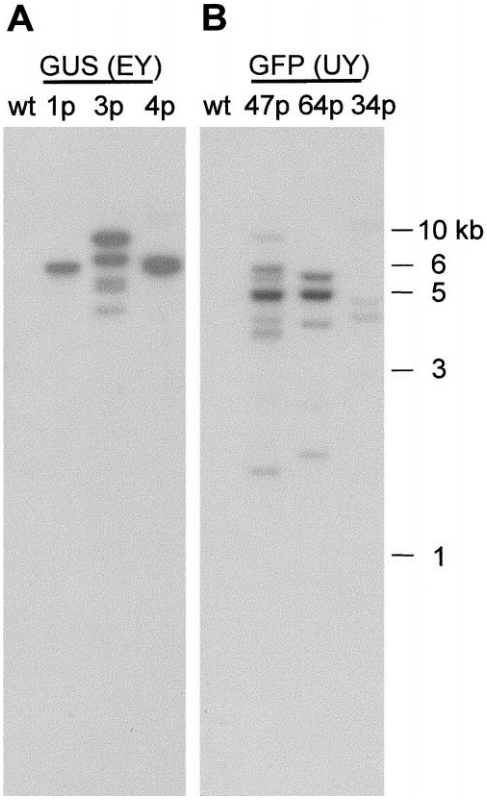

Fig. 2. Southern analysis of genomic DNA isolated from *ham34-gus* and *ham34-gfp P. palmivora* transformants. Genomic DNA of the *P. palmivora* wild-type recipient strain (wt), three *ham34-gus* transformants (EY1p, EY3p and EY4p) (A) and four *ham34-gfp* transformants (UY47p, UY64p and UY34p) (B) were digested with *Eco*RI, separated by electrophoresis and blotted onto a nylon membrane. The blot in A was hybridised with a probe of the GUS gene. The 5.96-kb hybridising band represents tandem integrations of the pHAMT35G construct. The blot in B was hybridised with a GFP probe and the 4.84-kb hybridising band represents tandem integrations of pVW2.

putative co-transformants contained and expressed the *ham34-gfp* construct, Southern analysis was performed and GFP production was analysed in sporangia and germinated cysts with a LSCM (Section 3.2). In total, 26 transformants were obtained that showed green fluorescence. Genomic DNA from three of these transformants (UY47p, UY64p and UY34p) was digested with *Eco*RI, recognising a single restriction site in pVW2. The digested DNA was blotted on to a membrane and hybridised with a probe specific for the GFP coding sequence (Fig. 2B). This resulted in a strong hybridising band of the size of the linearised plasmid in two *ham34-gfp* transformants (UY47p and UY64p), indicating that tandem integration of the pVW2 construct had occurred.

3.2. Detailed characterisation studies of the ham34-gfp and ham34-gus transformants

Eight *ham34-gus* transformants were tested for GUS activity by transferring pieces of agar containing mycelia into the GUS staining solution (Section 2.5). After 2 h of incubation, blue staining was analysed macroscopically. Hyphae of six *ham34-gus* transformants stained blue, indicating that GUS was produced in these transformants (data not shown). However, the level of GUS staining varied between all transformants. Some transformants stained light-blue (e.g. EY3p), whereas others stained more intensely (e.g. EY1p). Three transformants

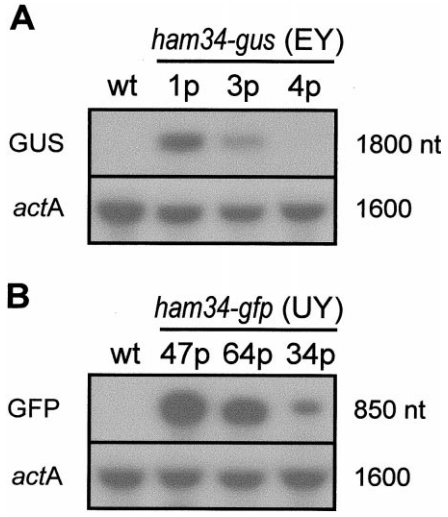

Fig. 3. Northern analysis of total RNA isolated from *ham34-gus* and *ham34-gfp P. palmivora* transformants. Total RNA (10 μg) was isolated from mycelium grown for 7 days of the *P. palmivora* wild-type recipient strain (wt), three *ham34-gus* transformants (EY1p, EY3p and EY4p) (A) and four *ham34-gfp* transformants (UY47p, UY64p and UY34p) (B). The RNA was separated by electrophoresis and blotted onto a nylon membrane. The blot in A was hybridised with a probe of the GUS gene and the *P. infestans* actin gene. The blot in B was hybridised with the GFP probe and the actin probe. Transcript lengths are indicated in nucleotides (nt).

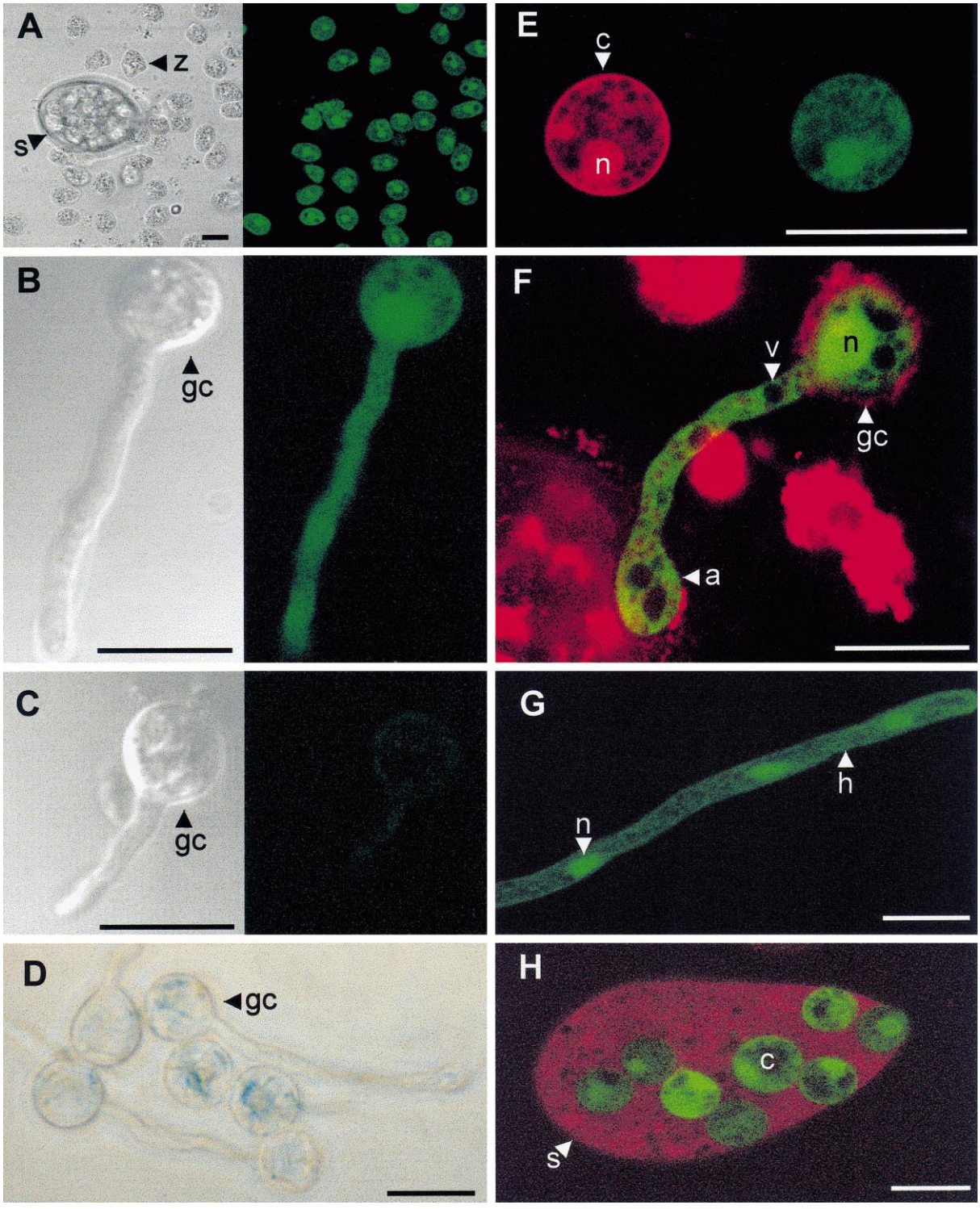
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Fig. 4. GFP localisation studies of *P. palmivora* transformant UY47p (A–C, E–H) and histochemical localisation of GUS activity in *P. palmivora* transformant EY1p (D). The panels show: (A) sporangium (s) releasing zoospores (z), (B–D) germinated cysts (gc) and (E) a cyst (c) with a GFP-rich nuclear region (n). (E) The picture on the left shows propidium iodide staining and the picture on the right shows GFP fluorescence of the same cyst. (F) A germinated cyst (gc) with an appressorium (a) stained with propidium iodide is shown, vacuoles (v) and nucleus are indicated. A multinucleated (n) hypha (h) and a propidium iodide-stained sporangium (s) filled with encysted zoospores (c) are shown in G and H, respectively. A–C show a bright field image (left) and fluorescent image (right). Scale bars represent 10 μm.
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showed GUS secretion or leakage of the blue stain, which has also been reported for *P. infestans* strains expressing GUS [8]. The wild-type *P. palmivora* strain did not show any endogenous GUS activity. Furthermore, we noted that within a single transformant, blue staining was not detected in all cells. For example, in one transformant (EY2p), less than 30% of the germinated cysts stained light-blue (data not shown).

The *ham34-gfp* (pVW2) transformants were tested for GFP production by analysing sporangia and germinated cysts with a LSCM. GFP produced in the transgenic strains fluoresces bright-green upon exposure to UV with an excitation maximum of 488 nm. 26 *ham34-gfp* transformants showed bright-green fluorescence. Among the *ham34-gfp* transformants, variation in the level of fluorescence was noted. Fluorescence was particularly bright in 12 transformants of which UY47p and UY64p were used for further studies.

Expression studies were performed, using Northern analysis, to investigate whether the level of green fluorescence in *ham34-gfp* transformants and light-blue staining in *ham34-gus* transformants correlated with GFP or GUS mRNA production, respectively. Total RNA was isolated from representative GFP (UY47p, UY64p and UY34p) and GUS (EY1p, EY3p and EY4p) transformants, blotted on to a membrane and hybridised with GFP or GUS probes (Fig. 3). To determine whether all lanes contained an equal amount of mRNA, a heterologous hybridisation was performed with a probe derived from the *P. infestans* actin gene (*act*A)[18]. The actin hybridisation showed similar amounts of radioactivity in all lanes, suggesting that equal amounts of mRNA were loaded. However, different levels of GFP or GUS mRNA were noted in the *ham34-gfp* or *ham34-gus* transformants, indicating that the *trans*-genes were expressed at different levels in different transformants.

3.3. Microscopic analysis of the ham34-gfp and ham34-gus transformants

GFP production in *ham34-gfp* transformant UY47p and UY64p was investigated in in vitro grown stages of the life cycle including sporangia, zoospores, cysts, germinating cysts, cysts producing appressoria and hyphae (Fig. 4). Green fluorescence was detected in all stages and was brightest around (or in) the position of nuclei (Fig. 4A, B, E and F). Vacuoles did not exhibit fluorescence (Fig. 4E–G). UY47p was the brightest fluorescing transformant, which correlated with the greatest amount of GFP mRNA detected by Northern analysis (Fig. 3B).

GFP production could be distinguished readily from background fluorescence using an objective lens of 10× magnification or greater. Best results were obtained using high magnification lenses of 40× or greater. Yellow-green autofluorescence varied among the analysed samples, but did not interfere with the examinations or image capturing (Fig. 4C). Fading of the green fluorescence was largely prevented by addition of the anti-fading agent *p*-phenylene diamine. Samples without *p*-phenylene diamine faded progressively. In general, after scanning for more than 20 times, green fluorescence was non-detectable due to photo-bleaching. Cells were usually scanned 3–5 times with the laser for image capturing. Zoospores were scanned only once to minimise blurring due to their movement.

To determine whether green fluorescence was found in living as well as in dead cells, propidium iodide was added to the *P. palmivora* cell suspensions. Propidium iodide is a viability stain that binds DNA from cells with non-intact or permeable cell

membranes [21]. When high concentrations are used, it also binds to cell wall material. In the majority of *P. palmivora* cells analysed, green fluorescence could only be detected in cells that did not stain with propidium iodide.

GUS activity of the *ham34-gus* transformants EY1p, EY3p and EY4p was analysed in cysts, germinated cysts, sporangia and hyphae using light microscopy. Transformant EY4p showed no GUS activity, which was in agreement with the observation that also no GUS mRNA was detected on the Northern blot (Fig. 3A). However, all cells of the analysed tissues from the *ham34-gus* transformants EY1p and EY3p contained localised clusters of blue crystals (Fig. 4D). Although the blue staining in the *P. palmivora* tissues was easily detected by the eye, it was often difficult to visualise by microscopy. This was probably due to the observation that the blue staining was only localised as crystals in certain areas within the cells. Furthermore, significant amounts of dye were lost from the cells during the staining procedure. This could be due to breakage of cell membranes during the infiltration stage of the enzymatic assay or secretion of the GUS enzyme.

4. Discussion

We describe the first successful transformation of the oomycete *P. palmivora* with two reporter gene constructs. One construct contained the open reading frame of a mammalian codon-optimised GFP gene fused to the constitutively expressed *ham34* promoter from *B. lactucae*. The second construct had the open reading frame of the *E. coli* GUS gene fused to the same promoter. Expression of the integrated transgenes was analysed by Northern analysis and histochemical GUS staining of the *ham34-gus* transformants. GFP localisation in the *ham34-gfp* transformants was analysed by LSCM.

GFP from the jellyfish *Aequoria victoria* is a versatile reporter for monitoring gene expression in vivo, in situ and in real time. GFP fluoresces bright-green upon exposure to UV light, unlike other bioluminescent reporters which require additional proteins, substrates or cofactors to emit light. Emission of light does not require an enzymatic reaction [22], which is for example needed for the LUC reporter gene system.

GFP fluorescence was stable enough to analyse *P. palmivora* cells without photo-bleaching. Addition of anti-fading agents to *P. palmivora* cell suspensions greatly reduced fading of the material, but in cases where fast analysis was desired, anti-fade could be omitted. GFP production can be assessed within minutes and additional viability stains, such as propidium iodide, are not required. Green fluorescence was detected predominantly in living cells. This can be an advantage for the analysis of single cells. However, it can also be a major disadvantage in those cases when a population of cells or cell-cell interactions are studied. Viability of *P. palmivora* cells can not be monitored with the GUS reporter gene system, because the enzymatic staining reaction must be performed on dead tissue. The ability to employ living material is especially useful for analysing GFP production in zoospores. The GUS reporter gene proved to be inappropriate for analysis of fragile zoospores, as all cells collapsed during the staining procedure. Therefore, we can conclude that GFP is the first reporter gene system to be described that can be used successfully to monitor gene expression in swimming *Phytophthora* zoospores. GFP will be suitable for the in vivo analysis of genes expressed during this and other life-cycle stages of *P. palmivora*.

The expression levels of the integrated GFP transgenes correlated well with the intensity of fluorescence. This correlation was also noted for the transgenic *ham34-gus* transformants. Hence, we can conclude that less fluorescence in some of the GFP transformants is likely to be the result of reduced mRNA production, rather than faster degradation of the GFP. Consequently, care must be taken in selecting appropriate transformants when GFP is used for detailed promoter activity studies or localisation studies.

We noted that not all cells of the transformants stained blue or showed green fluorescence in either the GUS or GFP transformants, respectively. An explanation could be that the expression level of the *trans*-genes in some cells are too low to be able to detect blue staining or green fluorescence. Another explanation could be that heterokaryotic strains were obtained after transformation with pHAMT35G or

pVW2 because some protoplasts may have multiple nuclei. Thus, transformation could result in cells that have genetically different nuclei, some with and some without the integrated construct. During zoosporogenesis, single nucleated zoospores are formed and segregation of nuclei takes place, leading to new cells that may have lost the integrated GUS or GFP gene.

We conclude that GFP is superior to GUS for high magnification microscopic studies. GFP is fairly stable, can be used in living cells and does not require substrates or any other chemicals to visualise. Furthermore, handling and analysis of the biological material does not require fixing or staining. It can also be used for double-labelling experiments with other fluorescent markers, such as propidium iodide or perhaps blue- or red-shifted fluorescent proteins. However, GFP production could not be visualised in UV light with low magnification imaging and its levels in the cell were too low to be detected using conventional fluorescence microscopy. For macroscopic studies, the GUS reporter system was superior, since the blue stain was visualised easily with the naked eye. Microscopic studies with the GUS transformants were less successful than the studies with the GFP-marked strains, since the blue staining was difficult to visualise and a large proportion of the *ham34-gus* transformants showed secretion of the enzyme or leakage of the blue crystals from the cells.

Acknowledgements

We thank the BBSRC (Grant 1/P07720) for P.v.W. and NERC for a studentship (GT04/96/FS/4) to T.C. for financial support.

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