

Agrobacterium tumefaciens-mediated transformation of *Leptosphaeria* spp. and *Oculimacula* spp. with the reef coral gene *DsRed* and the jellyfish gene *gfp*

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Abstract

Four filamentous ascomycetes, *Leptosphaeria maculans*, *L. biglobosa*, *Oculimacula yallundae* and *O. acufiformis*, were transformed via *Agrobacterium tumefaciens*-mediated transformation with the genes encoding DsRed and GFP. Using vectors pCAMDsRed and pCAMB_{gfp}, either germinated conidia of *Leptosphaeria* spp. and *O. yallundae* or physically fragmented cultures of *Oculimacula* spp. were transformed. In vitro, the expression of the two reporter proteins in mycelium of both *Oculimacula* and both *Leptosphaeria* species was sufficient to distinguish each species in co-inoculated cultures. In planta, transformants of *L. maculans* or *L. biglobosa* expressing DsRed or GFP could be observed together in leaves of *Brassica napus*. Either reporter protein could be used to view the colonization of leaf petioles by both *Leptosphaeria* spp. and growth in the xylem vessels could be clearly observed. With the generation of these transformants, further studies on interactions between pathogen species involved in disease complexes on various host species and between opposite mating types of the same species are now possible.

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Keywords: Pathogen species complex; *Tapesia* spp.; Eyespot; *Phoma lingam*; Stem canker; Reporter protein

1. Introduction

Plant diseases are often caused by pathogen species complexes [1]. When several closely related pathogens interact to produce symptoms, it is difficult to determine the contributions of each species. Two examples of global economic importance are phoma stem canker

(blackleg) of oilseed rape [2] and eyespot of cereals [3]. In Europe, phoma stem canker is associated with a complex of two co-existing species, *Leptosphaeria maculans* and *L. biglobosa*, although up to seven sub-species have been identified [2]. Eyespot is associated with a complex of at least two species, recently re-named as *Oculimacula yallundae* (formerly *Tapesia yallundae*) and *O. acufiformis* (*T. acufiformis*) [4]. For both pairs of related species, slight differences in epidemiology between the two species have been noted [5–7]. However, the details of the interactions between the species, in relation to environmental or agronomic factors, are difficult to study with existing methods, such as isolation onto selective culture media or species-specific PCR [6–9].

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The use of the *Aequorea victoria gfp* gene encoding green fluorescent protein (GFP) to create transgenic strains constitutively expressing this reporter protein has, since 1996, provided an excellent tool for the *in vitro* and *in planta* observation of several plant pathogenic fungi, including *O. yallundae* and *L. maculans* [10–12]. Recently, three filamentous ascomycete species have been transformed with the *Discosoma* reef coral gene *DsRed* [13,14]. Despite a small overlap in their emission spectra, DsRed offers a true alternative to GFP and discrimination between GFP and DsRed transformants is possible using fluorescence microscopy, without the need for confocal microscopy [13].

To our knowledge, *L. biglobosa* and *O. acufiformis* have not been transformed in the laboratory. *L. maculans* has been transformed using polyethylene glycol (PEG)-mediated transformation [11,15,16], electroporation [17] or *Agrobacterium tumefaciens*-mediated transformation [18]. *O. yallundae* has previously been transformed only by PEG-mediated transformation [10,19–21]. GFP-expressing transformants of *O. yallundae* and *L. maculans* have previously been generated by PEG-mediated transformation [10,11] and vectors currently available to modify ascomycetes with the *DsRed* gene require the use of PEG-mediated transformation. However, since the enzyme preparation Novozyme 234, used for the generation of protoplasts during PEG-mediated transformation, became commercially unavailable in the late 1990s, problems with preparation of protoplasts from *L. maculans* and *O. yallundae* have been encountered [18]. In our laboratory, it was not possible to modify *L. maculans* or *O. yallundae* using PEG-mediated transformation.

In this paper, we report the construction of a vector for *Agrobacterium*-mediated transformation of filamentous ascomycetes with the reef coral gene *DsRed*, the genetic modification of *Leptosphaeria* spp. and *Oculimacula* spp. with the genes encoding for GFP and DsRed and an evaluation of the potential use of these two reporter proteins to observe co-existing fungal pathogens *in vitro* and *in planta*.

2. Materials and methods

2.1. Bacterial strains and fungal isolates

Escherichia coli strain NovaBlue™ (Novagen, UK) was used during the construction and maintenance of plasmids. Electro-competent *A. tumefaciens* cells of strain Ag11 were prepared according to a standard protocol [22]. The fungal isolates used in this work are maintained within the Rothamsted culture collection (curated by Paul Hornby and Martin Urban). *L. maculans* ‘brassicae’ isolate ME24 and *L. biglobosa* ‘brassicae’ isolate B2003-2-8 were obtained from winter oilseed

rape stubble collected shortly before harvest in 2002 at Darrington, Yorkshire (ME24) or in 2003 at Orston, Nottinghamshire (B2003-2-8), using methods described [6]; species identification was confirmed by ITS-RFLP [23]. Isolates were maintained on 20% V8 medium and conidia harvested from 2-week-old cultures by flooding with sterile distilled water and filtration through two layers of sterile Miracloth (Calbiochem, USA). Opposite mating-type *O. yallundae* isolates 22-432 (*MATI-1*) and 22-433 (*MATI-2*) [24] were obtained from P.S. Dyer (University of Nottingham, UK); *O. acufiformis* isolate 185 was provided by G.L. Bateman (Rothamsted Research) and had been obtained from winter wheat collected in 1988 at Rothamsted. *Oculimacula* isolates were maintained on 20% PDA (Oxoid) medium and conidia of *O. yallundae* *MATI-2* were produced using methods previously described [25]. Stock cultures of fragmented *O. yallundae* *MATI-2* hyphae were prepared by suspending aerial hyphae from one 9 cm culture plate in 10 ml of Czapek Dox Broth (CDB, Oxoid), physically fragmenting the mycelium for 1 min using a Polytron (Ultra-Turrax® T25 Basic, Werke GmbH) at maximum speed and incubating shaking at 200 rpm and 17 °C for 3 days.

2.2. Plasmids and nucleic acid manipulations

Plasmids pGpdGFP [11] and pPgpD-DsRed [13] were obtained from B. Howlett (University of Melbourne, Victoria, Australia) and L. Mikkelsen (Royal Veterinary and Agricultural University, Frederiksberg, Denmark), respectively. Plasmid pCAMBIA1301 was purchased from CAMBIA (Canberra, Australia) and pCAMBgfp [26] was obtained from A. Sesma-Galarraga (John Innes Centre, Norwich, UK).

Standard molecular biology procedures were performed as previously described [27]. For the construction of pCAMDsRed, the T-DNA of pCAMBIA1301 was modified to include the *PtpC-hph* expression cassette from pGpdGFP and the *PgpDA-DsRed-Express-TtpC* expression cassette from pPgpD-DsRed. Primers PtpC-BstXI (5' CCACCATGTTGGAGCATTTTTGGGCTTGGCTGG 3') and hph-XhoI (5' CTCGAGCT-ATTCTTTGCCCTCGGACGAG 3') were used to amplify the *PtpC-hph* coding sequence from pGpdGFP using a proof-reading polymerase (Platinum® Pfx, Invitrogen). The PCR product was blunt-end ligated into pSTBlue-1 (Novagen) and sequenced to ensure that no errors had been incorporated during PCR. The *PtpC-hph* cassette was released from pSTBlue-1 by digestion with *BstXI* and *XhoI*. pCAMBIA1301 was digested with *BstXI* and *XhoI* to remove the existing CaMV35S promoter and hygromycin (*hph*) resistance gene; the *PtpC-hph* cassette was then ligated into the remaining backbone of the vector to create pCAMhph. Next, the *PgpDA-DsRed-Express-TtpC*

expression cassette was released from pPgpD-DsRed by digestion with *EcoRI* and *HindIII* and blunt-ended using T4 DNA Polymerase (Promega). pCAMhph was digested with *BstEII* and *BstXI* (removing the other CaMV35S promoter and the GUSA gene present in pCAMBIA1301), blunt ended using T4 DNA polymerase and de-phosphorylated using Calf Intestinal Alkaline Phosphatase (MBI Fermentas). The *PgpDA-DsRed-Express-TrpC* expression cassette was then ligated into the pCAMhph backbone to create pCAMDsRed (Fig. 1). Configuration of the construct was determined by restriction digestion with enzymes *EcoRI*, *NcoI* and *XhoI*.

Fungal genomic DNA was extracted using the CTAB protocol [28]. To determine copy number of GFP and DsRed-reporter genes a 543-bp GFP and a 579-bp DsRed fragment was amplified using primer pairs U61 (5' TAAACGGCCACAAGTTCA 3')/U62 (5' TGCTCAGGTAGTGGTTGT 3') and U32 (5' CTCCTCCGAGGACGTCATCAA 3')/U33 (5' ACGTAGTAGTAGCCGGGCAGCT 3'). Both fragments were $\alpha^{32}\text{P}$ -dCTP-labelled using the Rediprime II Random Prime Labelling System (Amersham Biosciences) following the instructions of the manufacturer. Twenty microgram aliquots of DNA of each transformant were restriction digested with *EcoRI* and then separated on a 1% agarose gel, blotted onto HybondTM N+ membrane (Amersham Biosciences). Blots were incubated for 8 h in Church buffer at 65 °C [29] and washed twice in 1% SDS/100 mM NaHPO₄ (pH 7.0) for 30 min at the same temperature. The radioactive image was visualized using a Typhoon 8600 Variable Mode Imager (Molecular Dynamics).

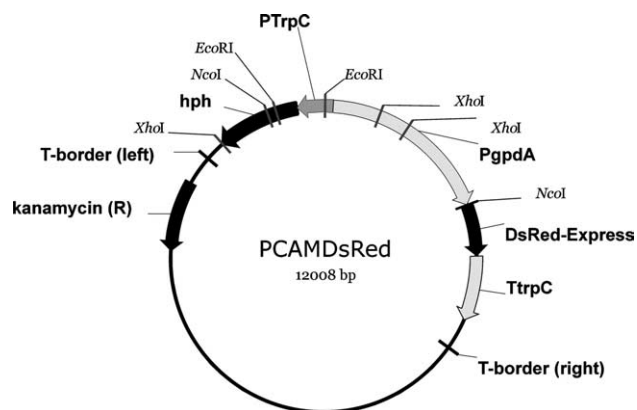


Fig. 1. Restriction enzyme map of pCAMDsRed. The plasmid was constructed for the *Agrobacterium*-mediated transformation of filamentous ascomycetes with the DsRed-Express gene placed under the constitutive *A. nidulans* glyceraldehyde 3-phosphate promoter (*PgpDA*) and followed by the 3' sequence of the *A. nidulans* *trpC* gene (*TrpC*). The plasmid also contains the hygromycin gene conferring resistance to hygromycin (*hph*) and the neomycin phosphotransferase II gene conferring resistance to kanamycin for selection in bacteria. The plasmid structure was drawn using Vector NTI 9.0.0 (2003) software (InforMax).

2.3. Transformation method

Agrobacterium-mediated transformation was done by a slightly modified version of an existing method [18]. *L. maculans* or *L. biglobosa* conidia (10^8) were incubated with shaking at 200 rpm in 40 ml of PDB (Sigma) at 28 °C until >50% of conidia had germinated (approximately 28 h for *L. maculans* and 18 h for *L. biglobosa*). Conidial concentrations were then adjusted to 10^7 conidia ml⁻¹ (including un-germinated conidia) in the induction medium (IM) [30], adjusted to pH 6. A 1:30 dilution of *O. yallundae* MAT1-2 physically fragmented mycelial stock culture or 10^4 conidia of *O. yallundae* MAT1-2 were inoculated into 10 ml of CDB. Approximately 50 or 30 cm² of PDA colonized by 4-week-old cultures of *O. yallundae* MAT1-1 or *O. acufiformis*, respectively, were transferred to 10 ml of CDB and physically fragmented. All *Oculimacula* cultures were incubated shaking at 200 rpm at 17 °C for 3 days, followed by re-suspension of the germinated conidia in 1 ml of IM. Alternatively, 500 μl of *O. yallundae* MAT1-2 mycelial culture or 100 μl of *O. yallundae* MAT1-1 or *O. acufiformis* mycelial cultures were re-suspended in 1 ml of IM.

To initiate transformation, 200 μl aliquots of prepared fungal material were mixed with 200 μl of log-phase *Agrobacterium* Agl1 cells carrying the transforming plasmid [18]. The cells were pelleted by centrifugation immediately after mixing, re-suspended in 50 μl of IM and applied to 3 × 3 cm pieces of sterile neutral nylon membrane (HybondTM-N, Amersham Bioscience, UK) placed on solid IM (1.5% agar). Plates were sealed, incubated at 22 °C in darkness for 3 days and the nylon membrane pieces were then transferred to selection medium (PDA containing 50 $\mu\text{g ml}^{-1}$ hygromycin and 250 $\mu\text{g ml}^{-1}$ cefotaxime). Alternatively, an overlay selection method [18] was used and transformants were selected by overlaying with PDA (or 20% V8 juice agar) containing 100 $\mu\text{g ml}^{-1}$ hygromycin and 500 $\mu\text{g ml}^{-1}$ cefotaxime. Plates were sealed and incubated at 22 °C in darkness until fungal colonies were visible.

2.4. Microscopy

A Leica MZ FLIII stereo-microscope, equipped with filters GFP1 (excitation 425/60 nm, emission 480 nm) and GFP3 (470/40 nm, 525/50 nm) from Leica Microsystems (Milton Keynes, UK) and the red shifted TRITC filter (545/30 nm, 620/60 nm) from Chroma (Rockingham, USA), was used both to screen colonies of transformants growing on nylon membranes for fluorescent protein expression and for observation of fungal colonization in plant tissues. A Zeiss Axiophot fluorescent microscope equipped with GFP fluorescence filters GFP1 (450–490 nm, LP 515 nm) and GFP3 (470/20 nm, BP 505–530 nm) from Zeiss Ltd. (Welwyn Garden City, UK) and the red shifted TRITC filter was used for

observation of GFP and DsRed fluorescence in spores and hyphae *in vitro* and *in planta*. Both microscopes are equipped with a mercury lamp and photomicrographs were taken by digital camera (Leica DC 300FX) operated with IM50 software (Leica DC Twain, v. 4.1.5.0).

3. Results and discussion

Four filamentous ascomycetes, *Leptosphaeria maculans*, *L. biglobosa*, *Oculimacula yallundae* and *O. acufornis*, were successfully transformed via *Agrobacterium tumefaciens*-mediated transformation with both GFP and DsRed, using vectors pCAMDsRed (Fig. 1) and pCAMBgf [26]. This is the first report that *L. biglobosa* and *O. acufornis* have been genetically modified in the laboratory. Furthermore, this is the first report of *Agrobacterium*-mediated transformation of filamentous ascomycetes with the *DsRed* gene, whereas PEG-mediated transformation has been used before [13,14]. The successful use of one *Agrobacterium*-mediated transformation protocol for the modification of all four species circumvents the need for PEG-mediated transformation, which often requires species-specific protocols and is also currently problematic [18].

Hygromycin-resistant transformants expressing either GFP or DsRed from the *ToxA* or *PgpdA* promoters, respectively, were obtained for all four species within 2–4 weeks after transformation. GFP or DsRed expression of colonies growing on nylon membranes was observed with a stereo-microscope (Fig. S1(a)–(d)). Strongly expressing colonies were removed from the membrane with a sterile tooth-pick and transferred to fresh solid media plates. The efficiency of transformation was 30–40 and 20–25 transformants per 10^7 spores for *L. maculans* and *L. biglobosa*, respectively. Around 10 times more *L. biglobosa* transformants were obtained when selection was done using a 20% V8 juice

agar + hygromycin overlay, whereas only 1–2 *L. biglobosa* transformants were obtained per 10^7 spores when a PDA + hygromycin overlay was used. On the contrary, more *L. maculans* transformants were obtained with a PDA + hygromycin overlay. This could indicate a greater requirement of *L. biglobosa* for nutrients during the recovery period after transformation. Individual *L. maculans* transformants expressed either reporter protein moderately or strongly, whereas individual *L. biglobosa* transformants expressed GFP weakly and DsRed either weakly or strongly. Curiously, few *L. biglobosa* transformants expressed either reporter protein moderately.

Transformation of both *Oculimacula* spp. and both mating-types of *O. yallundae* was successfully achieved using either germinated conidia or cultures of physically fragmented mycelium. No preference for either selection method was observed. However, in each transformation more *O. acufornis* than *O. yallundae* transformants were obtained (Fig. 2) and the two species differed in vector efficiency; i.e. numbers of transformants obtained with vectors pCAMBgf or pCAMDsRed. For *O. yallundae*, more GFP transformants were generated (GFP $n = 30$, DsRed $n = 6$), whereas for *O. acufornis* more DsRed transformants were generated (DsRed $n = 40$, GFP $n = 27$). The vector efficiency coincided with the strength of expression of the reporter protein; *O. yallundae* transformants expressed GFP strongly whereas *O. acufornis* transformants expressed DsRed strongly. The preference of both *L. biglobosa* and *O. acufornis* for DsRed expression could be due to greater compatibility of the *PgpdA* promoter, rather than preferential reporter protein synthesis. Similar strengths of GFP and DsRed expression had been observed previously in *Trichoderma* when the *PgpdA* promoter expressed either gene [13]. To determine whether there is a species-specific preference for one of the two reporter proteins, another plasmid could be created, in which the *ToxA* promoter regulates the expression of the *DsRed* gene.

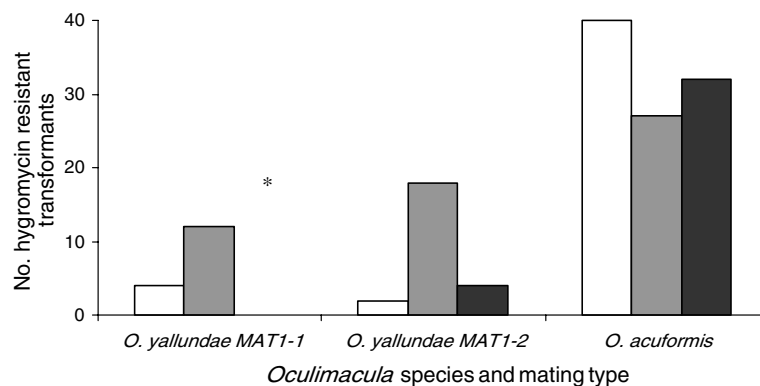


Fig. 2. Total numbers of hygromycin-resistant *MAT1-1 O. yallundae* (isolate 22–432), *MAT1-2 O. yallundae* (isolate 22–433) and *O. acufornis* (isolate 185) transformants obtained from two independent replicate transformations. Either germinated conidia or physically fragmented mycelium were co-cultivated with *Agrobacterium* strain Ag11 carrying plasmid pCAMDsRed (□), pCAMBgf (■) or pCAMhph (■) (* no data available).

In vitro, the reporter proteins GFP and DsRed were expressed in mycelium of both *Oculimacula* (Fig. 3(a)–(d) and Fig. S1(u) and (v)) and both *Leptosphaeria* (Fig. 3(e)–(n)) species. This made it possible to distinguish each species when they were co-inoculated onto the same piece of agar (Fig. 3(a)–(d)). The GFP and DsRed reporter proteins were also expressed in conidia of all four species (Fig. S1(e)–(t)). Older mycelium occasionally included segments of hyphae with reduced expression (Fig. 3(e)) or without expression (Fig. 3(h)). Light micrographs of *L. maculans* hyphae (Fig. 3(g)) indicated that non-expressing hyphal sections included collapsed cells. Growing hyphal tips always expressed GFP or DsRed strongly (Fig. 3(f)) and the GFP or DsRed expression of the transformants was continuous; i.e., no spontaneous loss of expression was observed for any of the transformants of the four species, even after continuous sub-culture over a period of one year.

GFP expression was visualized using GFP1 or GFP3 filters and some DsRed expression was observed with the GFP1 filter (Fig. 3(b) and Fig. S1(f) and (j)). Although DsRed was best viewed with the TRITC filter (Fig. 3(d) and Fig. S1(h) and (l)), the possibility of using one filter for in vitro co-visualization of two pathogen species (Fig. 3(b)) or two mating types of one species would allow faster analysis of samples without the need for computer-aided overlay of images taken with different filters. The transformation of both mating-types of a fungal species with different reporter genes and the ease of co-visualization of both species in one sample creates new opportunities for studying in vitro interactions between opposite mating types of filamentous fungi, such as *O. yallundae* [24].

Transformants expressing GFP or DsRed were observed together *in planta*. Using either GFP1 or TRITC filters, *L. maculans* and *L. biglobosa* were observed as germinating conidia on the surface of *B. napus* leaves (Fig. S2(a) and (b)), during different stages in stomatal penetration (Fig. S2(b)) and colonizing leaf tissues (Fig. 3(i)–(n) and Fig. S2(c) and (d)). Pycnidia were clearly visible in heavily colonized leaf tissue with either marker protein and their formation could be followed from initiation in the sub-stomatal cavity (Fig. S2(e)–(g)). Use of the GFP1 filter also gave information on the physiological state of plant tissues near to the fungal hyphae. Chloroplasts of healthy tissue autofluoresced red, whereas senescent plant cells either autofluoresced yellow-green or did not autofluoresce (Fig. 3(i) and (l)). A co-inoculation experiment on a *B. napus* leaf showed that extensive cell death occurred rapidly in regions colonized by *L. biglobosa*, whilst cells in areas colonized by *L. maculans* initially remained alive (Fig. 3(i)–(k)) and only subsequently died (Fig. 3(l)–(n)). These results indicate an initial hemi-biotrophic phase of leaf tissue colonization by *L. maculans*. The use of GFP and DsRed labelled isolates thus provides an opportunity

to investigate differences in spore germination, penetration, colonization and nutrient acquisition between closely related fungal species, such as *L. maculans* and *L. biglobosa*. Differences in any of these parameters could generate species-specific epidemiological changes under field conditions.

A key stage in the pathogenesis of phoma stem canker is the spread of the pathogens from the leaf to the stem [31]. Colonization of the vascular bundle in oilseed rape petioles by *Leptosphaeria* spp. was clearly observed using transformants expressing either reporter protein (Fig. 3(o)–(s)). In this case, with a strongly expressing DsRed transformant, more informative images were obtained using the GFP1 filter (Fig. 3(r)) than the TRITC filter (Fig. 3(s)). These images confirm that hyphae of both *Leptosphaeria* spp. can spread from leaves to stems down the xylem vessels of leaf petioles [31]. They offer new possibilities for studying interactions between *L. maculans*, *L. biglobosa* and *B. napus* during this symptomless phase of pathogen growth, which may be important in development of strategies for controlling the pathogens through host resistance or fungicides [1].

To determine GFP- and DsRed-reporter gene integration and copy number in the four filamentous ascomycetes, genomic DNA was isolated from 18 purified *Leptosphaeria* and 15 *Oculimacula* species transformants for Southern blot analysis (Fig. 4). The genomic DNA was digested with *EcoRI*, which cuts once the T-DNA of GFP transformants and twice within close vicinity the T-DNA of the DsRed transformants. The presence of different sized bands shows random integration, single bands confirm single integration, while two or more bands are indicative of multi-copy integration events. Hybridization patterns show that the majority of transformants have integrated only a single copy of the reporter gene similar to *A. tumefaciens* transformation systems of other filamentous fungi [30,32]. The expression strength of the GFP- and DsRed-reporter genes in the transformants was not found to be linked to the copy number of these genes, but may rather be dependent on the integration locus.

We have successfully used GFP and DsRed expressing transformants for the visualization, both in vitro and *in planta*, of two pairs of co-existing related pathogen species involved, respectively, in the oilseed rape phoma stem canker [1] and cereal eyespot [3] disease complexes. The development of *L. maculans*, *L. biglobosa*, *O. yallundae* and *O. aciformis* transformants strongly expressing these fluorescent reporter proteins will allow further studies on interactions between these pairs of species during host colonization and suggests that other filamentous fungi may be transformed with GFP and DsRed to investigate interactions between different pathogens involved in disease complexes.

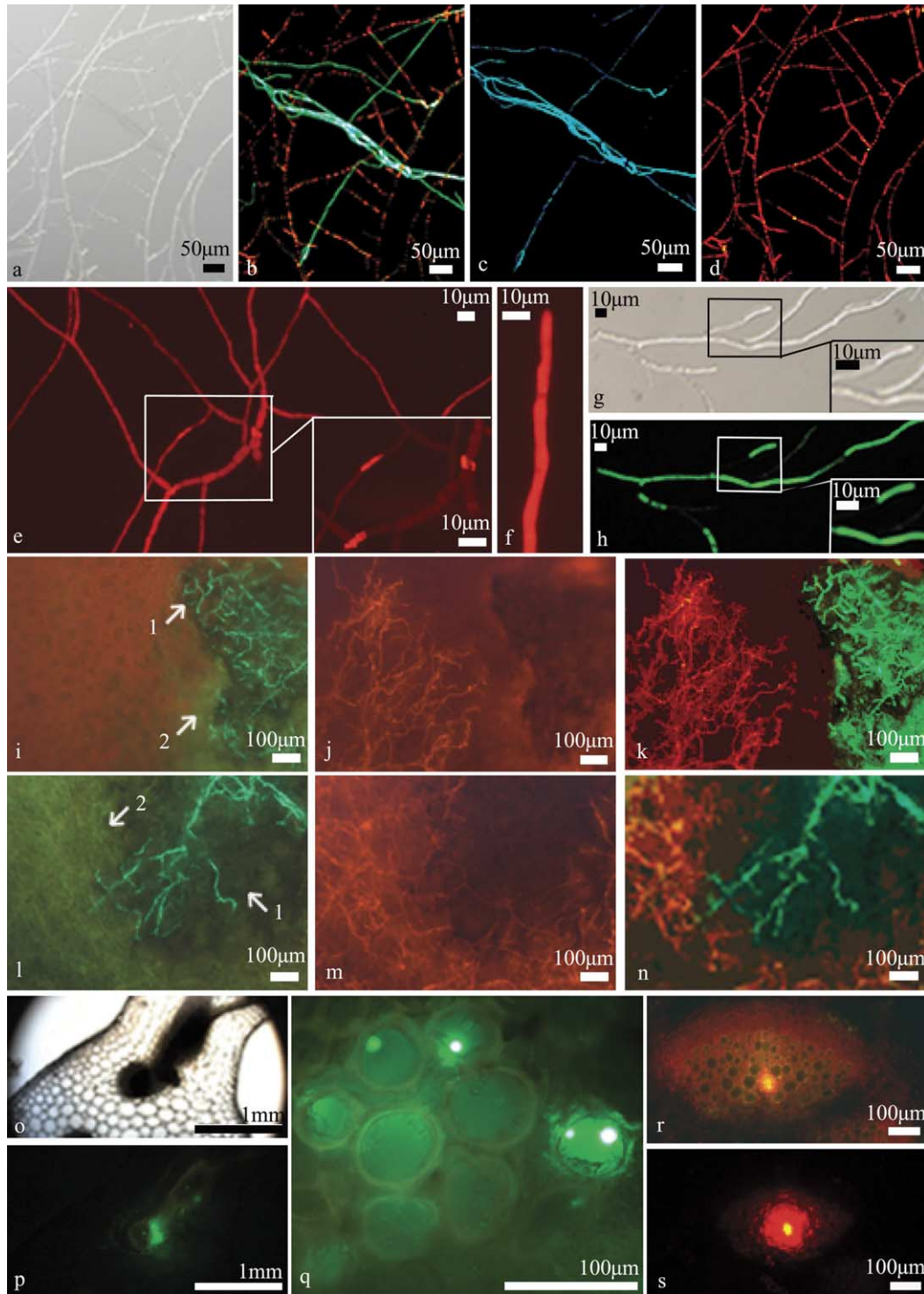


Fig. 3. Fluorescence micrographs; GFP visualized with GFP1 filter and DsRed visualized with TRITC filter, unless stated otherwise. (a)–(h) In vitro. (a)–(d) *Oculimacula yallundae* (GFP) and *O. acufiformis* (DsRed) co-inoculated onto an agar-covered microscope slide, allowing mycelium of the two species to grow into each other; (a) white light, no filter; (b) GFP 1 filter; (c) GFP3 filter and (d) TRITC filter. (e)–(h) Mature hyphae of *L. biglobosa* (DsRed) and *L. maculans* (GFP) growing on agar, showing areas of (e) ‘patchy’ reporter gene expression; (f) hyphal growing tip showing strong reporter gene expression or (g and h) parts of hyphae showing very weak or no reporter gene expression. (i)–(n) *L. maculans* (DsRed) and *L. biglobosa* (GFP) co-inoculated onto leaves of oilseed rape (*B. napus*) cv. Apex, at sites ca. 1 cm apart. Images k and n produced by merging images (i), (j) and (l), (m), respectively, using Adobe Photoshop version 7. (i)–(k) 7 dpi; leaf tissue colonized by *L. biglobosa* showing tissue necrosis (1) and senescence (2); leaf tissue colonized by *L. maculans* appeared healthy. (l)–(n) 14 dpi; leaf tissue colonized by *L. maculans* now showing necrosis (1) and senescence (2); *L. maculans* and *L. biglobosa* now growing into each other. (o)–(s) hand-cut sections ($\approx 300 \mu\text{m}$ width) of oilseed rape cv. Apex leaf petioles colonized by *L. maculans*, showing growth in the vascular bundle observed using (o)–(q) GFP or (r and s) DsRed (r, GFP1 filter; s, TRITC filter).

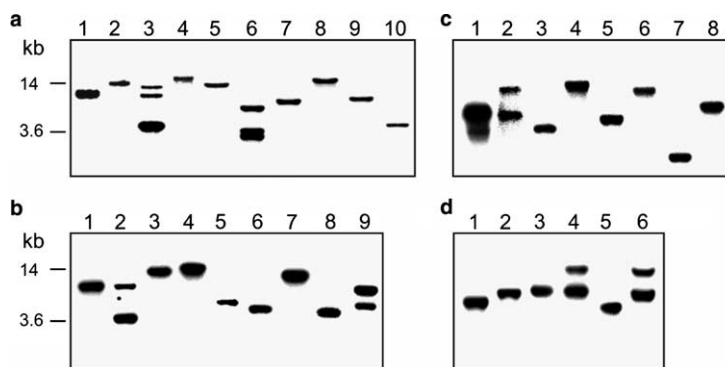


Fig. 4. Southern blot analysis of randomly chosen *L. biglobosa*, *L. maculans*, *O. yallundae* and *O. acuformis* GFP and DsRed transformants to determine copy number of T-DNA insertions. The genomic DNA was digested with *EcoRI*. Blots were hybridised using reporter-gene specific fragments. The presence of the GFP gene was detected (a) for *L. maculans* (lanes 1–5) and *L. biglobosa* (lanes 6–10) and (b) for *O. yallundae* (lanes 1–7) and *O. acuformis* (lanes 8 and 9). The presence of the DsRed gene was detected (c) for *L. maculans* (lanes 1–3) and *L. biglobosa* (lanes 4–8) and (d) for *O. yallundae* (lanes 1–3) and *O. acuformis* (lanes 4–6).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.femsle.2005.09.041](https://doi.org/10.1016/j.femsle.2005.09.041).

References

- [1] Rouxel, T., Mendes-Pereira, E., Brun, H. and Balesdent, M.-H. (2004) Species complex of fungal phytopathogens: *Leptosphaeria maculans*–*L. biglobosa* case study In: Plant Genome: Biodiversity and Evolution (Sharma, A.K. and Sharma, A., Eds.), pp. 33–75. Science Publishers, New Hampshire.
- [2] Mendes-Pereira, E., Balesdent, M.-H., Brun, H. and Rouxel, T. (2003) Molecular phylogeny of the *Leptosphaeria maculans*–*L. biglobosa* species complex. Mycol. Res. 107, 1287–1304.
- [3] Lucas, J.A., Dyer, P.S. and Murray, T.D. (2000) Pathogenicity, host-specificity, and population biology of *Tapesia* spp., causal agents of eyespot disease of cereals. Adv. Bot. Res. 33, 225–258.
- [4] Crous, P.W., Groenewald, J.Z. and Gams, W. (2003) Eyespot of cereals revisited: ITS phylogeny reveals new species relationships. Eur. J. Plant Pathol. 109, 841–850.
- [5] Huang, Y.J., Toscano-Underwood, C., Fitt, B.D.L., Hu, X.J. and Hall, A.M. (2003) Effects of temperature on ascospore germination and penetration of oilseed rape (*Brassica napus*) leaves by A- or B-group *Leptosphaeria maculans* (phoma stem canker). Plant Pathol. 52, 245–255.
- [6] West, J.S., Balesdent, M.-H., Rouxel, T., Narcy, J.P., Huang, Y.-J., Roux, J., Steed, J.M., Fitt, B.D.L. and Schmit, J. (2002) Colonization of winter oilseed rape tissues by A/Tox⁺ and B/Tox⁰ *Leptosphaeria maculans* (phoma stem canker) in France and England. Plant Pathol. 51, 311–321.
- [7] Wan, A.M., Bock, C.H., Fitt, B.D.L., Harvey, J.L. and Jenkyn, J.F. (2005) Development of *Oculimacula yallundae* and *O. acuformis* (eyespot) on leaf sheaths of winter wheat in the UK in relation to thermal time. Plant Pathol. 54, 144–155.
- [8] Nicholson, P., Turner, A.S., Edwards, S.G., Bateman, G.L., Morgan, L.W., Parry, D.W., Marshall, J. and Nuttall, M. (2002) Development of stem-base pathogens on different cultivars of winter wheat determined by quantitative PCR. Eur. J. Plant Pathol. 108, 163–177.
- [9] Williams, R.H. and Fitt, B.D.L. (1999) Differentiating A and B groups of *Leptosphaeria maculans*, causal agent of stem canker (blackleg) of oilseed rape. Plant Pathol. 48, 161–175.
- [10] Bowyer, P., Mueller, E. and Lucas, J. (2000) Use of an isocitrate lyase promoter-GFP fusion to monitor carbon metabolism of the plant pathogen *Tapesia yallundae* during infection of wheat. Mol. Plant Pathol. 1, 253–262.
- [11] Sexton, A.C. and Howlett, B.J. (2001) Green fluorescent protein as a reporter in the Brassica–*Leptosphaeria maculans* interaction. Physiol. Mol. Plant Pathol. 58, 13–21.
- [12] Spellig, T., Bottin, A. and Kahmann, R. (1996) Green fluorescent protein (GFP) as a new vital marker in the phytopathogenic fungus *Ustilago maydis*. Mol. Gen. Genet. 252, 503–509.
- [13] Mikkelsen, L., Sarrocco, S., Lübeck, M. and Jensen, D.F. (2003) Expression of the red fluorescent protein DsRed-express in filamentous ascomycete fungi. FEMS Microbiol. Lett. 223, 135–139.
- [14] Nahalkova, J. and Fatehi, J. (2003) Red fluorescent protein (DsRed2) as a novel reporter in *Fusarium oxysporum* f. sp. *lycopersici*. FEMS Microbiol. Lett. 225, 305–309.
- [15] Farman, M.L. and Oliver, R.P. (1988) The transformation of protoplasts of *Leptosphaeria maculans* to hygromycin-B resistance. Curr. Genet. 13, 327–330.
- [16] Cooley, R.N., Vangorcom, R.F.M., Vandenhondel, C. and Caten, C.E. (1991) Isolation of a benomyl-resistant allele of the

- beta-tubulin gene from *Septoria nodorum* and its use as a dominant selectable marker. *J. Gen. Microbiol.* 137, 2085–2091.
- [17] Chakraborty, B.N., Patterson, N.A. and Kapoor, M. (1991) An electroporation-based system for high-efficiency transformation of germinated conidia of filamentous fungi. *Can. J. Microbiol.* 37, 858–863.
- [18] Gardiner, D.M. and Howlett, B.J. (2004) Negative selection using thymidine kinase increases the efficiency of recovery of transformants with targeted genes in the filamentous fungus *Leptosphaeria maculans*. *Curr. Genet.* 45, 249–255.
- [19] Mueller, E., Bailey, A., Corran, A., Michael, A.J. and Bowyer, P. (2001) Ornithine decarboxylase knockout in *Tapesia yallundae* abolishes infection plaque formation in vitro but does not reduce virulence toward wheat. *Mol. Plant-Microbe Interact.* 14, 1303–1311.
- [20] Blakemore, E.J.A., Dobson, M.J., Hocart, M.J., Lucas, J.A. and Peberdy, J.F. (1989) Transformation of *Pseudocercospora herpotrichoides* using 2 heterologous genes. *Curr. Genet.* 16, 177–180.
- [21] Bunkers, G.J. (1991) Expression of the *Escherichia coli* beta-glucuronidase gene in *Pseudocercospora herpotrichoides*. *Appl. Environ. Microbiol.* 57, 2896–2900.
- [22] Wen-Jun, S. and Forde, B.G. (1989) Efficient transformation of *Agrobacterium* spp. by high-voltage electroporation. *Nucleic Acids Res.* 17, 8385.
- [23] Balesdent, M.H., Jedryczka, M., Jain, L., Mendes-Pereira, E., Bertrand, J. and Rouxel, T. (1998) Conidia as a substrate for internal transcribed spacer-based PCR identification of members of the *Leptosphaeria maculans* species complex. *Phytopathology* 88, 1210–1217.
- [24] Dyer, P.S., Nicholson, P., Rezanoor, H.N., Lucas, J.A. and Peberdy, J.F. (1993) Two allele heterothallism in *Tapesia yallundae*, the teleomorph of the cereal eyespot pathogen *Pseudocercospora herpotrichoides*. *Physiol. Mol. Plant Pathol.* 43, 403–414.
- [25] Bateman, G.L. and Taylor, G.S. (1976) Seedling infection of two wheat cultivars by *Pseudocercospora herpotrichoides*. *Trans. Br. Mycol. Soc.* 67, 95–101.
- [26] Sesma, A. and Osbourn, A.E. (2004) The rice leaf blast pathogen undergoes developmental processes typical of root-infecting fungi. *Nature* 431, 582–586.
- [27] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning – A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- [28] Xu, J.R. and Leslie, J.F. (1996) A genetic map of *Gibberella fujikuroi* mating population A (*Fusarium moniliforme*). *Genetics* 143, 175–189.
- [29] Church, G.M. and Gilbert, W. (1984) Genomic sequencing. *Proc. Natl. Acad. Sci. USA* 81, 1991–1995.
- [30] Bundock, P., Dendulkas, A., Beijersbergen, A. and Hooykaas, P.J.J. (1995) Transkingdom T-DNA transfer from *Agrobacterium tumefaciens* to *Saccharomyces cerevisiae*. *EMBO J.* 14, 3206–3214.
- [31] Hammond, K.E., Lewis, B.G. and Musa, T.M. (1985) A systemic pathway in the infection of oilseed rape plants by *Leptosphaeria maculans*. *Plant Pathol.* 34, 557–565.
- [32] de Groot, M.J.A., Bundock, P., Hooykaas, P.J.J. and Beijersbergen, A.G.M. (1998) *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. *Nat. Biotechnol.* 16, 839–842.