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Infection of *Rrs1* barley by an incompatible race of the fungus *Rhynchosporium secalis* expressing the green fluorescent protein

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Scald disease of barley, caused by the fungal pathogen *Rhynchosporium secalis*, is one of the most serious diseases of this crop worldwide. Disease control is achieved in part by deployment of major resistance (*Rrs*) genes in barley. However, in both susceptible and resistant barley plants, *R. secalis* is able to complete a symptomless infection cycle. To examine the *R. secalis* infection cycle, *Agrobacterium tumefaciens*-mediated transformation was used to generate *R. secalis* isolates expressing the green fluorescent protein or DsRed fluorescent protein, and that were virulent on an *Rrs2* plant (cv. Atlas), but avirulent on an *Rrs1* plant (cv. Atlas 46). Confocal laser scanning microscopy revealed that *R. secalis* infected the susceptible cultivar and formed an extensive hyphal network that followed the anticlinal cell walls of epidermal cells. In the resistant cultivar, hyphal development was more restricted and random in direction of growth. In contrast to earlier models of *R. secalis* infection, epidermal collapse was not observed until approximately 10 days post-inoculation in both cultivars. Sporulation of *R. secalis* was observed in both susceptible and resistant interactions. Observations made using the GFP-expressing isolate were complemented and confirmed using a combination of the fluorescent probes 5-chloromethylfluorescein diacetate and propidium iodide, in the non-transformed wild-type isolate. The findings will enable the different *Rrs* genes to be better characterized in the effect they exert on pathogen growth and may aid in identification of the most effective resistance.

Keywords: barley, GFP, *Hordeum vulgare*, plant pathogen, *Rhynchosporium secalis*, scald

Introduction

Scald or barley leaf blotch, caused by *Rhynchosporium secalis*, is a destructive disease of barley (*Hordeum vulgare*) prevalent in cool temperate climate zones (Shipton *et al.*, 1974). Typical visible symptoms on leaves begin as silvery areas which become darker and water-soaked and eventually develop into papery pale brown lozenge-shaped blotches with a dark brown rim (Caldwell, 1937; Davis & Fitt, 1990).

Infection of barley leaves begins following germination of conidia, when the germ tube directly penetrates the cuticle and hyphae form an inter-connected network, frequently following the outline of the anticlinal walls (at a right angle to the surface of the leaf) of epidermal cells (Jones & Ayres, 1974; Davis & Fitt, 1990). During this phase of growth the fungus has been described as biotrophic (Davis *et al.*, 1994; Zhan *et al.*, 2008) since no symptoms are observed and there is only localized degradation of the cell wall (Jones & Ayres, 1974). At this stage, it has been proposed that the hyphae absorb nutrients from leaky epidermal cells (Jones & Ayres, 1972). Other models of *R. secalis* infection have proposed that in the later stages of the infection process the fungus becomes nectrotrophic as first the epidermal cells and then the mesophyll cells collapse (Jones & Ayres, 1974; Lehnackers & Knogge, 1990). Conidia develop on extensions of the conidiogenous mycelium protruding through the leaf cuticle (Howlett & Cooke, 1987) with sporulation occurring with or without the development of symptoms visible to the unaided eye (Davis & Fitt, 1990; Davis *et al.*, 1994). The expression of symptoms may involve the production of fungal metabolites (Mazars *et al.*, 1990) since symptoms can occur beyond regions of the leaf colonized by *R. secalis* mycelium (Shipton *et al.*, 1974). Three necrosis-inducing peptides (NIPs) have been shown to cause cell death symptoms on leaves of both resistant and susceptible cultivars but are only produced by the fungus in compatible reactions (Wevelsiep *et al.*, 1991).

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The inheritance of resistance to scald has been investigated using a variety of barley cultivars and *R. secalis* races, leading to the conclusion that resistance to the pathogen is monogenetically inherited with several resistance loci being identified, although many quantitative trait loci have also been mapped where race-specificity is not known (Shipton *et al.*, 1974; Zhan *et al.*, 2008). The near-isogenic barley cultivars Atlas and Atlas 46 used in this study differ with respect to one particular resistance locus, *Rrs1*, located on chromosome 3H (Dyck & Schaller, 1961; Bockelman *et al.*, 1977; Søgaard & von Wettstein-Knowles, 1987). Five alleles at this locus have been described (Habgood & Hayes, 1971). Cultivar Atlas contains resistance locus *Rrs2* (Dyck & Schaller, 1961) whereas cv. Atlas 46 has, in addition, *Rrs1* (Dyck & Schaller, 1961; Habgood & Hayes, 1971). Although resistance to *R. secalis* appears to follow a gene-for-gene interaction, resistance is not mediated by the hypersensitive response. Indeed, infection by *R. secalis* may be symptomless in both susceptible and resistant cultivars, with *R. secalis* DNA detectable in resistant cultivars (Fountaine *et al.*, 2007; Atkins *et al.*, 2010).

Microscopy studies have shown the presence of subcuticular hyphae and sporulation taking place in the absence of visual symptoms (Davis & Fitt, 1990; Davis *et al.*, 1994). However, commonly used staining procedures in microscopy are often lethal to the pathogen and host and often do not reliably discriminate viable from non-viable pathogen tissue.

The ability to genetically manipulate fungal plant pathogens by insertion of foreign DNA has opened possibilities of using new molecular biology tools to dissect components of infection, as well as develop more sophisticated models of infection of host plants. In particular, transformation of fungal plant pathogens to express the green fluorescent protein (GFP) and other fluorescent proteins has been used extensively to track fungal infection of host plants (Bourett *et al.*, 2002; Lagopodi *et al.*, 2002; Fitt *et al.*, 2006; Campos-Soriano & San Segundo, 2009). *Rhynchosporium secalis* was first transformed by Rohe *et al.* (1996) to produce hygromycin-B and phleomycin resistance using polyethylene glycol (PEG)/$CaCl_2$ treatment of protoplasts. Since then, additional methods to transform fungi have become available. Of particular utility, due to its simplicity, has been the development of *Agrobacterium tumefaciens*-mediated transformation of fungi.

An enduring question in the development of *R. secalis* has been how the fungus grows within barley leaf tissue in resistant plants, completing its infection cycle, without producing symptoms. Here, *A. tumefaciens* transformation of *R. secalis* is used to generate transformants expressing GFP and DsRed fluorescent protein, to examine pathogen development in a resistant and susceptible barley cultivar. Infection time-courses, coupled with confocal laser scanning microscopy, were used to reveal differences in the development of the fungus in the near-isogenic barley cultivars. Findings with transgenic GFP-expressing *R. secalis* were complemented by a novel staining protocol in living plant tissue infected with *R. secalis*. The findings show that this strategy could also be used as a tool to identify different forms of resistance to scald in barley breeding programmes.

Materials and methods

Fungal culture conditions

Rhynchosporium secalis isolate R214 from the culture collection at the Scottish Crop Research Institute was grown on CZV8CM agar medium (Newton, 1989) at 17°C in the dark. This isolate causes no disease on barley plants carrying the *Rrs1* locus, but does cause disease on *Rrs2* plants (A.C. Newton, unpublished data). Cultures were maintained by transfer of mycelium to fresh CZV8CM agar plates every 2 weeks. Fungal conidia were harvested from approximately 14-day-old cultures by scraping the mycelial mat with a scalpel blade following the addition of 3 mL of sterile distilled water. The suspension was filtered through glass wool and resuspended in sterile distilled water at a concentration of 1×10^5 conidia per mL.

Agrobacterium tumefaciens transformation of *R. secalis*

Agrobacterium-mediated transformation was performed by modifying the methods described in Bundock *et al.* (1995), Gardiner & Howlett (2004) and Eckert *et al.* (2005). Cultures of *A. tumefaciens* strain AGL1 containing the plasmids pCAMBgfp or pCAMBDsRed used in Eckert *et al.* (2005) were received from Rothamsted Research, Harpenden, UK. The transformation was performed by co-cultivating log-phase *A. tumefaciens* containing the GFP or DsRed plasmid with *R. secalis* spores in induction medium (IM: 10 mM KH_2PO_4, 11·6 mM K_2HPO_4, 2·5 mM NaCl, 4 mM $MgSO_4$, 4 mM $(NH_4)_2SO_4$, 1 mM $CaCl_2$, 1 mM $FeSO_4$, 40 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) pH 5·3, 0·5% (w/v) glycerol, 0·2% (w/v) glucose) in the presence of acetosyringone (AS; 0·2 mM). Briefly, *A. tumefaciens* was grown in LB liquid medium (Sambrook *et al.*, 1989) at 28°C for 20 h. Bacterial cells were harvested by centrifugation at 16 100 ***g*** for 2 min, resuspended and grown in IM containing kanamycin (50 μg mL^{-1}) at 28°C until the optical density at 600 nm (OD_{600}) reached 0·25 or for 3 h. Spores were harvested from *R. secalis* isolate R214 and adjusted to a final concentration of 1×10^7–10^8 mL^{-1}. One hundred microlitres of spore solution, 100 μL of *A. tumefaciens* and 1 μL of 200 mM acetosyringone (AS) were mixed and spread on sterile cellophane discs (AA Packaging Ltd.), and placed on IM + AS (0·2 mM) agar plates. The plates were air dried, sealed and incubated in darkness at 17°C for 3 days. The cellophane discs were then transferred to minimal medium selection agar plates (IM without MES and glycerol) containing hygromycin (100 μg mL^{-1}) and ticarcillin

(150 μg mL^{-1}), and incubated in darkness at 17°C until fungal colonies were visible. These colonies were transferred to 2 mL of potato dextrose broth (PDB; Sigma-Aldrich) containing hygromycin (100 μg mL^{-1}) and ticarcillin (150 μg mL^{-1}) in a 24 well plate (Nunc) and incubated at 17°C for 2 days to kill any surviving *A. tumefaciens* cells. Fungal colonies from wells that remained clear were transferred to CZV8CM agar plates containing hygromycin (100 μg mL^{-1}).

Plant growth and inoculation conditions for *R. secalis*

Seeds of the barley (*Hordeum vulgare*) cvs Atlas and Atlas 46 were germinated and maintained in a glasshouse under 16 h daylength at 20°C during the day and 16°C at night for 10–15 days. Detached leaf assay was performed as described in Newton *et al.* (2001). Briefly, leaf sections approximately 3·5 cm in length were taken from near the base of either the first or second leaf, gently abraded near the centre of the adaxial surface with a sable hair paint-brush and placed in rectangular polystyrene boxes (79 × 47 × 22 mm) (Stewart Solutions) with the abaxial surface on 0·5% distilled water agar containing 120 mg L^{-1} benzimidazole (Sigma-Aldrich). The abraded area of each leaf was inoculated with 10 μL of spore suspension (1 × 10^5 spores per mL) and the boxes incubated in a controlled environment cabinet (Leec, model LT1201) at 17°C, light intensity 200 μmol m^{-2} s^{-1}. Leaf abrasion increases assay reliability and infection level but shows no interaction with cultivar (Newton *et al.*, 2001). Samples were taken for microscopic analysis at 10 different time-points after inoculation: 2, 3, 4, 7, 9, 10, 11, 16, 17 and 21 days post-inoculation (dpi). Six inoculated leaves were examined for each time-point. Infections were repeated on three separate occasions with different preparations of inoculum and plant leaves.

Fluorescent labelling

The CMFDA probe (CellTracker™ Green 5-chloromethylfluorescein diacetate; Invitrogen-Molecular Probes) was dissolved in dimethyl sulfoxide (DMSO) to 10 mM, and then diluted to 25 μM in water plus 1 μL mL^{-1} Silwet detergent (Vac-In-Stuff; Silwet L-77, Lehle Seeds) prior to use. The detergent was added to facilitate wetting of the leaf surface by the aqueous solution. The inoculated barley leaves were floated, adaxial (inoculated) surface down on this solution for at least 1 h in the dark at room temperature (approximately 20°C) before rinsing with water.

The leaf segments were further stained by floating on a solution of 1 mg mL^{-1} propidium iodide (PI) in water plus 1 μL mL^{-1} Silwet for 1 min before being blotted dry. The leaves were attached, adaxial surface up, to microscope slides and mounted in silicon oil (Dow Corning 200/100cs, VWR) under a coverslip for microscopic examination.

Confocal laser scanning microscopy

Hygromycin-resistant fungal colonies were initially viewed using a Leica MZ FLIII stereo-microscope under GFP1 Filter (excitation 425/60 nm, emission 480 nm) and GFP3 filter (470/40 nm, 525/50 nm) to identify those putative transformants expressing GFP or DsRed, respectively. Candidate transformants were then examined under the confocal microscope to confirm the typical excitation and emission spectra for GFP or DsRed. Confocal microscopy used either a Leica (Leica Microsystems) SP1 confocal laser scanning microscope (CLSM) mounted on a DMLFS microscope or an SP2 CLSM on a DM6000 microscope fitted with a FI/RH filter block (excitation filter BP 490/15, dichroic mirror 500, emission filter BP 525/20; excitation filter BP 560/25, dichroic mirror 580, emission filter BP 605/30) and water dipping lenses (HCX APO L10×/0·30 W U-V-1, L20×/0·50 W U-V-1, L40×/0·80 W U-V-1 or L63×/0·90 W U-V-1). Images for GFP fluorescence were collected using excitation at 488 nm with emission collected at 500–530 nm with the autofluorescent signal from chlorophyll being collected simultaneously at emission 650–700 nm. Images using 5-chloromethylfluorescein (CMF) and propidium iodide fluorescence were collected sequentially using excitation at 488 nm and emission 500–530 nm followed by excitation at 561 nm and emission 635–655 nm. Unless otherwise stated, images are presented as maximum intensity projections and were assembled and edited using Adobe Photoshop CS version 8.0.

Results

Transformation of *R. secalis*

Transformations using the GFP-encoding plasmid delivered by *A. tumefaciens* yielded two colonies that grew on hygromycin selection plates, while transformations using the DsRed-encoding plasmid yielded 21 colonies. These colonies were initially viewed under a fluorescence microscope to identify candidate transformants that were expressing the fluorescent proteins. Colonies that fluoresced were further examined under the confocal microscope to determine the emission spectra of each transformed isolate compared to the level of autofluorescence from the wild-type isolate. Although the wild-type isolate showed a low level of fluorescence when excited at 488 nm, both GFP and DsRed transformants demonstrated peaks in their emission spectra characteristic of GFP and DsRed, respectively (Fig. 1), thereby confirming their transformation. The growth rate of transformed isolates was compared to that of wild-type isolates *in vitro* and no significant difference was observed. All transformants retained full sporulation and pathogenicity when compared with non-transformed wild-type *R. secalis* isolate R214 (data not shown). The DsRed transformed isolates were not used in this experiment as the autofluorescence emission from chloroplasts was in the same

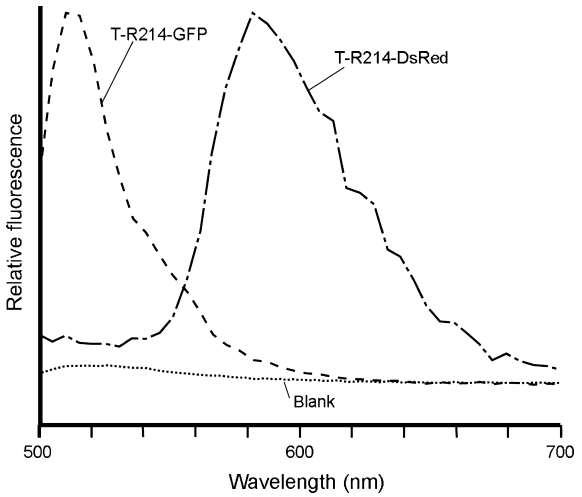

Figure 1 Fluorescence emission spectra for *Rhynchosporium secalis* transformants expressing GFP or DsRed collected at an excitation of 488 nm. The dashed line represents transformants expressing GFP (T-R214-GFP) with a peak in emission around 510 nm, characteristic of GFP. The broken line represents transformants expressing DsRed (T-R214-DsRed) with a peak in emission around 580 nm, characteristic of DsRed. The dotted line represents the non-transformed *R. secalis* isolate R214, which yielded only minimal levels of fluorescence in the wavelength range tested.

spectral range as DsRed emission. GFP-transformed isolate R214 was used further for this study and will be referred to as T-R214-GFP hereafter.

T-R214-GFP infection of barley cv. Atlas (*Rrs2*; compatible)

Although inoculated leaves were examined by confocal microscopy at 10 different time-points, four time-points representing major stages in *R. secalis* infection will be described here. Leaves were first observed by confocal microscopy at 2 dpi. The inoculum appeared to be well distributed with GFP-expressing spores observed at all places examined across the inoculation site and without mycelial debris. Germination of the spores at 2 dpi was clearly evident, (85 ± 0·8%) often producing branched mycelium (Fig. 2a).

The germinating mycelium had significantly elongated by 3 dpi, producing more branched mycelium, thereby establishing patches of hyphal network at different places within the inoculation site. The hyphae penetrated the cuticle without the formation of an appressorium or by invasion through stomata. The hyphae did not penetrate deep into the host tissue, but grew on the surface of the epidermal cells, frequently overlying the anticlinal walls, growing preferentially longitudinally and establishing a clear outline of mycelium around the epidermal cells (Fig. 2b).

By 10 dpi these patches of hyphae had progressed to form an extensive network that either spread throughout the inoculation site or in patches. The epidermal cells appeared intact as the sideways projection of the Z stacks (set of images acquired at different depths (focal planes) within a sample), showed a non-fluorescing layer between the GFP-fluorescing hyphae and auto-fluorescing chloroplasts in the mesophyll layer (Fig. 2c). There was also spread and growth of hyphae outwith the inoculation site at this point, although there were no visible symptoms of infection when viewed with the unaided eye.

After 21 dpi there was a confluent hyphal network observed at the inoculation site, which resulted in complete collapse of the underlying epidermis, since the sideways projection showed the mergence of the hyphal and chloroplast fluorescent layers (Fig. 2d). By this time there were necrotic spots on the leaf surface visible to the unaided eye. In contrast to the observations made at earlier time-points, the pattern of mycelial growth was denser, forming a closely packed mycelial network within the inoculation site (Fig. 2d). The margins of the advancing infection were similar to the observations at 3 dpi, with sub-cuticular hyphae aligned with the anticlinal walls, and no epidermal cell collapse. Spores were present on the hyphae emerging at the surface of the leaf throughout the entire inoculation site (Fig. 2e).

T-R214-GFP infection of barley cv. Atlas 46 (*Rrs1*, *Rrs2*; incompatible)

Initial observation of leaves at 2 dpi showed a reduction in spore germination in the incompatible compared with the compatible interaction (64 ± 2·4% cf. 85 ± 0·8%). However, the spores that did germinate developed normally producing branched hyphae (Fig. 2f). In contrast to infection of cv. Atlas at 3 dpi, there was no formation of a hyphal network. However, there was further, limited, and apparently undirected or disorganized, sub-cuticular hyphal growth without any penetration into the host cells (Fig. 2g) whilst the underlying epidermal cells remained intact.

By 10 dpi a hyphal network had developed but with a different pattern of development from that observed on cv. Atlas. The growth of mycelium did not predominantly follow the anticlinal walls connecting epidermal cells but rather grew randomly across the periclinal walls (parallel to the surface of the leaf) at the leaf surface (Fig. 2h). An additional obvious difference was the reduced number of colonies or infection sites formed. For example, on cv. Atlas there were several well established hyphal networks within the inoculation site at 10 dpi, but on cv. Atlas 46 there were fewer obvious infection sites and no extensive network of hyphae. Ungerminated spores were still observed at this time-point and there was no evidence of mycelial growth outwith the inoculation site on cv. Atlas 46.

After 21 dpi there was an increase in the extent of the hyphal network formed in cv. Atlas 46, compared with 10 dpi, although the extent of hyphal advancement was not as great as in cv. Atlas (Fig. 2i). Of the

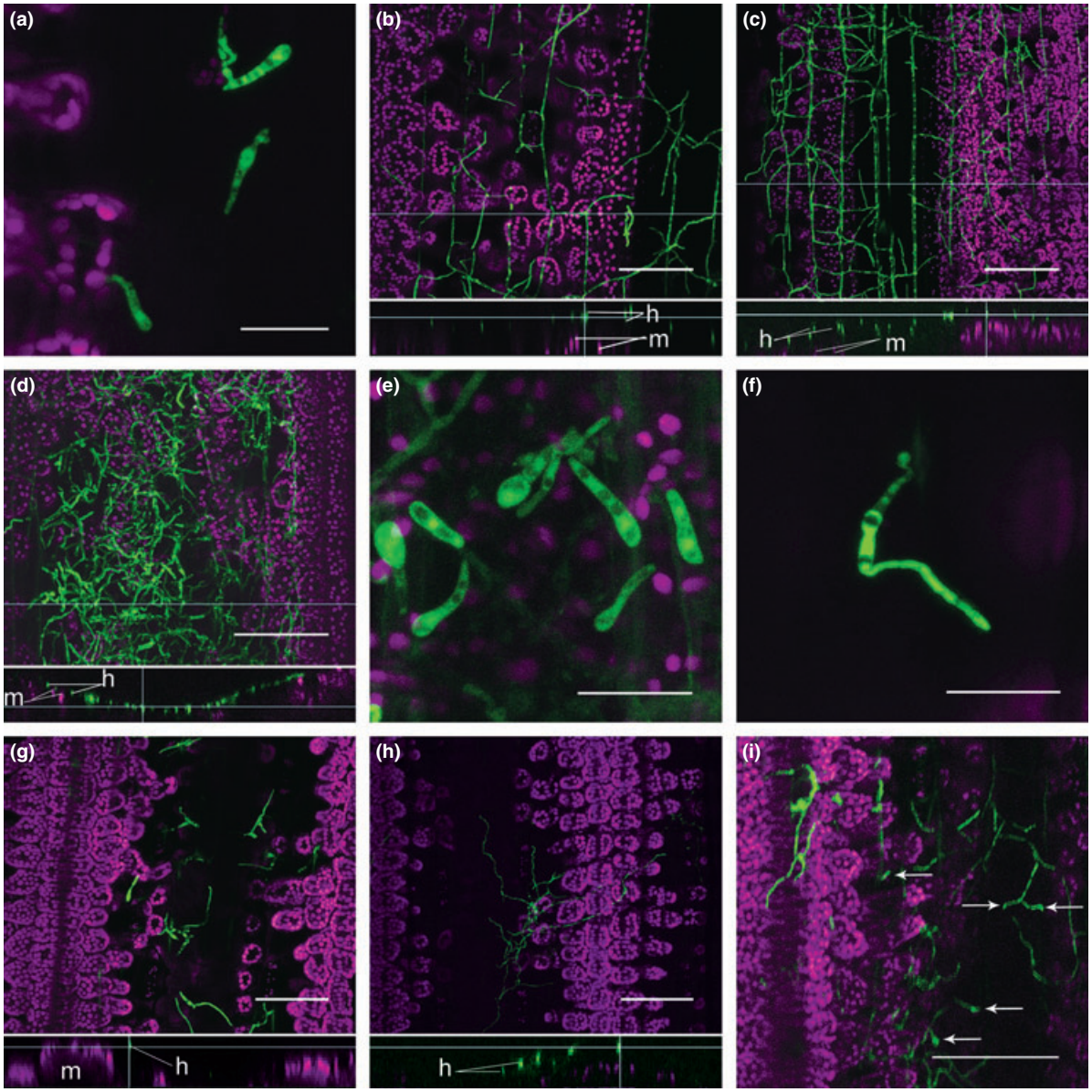

Figure 2 Confocal imaging infection of barley cvs Atlas and Atlas 46 by *Rhynchosporium secalis* transformant T-R214-GFP. At 2 dpi spores germinated on both cvs Atlas (a) and Atlas 46 (f). At 3 dpi, a hyphal network that followed the anticlinal cell walls was formed in cv. Atlas (b) overlying the epidermal cells. (b) Inset shows a sideways image or projection through the depth of the leaf at the position indicated by the horizontal line in (b) (m = mesophyll, h = hyphae) indicating a clear separation of hyphae from the mesophyll cells. By 10 dpi, the hyphal network was more extensive (c) but the epidermis had not collapsed as shown by the hyphae and mesophyll remaining spatially separated (c inset), The inoculation site on cv. Atlas was fully colonized by 21 dpi (d), the epidermal and mesophyll cells had collapsed (d inset), and sporulation was evident (e). In cv. Atlas 46 at 3 dpi, no organized hyphal network had formed (g) and this trend remained at 10 dpi (h). At 21 dpi in cv. Atlas 46, further colonization had occurred (i) with some sporulation evident (arrowed). Chloroplast autofluorescence is false-coloured magenta while the T-R214-GFP hyphae and spores are green. Scale bars represent 25 μm for a, e and f and 100 μm for all other images.

total number of infections observed at 21 dpi only 62% showed epidermal cell collapse, compared to total collapse in the compatible interaction. At this stage of infection ungerminated spores were still evident in most of the inoculated areas examined. In places where infection had established, spores were being produced (Fig. 2i).

Labelling of non-transformed *R. secalis* R214 infections of cvs Atlas and Atlas 46

Using a protocol involving CMFDA and PI, it was possible to label both the mycelia of non-transformed *R. secalis* R214 and plant cells of infection sites on either cv. Atlas or cv. Atlas 46. The green fluorescence which

accumulated in living cells of both the host and the fungus was presumed to be due to the cleavage of non-fluorescent CMFDA by intracellular esterases resulting in the formation of fluorescent CMF which initially accumulated in the cytoplasm, as in other organisms (Invitrogen-Molecular Probes Handbook section 14.2). Following conjugation to glutathione, the fluorescent product was transferred to the vacuole (Coleman *et al.*, 1997). Propidium iodide is recognized as a membrane-impermeant probe, and as such stained the cell walls of both the host and the fungus. However, during the later stages of infection, the probe also accumulated in the nuclei of plant and fungal cells, suggesting the presence of damaged or permeable plasma membranes.

As described for T-R214-GFP, a network of mycelia developed within 3 dpi on cv. Atlas, aligning with the anticlinal walls of the epidermal cells (Fig. 3a). By 10 dpi the mycelia formed a dense network associated with areas in which the epidermal cells had collapsed (Fig. 3b) with the nuclei of many plant cells accumulating PI, once again with spores forming on the hyphae within the inoculation site by 21 dpi (Fig. 3c and inset). Extensive areas of hyphae had accumulated PI, but not CMF, suggesting that membranes within these areas were compromised.

The growth of mycelia on cv. Atlas 46 was less rapid (Fig. 3d) than on cv. Atlas, resulting in a more random network of hyphae following periclinal walls by 10 dpi (Fig. 3e). Although at this stage there was no obvious sign of epidermal cell collapse, there was accumulation of PI by some nuclei, indicative of damage to the plasma membrane. As demonstrated using the transformed isolate, hyphal growth on cv. Atlas 46 was clearly less extensive (Fig. 3d–f), although some areas of epidermal cell collapse were visible by 21 dpi (Fig. 3f). Once again, areas of

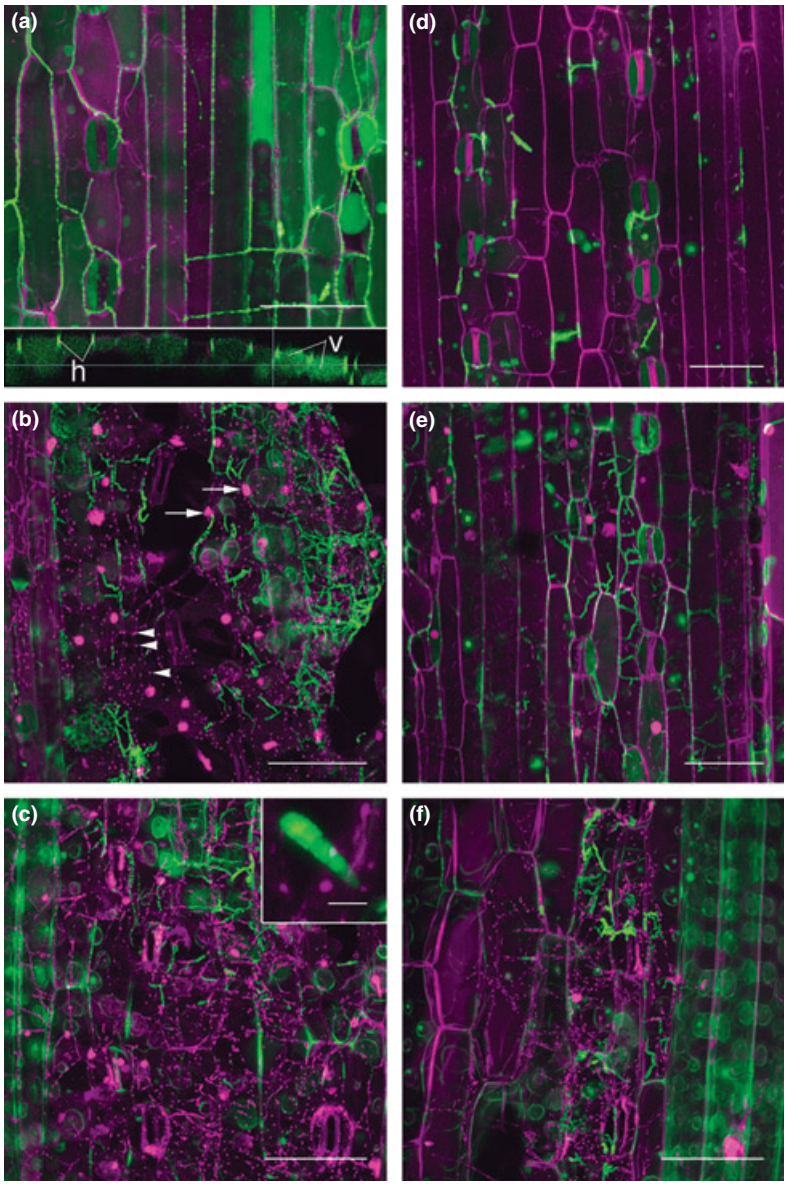

Figure 3 Confocal imaging infection of barley cvs Atlas and Atlas 46 by *Rhynchosporium secalis* using CMFDA and PI staining of wild-type R214. A hyphal network was established on cv. Atlas by 3 dpi (a) with hyphal growth overlying the epidermal cells (inset; h = hyphae, v = vacuole) and cellular integrity not compromised as shown by exclusion of PI. By 10 (b) and 21 (c) dpi on cv. Atlas, infection had established as in Figure 2c, and host cell integrity was compromised as shown by PI accumulation in plant cell nuclei (arrowed); PI also accumulating in the nuclei of some hyphae (darts); and sporulation was observed at 21 dpi (c and inset). The infection course in cv. Atlas 46 was also similar to that of the transformant (Fig. 2f–i) at 3 (d), 10 (e) and 21 dpi (f) although fewer host cells were damaged as shown by fewer PI-stained plant cell nuclei. CMFDA fluorescence is green and the PI staining is magenta. Scale bars represent 100 μm except for c inset, 5 μm.

the mycelium had not accumulated CMF but were stained with PI.

Discussion

Transformants of *R. secalis* were successfully obtained expressing either GFP or DsRed, and a labelling protocol for following infection in live plant tissue was developed. These resources have been used to examine the infection processes of *R. secalis* on near-isogenic lines of barley differing in the *Rrs1* resistance locus. This revealed that *R. secalis* can infect and grow within the resistant cultivar, although the colony morphology within the leaf tissue was markedly different to that in the susceptible cultivar.

Although *Rhynchosporium secalis* was first transformed by Rohe *et al.* (1996), using a protoplast-PEG/$CaCl_2$ protocol, this is the first report of *A. tumefaciens*-mediated transformation resulting in the expression of GFP or DsRed. The reliability of the protocol is supported by the successful transformation of one isolate with GFP and DsRed. The authors have since successfully transformed an additional isolate with GFP (unpublished), with many fluorescent colonies being generated. The formation of typical scald lesions on detached leaves from several susceptible cultivars grown under controlled environmental conditions verifies that the transformed isolates have retained their wild-type characteristics.

The labelling of infected tissue with the vital probe CMFDA provides a method to enable the study of early stages of *R. secalis* infection using non-transformed isolates under live conditions, with the advantage of indicating both live hyphae and intact epidermal cells. The results obtained here using non-transgenic R214 confirm that the pattern and extent of mycelium growth of the transformed isolate is representative of the wild-type on both cultivars. There was no significant difference in the rate of growth *in vitro* and both transformed and wild-type isolates showed similar sporulation and pathogenicity on differential cultivars. This transformation has therefore provided a valuable tool with which to evaluate the responses of susceptible and resistant cultivars to *R. secalis* infection during different stages of infection and disease development. In this instance the interaction between isolate R214 and the susceptible barley cv. Atlas and the resistant cv. Atlas 46 has been studied.

A greater proportion of spores observed on cv. Atlas had germinated than on cv. Atlas 46. This is consistent with the proposed host resistance mechanisms of inhibition of spore germination (Lehnackers & Knogge, 1990) and the prevention of cuticular penetration (Xi *et al.*, 2000).

In a previous investigation of the interaction between *R. secalis* race US238.1, and the same barley cultivars used here (cvs Atlas and Atlas 46), the collapse of epidermal cells occurred at an early stage (3 dpi), followed soon after by collapse of the mesophyll cells (Lehnackers & Knogge, 1990). Using the transformed isolate on cv. Atlas at 3 dpi, it appeared that the hyphae were growing on the surface of the epidermal cells although initially it was not possible to identify whether the cell walls had remained intact whilst the epidermal cell protoplasts may have collapsed (Ayesu-Offei & Clare, 1970). However, using the labelling protocol, the accumulation of CMF by the epidermal cell vacuoles confirmed that the cells were intact. Since PI remained excluded from these cells it was unclear whether the fungus had induced any alteration in membrane permeability by this stage (Jones & Ayres, 1972). At a later stage, approximately 10 dpi on cv. Atlas 46 infected with non-transformed R214, in spite of the vacuole of some epidermal cells clearly accumulating CMF, the staining of their nuclei with PI suggests that the plasma membrane had become permeable.

The most notable observation of this study was the marked difference in the morphology of *R. secalis* growth between the two cultivars, observable as early as 3 dpi. On cv. Atlas, as previously observed (Ayesu-Offei & Clare, 1970; Jones & Ayres, 1974; Lehnackers & Knogge, 1990), there was rapid development of a hyphal network following the anticlinal walls of the epidermal cells, preferentially in the longitudinal direction, resulting in the parallel alignment of hyphae. In marked contrast, the less extensive growth of hyphae on cv. Atlas 46 was less regular, growing apparently at random across the periclinal walls. This difference in morphology may indicate that part of the resistance demonstrated by cv. Atlas 46 results from the failure by R214 to establish a fully functioning mycelial network. This could result from recognition of the pathogen, leading to production of reactive oxygen species, secondary antimicrobial metabolites, host cell wall modification, or antimicrobial enzymes, all of which may result in the 'disorientation' of hyphal growth in cv. Atlas 46.

There was some variation in the timing of the later stages of infection on cv. Atlas between T-R214-GFP and R214; at 10 dpi there was no indication of cell wall collapse when infected with T-R214-GFP, but collapse of epidermal and mesophyll cells with R214 was clearly evident indicating that the transformed isolate may be slightly less aggressive than its parental wild-type, possibly due to the metabolic burden of over-expressing two additional proteins (hygromycin phosphotransferase and GFP). However, with both isolates, by 21 dpi there was extensive collapse of the epidermal cells along with some mesophyll cells and evident sporulation. In contrast to infections with race US238.1 on cv. Atlas 46 (Lehnackers & Knogge, 1990), restricted hyphal growth of both T-R214-GFP and R214 was observed, resulting in the formation of small patches of hyphal network, with a limited number of spores also being produced. In both cvs Atlas and Atlas 46, the production of spores did not necessarily coincide with the appearance of visible lesions, confirming previous reports of symptomless infection (Davis & Fitt, 1990; Davis *et al.*, 1994; Fountaine *et al.*, 2007; Atkins *et al.*, 2010).

As demonstrated previously (Xi *et al.*, 2000), it is clearly possible to differentiate between the susceptible and resistant interactions of R214 within 3 dpi, partially

on the basis of spore germination, but more significantly based on the formation of an effective hyphal network. It remains to be determined what factors are involved in restricting hyphal growth and development within the resistant interaction. However, the pathogen growth characteristics demonstrated clearly show that many host–parasite interactions occur at a cellular level, indicative of resistance mechanism differences that are not necessarily reflected in visible symptoms. A combination of fluorescently-labelled isolates, staining and confocal visualization could provide evidence of components of resistance complementary to those determined by symptoms alone. If extensive colonization correlates with yield penalties even in the absence of visual symptom development, these data may prove invaluable for identifying tolerant germplasm. Microscopic evaluation of germplasm using such techniques therefore represents a potentially important tool for plant breeders to obtain durably-resistant germplasm for sustainable agriculture.

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