Spatiotemporal changes in fungal growth and host responses of six yellow rust resistant near-isogenic lines of wheat

K. Saleem*abc, C. K. Sørensenad, R. Labouriaub and M. S. Hovmøllera

aDepartment of Agroecology, Aarhus University, Slagelse; bdDepartment of Mathematics, Aarhus University, Aarhus, Denmark; and cDepartment of Plant Protection Division, NIAB, Faisalabad, Pakistan

The objective of the present study was to investigate to what extent the macroscopic phenotype of incompatible host–pathogen interactions reflects differences in fungal development and host responses at the histological level. This was done by conventional and advanced microscopic analysis of six wheat near-isogenic lines differing by individual R genes and inoculated with an avirulent isolate of Puccinia striiformis. Wheat line AvocetYr15 had the lowest macroscopic infection type (IT) 0–1, in which fungal growth was stopped at early stages due to extensive expression of a hypersensitive response (HR) at all time points (4, 8 and 16 days post-inoculation, dpi) without any sign of haustorial bodies. AvocetYr5 and AvocetYr1 had IT 1, in which most fungal colonies developed secondary hyphae. Many colonies were encased by HR at 16 dpi, more extensively in AvocetYr1 than AvocetYr5. In AvocetYr6 (IT 2), HR was expressed after the formation of secondary hyphae at 4 dpi, after which fungal growth and HR increased simultaneously until most colonies became encased by HR. AvocetYr27 (IT 2–3) and AvocetYr17 (IT 4–5) showed similar fungal growth and HR at 4 dpi, where HR was only weakly expressed in a few host cells. Encasement of secondary and runner hyphae increased significantly in AvocetYr27, but at 16 dpi, HR was often circumvented by large, intermingled fungal colonies in both lines. No resistance responses were observed in Avocet S (susceptible control). The very different histological patterns conferred by the six R genes suggests differences in the timing of the host–pathogen recognition and onset of host defence pathways.

Keywords: histopathology, hypersensitive response, microscopy, Puccinia striiformis, R gene

Introduction

Yellow or stripe rust caused by the biotrophic fungus Puccinia striiformis f. sp. tritici (Pst) is one of the most destructive diseases of common wheat (Triticum aestivum) worldwide (Beddow et al., 2015). The ability of this biotrophic pathogen to have multiple asexual cycles in a single crop season associated with abundant spore production, long-distance spore migration capacity and a high rate of mutation may contribute to the escalating threat of yellow rust worldwide (Hovmøller et al., 2010). Host plant resistance is economical and environmentally friendly and the preferred disease management option. Host resistance can be conferred by R genes with major individual effects, which are often race-specific and expressed at all plant growth stages, or by multiple genes with minor effects, which are expressed quantitatively mainly at adult plant growth stages (Ellis et al., 2014). Up until now, 79 yellow rust resistance genes (R genes) have been permanently catalogued, while many additional temporarily designated Yr genes or quantitative trait loci (QTLs) have been reported (Wang & Chen, 2017; Feng et al., 2018). R genes usually encode cytoplasmic proteins that are directly or indirectly recognized by pathogen effector proteins (Jones & Dangl, 2006). Recognition of effectors by host R gene products may lead to effector-triggered immunity (ETI), which may be associated with a hypersensitive response (HR), i.e. death of infected and adjacent cells. Host cell autofluorescence is an indication of HR, often morphologically associated with distortion and disruption of cellular components (Mur et al., 2008; Sørensen et al., 2017). ETI-associated HR is an effective defence strategy for the host plant, particularly against biotrophic pathogens (Glazebrook, 2005), but effector recognition and the HR may vary significantly with respect to time and space (Wang et al., 2013). In a biotrophic host–pathogen interaction, the HR halts pathogen growth manifested by an incompatible phenotype, while in the absence of host–pathogen recognition, the pathogen may grow extensively and develop a compatible reaction.

The yellow rust–wheat interaction phenotype can be characterized through both macroscopic and microscopic assessment (Jagger et al., 2011; Sørensen et al., 2017). Macroscopically, the phenotype of the interaction involving an R gene with major effect is scored by infection types (IT), e.g. using a 0–9 scale (McNeal, 1971),
whereas partial resistance with minor effects is often scored by disease coverage using a percentage scale (Hovmøller, 2007). The phenotypic variation in R gene-mediated resistance may be governed by variation in fungal growth and host responses with respect to time and space under the given environmental conditions (Tang et al., 2015). The use of fluorescence and confocal microscopy have proven useful for investigating fungal growth patterns and host responses at the cellular level (Wang et al., 2013, 2015; Minker et al., 2016). Previous histological studies revealed that low IT of yellow rust-resistant wheat cultivars was associated with inhibition of fungal growth, delay of hyphal branching and decrease in the formation of haustorial mother cells (Kang et al., 2003). The Yr1-mediated seedling resistance was associated with extensive mesophyll cell death and early retardation of Pst infection (Bozkurtt et al., 2010). Similarly, seedling resistance in wheat cultivar Suwon 11 was associated with accumulation of hydrogen peroxide in an incompatible interaction (Wang et al., 2007). Further histological studies of the incompatible seedling response of Suwon 11 revealed the role of calcium ions, reactive oxygen species (ROS) and peroxidase in the HR (Wang et al., 2010; Yin et al., 2015). These previous studies highlighted different histological parameters but none of them investigated the spatiotemporal changes in fungal growth and extent of HR. More recently, the histological characterization of Yr2 revealed large variation in colony size, colony morphology and associated HR (Sørensen et al., 2017). The above studies provide motivation for histological characterization of widely used yellow rust resistance genes in agriculture. Different yellow rust resistance genes may display distinct spatiotemporal patterns of fungal growth and host HR as a phenotypic indication of their mode of action.

The present study investigated the hypothesis that differences in macroscopic phenotype (IT) conferred by a number of widely used Pst resistance genes reflect differences in fungal development and host responses at the histological level. The hypothesis was investigated by using six near-isogenic lines (NILs) of wheat variety Avocet and an avirulent P. striiformis isolate. The extent of fungal colony development and timing of associated host cellular responses are discussed in relation to recent discoveries of R gene function in the yellow rust–wheat pathosystem.

Materials and methods

Wheat lines and Pst isolate

Six near-isogenic Avocet lines containing Yr genes (Yr1, Yr5, Yr6, Yr15, Yr17 and Yr27) along with susceptible control Avocet S (Wellens et al., 2009) were used. Ten seeds of each wheat line were sown individually in small pots (7 × 7 × 7 cm) with standard peat-based mix (Pindstrup Mosebrug A/S). Plants were grown in spore-proof growth cabinets, 16 h day/8 h night, and provided with artificial light 50–100 μmol m⁻² s⁻¹ when daylight was <10 000 lux. The temperature was adjusted to 18 °C in the day and 12 °C at night. Seven seedlings were maintained in each pot.

The Pst isolate GB75/30 with avirulence for the six considered R genes (Hovmøller, 2007) was used in this study. Spore ampules were retrieved from liquid nitrogen (−196 °C) and subjected to heat shocked treatment at 40–42 °C for 2 min prior to inoculation. Approximately 5 mg of spores was suspended in 3 mL engineered fluid Novec 7100 (3M) and gently mixed. A standard GRRC differential set (Hovmøller et al., 2017) were spray inoculated with the spore suspension using an airbrush spray gun (standard class, Revell GmbH) in a laboratory fume hood to test isolate purity. At the same time, seedlings of wheat cultivar Anja were inoculated for spore multiplication. The seedlings were subsequently sprayed with mist water, and the pots were incubated in a dew chamber at 10–12 °C in darkness for 24 h, after which they were transferred to spore-proof greenhouse cabins at 18 °C day/12 °C night with a 16 h photoperiod of natural light and supplemental sodium light (100 μmol s⁻¹ m⁻²) and 8 h dark. Plants were watered automatically with added micronutrients for 10 min every 12 h. The multiplication pots were covered with cellophane bags (Helmut Schmidt Ver-packungsfolien GmbH) 7 days after inoculation (prior to sporulation). Spores for experimental use were harvested 14–16 days post-inoculation (dpi) by shaking the plants inside the cellophane bag and transferred to cryovials and dried in a desiccator for 3 days before the samples were preserved in a −80 °C freezer. The differential set was assessed for virulence phenotyping 17 days after inoculation using a 0–9 scale (McNeal, 1971) where infection types 0–6 were considered as incompatible, and 7–9 were considered compatible. The virulence phenotype of GB75/30 was confirmed and the spore samples were 100% pure.

Inoculation and sample collection

Six pots (replicates) each with seven seedlings were used per Avocet line. Six leaves from each pot were inoculated with GB75/30 and one leaf was mock-inoculated with Novec engineered fluid, in both cases using point inoculation (Sørensen et al., 2016) with little modification. In this inoculation method, the second leaf of 15–16-day-old seedlings was fixed on acrylic pedestals (22.5 × 15 × 15, length × width × height) using double-sided tape followed by overlay with surgical tape (Leukofix; BSN). Three pots (21 leaves) per pedestal were fixed and placed in plastic trays. Five microlitres of spore suspension (2 mg mL⁻¹ of Novec 7100) was applied to the 5 cm central part of each leaf using a micropipette. The micropipette was moved along the leaf surface to spread the spore suspension. Each small tray with acrylic pedestal was placed in a bigger tray (55 × 45 × 7 cm, length × width × height) and after inoculation, lids were misted with water and put on the tray to create a humid microenvironment. After inoculation, plants were shifted to a dew chamber at 10–12 °C in darkness for 24 h, after which they were transferred
Fixation, staining and microscopy

Leaf samples were collected and processed for microscopic investigation according to Moldenhauer et al. (2006) with little modification. In brief, leaf segments were collected and fixed in fixation solution (ethanol: chloroform: 3:1, v/v) + 0.15% (v/w) trichloroacetic acid) for at least 24 h. The fixation solution was changed and fresh solution added to tubes after 24 h for complete removal of chlorophyll. The leaf segments were washed twice in 50% ethanol for 10 min and then 0.05 M NaOH was added. Samples were kept in this solution for 30 min for clearing leaves followed by two washes with deionized water (DI). Afterwards, 0.1 M Tris.HCl buffer (pH 5.8) was added in Falcon tubes and left in a fume hood for 30 min. Leaf segments were then ready for staining with dye Uvitex 2B (Polysciences Inc.). Leaf samples were stained in 0.1% (w/v) Uvitex 2B (dissolved in 0.1 M Tris.HCl buffer) for 5 min. Samples were then washed four times in deionized water (DI) followed by one wash in 25% glycerol and left overnight in DI to remove the extra stain. Leaf samples were stored in 50% glycerol until further microscopic investigation.

For the microscopic investigation, leaf segments were mounted on a glass slide containing 75% glycerol. Colony size and extent of HR was measured with a calibrated eyepiece micrometer and the size was calculated as largest length × largest width × π/4 (Baart et al., 1991). The extent of HR was measured as a percentage of the area showing autofluorescence around the fungal colony and the description of quantitative measurements of HR is given in Figure S1. Images of representative colonies were recorded using a FV1200 confocal laser scanning microscope (Olympus). Leaf tissues and fungal structures were excited with laser beams of 405 and 515 nm lasers and two channels with filter settings of 460–425 nm and 535–470 nm, respectively, detected emitted light signals. Layers of confocal plane with 0.75 μm separation were collected for Z-stacks.

The confocal microscope in-built program IMARIS BITPLANE v. 6.2 was used to merge the images of two channels and execute their 3D projections. All resulting 2D images were adjusted for brightness and contrast to obtain an equal representation of the background signal from the healthy plant tissue using the free software PAINT.NET (https://www.getpaint.net/).

Statistical analysis

The responses in terms of colony sizes and HR recorded at different time points were compared for each R gene by the nonparametric Kruskal–Wallis test (Lehmann, 2006), because these responses were neither normally distributed and homoscedastic, nor adhered to any standard parametric statistical model. These parameters were characterized by their medians and the corresponding confidence interval obtained by nonparametric bootstrap (based on 10 000 bootstrap samples). The analyses were carried out using the R software (R Development Core Team, 2018); ad hoc pairwise comparisons and nonparametric bootstrap confidence intervals for the medians were calculated using the R package PAIRWISE.COMPARISONS. The simultaneous tests were adjusted for multiple comparisons using the FDR (controlled false discovery rate) method (Benjamini & Hochberg, 1995).

Results

Macroscopic phenotype

The temporal development of macroscopic phenotype was followed by scoring the IT on the six Avocet NILs and the susceptible parental line (Avocet S) at 4, 8 and 16 dpi. As expected, the IT changed according to time with respect to the level of chlorosis, necrosis and pustule development (Fig. S2). No differences among lines were detectable at 4 dpi, while at 8 dpi minor differences in the level of chlorotic and necrotic flecks were observed. The typical IT of the individual lines were observed at 16 dpi (Fig. 1a). AvocetYr15 had the lowest score (IT 0–1) followed by AvocetYr5 (IT 1), then AvocetYr1 (IT 3), with more chlorosis than the previous two. AvocetYr6 showed IT 2 with large necrotic areas whereas AvocetYr27 with IT 2–3 had minor sporadic pustules on some replicate leaves. AvocetYr17 showed an intermediate IT score of 4–5 in which patches of necrosis and sporulating pustules were observed. Avocet S was susceptible (IT 7) with abundant sporulation. The infection types of the seven Avocet lines were consistent across the six replicates, but the extent of necrotic and/ or chlorotic areas and sporulation varied as indicated by the box plots (Fig. 1b).

Fungal growth and host responses

The colony size distribution and HR relative to colony size were assessed at 4, 8 and 16 dpi for all incompatible interactions (Fig. 2). The susceptible parental line Avocet
S, observed at 2, 4 and 6 dpi, had exponential colony growth with no sign of HR (Fig. S3, Table 1). It was not possible to measure colony size beyond 6 dpi in this line due to merging of adjacent colonies. In AvocetYr15 almost all colonies appeared in the smallest size category at all time points, indicating that the growth of most colonies was stopped within 4 dpi. Relative HR was high already at 4 dpi, where 94% of colonies were at least 75% covered by autofluorescent host cells. At 16 dpi all colonies showed very high level of HR coverage. The results suggest that approximately half of the small colonies at 4 dpi were bigger at 8 dpi and they were all bigger at 16 dpi. About 60% of colonies at 16 dpi were stopped before they reached the third category of colony size (0.1–1 mm²) and no colony larger than 1 mm² was observed. The frequency of colonies covered by autofluorescent cells almost doubled from 52% at 4 dpi to >90% at 8 dpi and stayed high at 16 dpi. AvocetYr1 showed a similar pattern of colony growth as AvocetYr5, but the frequency of colonies with high degree of encasement (relative HR of 76–100%) was smaller at 8 and 16 dpi. In AvocetYr6 a wider range of colony sizes and relative HR at 4, 8 and 16 dpi were observed, which indicated that fungal growth was stopped during different stages of infection. At 4 dpi, 54% of colonies showed either no or low HR encasement (1–25%), which is probably the reason that a large proportion of colonies (>70%) were bigger at 8 dpi than at 4 dpi. At 16 dpi about 60% of colonies were stopped before reaching size category 1–10 mm² and 72% of colonies showed high levels of HR (76–100%). The pattern of colony growth and relative HR was similar in both AvocetYr27 and AvocetYr17, and colonies appeared to grow continuously bigger from 4 to 8 dpi. However, the frequency of colonies in size category 1–10 mm² and low HR coverage (1–25%) was higher at 8 dpi in AvocetYr17 than in AvocetYr27. In

**Figure 1** Yellow rust infection phenotype of six Avocet wheat lines carrying different R genes and susceptible control Avocet S at 17 days post-inoculation. Variation in macroscopic necrotic and or chlorotic area was observed on different wheat Avocet lines (a). Invisible to minute flecks were observed in AvocetYr15 while cleared chlorotic flecks were appeared in AvocetYr15. Extent of necrotic area was variable in AvocetYr1, AvocetYr6 and AvocetYr27 while large necrosis along with spores were observed on AvocetYr17. Variation in infection type was shown via boxplot (b) in which AvocetYr15, Yr5, Yr1, Yr6, Yr27 had an incompatible response (IT 0–3) and were consistent in all replications. AvocetYr17 had an intermediate response (IT 4–5) while Avocet S (control) was compatible in all replications.
both lines the colonies were intermingled at 16 dpi and could no longer be differentiated.

Median values and ranges of colony growth and relative HR for the different Avocet NILs were calculated for all time points to provide a summary of results (Table 1; Fig. 3). The median colony size changed significantly between time points in all lines, and significant differences were observed for most of these. The summaries stress the generally small colony sizes in AvocetYr15, AvocetYr5 and AvocetYr1 at all time points, and significantly larger colony sizes in AvocetYr6, AvocetYr27 and AvocetYr17. The most significant increase in median colony size was observed in AvocetYr17 where it changed from 0.014 mm² at 4 dpi to 1.366 mm² at 8 dpi. The patterns of median HR values mirrored these results, with the most extensive HR relative to colony size observed in AvocetYr15, AvocetYr5 and AvocetYr1. The maximum colony size changed significantly between time points in most lines (Table 1). The smallest increase in the maximum colony size was

Figure 2 Frequency distribution for colony size of Puccinia striiformis f. sp. tritici isolate GB/75/30 on six wheat Avocet lines at 4, 8 and 16 days post-inoculation (dpi) and host responses as relative hypersensitive response (HR). Colony sizes were measured in five leaves of each line and 50 colonies at each time point were visualized. Bars in histograms show number of colonies (%) under five category ranges. HR relative to colony size was estimated as percentage leaf area around the fungal structures which exhibited autofluorescence in five leaves of all Avocet lines. Bars indicate the number of colonies (%) associated with different levels of HR. In AvocetYr27 and AvocetYr17, colony size and HR was estimated only at 4 and 8 dpi because colonies were intermingled and unmeasurable thereafter.
observed in AvocetYr15. Further increases were observed in AvocetYr1 and AvocetYr5 and relatively large changes were seen in AvocetYr6 followed by AvocetYr27 and AvocetYr17. The overall spatial distribution of HR within and among colonies was further studied by confocal microscopy.

Table 1 Colony sizes of *Puccinia striiformis* isolate GB75/30 in seven Avocet wheat lines carrying different R genes and extent of host response as hypersensitive reaction (HR) recorded at 4, 8 and 16 days post-inoculation (dpi).

| Wheat line | dpi | Colony size (mm²) | HR (%) |
|------------|-----|------------------|--------|
|            |     | Median | Range | Median | Range |
| AvocetYr15 | 4   | 0.0045 A | 0.006-0.01 | 100 b | 75-100 |
|            | 8   | 0.0043 A | 0.007-0.01 | 100 b | 75-100 |
|            | 16  | 0.0064 B | 0.002-0.03 | 100 b | 90-100 |
| AvocetYr5  | 4   | 0.0059 A | 0.007-0.02 | 90 a | 10-100 |
|            | 8   | 0.0101 B | 0.018-0.10 | 100 b | 50-100 |
|            | 16  | 0.0865 C | 0.013-0.36 | 100 b | 50-100 |
| AvocetYr1  | 4   | 0.0073 A | 0.001-0.14 | 95 a | 0-100 |
|            | 8   | 0.0115 B | 0.002-0.18 | 100 a | 25-100 |
|            | 16  | 0.0793 C | 0.003-0.36 | 90 a | 50-100 |
| AvocetYr6  | 4   | 0.0471 A | 0.004-0.20 | 25 a | 0-100 |
|            | 8   | 0.1588 B | 0.02-1.03 | 75 b | 20-100 |
|            | 16  | 0.9444 C | 0.08-2.91 | 90 c | 50-100 |
| AvocetYr27 | 4   | 0.0093 A | 0.001-0.29 | 50 b | 0-100 |
|            | 8   | 0.5087 B | 0.005-3.36 | 25 a | 0-100 |
|            | 16  | NA a | NA | NA | NA |
| AvocetYr17 | 4   | 0.014 A | 0.001-0.19 | 62 b | 0-100 |
|            | 8   | 1.3688 B | 0.11-6.48 | 25 a | 0-90 |
|            | 16  | NA b | NA | NA | NA |
| Avocet S  | 2   | 0.0067 A | 0.002-0.014 | 0 | 0 |
|            | 4   | 0.0758 B | 0.012-0.18 | 0 | 0 |
|            | 6   | 3.335 C | 0.47-8.34 | 0 | 0 |

Differences in colony size and HR at individual time points were determined by Kruskal–Wallis tests. Columns followed by same letters are not statistically significantly different at a 5% confidence level (separate battery of tests made for each R gene).

*aNA, not applicable, colonies were intermingled and could no longer be differentiated.

Histological features

Representative colonies were selected for each interaction of Avocet NILs and Pst at three time points for a more detailed observation of colony morphology and associated host autofluorescence through confocal laser scanning microscopy (Fig. 4). All fungal colonies developed the basic structures which included substomatal vesicle (SSV), primary hyphae (PH), haustorial mother cell (HMC); for some interactions, haustoria (H), secondary hyphae (SH), runner hyphae (RH) and pustules (P) were also observed. HR was observed in association with fungal colonies in all Avocet NILs except Avocet S, but the extent varied between time points and lines. In general, HR was prominent in host cells in close connection with fungal structures where host cells often became distorted (Fig. 4). In AvocetYr15 most colonies only developed the basic structures with no sign of haustoria at any time point. The colonies were generally encased by autofluorescent host cells. For many colonies, autofluorescence was only observed in the host cells in direct contact with fungal structures, but in some cases the responses had spread to the adjacent cells. In AvocetYr5 all colonies had developed the basic structures at 4 dpi whereas at 8 and 16 dpi most colonies also had secondary infection hyphae and in some cases short runner hyphae. There was no sign of haustoria at any time point. All colonies were to a large extent encased by autofluorescent host cells, and at 8 and 16 dpi the responses had often spread to cells several layers away from the fungal structures. Colonies in AvocetYr1 showed a similar pattern to AvocetYr5, although small haustoria were observed at 4 dpi and most of the colonies formed secondary hyphae at 8 and 16 dpi. Many host cells around secondary hyphae were distorted and showed autofluorescence. The extensive autofluorescence was observed not only around the fungal structures but also in adjacent host cells. In AvocetYr6, host autofluorescence was observed at the periphery of secondary hyphae in most of the colonies at 4 dpi. At 8 dpi both fungal growth and host...
Figure 4  Variation in colony morphology of yellow rust fungus and associated host responses, in six Avocet wheat lines carrying different R genes along with Avocet S (control) recorded at 4, 8 and 16 days post-inoculation (dpi) with *Puccinia striiformis* f. sp. *tritici* isolate GB75/30. Leaves of all seven wheat NILs were stained with Uvitex 2B and images of fungal structures along with associated host autofluorescence were acquired by confocal laser scanning microscopy. Basal fungal colony structures include germ tube (GT) which penetrate through stomata (S) and develop into substomatal vesicles (SSV). SSV develop into two primary infection hyphae (PH) and haustorial mother cells (HMC). HMC develop into haustoria (H), secondary hyphae (SH) and runner hyphae (RH). Simultaneously, variable levels of autofluorescence (AF), as an indication of hypersensitive response (HR) are seen in close contact with fungal structures. (a–c): AvocetYr15: very small fungal colonies that consist of SSV, PH and HMC were observed with strong and robust autofluorescence. (d–f): Fungal growth on AvocetYr5: SSV, PH, HMC and SH were observed along with HR in host mesophyll cells that are in contact with fungal structures. HR was highly localized and no haustorium was observed. (g–i): Fungal colonies on AvocetYr1: SSV, PH, HMC, H and SH were seen whereas autofluorescence appeared to surround the entire colony at all time points. HR was observed in infected host cells and spread to adjacent uninfected cells. (j–l): AvocetYr6: large fungal colony along with many haustoria and secondary hyphae were seen but HR first observed at the edges and then escalated simultaneously with fungal colony. Fungal colony appeared as bunch of mycelium surrounded by HR as final observation. (m–o): Fungal colonies on AvocetYr27: many secondary and runner hyphae were seen at 16 dpi but growth was less vigorous and patchy because of weak and delayed host autofluorescence. (p–r): Fungal growth on AvocetYr17: colony was small with SSV, PH, HMC and H, along with weak autofluorescence. Delayed and weak HR resulted in rapid colony growth and HR appeared at the edges while large and intermingled mycelium along with few pustules were seen at 16 dpi. (s–u): Fungal development on Avocet S (control): rapid and vigorous colonies along with many pustules were observed without any sign of autofluorescence. Scale bar = 40 \( \mu \)m.
autofluorescence increased, and most fungal colonies had formed several runner hyphae with complete encasement by autofluorescent host cells at 16 dpi. In many colonies, autofluorescence was shown by host cells in direct contact with fungal structures. All fungal colonies in AvocetYr27 developed the basic structures at 4 dpi with weak autofluorescence in only a few host cells, while at 8 dpi many colonies had developed secondary and runner hyphae surrounded by extensive host autofluorescence. This autofluorescence spread to other host cells along the direction of fungal growth, and at 16 dpi the colonies had grown big with intermingled branched mycelia. The distribution of fungal colonies and host autofluorescence were similar in AvocetYr17 and AvocetYr27 at 4 dpi. At 8 dpi, the autofluorescent host cells were observed only at the edges of secondary and runner hyphae in AvocetYr17 while in AvocetYr27, most of the fungal structures were encased by extensive HR. At 16 dpi, typical colonies showed a highly branched mycelium with pustules and weak autofluorescence was shown by a few host cells. In the susceptible control, Avocet S, all colonies had developed secondary hyphae at 4 dpi, converting into many runner hyphae at 8 dpi without any sign of host autofluorescence. All colonies at 16 dpi showed a network of mycelia with abundant pustules.

Discussion

The present study links macroscopic phenotype with detailed histopathological characteristics of race-specific R gene-based resistances, which provides new insights to discern the various resistance functions of these genes against Pst. A pilot study, comprising 36 wheat lines carrying different R genes inoculated by six wheat-adapted Pst isolates, was executed prior to the main experiment. Most of the wheat lines were compatible to one or more of these Pst isolates. The isolate selected for the current study showed a high level of compatibility on susceptible control varieties, including the Avocet parental line, coupled with incompatibility for most of the resistant Avocet NILs. It is reasonable to assume that the phenotypes of the six resistant Avocet lines are due to the effect of the individual R genes. The selected isolates carry avirulence for several R genes, which makes it possible to study the effect of several R genes without having to interpret the possible influence of the genetic background of more isolates. The Pst isolate used in the current study was avirulent on all six Avocet NILs and showed a distinct infection type.

The distinct spatial and temporal pattern of fungal colony size and host defence responses clearly reflected the macroscopic infection type of six near-isogenic wheat lines with different R genes. AvocetYr15 had the minimum fungal growth and maximum HR coverage that was expressed at a very early stage of infection. AvocetYr3 and AvocetYr1 showed complete HR encasement at later stages of infection, mostly after the development of secondary hyphae. The pattern of AvocetYr6 appeared highly dynamic, in which expression of HR and fungal growth increased simultaneously. In AvocetYr17 and AvocetYr27 the expression of HR was delayed, resulting in bigger fungal colonies. Few big colonies became largely covered by HR in AvocetYr27; however, at later stages of infection the HR was often circumvented by large, intermingled fungal colonies in both lines. These results of large spatiotemporal variation in fungal growth and R genes resistance responses suggest differences in recognition and induction of the underlying resistance mechanism.

The use of histopathology for comparison of ITs and microscopic phenotype were previously described for leaf rust (Wang et al., 2013) and stem rust (Wang et al., 2015). Colony size has also been used to characterize the microscopic yellow rust phenotype (Moldenhauer et al., 2006; Jagger et al., 2011). In all of these studies, HR was measured as a qualitative parameter, whereas here, HR was measured quantitatively as the percentage of a fungal colony covered by autofluorescent host cells. During the expression of host autofluorescence in biotrophic interactions, the production of reactive oxygen species (ROS) is one of the earliest defence reactions activated after successful pathogen recognition and leads to the host cell death (Dickman & Fluh, 2013). ROS-induced HR does not only have antimicrobial activity but also spreads defence responses to adjacent host cells against avirulent pathogen isolates (Coll et al., 2011). The HR expression of host cells during earlier stages of fungal growth may result in a low IT whereas delayed HR may result in more fungal growth and a higher IT. In the present study, the spatiotemporal changes showed distinct patterns of colony size and HR in the six Avocet NILs.

AvocetYr15 (IT 0–1) was histologically characterized by small fungal colonies that were stopped at an early infection stage and largely covered with host autofluorescence without any haustoria. The microscopic phenotype of Yr15 showed that the resistance response of this gene was distinct from other R genes and activated shorty after inoculation. This result supports the finding of the Yr15 cloning studies (Klymiuk et al., 2018). The cloning of Yr15 revealed that it encodes a putative kinase-pseudokinase protein, designated as a wheat tandem kinase 1 (WTK1) belonging to the family of receptor-like cytoplasmic kinases (RLCK) (Klymiuk et al., 2018). The decoy role, where host R protein mimics effector target to trap the pathogen, has been proposed as one of the potential mechanisms of function of RLCK family members in plant immunity (van der Hoorn & Kamoun, 2008). The clear role of RLCK in wheat is not known; however, in other pathosystems, RLCK is postulated to elicit recognition and/or signalling (Kourelis & van der Hoorn, 2018). In the cloning studies the haustorium was observed in Yr15 microphenotype at 3 dpi without any sign of HR, but in the present study no visible haustorium was observed in AvocetYr15 at any time point. Usually, the yellow rust fungus develops visible haustoria within 24 h post-inoculation in susceptible wheat seedlings (Sørensen et al., 2012). One of the reasons for not
observing haustoria could be a degeneration by ROS or by secondary phytotoxic compounds produced during host cell death (Kang et al., 2002). There is a possibility that a degeneration of haustoria occurred at an early infection stage before observation at the first time point (4 dpi). The microphenotype of Yr15 showed the spread of host autofluorescence to adjacent host cells, which may create a hostile environment for the fungus to penetrate into surrounding cells. Overall the results of microscopic and cloning studies reinforce the unique features of Yr15; however, further studies are required to elucidate its mechanism of resistance in wheat. At a histological level, the assessments of fungal growth and host autofluorescence over short time intervals and early stages of infection may provide even better understanding of the Yr15 resistance mechanism.

The colony size and associated HR patterns in AvocetYr5 (IT 1) were different from AvocetYr15. The histological observations showed that most of the fungal colonies developed secondary hyphae, which were arrested by localized expression of host autofluorescence. The rapid cell death and early retardation of Pst infection was previously found in race-specific seedling reactions of Yr1 (Bozkurt et al., 2010) and Yr5 (Coram et al., 2008), which are in line with the results presented here. In AvocetYr5, the expression of host autofluorescence at an early stage of infection without any visible haustoria supports a previous transcriptomic study in which the Yr5–Pst interaction revealed the induction of defence-related transcripts during prepenetration of the fungal pathogen (Coram et al., 2008). However, the development of secondary hyphae suggests that the Yr5 interaction is probably associated with development of haustoria although it was not observed. The reasons for not observing haustoria could be degradation as suggested in the case of AvocetYr15, or they had become invisible due to early and extensive HR. To ensure the detection of haustoria in future studies, two-photon laser microscopy can be used as suggested by Sørensen et al. (2012). Recently, the cloning of Yr5 revealed an additional N-terminal zinc-finger BED domain (termed as BED-NLRs) (Marchal et al., 2018). The clear role of the additional BED domain in Yr5-mediated yellow rust resistance is not well defined; however, Marchal et al. (2018) postulate that it may have a decoy role as well as a potential role in signalling of the resistance response.

In AvocetYr1, the colony size and associated HR were mostly similar to AvocetYr5 in addition to similar macroscopic infection type (IT 1). However, the autofluorescence was more extensive in AvocetYr1 and completely encased the fungal colonies, and most of the autofluorescent host cells showed distortion. The morphological cellular changes associated with HR were host cell distortion, dilation and disintegration of cellular components, and all these are an indication of cell death, starting with the production of ROS (Mur et al., 2008). The ROS not only ensure cell death but also act as a signal for neighbouring cells, resulting in the autofluorescence of adjacent host cells. In the previous histological study of Yr1, granulation and collapse of autofluorescing mesophyll cells was observed (Bozkurt et al., 2010), which is in line with the results here. The extensive hypersensitive cell death (HCD) in a previous study was categorized as primary HCD (seen in mesophyll cells in direct contact with primary infection hyphae) and secondary HCD (in mesophyll cells that were not in direct contact with fungal structures). For future studies, the number of colonies associated with one or both categories of HCD can be used to differentiate fungal growth with respect to time and Pst isolate. The pattern of distorted host cells with autofluorescence in close proximity of fungal colonies were also reported in Yr2 resistance (Sørensen et al., 2017). The microphenotype of extensive cell death and distortion may result in the manifestation of large chlorotic areas on AvocetYr1 leaves compared to the leaves of AvocetYr5.

The Yr6 gene has been widely used in developing yellow rust resistant varieties in the past but the microphenotype of wheat with Yr6 has not previously been investigated. The pattern of fungal colonies and HR expression in AvocetYr6 was distinct from other Avocet wheat lines and reflected the larger necrotic areas, resulting in a macroscopic IT 2. The microscopic phenotype exhibited relatively large fungal colonies with branched mycelium. HR was initially delayed but in later stages, autofluorescence was recorded in almost all surrounding host cells for the majority of fungal colonies. The delay in HR could be due to weak recognition of some of the effectors initially, which allowed the fungus to consistently develop up to secondary hyphae and beyond. There is the possibility of indirect recognition of effectors in AvocetYr6 resistance which may cause delay in expression of host responses. The indirect recognition of pathogen products and subsequent activation of resistance was previously reported as one of the resistance functions of R genes (Kourelis & van der Hoorn, 2018). Such indirect and delayed recognition may explain the large variation in colony size and HR between time points for AvocetYr6.

The AvocetYr17 and AvocetYr27 NILs have not been previously investigated for their histological phenotype and the present study is an effort to link their respective macroscopic phenotype (IT 4–5 and IT 2–3) with their histological features. AvocetYr17 and AvocetYr27 displayed small colonies with weak signs of HR at 4 dpi, but at later stages of infection, differences between colony size and the associated host autofluorescence were observed. The delay in recognition and induction of host defence responses at initial stages of infection allowed the fungus to extend its growth; however, at later stages, HR increased significantly in AvocetYr27. The extent of delay in HR and extensive fungal growth in AvocetYr17 compared to AvocetYr27 is reflected in their different IT. The variation in macroscopic IT and microscopic phenotype between AvocetYr17 and AvocetYr27 are in line with previous studies, according to which the effect of delayed recognition and induction of HR on phenotype vary between different R genes (Wang et al., 2013).
AvocetYr27, despite the high HR coverage at 8 dpi, many colonies continued their growth and formed branched mycelium. The different microscopic phenotype provide evidence that AvocetYr17 and AvocetYr27 have different mechanisms compared to other Yr genes in this study. A possible difference could be that Yr17 and Yr27 recognizes an effector that is expressed late in the infection process or an effector that is only weakly expressed. The phenotype of Yr17 has been reported to be environment-dependent (Milus et al., 2015), therefore the histological investigation under different temperature, light regime and genetic background can provide better understanding of the resistance mechanism of Yr17.

Overall this study has shown that R genes conferring almost identical macroscopic phenotypes may result in very diverse patterns of fungal growth and host responses at the cellular level. The present histological studies are complementary to transcriptomic, genetic and biochemical approaches, providing new insights into the wheat–Pst interaction. For a more comprehensive understanding of the overall level of diversity of such wheat–Pst cellular responses, additional R gene resources, pathogen isolates and environments could be explored. This may help to combine genes of diverse resistance mechanisms in wheat to enhance the durability of disease resistance. The macroscopic and microscopic phenotyping approaches used in this study can be very useful as a framework for identification and characterization of new sources of yellow rust resistance in research and breeding programmes.

Acknowledgements

This work is part of a PhD project funded by Innovation Fund Denmark, Ministry of Higher Education and Science (grant no. 19052, MultiRes) and Aarhus University. The authors are very grateful to Annemarie Fejer Justesen for valuable suggestions during the course of the study and to Ellen Jørgensen, Janne Holm Hansen and Steen Meier, Aarhus University, for technical assistance during experiments. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

Baart PGJ, Parlevliet JE, Limburg H, 1991. Effects of infection density on the size of barley and wheat leaf rust colonies before and on the size of uredia after the start of sporulation. Journal of Phytopathology 131, 59–64.
Beddow JM, Pardey PG, Chai Y, et al., 2015. Research investment implications of shifts in the global geography of wheat stripe rust. Nature Plants 1, 15132.
Benjamin Y, Hochberg Y, 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society: Series B (Methodological) 57, 289–300.
Bozkurt TO, McGrann GRD, MacCormack R, Boyd LA, Akkaya MS, 2010. Cellular and transcriptional responses of wheat during compatible and incompatible race-specific interactions with

Puccinia striiformis f. sp. tritici. Molecular Plant Pathology 11, 625–40.
Coll N, Eppl P, Dang J, 2011. Programmed cell death in the plant immune system. Cell Death and Differentiation 18, 1247–56.
Coram TE, Wang M, Chen X, 2008. Transcriptome analysis of the wheat–Puccinia striiformis f. sp. tritici interaction. Molecular Plant Pathology 9, 157–69.
Dickman MB, Fluur R, 2013. Centrality of host cell death in plant–microbe interactions. Annual Review of Phytopathology 51, 543–70.
Ellis JG, Lagudah ES, Spielmeyer W, Dodds PN, 2014. The past, present and future of breeding rust resistant wheat. Frontiers in Plant Science 5, 641.
Feng J, Wang M, See DR, Chao S, Zheng Y, Chen X, 2018. Characterization of novel gene Yr79 and four additional quantitative trait loci for all-stage and high-temperature adult-plant resistance to stripe rust in spring wheat PI 182103. Phytopathologia Mediterranea 108, 737–47.
Glazbrook J, 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annual Review of Phytopathology 43, 205–27.
van der Hoorn RAL, Kamoun S, 2008. From guard to decoy: a new model for perception of plant pathogen effectors. The Plant Cell 20, 2009–17.
Hovmøller MS, 2007. Sources of seedling and adult plant resistance to Puccinia striiformis f. sp. tritici in European wheats. Plant Breeding 126, 225–33.
Hovmøller MS, Walter S, Justesen AF, 2010. Escalating threat of wheat rusts. Science 329, 369.
Hovmøller MS, Rodriguez-Algaba J, Thach T, Sørensen CK, 2017. Race typing of Puccinia striiformis on wheat. In: Perryman S, ed. Wheat Rust Diseases: Methods and Protocols. New York, NY, USA: Springer, 29–40.
Jagger LJ, Newell C, Berry ST, MacCormack R, Boyd LA, 2011. Histopathology provides a phenotype by which to characterize stripe rust resistance genes in wheat. Plant Pathology 60, 640–8.
Jones JDG, Dangl L, 2006. The plant immune system. Nature 444, 323–9.
Kang Z, Huang L, Buchenauer H, 2002. Ultrastructural changes and localization of lignin and callose in compatible and incompatible interactions between wheat and Puccinia striiformis. Journal of Plant Diseases and Protection 109, 25–37.
Kang ZS, Wang Y, Huang LL, Wei GR, Zhao J, 2003. Histology and ultrastructure of incompatible combination between Puccinia striiformis and wheat with low reaction type resistance. Cell Death and Differentiation 6, 1102–13.
Klymivuk V, Yaniv E, Huang L et al., 2018. Cloning of the wheat Yr15 resistance gene sheds light on the plant tandem kinase-pseudokinase family. Nature Communications 9, 3735.
Kourelis J, Van Der Hoorn RAL, 2018. Defended to the nines: 25 years and future of breeding rust resistant wheat. The Plant Cell 30, 285–99.
Lehmann EL, 2006. Nonparametric: Statistical Methods Based on Ranks. New York, NY, USA: Springer.
Marchal C, Zhang J, Zhang P et al., 2018. BED-domain-containing immune receptors confer diverse resistance spectra to yellow rust. Nature Plants 4, 662–8.
McNeal FH, 1971. A Uniform System for Recording and Processing Cereal Research Data. Beltsville, MD, USA: Agricultural Research Service, United States Department of Agriculture.
Milus EA, Lee KD, Brown-Guedira G, 2015. Characterization of stripe rust resistance in wheat lines with resistance gene Yr79 and implications for evaluating resistance and virulence. Phytopathology 105, 112–30.
Minker KR, Brzdycki ML, Kolagunda A et al., 2016. Semi-automated confocal imaging of fungal pathogenesis on plants: microscopic analysis of macroscopic specimens. Microscopy Research and Technique 81, 141–52.
Moldenhauer J, Moerschbacher BM, van der Westhuizen AJ, 2006. Histological investigation of stripe rust (Puccinia striiformis f. sp.
temporal and spatial development of *Puccinia striiformis* haustoria in wheat. *Mycologia* 104, 1381–9.

Sørensen CK, Thach T, Hovmøller MS, 2016. Evaluation of spray and point inoculation methods for the phenotyping of *Puccinia striiformis* on wheat. *Plant Disease* 100, 1064–70.

Sørensen CK, Labavintia R, Hovmøller MS, 2017. Temporal and spatial variability of fungal structures and host responses in an incompatible rust–wheat interaction. *Frontiers in Plant Science* 8, 484.

Tang C, Wang X, Cheng Y, Liu M, Zhao M, Wei J, 2015. New insights in the battle between wheat and *Puccinia striiformis*. *Frontiers of Agricultural Science and Engineering* 2, 101–14.

Wang M, Chen X, 2017. Stripe rust resistance. In: Chen X, Kang Z, eds. *Stripe Rust*. Dordrecht, Netherlands: Springer, 353–8.

Wang C-F, Huang L-L, Buchenauer H, Han Q-M, Zhang H-C, Kang Z-S, 2007. Histochemical studies on the accumulation of reactive oxygen species (O2•− and H2O2) in the incompatible and compatible interaction of wheat–*Puccinia striiformis f. sp. tritici*. *Physiological and Molecular Plant Pathology* 71, 230–9.

Wang C-F, Huang L-L, Zhang H-C, Han Q-M, Buchenauer H, Kang Z-S, 2010. Cytological studies of reactive oxygen species (O2•− and H2O2) and peroxidase in the incompatible and compatible interaction of wheat–*Puccinia striiformis f. sp. tritici*. *Physiological and Molecular Plant Pathology* 74, 221–9.

Wang X, McCallum BD, Fetch T, Bakkeren G, Marais GF, Saville BJ, 2013. Comparative microscopic and molecular analysis of Thatcher near-isogenic lines with wheat leaf rust resistance genes Lr2a, Lr3, LrB or Lr9 upon challenge with different *Puccinia triticina* races. *Plant Pathology* 62, 698–707.

Wang X, McCallum BD, Fetch T, Bakkeren G, Saville BJ, 2015. Sr36- and Sr3-mediated resistance response to *Puccinia graminis f. sp. tritici* is associated with callose deposition in wheat guard cells. *Phytopathology* 105, 728–37.

Welling CR, Singh RP, Yahyaoui AH, Nazari K, McIntosh RA, 2009. The development and application of near-isogenic lines for monitoring cereal rust pathogens. In: McIntosh R, ed. *Proceedings of the Borlaug Global Rust Initiative: Oral Papers Technical Workshop*. Ithaca, NY, USA: Borlaug Global Rust Initiative, 77–87.

Yin S, Wang C, Jiao M et al., 2015. Subcellular localization of calcium in the incompatible and compatible interactions of wheat and *Puccinia striiformis f. sp. tritici*. *Protoplasma* 252, 103–16.

**Supporting Information**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site.

**Figure S1.** Scale for HR observation as an area of host tissue in percentage around the fungal structures showing autofluorescence. Infected leaf samples were stained with Uvitex 2B visualized under epifluorescence microscope for recording HR around fungal colonies. (a) HR is categorized as 0 when a fungal colony grew without any sign of host autofluorescence. (b) HR was recorded as up to 25% if HR was apparent around one hypha of a fungal colony. (c) HR was recorded as up to 50% when autofluorescence expressed along one side of a colony. (d) HR is 51–75% when most of the fungal colony was covered with host autofluorescence. (e) HR was recorded as 76–100% if the fungal colony grew bigger while HR also increased and covered most of the colony. (f) A fungal colony completely surrounded by HR was assigned as 100%.

**Figure S2.** Temporal variation in macroscopic phenotype of seven wheat lines recorded at 4, 8 and 16 days post-inoculation (dpi). Macroscopically, no difference was observed at 4 dpi (a) while chlorotic flecks were apparent at 8 dpi except on AvocetYr5 and AvocetYr15 (b). Minute flecks on AvocetYr15 and AvocetYr15 while large necrotic areas were observed on AvocetYr1, AvocetYr6 and AvocetYr27 at 16 dpi (c). Necrosis along with sporulation was recorded on AvocetYr17; AvocetS showed complete sporulation.

**Figure S3.** Temporal growth of fungal colony size on susceptible parental line AvocetS. Increase in colony size from 2 to 4 days post-inoculation (dpi) was not large but afterwards it increased rapidly and become large colonies at 6 dpi. After 6 dpi, all colonies were intermingled and not measureable. The colony size at individual time points followed by same letter is not significantly different at a 5% confidence level.