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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

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Thatcher near-isogenic lines (NILs) of wheat carrying resistance gene *Lr2a*, *Lr3*, *LrB* or *Lr9* were inoculated with *Puccinia triticina* races of virulence phenotype BBBD, MBDS, SBDG and FBDJ. *Puccinia triticina* infection structures were analysed under the fluorescence microscope over a course of 14 days after inoculation (dai). The relative proportion of *P. triticina* and wheat genomic DNA in infected leaves was estimated with a semiquantitative multiplex PCR analysis using *P. triticina*- and wheat-specific primers. The occurrence of a hypersensitive response (HR), cellular lignification and callose deposition in inoculated plants was investigated microscopically. In interactions producing highly resistant infection type (IT) '0;', a maximum of two haustorial mother cells per infection site were produced, and there was no increase in the proportion of *P. triticina* genomic DNA in infected leaves, indicating the absence of *P. triticina* growth. In comparison, sizes of *P. triticina* colonies increased gradually in interactions producing moderately resistant IT '1' and '2', with the highest proportion of *P. triticina* genomic DNA found in leaves sampled at 14 dai. In interactions producing susceptible IT '3–4', the highest proportion of *P. triticina* genomic DNA was found in leaves sampled at 10 dai (45·5–51·5%). HR and cellular lignification were induced in interactions producing IT '0;' and '1' at 1 dai but they were not observed in interactions producing IT '2' until 2 dai. No HR or cellular lignification were induced in interactions producing susceptible IT '3–4'. Furthermore, a strong deposition of callose was induced in *Lr9* + BBBD and *Lr9* + FBDJ (IT '0;'), whereas this defence response was not induced in resistant or susceptible interactions involving *Lr2a*, *Lr3* or *LrB*, indicating that *Lr9* mediated resistance was different from that conditioned by *Lr2a*, *Lr3* or *LrB*.

Keywords: callose deposition, cellular lignification, hypersensitive response, *Puccinia triticina*, wheat leaf rust

Introduction

Wheat leaf rust, caused by *Puccinia triticina*, is a major threat to the production of wheat worldwide. In Canada, *P. triticina* causes an annual yield loss of up to 25%, depending on the growth stage of crops at the time of initial infection and resistance of cultivars in production (Kolmer, 2005). Breeding for durable resistance to *P. triticina* in wheat cultivars has met with only partial success because *P. triticina* is highly variable and capable of migrating over large geographical regions (Kolmer, 1996). Wheat cultivars often lose their resistance against *P. triticina* a few years after their release, which suggests that the population of *P. triticina* is under a strong selection pressure for virulence against newly deployed resistance genes (Johnson, 1961).

To date, wheat leaf rust resistance genes (*Lr*) *Lr1* to *Lr67* have been described (Kolmer, 2005). Most *Lr* genes are race-specific and condition hypersensitive responses (HR) to *P. triticina* races carrying genetically matching avirulence genes (Bolton *et al.*, 2008). Although these race-specific *Lr* genes are initially effective against *P. triticina*, many are rapidly overcome by *P. triticina* in the field (Kolmer, 2005). Another major type of wheat leaf rust resistance is non-race-specific resistance, which is exemplified by adult plant resistance mediated by *Lr34*, *Lr46* or *Lr67* (Bolton *et al.*, 2008). This type of resistance is often less effective but considered to be more durable. Resistance conditioned by *Lr34* is not associated with an HR but is characterized by fewer and smaller uredinia,

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along with the absence of chlorosis and necrosis showing on flag leaves (Dyck *et al.*, 1985). *Lr46* conditions a resistance phenotype typified by fewer and smaller uredinia with varying amount of chlorosis present on adult plant leaves (Singh *et al.*, 1998). In addition, a form of pre-haustorial resistance, which acts before pathogen penetration of host cell walls to establish the haustorium, has been described in interactions between rust fungi and non-host plants (Heath, 1981). In this pre-haustorial resistance, the development of fungal sporelings is often arrested before the formation of haustoria due to callose deposition at the site of cell wall penetration (Niks, 1982).

In 1986, a nomenclature system for designating virulence combinations of *P. triticina* in North America was developed based on infection types (IT) of *P. triticina* on a set of wheat near-isogenic lines (NILs) carrying different *Lr* genes (Long & Kolmer, 1989). IT designations such as ';', '0', '1' and '2' were rated as avirulent interactions whereas infection types '3' and '4' were rated as virulent interactions (McCallum & Seto-Goh, 2004; Kolmer *et al.*, 2005). Many *Lr* genes produce specific ITs when infected by *P. triticina* races. For example, wheat lines carrying *Lr2a* produce very light flecks when inoculated with avirulent *P. triticina* races, whereas the resistance response mediated by *Lr3* shows clearly defined hypersensitive flecks (McIntosh *et al.*, 1995). Other race-specific resistance genes, such as *LrB* and *Lr3Ka,* result in small uredinia surrounded by chlorosis (Bolton *et al.*, 2008).

Although the early infection process of *P. triticina* in susceptible and resistant wheat lines has been previously investigated using scanning and transmission electron microscopy (Hu & Rijkenberg, 1998a,b), the molecular basis of *P. triticina* pathogenicity remains unclear and effects of different *Lr* genes on the infection process of *P. triticina* are poorly described. In this regard, Thatcher NILs carrying different *Lr* genes are extremely valuable for conducting in-depth analyses of interactions between *Lr* genes and different races of *P. triticina*.

The aim of this study was to compare the sequence of cellular interactions between various *P. triticina* races and Thatcher NILs carrying *Lr9*, *Lr2a*, *Lr3* or *LrB*, representing a range of ITs from a near-immune resistant response (IT 0;) to a highly susceptible IT '4'. The events occurring during *P. triticina* infection were investigated over time through visual observation, fluorescent microscopy and through estimates of relative fungal biomass based on the relative proportion of *P. triticina* genomic DNA to wheat genomic DNA extracted from infected leaves. In addition, the occurrence of host cellular defence responses, such as HR, cellular lignification and callose deposition, were monitored in relation to *P. triticina* development in these Thatcher NILs.

Materials and methods

Plant and fungal material

Four Thatcher NILs including Thatcher-*Lr2a* (RL6016), Thatcher-*Lr3* (RL6002), Thatcher-*LrB* (RL6051) and Thatcher-*Lr9* (RL6010) and *P. triticina* races with virulence phenotype BBBD (strain designation 1-1), SBDG (9-1), FBDJ (161-1) and MBDS (12-3) were used. Infection types produced by *P. triticina* races on Thatcher NILs were rated 10 days after inoculation (dai) and are summarized in Table 1. Wheat plants were grown in 25 × 25 cm flats at 18–25°C in a glasshouse. Each flat contained 100 seeds planted in four rows. *Puccinia triticina* isolates were increased from a single pustule inoculation and virulence of *P. triticina* isolates was verified on a differential set containing 16 Thatcher NILs using the method described by McCallum & Seto-Goh (2004).

Plant growth and inoculation conditions

Puccinia triticina urediniospores were suspended in a light mineral oil (Bayol®; Esso Canada) and adjusted to a concentration of 5×10^6 urediniospores mL^{-1}. Seven-day-old wheat plants at the two-leaf stage were inoculated with *P. triticina* by spraying with 700 μL of urediniospore–oil suspension per flat. After inoculation, wheat plants were incubated in a moisture chamber (Percival model I-60D; Percival Scientific) at 100% relative humidity overnight at 18°C and then transferred to a growth chamber with a light/dark cycle of 18 h at 24°C/6 h at 18°C. Twenty inoculated first leaves were sampled at 0, 1, 2, 3, 4, 5, 7, 10 and 14 dai for each race × NIL and used for further analysis.

Microscopy techniques

Infection structures of *P. triticina* in wheat leaves were visualized using the method described by Moldenhauer

Table 1 Infection types (IT) of *Puccinia triticina* races on wheat Thatcher near-isogenic lines

Race	Thatcher-*Lr9*	Thatcher-*Lr2a*	Thatcher-*Lr3*	Thatcher-*LrB*	Thatcher
P. triticina BBBD	0;[a]	1[b]	2[c]	2	3–4[d]
P. triticina FBDJ	0;	2	3–4	2	3–4
P. triticina MBDS	1	1	3–4	3–4	3–4
P. triticina SBDG	1	3–4	2	2	3–4

[a]IT '0;', no macroscopic sign of infection or only few faint flecks.
[b]IT '1', small uredinia surrounded by a necrosis are visible.
[c]IT '2', small- to medium-sized uredinia are visible, surrounded by chlorosis.
[d]IT '3–4', medium- or large-sized uredinia without chlorosis or necrosis showing.

et al. (2006). Wheat leaves were first fixed in an ethanol:trichloromethane (3:1, v/v) solution containing 0·15% trichloroacetic acid for 24 h and subsequently washed once with 50% ethanol (v/v). Fixed leaf samples were incubated at 90°C for 30 min in 0·5 M NaOH and then rinsed once with water, followed by incubation in 0·1 M Tris–HCl (pH 5·8) for 30 min. Subsequently, leaves were stained with 0·1% Uvitex 2B (Analychem Corp. Ltd) for 5 min followed by five washes with distilled water. Specimens were mounted in 50% (v/v) glycerol and observed under a Leica fluorescent microscope. The number of haustorial mother cells produced by *P. triticina* was counted visually under the microscope.

Wheat cell necrosis induced by the infection of *P. triticina* was monitored by staining freshly collected first wheat leaves with lactophenol–trypan blue using the method described by Koch & Slusarenko (2011). The sample was then mounted on a microscope slide in 50% glycerol and examined using a light microscope (Leica DMRB microscope). Staining of lignin was done using phloroglucinol-HCl as described by Sherwood & Vance (1976) using leaves prepared as for Uvitex2B staining described above. Leaves were first fixed in an ethanol: trichloromethane (3:1, v/v) solution as described above and then soaked in phloroglucinol (4% w/v in 20% ethanol) for 30 min. The sample was mounted on a microscope slide and one drop of 6 M HCl was added to the sample, after which the slide was heated on a hot plate until the red lignin coloration appeared. The accumulation of callose in infected leaves was revealed by aniline blue staining as described by Adam & Somerville (1996). Briefly, leaf samples were fixed in an ethanol:trichloromethane (3:1, v/v) solution containing 0·15% trichloroacetic acid for 24 h. Leaves were then washed once with 50% ethanol (v/v) followed by a rinse with distilled water. Subsequently, leaf samples were stained in 150 mM H_2HPO_4 (pH 9·5) containing 0·01% aniline blue for 30 min. Samples were mounted in 50% glycerol and examined by epifluorescent illumination.

For leaf samples stained with Uvitex2B and aniline blue, fluorescence was examined using a Leica DMRB microscope equipped with a Leica fibre-optic light source and a Leica DAPI filter cube (excitation range 400 nm), and images were recorded with a Leica DFC360FX camera. The autofluorescence emitted from necrotic cells was observed with a Leica Fred filter cube (excitation range 550 nm). Images were then overlaid using Leica image overlay software (v. 3.6). For each time point, over 150 infection sites (microscopic fields of view) from 15 inoculated first leaves were examined. The experiment was repeated three times and only representative pictures are shown in Figs 1–4. The experiment visualizing haustorial mother cell formation was repeated twice. Each replicate included the examination of five first leaves randomly

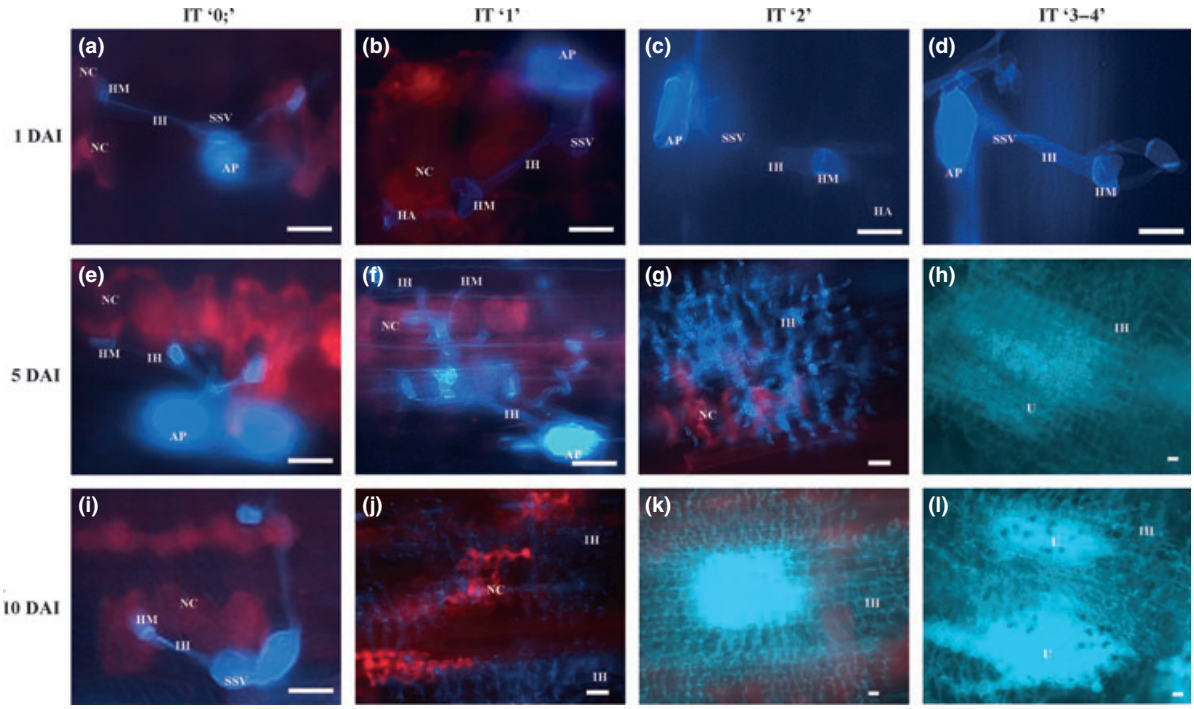

Figure 1 Representative cellular responses illustrating various infection types (IT) during *Puccinia triticina* infection of different wheat near-isogenic lines. IT '0;' represented by *Lr9+BBBD and Lr9+FBDJ; IT '1' represented by Lr2a*+BBBD, *Lr2a*+MBDS, *Lr9+MBDS and Lr9+SBDG; IT '2' represented by Lr2a*+FBDJ, *Lr3+BBBD, Lr3+SBDG, LrB*+BBBD, *LrB*+SBDG and *LrB*+FBDJ; IT '3–4' represented by *Lr2a*+SBDG, *Lr3+FBDJ, Lr3+MBDS, LrB*+MBDS, *LrB*+FBDJ, Thatcher+BBBD, Thatcher+SBDG, Thatcher+FBDJ and Thatcher+MBDS. NC: necrotic cell; HM: haustoria mother cell; IH: infection hyphae; AP: appresorium; SSV: substomatal vesicle; HA: haustoria. Scale bar is 5 μm.

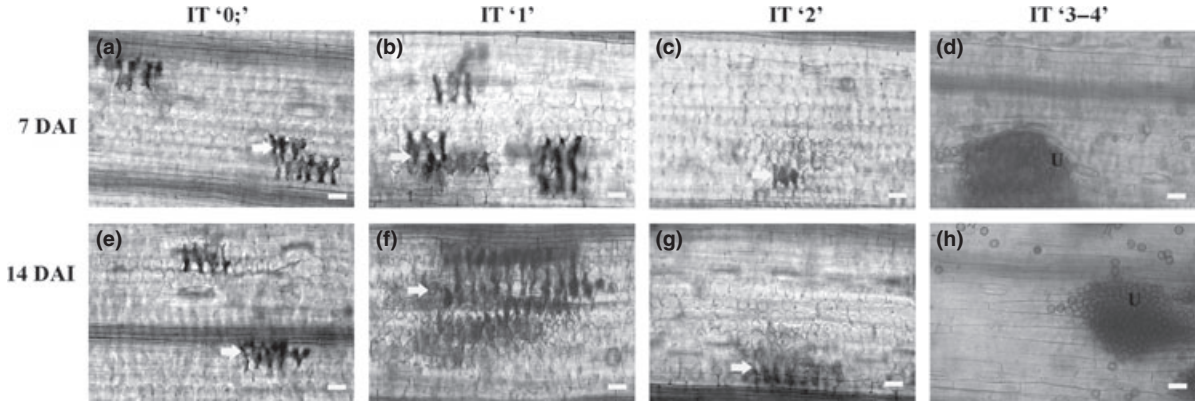

Figure 2 Occurrence of hypersensitive cell death during *Puccinia triticina* infection of wheat near-isogenic lines with resistance genes *Lr2a* and *Lr9*. IT '0;' represented by *Lr9+BBBD and Lr9+FBDJ; IT '1' represented by Lr2a*+BBBD, *Lr2a*+MBDS, *Lr9+MBDS and Lr9+SBDG; IT '2' represented by Lr2a*+FBDJ, *Lr3+BBBD, Lr3+SBDG, LrB*+BBBD, *LrB*+SBDG and *LrB*+FBDJ; IT '3–4' represented by *Lr2a*+SBDG, *Lr3+FBDJ, Lr3+MBDS, LrB*+MBDS, *LrB*+FBDJ, Thatcher+BBBD, Thatcher+SBDG, Thatcher+FBDJ and Thatcher+MBDS. Arrows point to regions of cells responding hypersensitively as revealed by trypan blue staining. U: urediniospores. Scale bar is 10 μm.

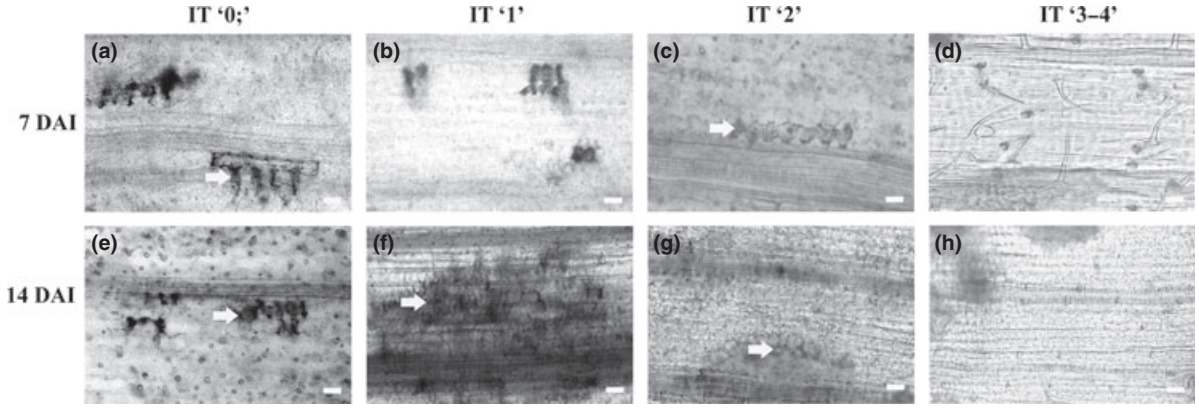

Figure 3 Accumulation of lignin during *Puccinia triticina* infection of different wheat near-isogenic lines. IT '0;' represented by *Lr9+BBBD and Lr9+FBDJ; IT '1' represented by Lr2a*+BBBD, *Lr2a*+MBDS, *Lr9+MBDS and Lr9+SBDG; IT '2' represented by Lr2a*+FBDJ, *Lr3+BBBD, Lr3+SBDG, LrB*+BBBD, *LrB*+SBDG and *LrB*+FBDJ; IT '3–4' represented by *Lr2a*+SBDG, *Lr3+FBDJ, Lr3+MBDS, LrB*+MBDS, *LrB*+FBDJ, Thatcher+BBBD, Thatcher+SBDG, Thatcher+FBDJ and Thatcher+MBDS. Arrows indicate regions of cells showing cellular lignification as revealed by pholoroglucinol-HCl staining. Scale bar is 10 μm.

selected from three flats, and five random microscopic views were chosen per leaf for counting of haustorial mother cells. Only one set of data is presented in Table 2 because both replicates were highly similar. Table 4 summarizes microscopic observations of the five first leaves and five microscopic views per leaf for each race × NIL interaction and given time point. The experiment was repeated three times.

Semiquantitative multiplex PCR analysis of *P. triticina* DNA content in infected wheat leaves

One gram of infected leaf samples was ground in liquid nitrogen and genomic DNA was extracted using the method described by Kolmer (2001). DNA concentration was determined by spectrophotometer readings at A_{260} and A_{280} and a working solution of 100 ng μL^{-1} was made. A semiquantitative PCR protocol was used for the estimation of *P. triticina* DNA content in infected wheat leaves using primer pairs specific to *P. triticina* and wheat. For *P. triticina*, a previously employed, single-copy gene encoding succinate dehydrogenase (Song *et al.*, 2011; Broad Institute, PTTG_01208.1) was used requiring primers 5′-GATCCTCGCCATAGGAATCA-3′ (forward) and 5′-CATCCTATGTGCATGCTGCT-3′ (reverse). For wheat, a gene encoding puroindolin β (Gautier *et al.*, 2000; GenBank AJ302100) was chosen requiring primers 5′-TGCAAGGATTACGTGATGGA-3′ (forward) and 5′-GCTATCTGGCTCAGCTGCTT-3′ (reverse). The specificity of these primers was tested against pure wheat and *P. triticina* genomic DNA samples and no cross-amplification between the two species was observed.

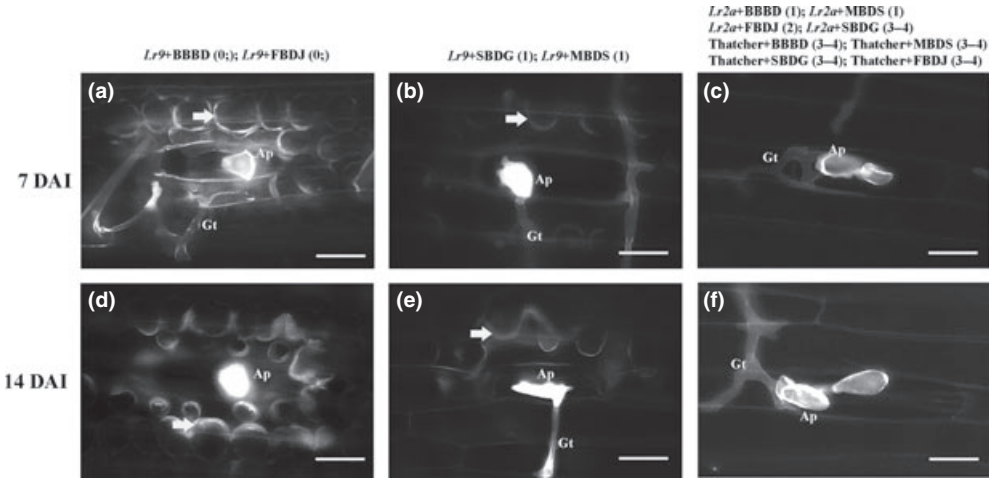

Figure 4 Accumulation of callose during *Puccinia triticina* infection of Thatcher wheat near-isogenic lines with *Lr2a* or *Lr9*. The interactions for which representative pictures are shown are given above the figure, with infection type in parentheses. Arrows indicate callose deposition as revealed by aniline blue staining. Ap: appresorium; Gt: germ tube. Scale bar is 5 μm.

Table 2 Number of haustorial mother cells produced by *Puccinia triticina* at the early infection stage

		No. of haustorial mother cells per infection site			
IT[a]	Treatment[b]	1 dai[c]	2 dai	3 dai	4 dai
0;	*Lr9*+BBBD	1 ± 1[d]	1 ± 1	1 ± 1	1 ± 1
	Lr9+FBDJ	1 ± 1	1 ± 1	1 ± 1	1 ± 1
1	*Lr9*+SBDG	1 ± 1	3 ± 1	9 ± 2	18 ± 4
	Lr9+MBDS	1 ± 1	4 ± 1	10 ± 1	20 ± 4
	Lr2a+BBBD	1 ± 1	4 ± 2	8 ± 2	17 ± 3
	Lr2a+MBDS	1 ± 1	3 ± 2	8 ± 1	16 ± 2
2	*Lr2a*+FBDJ	1 ± 1	12 ± 2	27 ± 4	91 ± 22
	Lr3+BBBD	1 ± 1	15 ± 4	31 ± 4	101 ± 15
	Lr3+SBDG	1 ± 1	12 ± 3	34 ± 4	97 ± 12
	LrB+BBBD	1 ± 1	13 ± 4	41 ± 6	150 ± 21
	LrB+SBDG	1 ± 1	16 ± 4	45 ± 5	166 ± 15
	LrB+FBDJ	1 ± 1	17 ± 5	44 ± 5	137 ± 19
3–4	*Lr2a*+SBDG	1 ± 1	18 ± 2	82 ± 7	536 ± 39
	Lr3+FBDJ	1 ± 1	19 ± 2	92 ± 10	524 ± 32
	Lr3+MBDS	1 ± 1	18 ± 3	89 ± 8	521 ± 42
	LrB+MBDS	1 ± 1	17 ± 2	84 ± 7	551 ± 45
	Thatcher+BBBD	1 ± 1	20 ± 2	85 ± 6	541 ± 35
	Thatcher+FBDJ	1 ± 1	21 ± 3	91 ± 6	515 ± 39
	Thatcher+SBDG	1 ± 1	20 ± 2	89 ± 10	535 ± 31
	Thatcher+MBDS	1 ± 1	20 ± 4	91 ± 5	542 ± 36

[a]Infection types as indicated in Table 1.
[b]Observations are based on the result from 25 infection sites from five first leaves.
[c]dai, days after inoculation.
[d]± represents the standard deviation.

Multiplex PCR was carried out in a 12·5 μL reaction volume containing 1 × GeneAmp PCR buffer (50 mM KCl, 15 mM Tris–HCl pH 8·0; Applied Biosystems), 1·5 mM $MgCl_2$, 0·2 mM dNTPs, 2·5 mM of each primer, 0·5 U *Taq* DNA polymerase (Invitrogen) and 100 ng sample DNA. Amplifications were performed in a thermal cycler (GeneAmp PCR 9700; Applied Biosystems) using the following temperature profile: initial denaturation step at 95°C for 2 min, then 25 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by a final extension step at 72°C for 10 min. Five microlitres of the amplification product was mixed with 1 μL of 6 × loading buffer and then separated in a 2% agarose gel (Sigma) at a voltage of 5 V cm^{-1}. Images of gels were captured with an AlphaImager mini system (Cell & Bioscience) and analysed with AlphaEase@FC (v. 6.0). The integrated densities of the *Puccinia*- and wheat-specific DNA bands, each representing a unique house keeping gene, were used to calculate the relative proportion of *P. triticina* total genomic DNA to wheat total genomic DNA.

For the semiquantitative estimation of the relative portion of *P. triticina* DNA in infected leaves, the multiplex PCR was performed on 100 ng of standard DNA samples containing increasing proportions of 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% *P. triticina* DNA in a *P. triticina*/wheat genomic DNA mix. A standard curve was constructed using the ratio of integrated density of *P. triticina*- and wheat-specific bands amplified from these DNA standards. For each race × NIL and time point, three DNA extractions were performed using first leaves sampled from three different flats. The average of the integrated density of PCR product from three replicates was used for estimation of the portion of *P. triticina* genomic DNA in infected leaves.

Results

Puccinia triticina growth in infected wheat leaves

IT '0;' was only found in Thatcher-*Lr9* inoculated with *P. triticina* races BBBD and FBDJ, and the most resistant IT achieved with Thatcher-*Lr2a* was IT '1'. In comparison, Thatcher-*Lr3* and Thatcher-*LrB* only pro-

duced IT '2' when infected by avirulent *P. triticina* races (Table 1).

The number of haustorial mother cells per infection site was investigated from 1 to 4 dai (Table 2). At 1 dai, the number of haustorial mother cells per infection site was similar in interactions producing IT '0;', '1', '2' and '3–4', with one or two haustorial mother cells produced per infection site. After 1 dai, the infection process of *P. triticina* started to differentiate among different ITs. No increase was found in interactions producing IT '0;' but the number increased gradually in interactions producing moderately resistant IT '1', '2' and susceptible IT '3–4' from 2 to 4 dai. The highest number of haustorial mother cells per infection site was found in interactions producing susceptible IT '3–4' at 4 dai. Additionally, haustoria were not observed in two interactions producing IT '0;' from 1 to 4 dai but they were commonly observed in interactions producing resistant IT '1', IT '2' and susceptible IT '3–4' (data not shown).

In interactions producing IT '0;', a maximum of two infection hyphae were formed per infection site at 1, 5 and 10 dai (Fig. 1a,e,i) and no amplification of *P. triticina* DNA was achieved using *P. triticina*-specific primers in leaves collected at 5, 7, 10 and 14 dai (Table 3). A strong autofluorescence emitted from necrotic wheat cells adjacent to *P. triticina* haustorial mother cells was observed at 1 dai. In interactions producing IT '1' (Fig. 1b,f,j) and IT '2' (Fig. 1c,g,k), *P. triticina* grew slowly beyond 1 dai and the highest proportion of *P. triticina* genomic DNA was found in leaves sampled at 14 dai (IT '1', 9·2–12·5% and IT '2', 26·4–40·2%; Table 3). Although autofluorescence was observed in wheat cells near *P. triticina* haustorial mother cells at 1 dai in IT '1', this infection type produced subsequent runner hyphae which grew past these initial host cells into the region where no visible autofluorescence was observed (Fig. 1b). In comparison, no autofluorescence was detected in wheat cells surrounding *P. triticina* haustorial mother cells in interactions producing IT '2' at 1 dai (Fig. 1c). In interactions producing susceptible IT '3–4', the proportion of *P. triticina* DNA reached 31·5–35·4% in leaves sampled at 5 dai and increased quickly to approximately 45% at 7 dai, staying in that range up to 10 dai. In some interactions, such as MBDS on Thatcher-*Lr3* and Thatcher-*LrB*, the increase of *P. triticina* DNA was slightly lower. At 14 dai, fungal biomass started to decrease slowly, probably due to the sporulation of *P. triticina* which could affect its vegetative growth in infected leaves. In addition, the senescence of wheat leaves and subsequence degradation of DNA could also contribute to decrease of fungal DNA percentage in infected leaves at 14 dai. No autofluorescence was observed in necrotic wheat cells in interactions producing IT '3–4' at 1, 5 and 10 dai (Fig. 1d,h,m).

Table 3 Semi quantitative PCR analysis of the percentage of *Puccinia triticina* genomic DNA in total genomic DNA extracted from infected wheat leaves

Infection type[a]	Treatment[b]	Percentage of *P. triticina* DNA in total DNA extracted from infected leaves			
		5 dai[c]	7 dai	10 dai	14 dai
0;	*Lr9*+BBBD	–	–	–	–
	Lr9+FBDJ	–	–	–	–
1	*Lr2a*+BBBD	2·3 ± 1·2[d]	3·4 ± 1·9	7·5 ± 2·5	9·4 ± 2·1
	Lr2a+MBDS	1·0 ± 0·5	2·1 ± 1·1	5·3 ± 1·7	9·2 ± 2·1
	Lr9+MBDS	1·9 ± 0·2	3·5 ± 1·5	6·6 ± 2·3	12·5 ± 2·5
	Lr9+SBDG	1·3 ± 0·6	3·1 ± 1·2	5·9 ± 2·1	10·6 ± 3·5
2	*Lr2a*+FBDJ	10·3 ± 3·3	18·2 ± 4·7	23·6 ± 3·4	26·4 ± 2·1
	Lr3+BBBD	11·5 ± 2·4	18·5 ± 3·2	23·5 ± 3·7	29·2 ± 4·6
	Lr3+SBDG	12·5 ± 1·9	16·7 ± 2·1	22·5 ± 3·2	31·5 ± 3·2
	LrB+BBBD	21·5 ± 3·6	31·1 ± 4·5	35·9 ± 4·1	37·5 ± 3·6
	LrB+SBDG	20·5 ± 1·5	29·2 ± 3·2	35·6 ± 3·2	36·5 ± 5·1
	LrB+FBDJ	22·2 ± 4·6	32·3 ± 2·6	36·4 ± 5·6	40·2 ± 3·2
3–4	*Lr2a*+SBDG	34·5 ± 5·1	44·5 ± 4·5	44·5 ± 3·2	41·5 ± 4·6
	Lr3+FBDJ	35·4 ± 4·1	44·2 ± 4·1	43·5 ± 3·5	42·5 ± 3·5
	Lr3+MBDS	32·1 ± 3·5	38·6 ± 6·3	42·3 ± 5·4	41·2 ± 3·1
	LrB+MBDS	31·5 ± 1·5	37·5 ± 3·4	42·2 ± 2·5	38·3 ± 3·1
	LrB+FBDJ	34·5 ± 2·5	43·5 ± 3·5	42·1 ± 2·5	38·4 ± 3·5
	Thatcher+BBBD	34·5 ± 3·5	45·1 ± 2·3	43·5 ± 3·5	39·1 ± 1·5
	Thatcher+SBDG	35·1 ± 2·4	43·4 ± 3·1	43·5 ± 2·3	40·2 ± 2·5
	Thatcher+FBDJ	33·4 ± 4·1	46·3 ± 5·4	42·4 ± 4·2	37·6 ± 1·5
	Thatcher+MBDS	35·1 ± 4·3	42·7 ± 5·1	44·5 ± 1·5	42·2 ± 2·5

[a]Infection types as indicated in Table 1.
[b]The average is calculated from three biological repeats including 15 infected first leaves.
[c]dai, days after inoculation.
[d]± indicates the standard deviation.

Hypersensitive response, cellular lignification and callose deposition

In interactions producing IT '0;', an HR and cellular lignification were induced at 1 dai, whereas callose deposition was not detected until 2 dai (Table 4). In interactions producing IT '1', an HR and cellular lignification were also observed at 1 dai. Although the deposition of callose was observed in two of the interactions producing IT '1' (*Lr9* + SBDG and *Lr9* + MBDS) at 2 dai, it was significantly weaker than in *Lr9* + BBBD and *Lr9* + FBDJ (IT '0;', data not shown). In comparison, callose deposition was not detected in IT '1' interactions involving *Lr2a* (*Lr2a* + BBBD and *Lr2a* + MBDS; Table 4). In interactions producing IT '2', an HR and cellular lignification were observed at 2 dai but no deposition of callose was observed from 1 to 4 dai. In interactions producing IT '3–4', no induction of HR, cellular lignification or callose deposition were observed from 1 to 4 dai.

Inductions of HR, cellular lignification and callose deposition were also investigated in infected leaves sampled at 7 and 14 dai (Figs 2–4). In interactions producing IT '0;', the size of leaf area showing HR was similar at 7 and 14 dai (Fig. 2a,e), but it became larger in interactions producing IT '1' at 14 dai (Fig. 2b,f). In comparison, HR was weaker and more diffuse in interactions producing IT '2' (Fig. 2c,g) and it was not observed in interactions

Table 4 Occurrence of host defence responses during the infection of *Puccinia triticina* in wheat Thatcher near-isogenic lines carrying *Lr2a, Lr3, LrB* or *Lr9*

		1 dai[b]			2 dai			3 dai			4 dai		
IT[a]	Treatment	Callose[c]	HR	Lignin	Callose	HR	Lignin	Callose	HR	Lignin	Callose	HR	Lignin
0;	*Lr9*+BBBD	–	+	+	+	+	+	+	+	+	+	+	+
0;	*Lr9*+FBDJ	–	+	+	+	+	+	+	+	+	+	+	+
1	*Lr9*+SBDG	–	+	+	+	+	+	+	+	+	+	+	+
1	*Lr9*+MBDS	–	+	+	+	+	+	+	+	+	+	+	+
1	*Lr2a*+BBBD	–	+	+	–	+	+	–	+	+	–	+	+
1	*Lr2a*+MBDS	–	+	+	–	+	+	–	+	+	–	+	+
2	*Lr2a*+FBDJ	–	–	–	–	+	+	–	+	+	–	+	+
2	*Lr3*+BBBD	–	–	–	–	+	+	–	+	+	–	+	+
2	*Lr3*+SBDJ	–	–	–	–	+	+	–	+	+	–	+	+
2	*LrB*+BBBD	–	–	–	–	+	+	–	+	+	–	+	+
2	*LrB*+SBDG	–	–	–	–	+	+	–	+	+	–	+	+
2	*LrB*+FBDJ	–	–	–	–	+	+	–	+	+	–	+	+
3–4	*Lr2a*+SBDG	–	–	–	–	–	–	–	–	–	–	–	–
3–4	*Lr3*+MBDS	–	–	–	–	–	–	–	–	–	–	–	–
3–4	*Lr3*+FBDJ	–	–	–	–	–	–	–	–	–	–	–	–
3–4	*LrB*+MBDS	–	–	–	–	–	–	–	–	–	–	–	–
3–4	Thatcher+BBBD	–	–	–	–	–	–	–	–	–	–	–	–
3–4	Thatcher+FBDJ	–	–	–	–	–	–	–	–	–	–	–	–
3–4	Thatcher+SBDG	–	–	–	–	–	–	–	–	–	–	–	–
3–4	Thatcher+MBDS	–	–	–	–	–	–	–	–	–	–	–	–

[a]Infection types as indicated in Table 1.
[b]dai, days after inoculation.
[c]–/+ represents absence/presence of host defence responses.

producing IT '3–4' at 7 and 14 dai (Fig. 2d,h). Similarly, a significant accumulation of lignin was found at 7 dai in interactions producing IT '0;' and '1' (Fig. 3a,b,e,f) and the area containing lignified cells became larger in interactions producing IT '1' at 14 dai (Fig. 3f). Cellular lignification was barely detectable in interactions producing IT '2' (Fig. 3c,g) and it was not detected at all in interactions producing susceptible IT '3–4' at 7 and 14 dai (Fig. 3d,h).

Callose deposition was only observed in Thatcher-*Lr9* during interactions producing IT '0;' and '1' (Table 4). However, there seemed to be a differential response. To determine whether this response was truly differential, an in-depth microscopic analysis was performed. When inoculated with *P. triticina* BBBD and FBDJ (IT '0;'), large amounts of callose were detected at 7 and 14 dai in cells surrounding the stomata penetrated by *P. triticina* (Fig. 4a,d). In comparison, the deposition of callose was weaker in leaves inoculated with *P. triticina* SBDG and MBDS (IT '1'), as revealed by fainter fluorescence near stomata (Fig. 4b,e). No deposition of callose was found in leaves of Thatcher and Thatcher-*Lr2a* inoculated with *P. triticina* BBBD, SBDG and FBDJ at 7 and 14 dai (Fig. 4c,f). Additionally, no deposition of callose was observed in Thatcher-*Lr3* and Thatcher-*LrB* inoculated with different *P. triticina* races (data not shown).

Discussion

Multiple resistant infection types and differentially regulated host defence responses were observed on Thatcher-*Lr9* or Thatcher-*Lr2a* inoculated with different races of *P. triticina* carrying corresponding avirulence genes. Similarly, it has been previously noted that the resistance of Thatcher NILs carrying *Lr2a*, *Lr2c*, *Lr3a*, *Lr11* or *Lr17* ranged from completely dominant to recessive, depending on *P. triticina* avirulent genotypes (homozygous or heterozygous; Kolmer & Dyck, 1994). It is possible that this difference in the expression of resistance is caused by the heterozygosity of corresponding avirulence genes in *P. triticina* races (Kolmer, 1996, 2005). Homo- or heterozygosity of avirulence genes may dictate the level of elicitors produced and in turn affect the timing and strength of host defence response triggered. Another possibility is that the mutation in *P. triticina* avirulence alleles contributes to a difference in the triggering and strength of host resistance. In a previous mutation study conducted by Statler (1985), mutants with increased virulence were obtained from *P. triticina* races heterozygous for avirulence genes. In this case, *P. triticina* avirulence alleles may be altered in such a way that their products are still recognized by the product of the corresponding host resistance genes, but have lost their normal functions during infection.

Variations in the expression of resistance were also observed in different Thatcher NILs inoculated with the same race. The effectiveness of wheat leaf rust resistance varies, depending on the combination of a particular race-specific *Lr* gene and a corresponding *P. triticina* avirulence gene (Bolton *et al.*, 2008). Manickavelu *et al.* (2010) showed that phenotypic differences between incompatible and compatible interactions in wheat NILs

carrying different *Lr* genes were related to the differential expression of defence-related genes during the infection process. Nevertheless, the correlation between infection types and the induction of host defence responses in this pathosystem has not been established. In the present study, the resistance mediated by *Lr3* and *LrB* is expressed at a later stage of the infection process compared to the resistance mediated by *Lr2a*, which indicates that the timing of the host defence response affects the infection type caused by *P. triticina*.

The vegetative growth of *P. triticina* was completely suppressed in *Lr9* + BBBD and *Lr9* + FBDJ (IT '0;'). Similarly, Ortelli *et al.* (1996) reported that host cells of a near-isogenic line of Swiss cultivar Arina carrying *Lr9* died 24–44 h after inoculation with avirulent *P. triticina* isolates, and *P. triticina* only succeeded in forming one to two haustorial mother cells per infection site. Such near-immune IT has also been described for wheat cultivars carrying *Sr5* (a stem rust resistance gene) when inoculated with the corresponding avirulent races of *P. graminis* f. sp. *tritici* (Lennox & Rijkenberg, 1994). *Lr9* was first introduced into wheat from *Triticum umbellulatum*, which is a diploid wild wheat species, whereas *Lr2a*, *Lr3* and *LrB* were all introduced from common wheat. In diploid wild wheat species, a form of pre-haustorial resistance has been previously described in which rust urediniospores develop normal haustorial mother cells after the initial penetration but further development of haustoria is arrested due to callose deposition on the cell wall near the penetration site (Niks, 1982). This form of resistance is also very common in non-host interactions involving rust pathogens such as in (diploid) barley inoculated with rye leaf rust (Niks, 1989). The early abortion of *P. triticina* infection structures in *Lr9* + BBBD and *Lr9* + FBDJ, coinciding with the deposition of callose around the site of penetration, shares some similarities with the previously described pre-haustorial resistance (Niks, 1982; Hu & Rijkenberg, 1998a). Nevertheless, the induction of an HR and cellular lignification is also involved in the resistance mediated by *Lr9,* which is different from the previously described pre-haustorial resistance.

The HR and cellular lignification have been commonly regarded as important defence mechanisms in resistance against cereal rusts, resulting from the accompanying up-regulation of a multitude of defence-related genes and assumed loss of the essential nutrient base when host cells bearing fungal haustoria collapse (Leonard & Szabo, 2005). In this study, most host cells undergoing HR and cellular lignification in interactions producing resistant IT '0;' and '1' were adjacent to *P. triticina* haustorial mother cells, and the suppression of haustorial mother cell production occurred after induction of an HR and cellular lignification. These results suggest that these defence responses are also important in the resistance mediated by dominant race-specific *Lr* genes. However, it is unlikely that the complete suppression of *P. triticina* growth in IT '0;' is due to an HR and cellular lignification alone, because a limited growth of *P. triticina* is observed in IT '1' in the presence of a rapid induction of an HR and cellular lignification. It is possible that the antifungal compounds produced during an HR and cellular lignification are not released quickly enough or not in sufficient quantity to completely suppress the growth of *P. triticina*. The complete suppression of *P. triticina* development in IT '0;' also requires the deposition of callose.

Callose can be deposited between the plasma membrane and cell wall after the exposure of plants to a range of biotic or abiotic stresses. During fungal infections, callose can be deposited in papillae formed beneath infection sites, which provides a physical barrier preventing the penetration of a pathogen (Nishimura *et al.*, 2003). The most well-characterized papillae-based defence response is described in the reaction of barley to infection by *Blumeria graminis*, the powdery mildew fungus. In this interaction, the formation of papillae confers complete protection against the invading pathogen (Zeyen *et al.*, 2002). The deposition of callose around intercellular fungal structures is also one of the resistance responses in the incompatible interaction between cowpea plant and cowpea rust fungus (*Uromyces vignae*), resulting in the complete encasement of intercellular fungus (Heath, 1971). In cereal–rust interactions, the deposition of callose in host mesophyll cells has been described in the penetration resistance against *P. triticina* and *P. striiformis* (Jacobs, 1989; Ma & Shang, 2004). Nevertheless, the involvement of callose in the resistance mediated by dominant race-specific *Lr* genes has not been previously reported.

HR and cellular lignification are induced earlier than the deposition of callose in *Lr9* + BBBD and *Lr9* + FBDJ, which suggests that the induction of these host defence responses in the resistance mediated by *Lr9* are triggered by different effectors. One possibility is that the callose deposition is induced by signal(s) released from cells undergoing HR and cellular lignification. Skalamera & Heath (1996) proposed that plants could respond to damage to cell walls rather than the presence of fungus during fungus-triggered callose deposition. Support for this hypothesis comes from studies on the action of cellular components of pathogens which act separately from products of *Avr* genes corresponding to race-specific *R* genes. For instance, in the interaction between tobacco (*Nicotiana tabacum*) and *Phytophthora cryptogea,* diffusible factor(s) were released from tobacco cells treated with elicitors over a 20-h period which induced a more rapid expression of defence-related genes than did the direct treatment of cell cultures with pure elicitors (Chappell *et al.*, 1997).

The deposition of callose is stronger in *Lr9* + BBBD and *Lr9* + FBDJ (IT '0;') than in *Lr9* + SBDG and *Lr9* + MBDS (IT '1') (Fig. 4). All these four races are avirulent on *Lr9*. During the infection of a susceptible cowpea cultivar by *Uromyces vignae*, deposition of callose on the plant membrane is normally inhibited but did occur when the fungus was killed by an heat shock treatment (Skalamera & Heath, 1996). By analogy, it is possible that *P. triticina* may inhibit this deposition through the production of effectors targeting this specific response.

As very little is known about the mechanism of callose deposition during the resistance mediated by *Lr9*, it is not clear whether *P. triticina* is capable of suppressing the accumulation of callose during its infection process. Further investigation is required to better understand the role of callose in the resistance mediated by *Lr9* and the signalling pathway(s) involved.

In summary, this study demonstrates that *P. triticina* development after initial penetration into the substomatal cavity is completely suppressed in highly resistant interactions producing IT '0;' and is only quantitatively reduced, but not stopped, in moderately resistant infection types IT '1' and '2', in which *P. triticina* is able to sustain a certain level of growth despite the presence of an induced HR and cellular lignification. The difference in resistant infection types, such as IT '1' and IT '2', is related to a delay in the expression of the host defence response. The resistance response mediated by *Lr9* is different from that mediated by *Lr2a, Lr3* and *LrB*, and the deposition of callose is important in resistance mediated by *Lr9*. These results suggest that the timing of induction of the host defence response plays a role in the interaction between *P. triticina* and wheat. Defence mechanisms other than HR and cellular lignification are also involved in the resistance against *P. triticina*. It is plausible to suggest that pyramiding of resistance genes with defence responses activated at different stages of the infection process of *P. triticina,* and/or resistance genes involving different defence mechanisms, could be an effective alternative in the breeding of durable resistance against *P. triticina*.

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