Histopathology and PR-protein markers provide insight into adult plant resistance to stripe rust of wheat

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SUMMARY

Stripe rust, caused by *Puccinia striiformis* f. sp. *tritici*, is a serious disease of wheat. The spring wheat cultivar Kariega expresses complete adult plant resistance to stripe rust, whereas Avocet S is susceptible. In former studies, quantitative trait loci (QTL) analysis of doubled haploid lines derived from a Kariega × Avocet S cross revealed two major QTL (*QYr.sgi-7D* and *QYr.sgi-2B.1*) and two minor QTL (*QYr.sgi-1A* and *QYr.sgi-4A.1*) responsible for the adult resistance of Kariega in the field. Avocet S contains none of these QTL. In the present study, stripe rust development was compared, by means of fluorescence and confocal laser scanning microscopy, in flag leaves of Kariega, Avocet S and six doubled haploid (DH) lines, containing all four, none or one QTL. Depending on the QTL present, the infection types of the DH lines ranged from resistant to fully susceptible. No differences in fungal growth were observed during the first 5 days post inoculation (dpi), whereas the mean length of the fungal colonies started to differ at 6 dpi. Interestingly, MP 51 carrying *QYr.sgi-7D* responded with lignification to the fungal growth without restricting it, whereas MP 35 containing *QYr.sgi-2B.1* did not show lignified host tissue, but fungal growth was restricted. RT-PCR experiments with sequences of pathogenesis-related (PR) proteins resulted in a slightly stronger induction of PR 1, 2 and 5, known markers for the hypersensitive reaction, and peroxidases in MP 51, whereas a second band for chitinases was detected in MP 35 only.

INTRODUCTION

Stripe rust, caused by *Puccinia striiformis* f. sp. *tritici*, is one of the most important diseases of wheat (*Triticum aestivum* L.). In South Africa, stripe rust was first detected in 1996 in the Western

Cape and since then has spread to all wheat production areas in the country (Boshoff *et al.*, 2002). A similar dispersion of this obligate biotrophic fungus was recorded in Australia where it was first detected in Victoria in 1979 (O'Brien *et al.*, 1980) before spreading to most Australian wheat-growing areas (McIntosh *et al.*, 2001; Wellings *et al.*, 2003). In recent years, races with new virulence combinations have been introduced or have evolved and threaten wheat production in many areas of the world (Kolmer, 2005). Although chemical control can be very effective, expenses are high and, therefore, genetic resistance is the preferred method to reduce losses (Knott, 1989).

In susceptible plants wheat rusts penetrate the leaf through a stoma, followed by differentiation of a substomatal vesicle, infection hyphae, haustorial mother cells and haustoria. As obligate biotrophic fungi, rusts depend on living host cells and take up nutrients from the host through their haustoria. During the later part of the disease cycle, pustules are formed and large amounts of spores produced.

Reactions of wheat to rusts can range from fully resistant over moderately resistant and moderately susceptible to fully susceptible (McIntosh *et al.*, 1995; Parlevliet and Van Ommeren, 1975). Fully resistant or immune wheat cultivars typically exhibit a hypersensitive reaction (HR) which can prevent further fungal growth at a very early stage of infection. HR in wheat against rust fungi is monogenic, post-haustorial and often cell autonomous: localized plant cell death is rapidly induced after the formation of a fungal haustorium, preventing the uptake of nutrients by the pathogen. In contrast, partial resistance can be mono- or polygenic: a portion of the infection sites in partially resistant wheat may be aborted pre-haustorially, before the formation of haustoria by the fungus, whereas other infection sites may continue to grow, albeit usually at a reduced rate compared with fully susceptible plants, and may even form pustules.

Resistance can be expressed in seedlings and throughout all ensuing stages or at later growth stages only. Adult plant resistance (APR) can be complete or partial, and can be either monogenic (McIntosh *et al.*, 1995) or multigenic (Bariana and McIntosh,

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1995). While seedling resistance against stripe rust can be very effective, it is usually race-specific and, thus, easily overcome by the pathogen. In contrast, APR may be a potentially durable source of resistance (Johnson, 1988). Thus far, little physiological, biochemical or molecular work has been done on APR, and the mechanisms behind it are not well understood (Broers and López-Atilano, 1996). This is mainly due to less discrete infection types and numerous recessive, often temperature-sensitive host resistance genes, typically with additive effects (Roelfs *et al.*, 1992).

Kariega is a South African bread wheat cultivar that exhibits complete APR to stripe rust. In spite of concern that accumulating partial and adult plant forms of resistance may be incompatible with high yields (Brown, 2002), Kariega is a high-yielding cultivar and of particular interest to wheat breeders. To gain further insight into the genetics of Kariega, Prins *et al.* (2005) developed a doubled haploid (DH) mapping population of 150 lines from a Kariega × Avocet S cross. Quantitative trait loci (QTL) analysis of these DH lines has identified two major QTL, located on chromosomes 7D (*QYr.sgi-7D*) and 2B (*QYr.sgi-2B.1*), and two minor QTL (*QYr.sgi-1A* and *QYr.sgi-4A.1*), responsible for the APR to stripe rust in Kariega (Ramburan *et al.*, 2004).

Previously, we described differences in the timing of host-pathogen responses and in development of stripe rust in infected flag leaves of the parental cultivars Kariega and Avocet S. We showed that fungal growth was similar in both cultivars during the first days post inoculation, followed by exponential growth of the pathogen in the susceptible line only. Four days post inoculation (dpi), induced cellular lignification was observed in Kariega and, 2 days later, lignified tissue surrounded the fungal infection sites. Only isolated lignified host cells in Avocet S occurred at 6 dpi and long, unbranched fungal hyphae outgrew the resistance reaction (Moldenhauer *et al.*, 2006). Here, we compare stripe rust development in infected flag leaves of the parental lines Kariega and Avocet S to that in six DH lines, containing all four QTL, none of the QTL or one QTL each. Using fluorescence and confocal laser scanning microscopy, we have focused on DH lines with the major *QYr.sgi-2B.1* and *QYr.sgi-7D* QTL. In addition to microscopically and cytochemically visible host-responses, RT-PCR results for genes of pathogenesis-related (PR) proteins typically involved in different types of plant disease resistance reactions are presented.

RESULTS

The infection types of Kariega, Avocet S and six DH lines are presented in Fig. 1 and Table 1. The resistant cultivar Kariega (Fig. 1a) showed only rare hypersensitive flecks on heavily infected flag leaves, thus exhibiting successful adult plant resistance. The DH line MP 6 containing all four QTL (Fig. 1c) responded similarly. In contrast, more than two-thirds of the infected flag leaf area of the susceptible parental cultivar Avocet S (Fig. 1b) was blotched

Fig. 1 Stripe rust-infected flag leaves (15 dpi) of wheat cultivars Kariega (a) and Avocet S (b) and six doubled haploid lines derived from a cross Kariega × Avocet S, containing all four (MP 6, c), none (MP 145, h) and one QTL each (d = MP 35, *QYr.sgi-7D*; e = MP 51, *QYr.sgi-2B.1*; f = MP 107, *QYr.sgi-1A*; g = MP 119, *QYr.sgi-4A.1*).

Table 1 Infection types of stripe rust-infected wheat lines containing defined combinations of four QTL responsible for adult plant resistance in Kariega, 15 dpi.

Wheat line	Major QTL		Minor QTL		Flag leaf infection response	
	<i>QYr.sgi-7D</i>	<i>QYr.sgi-2B.1</i>	<i>QYr.sgi-1A</i>	<i>QYr.sgi-4A.1</i>	Flag leaf infection type	Severity and reaction type
Kariega	+	+	+	+	;	1% TR
Avocet S	–	–	–	–	3C	70% MS
MP 6	+	+	+	+	;	1% TR
MP 35	+	–	–	–	;1C	25% R-MR
MP 51	–	+	–	–	1CN	30% MR
MP 107	–	–	+	–	3C	40% MS
MP119	–	–	–	+	4	70% S
MP 145	–	–	–	–	4	70% S

Evaluation of adult plant resistance was done on the disease intensity of 10–15 randomly chosen plants per wheat line. Flag leaf infection types were determined according to a 0 to 4 scale (McIntosh *et al.*, 1995), and disease severity resulting from the primary infection cycle was rated according to the modified Cobb scale (Peterson *et al.*, 1948), as indicated in the Experimental procedures. ; = flecking, T = trace, R = resistant, MR = moderately resistant, MS = moderately susceptible, S = susceptible, C = chlorosis, N = necrosis.

with rust pustules and some chlorosis. Line MP 145, containing no QTL according to field data, behaved similarly (Fig. 1h).

DH line MP 35, containing the major QTL *QYr.sgi-7D*, exhibited a resistant to moderately resistant infection type with limited sporulation (Fig. 1d). The flag leaves responded to fungal infection with visible hypersensitive flecks, chlorosis and leaf tip necrosis, affecting about one-quarter of the leaf area in the primary infection cycle. Stripe rust inoculation of DH line MP 51, containing the

major QTL *QYr.sgi-2B.1*, resulted in a moderately resistant infection type with large-scale chlorosis and necrosis affecting about one-third of the leaf area, and some limited sporulation (Fig. 1e).

DH line MP 107, containing the minor QTL *QYr.sgi-1A*, was rated as moderately susceptible where about 40% of the leaf area was blotched with stripe rust pustules and chlorosis (Fig. 1f). In contrast, minor QTL *QYr.sgi-4A.1* in DH line MP 119 did not have any visible effect on stripe rust infection under these controlled environmental conditions. MP 119 was recorded as fully susceptible with 70% of the leaf area covered with rust pustules without any chlorotic or necrotic plant responses (Fig. 1g).

To gain further insight into the resistance mechanism underlying the four QTL, stripe rust-inoculated flag leaves were subjected to histological analysis. Early infection structures of the stripe rust fungus inside flag leaves of all eight wheat lines were compared, and the number of haustorial mother cells (HMCs) per fungal colony were counted from 2 to 4 dpi. Two days post inoculation, two to three HMCs per fungal colony had formed in all cases, and five to seven HMCs per colony were counted 1 day later. Four days post inoculation, 18–25 HMCs per colony had developed, but no consistent significant difference could be detected between the wheat lines (data not shown). Beyond this time, colonies became too large for counting the number of HMCs.

The average length of fungal colonies parallel to the long axis of the wheat leaf was measured as a second parameter for fungal growth (Fig. 2). Although two of the wheat lines (Kariega and

Fig. 2 Mean length of stripe rust colonies in flag leaves of cvs Kariega (closed circle, resistant) and Avocet S (open square, susceptible) and six doubled haploid wheat lines containing all (closed star, MP 6), none (open upwards triangle, MP 145) or one QTL each (closed downwards triangle, MP 35, *QYr.sgi-7D*; closed upwards triangle, MP 51, *QYr.sgi-2B.1*; open star, MP 107, *QYr.sgi-1A*; open downwards triangle, MP 119, *QYr.sgi-4A.1*) as a function of time after inoculation. On average, 80 colonies from three to five flag leaves per wheat line and time point were analysed. The fungal growth between all cultivars was significantly different at 7 dpi except for growth in lines MP 51, Avocet S and MP 145, which exhibited a similar average colony length (*t*-test, $P < 0.01$).

MP 6) responded with hypersensitive flecking to infection, limited fungal growth was observed inside the flag leaves. Again, no significant differences were seen up to 5 dpi. However, 1 day later, fungal growth started to differ between lines, showing large differences in the mean length of the rust colonies at 7 dpi. At this time, the average rust colony in flag leaves of the resistant parental wheat cultivar Kariega reached about 300 μm in length, and about 460 μm in the comparable line MP 6 containing all four QTL. Fungal colonies reached lengths of c. 625 μm and c. 900 μm in the two DH lines containing one of the two major QTL, *QYr.sgi-7D* in MP 35 and *QYr.sgi-2B.1* in MP 51, respectively. In the two DH lines containing one of the two minor QTL, *QYr.sgi-1A* in MP 107 and *QYr.sgi-4A.1* in MP 119, fungal colonies grew to lengths of c. 750 μm and c. 1280 μm, respectively. At 7 dpi, average colony lengths of c. 1025 μm and c. 1050 μm were measured in the susceptible parental wheat cultivar Avocet S and in MP 145 possessing no QTL, respectively.

The contrasting results obtained with the two DH lines carrying single major QTL, i.e. rather small colonies with hypersensitive flecking and little chlorosis and necrosis formed in MP 35 containing *QYr.sgi-7D*, and rather large colonies with heavy chlorosis and necrosis formed in MP 51 containing *QYr.sgi-2B.1*, prompted further examinations using confocal laser scanning microscopy. Figure 3 shows the UV-autofluorescent host cell response to stripe rust infection in flag leaves of these two DH lines, 7 dpi. In spite of the hypersensitive flecking observed in MP 35, no or only very few autofluorescent host cells were seen in MP 35 (Figs 1 and 3b). In contrast, a strong autofluorescent host cell response was seen in MP 51, which exhibited large-scale chlorosis and necrosis upon infection (Figs 1 and 3a). The occurrence of autofluorescent, necrotic host cells per fungal colony was, therefore, quantified in both of the parental cultivars and in all six DH lines over the course of infection (Fig. 4).

Four days post inoculations the first necrotic cells occurred in wheat line MP 6 (all four QTL) followed 1 day later by MP 51 (*QYr.sgi-2B.1*) and Kariega, around the substomatal vesicle (SSV), with a mean of two to three necrotic cells per fungal colony. Seven days post inoculation, about five necrotic host cells per fungal colony were counted in Kariega and in MP 6, following the pathogenic growth and surrounding the infection site completely or partially, while on average c. 17 necrotic host cells per colony were detected in MP 51, also following fungal growth but not necessarily starting from the SSV. No or only few autofluorescent host cells around the SSV were obtained in line MP 35 (*QYr.sgi-7D*), in the lines containing minor or no QTL, and in the susceptible cultivar Avocet S (Fig. 4 and data not shown).

As the yellow autofluorescence of hypersensitively necrotic host cells in rust-infected wheat leaves is typically caused by cellular lignification (Menden *et al.*, 2007; Tiburzy and Reisener, 1990), inoculated flag leaves were analysed following phloroglucinol/HCl staining. Figure 5 shows fungal development and host cell

Fig. 3 Differences of wheat stripe rust development and host response in two doubled haploid wheat lines (MP 51 containing major-QTL *QYr.sgi-2B.1*; MP 35 containing major-QTL *QYr.sgi-7D*) derived from a Kariega (resistant) × Avocet S (susceptible) cross, 7 dpi. Infection structures shown (SSV = substomatal vesicle) are typical for these wheat lines, but the amount of necrotic host cells in line MP 51 shown here is higher than the average value of dead cells presented in Fig. 4. The fungus was stained with Uvitex 2B and analysed using confocal laser scanning microscopy. In the immediate vicinity of the fungus (excitation 351/364 nm, filter 400–500 nm, depicted in green), autofluorescing necrotic tissue (excitation 514/543 nm, filter 560–680 nm, depicted in yellowish red) was visible in MP 51 (a) but only sporadic in MP 35 (b).

reactions in the two DH lines carrying one major QTL each. Nine days post inoculation, the Uvitex-stained, blue-fluorescing fungal colonies occupied large areas of flag leaves of MP 51 (Fig. 5a), whereas much smaller colonies occurred in flag leaves of MP 35 (Fig. 5d). The autofluorescence of the necrotic tissue (Fig. 5b) associated with the fungal colonies resisted alkaline extraction,

Fig. 4 Average number of necrotic host cells per stripe rust colony in flag leaves of cvs Kariega (closed circle, resistant) and Avocet S (open square, susceptible) and doubled haploid wheat lines containing all (closed star, MP 6), none (open upwards triangle, MP 145) or one QTL each (closed downwards triangle, MP 35, *QYr.sgi-7D*; closed upwards triangle, MP 51, *QYr.sgi-2B.1*; open star, MP 107, *QYr.sgi-1A*; open downwards triangle, MP 119, *QYr.sgi-4A.1*), depicted as a function of time post inoculation. On average, 80 colonies from three to five flag leaves per wheat line and time point were analysed. Seven days post inoculation, the number of necrotic host cells on MP 51 differed significantly from all investigated lines, whereas the host reaction was similar in MP 6 and Kariega, but also significantly different to all investigated wheat lines (*t*-test, $P < 0.001$).

and a positive outcome of the stain (Fig. 5c) indicated the deposition of lignin or lignin-like material in the necrotic cells. Sporadically occurring autofluorescent cells in infected flag leaves of MP 35 (Fig. 5e) also responded positively to the phloroglucinol/HCl stain (Fig. 5f). Similarly, necrotic host cells obtained in all other investigated wheat cultivars and lines responded with a positive outcome of the stain (data not shown).

Clearly, hypersensitive necrosis is not involved in restricting fungal growth in DH line MP 35 carrying *QYr.sgi-7D*. To investigate the involvement of other metabolic pathways in the resistance mechanisms governed by *QYr.sgi-7D*, RT-PCR analyses were carried out (Fig. 6). For comparison, DH line MP 51 carrying *QYr.sgi-2B.1* and exhibiting large-scale necrosis without restricting fungal growth during the first 7 days post inoculation was included. Pooled RNA from days 5, 6 and 7 post inoculation was used as templates, and primers were derived from known PR proteins. Transcript accumulation for PR-1, PR-2 (glucanase) and PR-5 (thaumatin) was greater in rust-infected flag leaves of the necrosis-exhibiting line MP 51 than in leaves of the non-necrotic line MP 35. The same behaviour was seen with transcript accumulation for PR-9 (peroxidase). In contrast, the transcripts for PR-4 (chitinase) accumulated to similar levels in infected leaves of both lines, but a second, smaller chitinase band was obtained in the non-necrotic MP 35 only. No expression of PR proteins was seen in any of the mock-inoculated control plants

Fig. 5 Comparison of lignin formation in stripe rust-infected flag leaves of doubled haploid wheat lines MP 51 (a–c) and MP 35 (d–f), 9 dpi. Under the epifluorescence microscope (excitation 330–380 nm, barrier filter 420 nm), Uvitex-stained fungal colonies were visible in infected flag leaves (a, d). The autofluorescing mesophyll cells of MP 51 (b) showed a positive phloroglucinol/HCl-stain under the transmission light microscope (c), indicating the deposition of lignin or lignin-like material, whereas in MP 35, cells responded only sporadically with autofluorescing mesophyll cells (e) and a positive phloroglucinol/HCl stain (f).

except for a slight expression of PR-5 in both lines, and of PR-9 in MP 51 only.

DISCUSSION

Clear differences in infection responses were observed among parental cultivars and DH lines at all levels of assessment. Furthermore, the two DH lines containing one of the major QTL each, differed in the accumulation of transcripts of known PR proteins.

As expected, the response of DH line MP 6 carrying the four known Kariëga-QTL for APR was similar to Kariëga. The slightly larger size of fungal colonies in the DH line MP 6 compared with the resistant parent might point to additional resistance in Kariëga, or a change in gene interactions in the more susceptible background contributed by Avocet S.

Unexpectedly, the DH line MP 145 carrying none of the Kariëga-QTL was even more susceptible than the susceptible parent Avocet S. In previous studies, it has been shown that Avocet S carries minor stripe rust QTL on chromosome 6A (Singh *et al.*, 2001) and 4A (*QYr.sgi-4A.2*; Ramburan *et al.*, 2004). The latter was only detected in growth chamber conditions and was not consistently detected in all environments. MP 145 has Avocet S alleles spanning the whole chromosome region containing the two 4A QTL, thus implying that it actually should have the *QYr.sgi-4A.2* originating from Avocet S. The observed difference

could therefore possibly be ascribed to a minor Avocet S-QTL for APR such as 6A not being inherited by MP 145. The 6A QTL found in Avocet S (Singh *et al.*, 2001) was below detection level in the Kariëga × Avocet S population. This may have resulted from poor coverage of the 6AS region as the marker Gwm356, which is linked to the 6A QTL (Singh *et al.*, 2001), was not mapped in the current population. Interestingly, both MP 145 and MP 119 contain Kariëga alleles spanning most of chromosome 6AL, thus lacking the stem rust resistance gene *Sr26* from Avocet S. Although this region reduced stripe rust severity in an Avocet S × Pavon cross (Singh *et al.*, 2001), it did not influence stripe rust resistance in the Kariëga × Avocet S population.

Similarly, line MP 119 carrying the minor Kariëga QTL *QYr.sgi-4A.1* was as susceptible as MP 145 and thus more susceptible than Avocet S, a result that is in accordance with the field data (Ramburan *et al.*, 2004). In this line, the *QYr.sgi-4A.2* minor QTL derived from Avocet S is absent. Apart from the possible absence of undetected Avocet S QTL, it is likely that *QYr.sgi-4A.1* is only expressed at a measurable level in the presence of the other Kariëga QTL as their interactions are as yet unknown. Although most of the markers spanning the 4A region are less than 5 cM apart, the possibility that the QTL has been recombined by a double crossover cannot be ruled out. This can only be detected if marker density is increased. In contrast, DH line MP 107 carrying the second minor QTL *QYr.sgi-1A*, in which fungal development was somewhat delayed compared with the susceptible parent

Fig. 6 Expression pattern of different PR proteins in stripe rust-infected (inf.) and mock-infected (contr.) flag leaves of two doubled haploid wheat lines carrying major QTL *QYr.sgi-7D* (MP 35) and *QYr.sgi-2B.1* (MP 51), respectively. Total RNA was isolated and pooled 5, 6 and 7 dpi and RT PCRs were performed by using primer sequences of known PR proteins: PR-1, PR-2 (glucanase), PR-5 (thaumatin), PR-9 (peroxidase) and PR-4 (chitinase). Primers for tubulin were used as control for equal amounts of cDNA during RT PCRs.

cultivar, eventually showed the same infection type as the latter. In a previous field trial, *QYr.sgi-1A* was consistently detected whereas *QYr.sgi-4A.1* was only detected early during the season. This QTL was poorly expressed according to terminal scores and did not explain a significant amount of variation. In the same study, both QTL were not detected in growth chamber scores (Ramburan *et al.*, 2004). Some APR genes are known to be influenced by the environment, temperature and light conditions, and their expression is known to be dependent on the growth stage of the plants (Drijepontd and Pretorius, 1989, 1991; Park and Rees, 1989). In our experiments, greenhouse conditions seemed to be sufficient for expression of QTL *QYr.sgi-1A*, whereas QTL *QYr.sgi-4A.1* obviously was not expressed. More likely, the minor QTL detected under controlled conditions is environmentally

specific in its expression and not always representative of field conditions. As the growing of plants in the present study also differed from the procedures of Ramburan *et al.* (2004) (plants were grown as dwarf adult plants), deviation in minor QTL detection is quite possible. Further analyses are needed to confirm the existence of these minor QTL as it is well known that the accuracy of QTL mapping is influenced by several factors, such as the size of the explained variance (Van Ooijen, 1992, 1999).

The two DH lines carrying single major QTL, MP 51 and MP 35, showed moderately resistant infection types 2 weeks after inoculation. During the first week after inoculation, however, fungal growth was restricted in line MP 35 but not in MP 51 in which the fungus grew as well as in the susceptible lines. This is in agreement with the QTL analysis of field disease scores, where *QYr.sgi-7D* explained a higher proportion of the variance in proportion of leaf area infected (27–29%) than host reaction type (9–18%) (Ramburan *et al.*, 2004). Another striking difference between the two lines was the extensive occurrence of necrotic host cells associated with fungal colonies in MP 51 but not in MP 35. This confirms the QTL analysis finding that *QYr.sgi-2B.1* explained a higher proportion of the variance in host reaction type (33–46%) than percentage leaf area infected (17–30%) (Ramburan *et al.*, 2004). While the necrotic host cells in MP 51 had the appearance of a hypersensitive reaction and stained positively for lignin, which has been shown to be related to hypersensitive cell death (Hijwegen, 1963; Moerschbacher *et al.*, 1990; Vance *et al.*, 1980), this reaction does not appear to retard fungal growth in this case during the first 7 days post inoculation. Cell death here appears to be normosensitive rather than hypersensitive, i.e. the slow, normosensitive plant cell death spreads beyond the site of infection and does not effectively prevent pathogen growth or spread and is therefore not associated with local resistance (He, 1996; Klement, 1982). In a recent study by Tao *et al.* (2003), a large-scale mRNA expression profile analysis of *Arabidopsis* responses to compatible and incompatible strains of *Pseudomonas syringae*, the overall similarity of the responses was evaluated. A broad range of defence responses in early incompatible interactions have close similarity to those in late compatible interactions, indicating that a common signal transduction pathway underlies both responses, but they differ in their timing. However, sporulation of the fungus in MP 51 was limited 15 days post inoculation. It is unclear if the normosensitive cell death deprives the pathogen of nutrients, has another effect on repressing fungal sporulation or if other resistance mechanisms not involved in cell death are responsible for inhibited sporulation. A similar phenomenon as observed in our experiments has been described previously for leaf rust-infected wheat (Lee and Shaner, 1984) and barley (Niks and Kuiper, 1983) plants.

Interestingly, larger amounts of necrotic host cells were observed only in those plants carrying *QYr.sgi-2B.1*. Line MP 6 carrying all four QTL, as well as the resistant parent line Kariaga, developed

fewer necrotic host cells but was more resistant than MP 51 carrying *QYr.sgi-2B.1* only. First necrotic host cells in Karioga, MP 6 and MP 35 were obtained close to the substomatal vesicle, whereas necrosis in MP 51 containing *QYr.sgi-2B.1* sometimes started away from the SSV. This major QTL thus appears to convey the ability to react with a necrotic response to the presence of the rust pathogen, but this response is too slow to effectively prevent fungal growth. By contrast, fungal infection sites in MP 6 and Karioga could possibly be surrounded by lignified host cells, because the fungal growth was, unlike in MP 51, restricted by a different resistance reaction, which might not involve the lignification mediated by *QYr.sgi-2B.1*. *QYr.sgi-7D* in MP 35 seems to convey this kind of resistance; fungal growth was to some extent restricted, but no or only few necrotic cells were obtained close to the SSV. In the presence of all four QTL, their combined action is effective enough to slow down fungal growth sufficiently to prevent an excess necrotic reaction from occurring. It would be interesting to analyse infection type and fungal growth in a DH line lacking *QYr.sgi-2B.1* but carrying the other three QTL.

QYr.sgi-7D has been suggested to be closely linked or identical to the major stripe rust resistance gene *Yr18*, a highly interesting locus conveying resistance also to leaf rust due to the *Lr34* gene (McIntosh, 1992; Singh, 1992) and to three other wheat pathogens (Spielmeyer *et al.*, 2005). The resistance mechanism, in this case, clearly does not involve necrosis. Papilla formation, which has been described to be involved in the non-hypersensitive type of resistance of some wheat and barley cultivars to leaf rust (Jacobs, 1989; Niks, 1983, 1986), was not observed in this study. Similarly, two other studies on *Lr34*-mediated leaf rust resistance reported on restricted fungal growth in the absence of both host cell necrosis and papilla formation (Rubiales and Niks, 1995; Singh and Huerta-Espino, 2003).

To assess other possible resistance mechanisms not visible microscopically, such as phytoalexin production (Bücker and Grambow, 1990; Mansfield, 2000) or the secretion of PR proteins (Münch-Garhoff *et al.*, 1997), biochemical and molecular studies are needed. In an exploratory study, we have focused on genes for PR 1, 2 and 5, which have been used as markers for hypersensitive resistance in *Arabidopsis* (Uknes *et al.*, 1992), PR 9 for peroxidases possibly involved in lignin formation, and PR 4 coding for chitinases. The expression of these genes was analysed in the two DH lines carrying one of the two major QTL each. Infection induced expression of HR and lignin-related PR proteins seemed to be slightly stronger in line MP 51 showing extensive necrosis, while an additional chitinase gene appears to be induced in MP 35 only. We are currently extending these molecular studies using DNA macroarrays based on differential cDNA libraries obtained from infected and healthy Karioga and Avocet S plants. Using mRNA from the DH lines described here, these studies will allow us to dissect molecular aspects of QTL-based adult plant resistance of Karioga to stripe rust.

EXPERIMENTAL PROCEDURES

Wheat (*Triticum aestivum* L.) cvs Karioga and Avocet S were grown in sterilized soil in $3.8 \times 3.8 \times 10$ -cm³ plastic cones at 18–25 °C in a greenhouse. Additionally, six DH wheat lines derived from a Karioga $\times$ Avocet S cross (Prins *et al.*, 2005) with four, none or one QTL each (*QYr.sgi-7D*, *QYr.sgi-2B.1*, *QYr.sgi-1A* and *QYr.sgi-4A.1*) responsible for the APR in Karioga were grown alongside the parents. Each cone contained five to seven seeds. Plants were inoculated 35 days after planting when most plants were heading (growth stage 54) (Zadoks *et al.*, 1974), by spraying with a fine mist of sterile, distilled water containing fresh urediospores of *Puccinia striiformis* Westend. f. sp. *tritici* Eriks. pathotype 6E22A- (c. 200 000 spores/mL) and Tween 20 (0.03%, v/v) as a surfactant. After incubation at 10 °C in a high-humidity chamber for 48 h, plants were returned to a greenhouse cubicle at 20/15 °C. Two to 8 dpi, flag leaves were harvested daily and combined for further histological and molecular analyses.

Evaluation of adult plant resistance was done on the disease intensity of 10–15 randomly chosen plants per wheat line 15 dpi. Flag leaf infection types were determined according to a 0 to 4 scale (McIntosh *et al.*, 1995) where 0 and 4 indicate the absence of visible symptoms and full susceptibility, respectively. Disease severity resulting from the primary infection cycle was rated according to the modified Cobb scale (Peterson *et al.*, 1948) where the numerical value indicates the percentage flag leaf area affected (100% is equal to the entire flag leaf rusted). Accompanying reaction types indicate resistance (R), moderate resistance (MR), moderate susceptibility (MS) and susceptibility (S).

For histology, three to five flag leaves per sample were fixed and cleared in ethanol/trichloromethane (3:1, v/v) containing 0.15% (w/v) trichloroacetic acid, for at least 18 h. After washing with 50% (v/v) ethanol, specimens were incubated at 95 °C in 0.5 M sodium hydroxide for 30 min. Leaves were then rinsed twice with water and soaked in 0.1 M Tris/HCl buffer (pH 5.8) for 30 min. Subsequently, leaves were stained in 0.1% (w/v) Uvitex 2B (Ciba-Geigy, Basel, Switzerland) for 5 min, thoroughly rinsed four times (10 min each) with water and once (15 min) with 25% (v/v) glycerol (protocol modified after Rohringer *et al.*, 1977). Specimens were stored in 50% (v/v) glycerol.

Leaves were examined using a Nikon Labophot fluorescence microscope equipped with epifluorescence optics. A UV-1A filter (excitation filter 330–380 nm, barrier filter 420 nm) was used to visualize fungal structures by their light blue fluorescence. To analyse the size of endophytically growing infection structures of stripe rust in wheat flag leaves, the maximum length of the fungal colonies parallel to the length of the leaf was measured, and HMCs were counted. Using a confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany), Uvitex 2B-stained leaves were excited with UV-laser beams at 351 and 364 nm and scanned with filter settings at 400–500 nm for fungal infection structures,

or with argon-laser beams at 514 and 543 nm and scanned with filter settings at 560–680 nm for necrotic mesophyll cells.

Lignin was stained using phloroglucinol/HCl (Sherwood and Vance, 1976), in a procedure modified for leaves prestained with Uvitex 2B (see above). Specimens were soaked in phloroglucinol (4%, in distilled water) for at least 20 min prior to localization of fungal hyphae and hypersensitive host cells using UV-fluorescence microscopy. The cover glass was then removed carefully, and a drop of 37% HCl was added to the leaf. The slide was heated slowly on a hot plate until the red lignin coloration appeared, and leaves were washed very gently with water prior to microscopic observation of lignin formation.

The infection types obtained in this study were fully consistent with those observed in at least two field seasons (Ramburan *et al.*, 2004; V. P. Ramburan, unpublished data) and in greenhouse-grown plants of the parent lines (Moldenhauer *et al.*, 2006). Infection levels were excellent in all cases, and all plants within a treatment were consistent in expression. On average, 80 colonies from three to five infected flag leaves per wheat line and time point were analysed. Statistical treatment of the results was done by unpaired Student's *t*-test.

RT-PCRs were performed on 1 µg of total RNA, isolated and pooled 5, 6, and 7 dpi from stripe rust-infected and mock-infected flag leaves of DH lines MP 35 and MP 51, by using the following primer combinations: Pr-1 (T_m 50 °C) 5'-AGTAATGGA-GACGCCAA-3' and 5'-ACTGCGGAGCAATACA-3', glucanase (T_m 59 °C) 5'-GGCGTCTCTTCGCGTACAT-3' and 5'-CAGCAAGCT-GGGCTTCTTCG-3', thaumatin (T_m 50.7 °C) 5'-TCAACAYCAA-GAACAAT-3' and 5'-AGATGTCGTARAAGTCCTG-3', peroxidase (T_m 58.2 °C) 5'-ATCAGACCGTCTCCTGCG-3' and 5'-GCAGCT-GAGCCTGATCTG-3', chitinase (T_m 59 °C) 5'-GGCGTGTCTCCAT-TATCTC-3' and 5'-CGGCCGTAGTTGTAGTTGT-3', tubulin (T_m 56 °C) 5'-CACACAGCAGATGTGGGACT-3' and 5'-GTGGAGTTGCCAAT-GAAGAT-3'. Degenerate primers were designed from conserved regions of the PR proteins except for glucanase, which was detected by specific primers. RT PCRs were performed in an Eppendorf cyclor. General cycling conditions were 94 °C for 2 min, 30–34 cycles of 94 °C for 30 s, annealing at primer-specific T_m for 30 s, and 72 °C for 30 s per 500-bp PCR fragment, followed by a final extension of 7 min at 72 °C.

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