Unraveling the Wheat Stem Rust Infection Process on Barley Genotypes Through Relative qPCR and Fluorescence Microscopy

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ABSTRACT

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The infection process of wheat stem rust (*Puccinia graminis* f. sp. *tritici*) on barley (*Hordeum vulgare*) is often observed as a mesothetic infection type at the seedling stages, and cultivars containing the same major resistance genes often show variation in the level of resistance provided against the same pathogen race or isolate. Thus, robust phenotyping data based on quantification of fungal DNA can improve the ability to elucidate host–pathogen interaction, especially at early time points of infection when disease symptoms are not yet evident. Quantitative real-time polymerase chain reaction (qPCR) was used to determine the amount of fungal DNA relative to host DNA in infected tissue, providing new insights about fungal development and host resistance during the infection process in this pathosystem. The stem rust susceptible ‘Steptoe’, resistant cultivars containing only *Rpg1*

(‘Beacon’, ‘Morex’, and ‘Chevron’), and the resistant line Q21861 containing *Rpg1* and the *rpg4/Rpg5* complex were evaluated using the traditional 0-to-4 rating scale, fluorescence microscopy, and qPCR. Statistical differences ($P < 0.05$) were observed in fungal development as early as 24 h postinoculation using the qPCR assay. Fungal development observed using fluorescence microscopy displayed the same hierarchical ordering observed using the qPCR assay. The fungal development occurring at 24 and 48 h postinoculation was vastly different than what was expected using the traditional disease phenotyping methodology; with Steptoe appearing more resistant than the barley lines harboring the known *Rpg1* and *rpg4/Rpg5* resistance complex. These data indicate potential early prehaustorial resistance contributions in a cultivar considered susceptible based on infection type. Moreover, the temporal differences in resistance suggest pre- and post-haustorial resistance mechanisms in the barley–wheat stem rust infection process, indicating potential host genotype contributions related to basal defense during the wheat stem rust infection process.

In the early 20th century, many epidemics of wheat stem rust, caused by *Puccinia graminis* f. sp. *tritici* Erikss. & Henning, caused large yield losses for common wheat (*Triticum aestivum* L.), durum wheat (*T. turgidum* subsp. *durum* (Desf.) Husn.), and barley (*Hordeum vulgare* L.). Since then, *P. graminis* has been primarily managed through the removal of its alternate host, the common barberry (*Berberis vulgaris* L.), and the introduction of resistant wheat and barley cultivars (17). The barberry eradication program in the United States was particularly effective by eliminating the sexual stage of *P. graminis* (17). The removal of the pathogen’s sexual stage precludes genetic recombination, dramatically slowing the emergence of new races and allowing for the prediction of race prevalence on a yearly basis (29).

The distribution of resistant barley varieties to farmers has mitigated damage caused by *P. graminis* f. sp. *tritici* since the 1940s (34). This is mainly due to the incorporation of the gene *Rpg1*, which was acquired from ‘Kindred’ in the United States and ‘Peatland’ in Canada, and is now fixed in all Midwestern barley cultivars (6). *Rpg1* provides the majority of resistance to endemic races of the stem rust pathogen in the Midwestern United States and Prairie Provinces of Canada (6,9). Furthermore, significant effort has been devoted toward the characterization of *Rpg1* due to its longstanding success as a durable resistance gene. The emergence of the highly virulent Ug99 lineage in Eastern Africa in 1999 and races virulent on *Rpg1* from the Pacific Northwest in 1989 and the ensuing identification of the *rpg4/Rpg5* resistance complex

effective against these races have warranted a need to better understand the barley–wheat stem rust pathosystem (26,34,37).

Rpg1-containing cultivars often exhibit varying resistant infection types due to their unique genetic backgrounds (21,34,39,44). The quantitative interactions of genes influencing this phenomenon are not currently fully understood. The use of a traditional 0-to-4 scoring scale on barley seedlings is often complicated by a mesothetic reaction to many pathotypes of *P. graminis* (11,34,36). Quantification of fungal DNA by quantitative real-time polymerase chain reaction (qPCR) is an effective way to overcome the limitations of evaluating host–pathogen interaction solely based on categorical data collected using the 0-to-4 scale. This method also allows for a more robust assessment of temporal interactions occurring during the infection process because of the ability to assess early stages of the infection process.

The use of absolute qPCR for resistance phenotyping has been used successfully for other cereal pathosystems, such as crown rust (caused by *Puccinia coronata* f. sp. *avenae* P. Syd. & Syd.) in *Avena sativa* L. (15). The process described by Jackson et al. (15) involved a series of laborious measurements which led to the development of a relative assay which overcame the measurement issues by using specific primer and probe combinations for a reference gene instead of relying on dry weight measurements (2). The use of relative qPCR assays has been shown to provide an accurate way to characterize a cultivar’s phenotype, as well as provide information about cultivar resistance prior to the appearance of symptoms in numerous pathosystems (2–4,14,25,27). Additionally, relative qPCR has been successfully utilized to map quantitative trait loci (QTL) associated with adult partial resistance (1).

Because disease symptoms are not visible until 8 or 9 days after inoculation, it is difficult to determine how the wheat stem rust

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infection progresses during early stages of development. Currently, the best methodology for assessing the infection process at early stages is through the use of fluorescence microscopy. Fluorescence microscopy has been used to evaluate the infection process of wheat rusts; however, the methodology surrounding sample preparation is laborious for large sample sizes (16,18,30,31,42). Thus, the objective of this project was to develop a qPCR assay to assess the infection process at multiple time points, determine the accuracy of the qPCR assay at early stages using fluorescence microscopy and late stages using traditional phenotyping, and evaluate cellular and physical differences in host resistance at the early stages of the infection process.

MATERIALS AND METHODS

Host and inoculum. Four barley cultivars (‘Steptoe’, ‘Beacon’, ‘Morex’, and ‘Chevron’) and the line Q21861 were grown in a completely randomized design with an experimental unit consisting of two plants. The experimental units were replicated four times for each time point to be evaluated for the qPCR assay. The experiment was performed twice to account for biological and experimental variation. An independent set of the same varieties consisting of 12 replications of one plant per genotype per time point was also planted for evaluation using fluorescence microscopy. Steptoe does not contain any previously characterized resistance genes to *P. graminis*; Beacon, Morex, and Chevron have the major resistance gene *Rpg1*, which is effective against *P. graminis* f. sp. *tritici* race MCCFC used in this study; and Q21861 contains both *Rpg1* and the *rpg4/Rpg5* resistance complex, which is also effective against *P. graminis* f. sp. *tritici* race MCCFC (7,21,22). Plants were grown in a growth chamber with a day-and-night cycle of 14 and 10 h and a temperature of 22 and 18°C, respectively. Seven days after planting, the seedlings were inoculated via a spray inoculation with *P. graminis* f. sp. *tritici* race MCCFC at a concentration of 7 × 10⁶ urediniospores/ml of Soltrol 170 (Chevron Phillips Chemical Company LLC, Woodlands, TX).

Sample handling, phenotypic evaluation, and standard development for qPCR. Four tissue samples were collected for the qPCR assays at five time points for each barley genotype, reflecting a natural progression of the infection process. The temporal points for the first experiment included prior to inoculation, immediately after inoculation, 48 h postinoculation (HPI), 6 days postinoculation (DPI), and 14 DPI. The temporal points for the second experiment included prior to inoculation, immediately after inoculation, 24 and 48 HPI, and 6 DPI. At each time point, 5 cm of tissue was collected from the center of the leaves and stored in a 2-ml microtube at -80°C until all samples were collected. At 12 DPI, all plants were scored prior to tissue collection using a 0-to-4 scale and the median infection type observed was used to establish resistance levels to *P. graminis* f. sp. *tritici* race MCCFC for each cultivar. The 0-to-4 scale used was modified from the scale used to evaluate *P. graminis* f. sp. *tritici* infection in wheat (33). In the scale, an infection type of 0 indicates the absence of symptoms, a fleck (:) indicates necrosis in the absence of visual uredinial pustules, 1

indicates a pustule surrounded by necrosis, 2 indicates a pustule surrounded by chlorosis, 3 is defined as a round pustule without chlorosis, and 4 is defined as a large and often diamond-shaped pustule. Additionally, a plus (+) or minus (-) sign was used to indicate increased or decreased sporulation that deviate from what is typically observed for an infection type, and a mesothetic reaction was denoted by X. In cases where multiple infection types were observed on a leaf, each infection type was recorded in order of frequency observed on the leaf. Infection types of 0, fleck (:), 1, 2, and X are considered resistant and infection type of 3 and 4 are susceptible.

DNA for the standards and samples was extracted using a modified cetyltrimethylammonium bromide method (38). Host and pathogen standards for this experiment consisted of DNA extracted from Steptoe barley and *P. graminis* f. sp. *tritici* race MCCFC urediniospores, respectively. Five-point serial host and pathogen standards were developed by diluting genomic DNA to concentrations ranging from 200 to 2.47 ng/μl along a threefold serial dilution in accordance with the MIQE guidelines (8).

Evaluation of the stem rust infection progression via qPCR. Fungal DNA was amplified utilizing a previously developed hydrolysis probe and species-specific markers (5). Primers and hydrolysis probes for the reference gene (Table 1) were developed based on a barley ubiquitin gene (GenBank accession M60175) identified by Rostoks et al. (32). Primers were obtained from Sigma-Aldrich (St. Louis, MO) and hydrolysis probes were obtained from Life Technologies (Carlsbad, CA). The expected amplicon size is 242 and 270 bp for the barley (Hvubi-F1 and Hvubi-R1) and rust primers (ITS1rustF10d and ITS1rustR3c), respectively. Amplicon size was confirmed through conventional PCR and agarose gel electrophoresis. Reactions were completed at a volume of 20 μl containing 1× PCR buffer, 2.5 mM MgCl₂, 187.5 μM dNTPs, 500 nM primer, 60 ng of DNA, and 1 unit of GoTaq (Promega Corporation, Madison, WI). Amplification was performed under the following conditions: initial denaturation at 94°C for 5 min; followed by 40 cycles of 30 s of denaturation at 94°C, a 60°C annealing step for 30 s, and an extension step of 72°C for 45 s; followed by a final extension at 72°C for 7 min. The specificity of the fungal and barley primers and probes was confirmed on the standards through qPCR. This was accomplished by running the markers with their corresponding template DNA, with the opposite DNA, and as a multiplex reaction with both barley and fungal DNA present to insure that no reaction inhibition or nonspecific amplification occurred. All qPCR reactions were performed in a Bio-Rad Laboratories C1000 thermocycler with an attached CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) utilizing the following protocol: denaturation of 95°C for 3 min followed by 40 cycles of 15 s of denaturation at 95°C and a 1 min annealing step at 60°C. The reactions were optimized to a volume of 25 μl in clear 96-well PCR plates (Bio-Rad Laboratories) using iQ multiplex powermix (Bio-Rad Laboratories), with each reaction containing 8.75 pmol of each ubiquitin primer and probe and 5 pmol of each fungal primer and probe. The efficiency (efficiency = 10^(-1/slope) - 1) of the experiment was then calculated to determine multiplex suitability and repeatability (8).

The samples for each time point were run in replicate using the previously mentioned conditions and the assay was repeated twice. Each assay consisted of the samples from one time point and the standards. Samples where quantification cycle (Cq) values were greater than 37 or where Cq values for a primer set differed by more than one cycle between replicates within an assay were removed from analysis. The varieties were evaluated by subtracting the Cq value of the barley hydrolysis probe from the Cq value of the fungal hydrolysis probe for each sample to normalize for the amount of barley tissue present. A Shapiro-Wilk test was used to establish population normality at each time point followed by an analysis of variance (ANOVA) for the cultivars and line to determine whether

TABLE 1. Barley ubiquitin and *Puccinia graminis* f. sp. *tritici* internal transcribed spacer primer and probe sequences

Primer/probe	Sequence (5'-3')
Hvubi-F1	ACTACAACATCCAGAAGGAGTCCAC
Hvubi-R1	GTCGAAGTGGTTGGCGGCCATGAAGGTC
Hvubi VIC	VIC-CGCCAAGAAGCGCAAGAAGAAGACGTACACC-MGBNFQ
ITS1rustF10d	TGAACCTGCAGAAGGATCATT
ITS1rustR3c	TGAGAGCCTAGAGATCCATTGT
Pg FAM 1	6FAM-TTGTGGCTCGACTCTCTTATAACCAAACC-MGBNFQ

statistical differences were observed. Tukey's honest significant difference test was performed to establish statistical differences between cultivars and provide a ranking from most to least amount of fungal DNA present. All statistical analysis was performed using JMP 10.0 (SAS Institute, Cary, NC).

Evaluation of stem rust infection progression via fluorescence microscopy. Twelve 2-cm tissue segments from the center of the primary leaf were collected for each barley genotype at each time point. The time points 24 and 48 HPI were evaluated using a modified Uvitex 2B fluorescence microscopy protocol developed by S. Dugyala, P. Borowicz, R. Brueggeman, and M. Acevedo (unpublished data) (16,18,31). The samples were cleared in chloroform-methanol (2:1, vol/vol) for 3 h at 22°C. The chloroform-methanol solution was replaced with a lactophenol-ethanol (1:2, vol/vol) solution and incubated at 70°C for 2 min, then stored overnight at 22°C. After washing in 50% ethanol twice for 15 min, twice in 0.5 M NaOH for 15 min, and twice in distilled water for 15 min, samples were incubated in 0.1 M Tris/HCl buffer for 30 min at 22°C and 0.3% Uvitex 2B for 5 min at 22°C, then washed four times in distilled water for 10 min. The multiple wash steps were followed by incubating the samples in 25% glycerol for 20 min at 22°C and then storing them in 50% glycerol at 4°C in the dark until they were mounted between a glass slide and a coverslip in a lactophenol and glycerol (1:200, vol/vol) solution. All mounted tissue samples were viewed using a structured illumination technique using a Zeiss ApoTome 2 module on a Zeiss Axio Imager M2 fluorescence microscope with the BP379-401 excitation filter and FT420 beam splitter (Carl Zeiss AG, Oberkochen, Germany).

RESULTS

Evaluation of stem rust infection process via qPCR assay. The primers utilized for qPCR were found to be specific and only produced amplicons from the expected DNA sources (Fig. 1). Additionally, the fungal and barley primers and probes did not perform significantly differently ($P = 0.65$) under single or multiplex conditions (Fig. 2). Cross amplification was not observed between the barley DNA and fungal primers and probes or the fungal DNA and barley primers and probes during the qPCR assay. The Cq values for the fungal standards ranged from 19.5 ± 0.3 for the 200 ng/μl standard to 25.7 ± 0.4 for the 2.47 ng/μl standard in the first experiment and from 21.1 ± 0.3 for the 200 ng/μl standard to 25.8 ± 0.2 for the 2.47 ng/μl standard in the second experiment. The Cq values for the barley standards ranged from 27.0 ± 0.3 for the 200 ng/μl standard to 32.9 ± 0.4 for the 2.47 ng/μl standard in the first experiment and from 21.1 ± 0.3 for the 200 ng/μl standard to 31.6 ± 0.2 for the 2.47 ng/μl standard in the second experiment. The average reaction efficiencies for the first experiment were 100.2 ± 4.8 and $106.7 \pm 5.4\%$ for the fungal and barley primer and probe combination, respectively. The average reaction efficiencies for the

second experiment were 100.4 ± 6.0 and $108.5 \pm 7.4\%$ for the fungal and barley primer and probe combination, respectively. The prior-to-inoculation time point produced fungal Cq values greater than 37 in all samples for both the first and second experiment. As such, 37 cycles was utilized as a cutting-off point for the presence of the pathogen, and the prior-to-inoculation time points were excluded from further analysis. In addition to the control time point, 20 data points (6.25%) from the first experiment and 51 data points (15.94%) from the second experiment were removed due to Cq values differing by more than one for intra-assay replicates or for Cq values of one of the primers being greater than 37. The Shapiro-Wilk test established that a normal distribution was present for each population at each time point. The ANOVA analysis showed significant differences ($P < 0.05$) for the barley varieties at each time point for both the first and second experiment (Table 2). During the first experiment, at the 48 HPI time point, the least amount of fungal DNA was present on resistant Beacon followed by the traditionally susceptible Steptoe, and the largest amount of fungal DNA was present on the highly resistant Q21861. This hierarchical order changed by the 12 DPI time point and susceptible Steptoe had the most fungal DNA and resistant Morex and Q21861 had the least fungal DNA. A similar pattern was observed for the second experiment. At the 48 HPI time point, the least amount of fungal DNA was detected on susceptible Steptoe and the most was detected on resistant Chevron and Q21861. The hierarchical ordering change observed in the first experiment was also observed in the second experiment by the 6 DPI time point. The most fungal DNA was detected on susceptible Steptoe and the least amount of fungal DNA was found on resistant Morex, Chevron, and Q21861.

Evaluation of stem rust infection process via fluorescence microscopy. Phenotypic differences at the microscopic level, including timing of pathogen structure development, number of established infection points, and host resistance responses, were observed among cultivars. For each sample, 100 infection sites were observed for each sample at the 24 and 48 HPI time points. At 24 HPI, appressoria were observed on Steptoe while both appressoria and haustorial mother cells (HMC) were observed on Morex, Beacon, Chevron, and Q21861 (Fig. 3). By 24 HPI, 100% of the observed *P. graminis* spores had formed appressoria and 20% formed substomatal vesicles for Steptoe. The *P. graminis* growth had progressed further in the infection process by 24 HPI in all *Rpg1*-containing varieties. HMC were observed for 80, 100, 90, and 70% of the *P. graminis* spores inoculated on Beacon, Morex, Chevron, and Q21861, respectively. Necrosis associated with a hypersensitive response was observed for Chevron and Q21861 at 48 HPI. At 48 HPI, HMC were formed for 30% of the *P. graminis* infection sites

Fig. 1. Results of the specificity test of the rust primers (ITS1rustF10d and ITS1rustR3c) and the barley primers (Hvubi-F1 and Hvubi-R1). NTC = nontemplate control where water was used in place of DNA.

Fig. 2. Efficiency curves for the fungal and barley DNA standards under single and multiplex conditions. Fungal: $y = -3.173x + 23.907$, $R^2 = 0.993$, efficiency = 106.6%; Fungal multiplex: $y = -3.101x + 23.164$, $R^2 = 0.989$, efficiency = 110.1%; Barley: $y = -3.060x + 34.424$, $R^2 = 0.997$, efficiency = 112.2%; and Barley multiplex: $y = -2.929x + 34.549$, $R^2 = 0.991$, efficiency = 119.5%.

inoculated on Steptoe, 80% for Beacon, and 100% for Morex, Chevron, and Q21861.

Phenotypic evaluation. During the phenotypic evaluation of experiment 1, the lowest infection type observed was a 0; and the highest was a 3+ (Table 2). Susceptible Steptoe's infection type ranged from 3 to 3+. The infection types of the resistant Beacon, Morex, and Q21861 ranged from 12 to 2, 12 to 2, and 0; to ;1-, respectively. During the evaluation of experiment 2, the lowest infection type observed was a 0; and the highest was a 3+ (Table 2). Infection types of Steptoe ranged from 3- to 3+, Beacon from 12 to 2, Morex from 12 to 21, and Q21861 from 0; to ;1-. All Chevron individuals presented with a mesothetic reaction (X) that included the infection types 0;, 1, and 2 in both experiments 1 and 2.

DISCUSSION

The qPCR assay reported in the current study was shown to effectively separate cultivars by the relative amounts of fungal DNA at early stages of the infection process. The hierarchal order produced by the analysis method at 24 and 48 HPI was not the same as the order by the level of resistance determined using the traditional phenotyping methodology at 12 DPI. However, the qPCR assay demonstrated similarity to qualitative microscopic observations a 48 HPI, allowing for early phenotyping and the ability to separate temporal differences in resistance responses.

The phenotyping at 12 DPI was consistent with what has been previously reported for all of the lines except Chevron (22,34). Chevron has been previously reported to have an infection type that ranges between 0; to 1; when inoculated with race MCC. It is unknown why Chevron displayed a mesothetic reaction; however, the infection types observed were consistent between experiments 1 and 2 in the present study. It is possible that there are virulence differences between the MCCFC isolate used in the current study and the MCC isolates used in previous studies which affect Chevron's infection type. At 12 DPI, the qPCR assay was able to distinguish susceptible Steptoe from the resistant cultivars and lines; however, the hierarchal ordering of resistant cultivars for the qPCR assay and the order based solely on infection types was not the same. This lack of similarity may be due to the qPCR assay being able to account for additional components of resistance and the nature of the stem rust infection process such as disease severity, infection efficiency (receptivity), mycelial growth, latent period, and aggressiveness of the pathogen (12,24,35,40). The ability to differentiate these components may lead to the identification and incorporation of underutilized and underappreciated resistance mechanisms.

The ability to phenotype barley at early time points can provide new and useful information toward understanding the mechanisms involved in stem rust resistance. Upon completion of the first experiment, the time point of 24 HPI was added to determine whether statistical differences could be detected earlier than 48 HPI. It was found that the hierarchal order of the 24 HPI time point was similar to that of the 0 HPI time point in the second experiment because statistical differences were observed between the cultivars

at 0 HPI (Table 2). Furthermore, this ordering did not correlate with the microscopic observations at 24 HPI. These variations may be due to the number of spores on the leaf surface or the amount of leaf tissue present and suggests that the qPCR assay may not be sensitive or accurate enough at very low levels of fungal DNA in planta.

In barley, the most well-characterized resistance gene to wheat stem rust is *Rpg1* (7,21–23). The phosphorylation of *Rpg1* has been shown to occur within 5 min of spore contact with the leaf when inoculated with *P. graminis* f. sp. *tritici* race MCC (22,23). The early phosphorylation in response to spore contact suggests the *Rpg1*-mediated resistance response would be a pre-haustorial resistance response (20,41). Microscopic observations in the present study, however, show that necrosis occurs between 24 and 48 HPI in *Rpg1*-containing varieties after the formation of HMC within the first 24 HPI. As such, the *Rpg1*-mediated resistance pathway does not appear to impede the formation of haustoria. Furthermore, the evidence presented by Nirmala et al. (21) supporting *Rpg1* phosphorylation and subsequent degradation prior to the 24 HPI time point suggests that early spore detection may prime the later post-haustorial formation defense response. Thus, *Rpg1*-mediated resistance may involve both pre- and post-haustorial components.

Interestingly, in the current study, susceptible Steptoe displayed greater resistance to *P. graminis* race MCCFC at both 24 and 48 HPI compared with the varieties containing known stem rust resistance genes. As a susceptible cultivar, it would be expected that fungal growth and structural development would occur at a greater rate on Steptoe than on resistant cultivars. However, at 24 and 48 HPI, the fungal development in Steptoe was delayed, indicating that there may be a minor gene or genes providing a stronger initial resistance than the barley varieties containing *Rpg1* and *rpg4/Rpg5*. This basal resistance could be provided via physiological differences in the leaf epidermal chemistry of Steptoe. Variations of cuticle surface chemistry between cultivars have been shown to have an effect on the infection process of *Blumeria graminis* (DC) Speer f. sp. *hordei* on barley and *B. graminis* (DC) Speer f. sp. *tritici* on wheat (43). Surface topology and chemistry have also been shown to play important roles in inducing the formation of appressoria for *P. graminis* f. sp. *tritici* (10,28). Conversely, the resistance may be provided by a minor-effect resistance gene or a minor-effect gene that provides an additive effect to other resistance genes. Two minor additive effect stem rust resistance QTL have been previously identified in barley. Both of these QTL were located on chromosome 2H; one was identified in Morex by Druka et al. (13) and the other identified in SM89010 by Moscou et al. (19). Both QTL were identified via expression (e)QTL methodology and do not appear to provide resistance while unaccompanied by a major-effect resistance gene such as *Rpg1* or *rpg4/Rpg5*. The eQTL identified by Druka et al. (13) was mapped using the Steptoe/Morex double haploid population and found to be conferred by Morex. The eQTL in Morex was identified using mRNA from noninoculated plants, which would only allow for identification of genes that are constitutively expressed. Furthermore, the eQTL was detected using the infection type 12 to 14

TABLE 2. Comparison of mean relative fungal DNA in planta for each time point and the observed median infection type (IT) for each barley genotype during the two experiments²

Cultivar or line	Experiment 1					Experiment 2				
	0 HPI	48 HPI	6 DPI	12 DPI	IT	0 HPI	24 HPI	48 HPI	6 DPI	IT
Steptoe	2.64 a	2.23 b	0.93 a	−7.13 c	3	2.51 a	1.70 ab	1.16 a	−6.02 c	3
Beacon	3.29 a	4.96 a	−2.22 b	−4.44 b	12	1.84 ab	2.15 a	1.04 ab	−4.55 b	21
Morex	0.76 b	1.59 bc	1.37 a	−0.54 a	21	0.94 c	0.59 c	0.24 bc	−1.98 a	12
Chevron	1.5 ab	1.31 bc	−4.24 c	−3.56 b	X (0;21)	1.29 bc	0.86 bc	−0.45 c	−1.15 a	X (0;12)
Q21861	0.61 b	0.81 c	−1.46 b	−1.34 a	0;	2.37 ab	0.97 bc	−0.31 c	−3.05 a	0;

² Barley genotypes with the same letter in a column are not statistically different. Letters of the statistical group from “a” to “d” describes the relation from low to high amounts of fungal DNA relative to host DNA. The time point 0 h postinoculation (HPI) consists of tissue collected immediately after inoculation; DPI = days postinoculation. Mean of relative fungal DNA was calculated by subtracting the barley quantification cycle (Cq) from the fungal Cq.

DPI, which may not allow for detection of the defense response in Steptoe observed at 24 and 48 HPI in this study. Despite the presumed presence of this resistance component gene in Steptoe, the effect is not enough to provide resistance during later stages of the infection, resulting in susceptibility at 12 DPI. Future

mapping experiments will be needed to identify the gene or genes involved. The observations in the present study demonstrate the variability of resistance at different stages and the need for additional methods of evaluation to unravel the complexities of disease resistance.

Fig. 3. Fluorescence microscopy images showing infection structure development for *Puccinia graminis* f. sp. *tritici* race MCCFC on barley. All images were taken at $\times 63$ magnification. The color of some images was adjusted to green to provide better contrast. Haustorial mother cell (HMC) development was difficult to observe for Chevron and Q21861 at 48 h postinoculation (HPI) due to a masking effect resulting from host necrosis.

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