Polymerase Chain Reaction Detection of *Ustilago hordei* in Leaves of Susceptible and Resistant Barley Varieties

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ABSTRACT

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Although *Ustilago hordei* infects barley seedlings, symptoms of the disease covered smut are not visible until heading. Natural or artificial inoculation usually results in inconsistent infection, even in highly susceptible lines. Thus, breeding for resistance to covered smut is time consuming and difficult. The ribosomal DNA internal transcribed spacer (ITS) regions of *U. hordei* were sequenced and a primer pair was developed for polymerase chain reaction (PCR). These primers amplified a 574-bp fragment from DNA of *Ustilago* spp., but did not amplify DNA from barley or other com-

mon barley pathogens. DNA extracted from as few as four *U. hordei* sporidia was detected by this method. *U. hordei* DNA was amplified from leaf tissue of inoculated susceptible and resistant plants at different stages of plant development in differential varieties. Growth of the fungus in different leaves of an individual plant was variable. Several highly resistant varieties were shown to contain *U. hordei* DNA in the first three to four leaves, but not in later leaves. Thus, although the fungus can infect many resistant plants, the plants remain symptomless. Detection of *U. hordei* prior to heading should assist efforts for breeding for resistance and provide clues concerning the mechanisms of resistance employed by different resistance genes.

Additional keywords: fungi, *Hordeum vulgare*.

Ustilago hordei (Pers.) Lagerh. is a basidiomycete fungus that causes covered smut of barley (*Hordeum vulgare* L.) (8). Diploid fungal teliospores germinate to form a promycelium with four basidiospores or sporidia (23). These sporidia form conjugation tubes that fuse with the opposite mating type to form the infectious dikaryotic mycelium (22). This mycelium penetrates the coleoptile of barley seedlings prior to emergence and grows intercellularly through the plant behind the apical meristem. At the time of flowering, diploid teliospores develop in place of the barley kernels, resulting in a black mass of spores in the infected head and, in some cases, in the upper leaves of the plant (23,31). The teliospores are covered with a membrane that ruptures during harvest, allowing their spread onto healthy seeds or into the soil.

Covered smut has worldwide distribution, but can be effectively controlled by sowing resistant cultivars or by the application of fungicides such as carboxin to the seed (23). Most barley-growing regions of the world plant cultivars that are susceptible to covered smut infection and use fungicides to control the disease (2,23,30). In regions that routinely plant untreated seed because of economics or unavailability of the chemicals, covered smut can cause economic losses. Economic loss is not only due to decreased yield but also to contamination of healthy seed with smut spores (23). Cultivars resistant to *U. hordei* would be of great importance if the pathogen developed a tolerance to fungicides. Induced mutants tolerant to four different fungicides including carboxin have been identified (4,14). Constant fungicide use could eventually select for fungicide-tolerant mutants (5). In France, carboxin resistance was described for two strains of *U. nuda* on winter barley crops in 1986 (20).

Even though several resistance genes are known, breeding for resistance to covered smut is not routinely done because of the lengthy process involved and the effectiveness of fungicides (2,23,31). Plants need to grow 2 to 3 months before disease symptoms become apparent (11). Additionally, infection is inconsistent even

in highly susceptible lines, so that a symptomless plant may not necessarily be resistant. Thus, additional generations have to be tested to determine if the F₂ parent was truly resistant. Another hindrance of breeding for resistance is that many discrepancies are found in the literature concerning the genetics of resistance to *U. hordei* (31). Resistant cultivars with good agronomic qualities would benefit regions that use untreated seed and provide an alternative to fungicides. Methods that could hasten the breeding process may encourage the incorporation of covered smut resistance into elite cultivars.

Polymerase chain reaction (PCR) has been used for detection of a number of fungal plant pathogens based on the internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) (15). The 18S, 5.8S, and 28S nuclear rDNA genes are highly conserved for different fungal species and have been used for phylogenetic studies. Universal primers have been designed from these conserved regions for amplification of the ITS regions between the genes. The ITS regions evolve more quickly than the genes themselves and may vary among species within a genus (32), allowing the development of PCR primers that uniquely amplify a given species (15). High copy number of the rDNA genes increases sensitivity of this detection strategy so that rDNA can be amplified from a mixed-DNA preparation (32). Fungal DNA can be detected from a DNA extraction of diseased plant tissue when using primers that amplify the rDNA regions (15).

In this study, the ITS region of *U. hordei* rDNA was cloned and sequenced, and primers that amplify this region were developed. The sensitivity and specificity of this detection method were determined and optimized. A procedure to amplify *U. hordei* DNA from infected plant leaves was developed. This method was used to investigate the distribution of *U. hordei* in leaves of susceptible and resistant barley varieties.

MATERIALS AND METHODS

Fungal strains. Teliospores of *U. hordei*, *U. avenae*, *U. bullata*, *U. kolleri*, *U. tritici*, and *U. nuda* were obtained from W. Pedersen, University of Illinois, or D. Mills, Oregon State University. Race 8 sporidial cultures were maintained on Holliday's complete media

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(HCM) agar (16). *Tilletia controversa* and *T. laevis* teliospores; plant samples infected with *Rhynchosporium secalis*, *Pyrenophora teres*, *Puccinia striiformis*, and *Erysiphe graminis*; and *Cochliobolus sativus* cultures were provided by D. Mathre, M. Johnston, and A. Scharen, respectively, Montana State University.

Cloning and sequencing of the PCR-amplified ITS regions.

The ITS regions of *U. hordei* were amplified using PCR and the universal ITS primers 1, 3, and 4 (32) provided by H. Leung (Washington State University). *U. hordei* DNA was extracted by boiling 2 mg of teliospores in 1 ml of 10 mM Tris, pH 8.0, for 20 min and using 5 μ l of the supernatant in a 50- μ l PCR reaction (15). PCR reagents and *Taq* DNA polymerase were used according to manufacturer's specifications (Perkin-Elmer Cetus, Branchburg, NJ). The final concentrations were 1.6 μ M for each ITS primer, 5 mM $MgCl_2$, 200 μ M for each deoxynucleoside triphosphate (dNTP), and 1.25 U of *Taq* DNA polymerase. PCR cycling conditions consisted of denaturation at 94°C for 1.5 min, annealing at 55°C for 2.5 min, and extension at 72°C for 3 min for 40 cycles, with a final extension at 72°C for 10 min. PCR products (10 μ l) amplified using ITS1 and ITS4, and ITS3 and ITS4, were separated on a 1.5% agarose gel stained with ethidium bromide and viewed with ultraviolet light. PCR product (1 μ l of each) was ligated into pCRII using the TA cloning kit (Invitrogen Corp., San Diego, CA) and transformed into *Escherichia coli* strain DH5 α . The clones were screened for the presence of the desired insert and prepared for sequencing using a Qiagen plasmid miniprep kit (Qiagen Inc., Chatsworth, CA). Double-stranded DNA was denatured by an alkaline method (13) and sequenced by the dideoxy chain termination method (25) using Sequenase version 2.0 (United States Biochemical Corp., Cleveland, OH) according to manufacturer's instructions with the M13 universal forward and reverse primers.

The resulting ITS sequence was entered into the Wisconsin Genetic Computer Group (GCG) sequence analysis package (6) and the Prime program was used to identify primer pairs. Primer Uh1, located 42 bases from ITS1, and Uh4, located 137 bases from ITS4, were synthesized (Gibco-BRL, Gaithersburg, MD). The T_m value for the primer pair was 58°C. PCR conditions were optimized by adjusting primer concentrations, $MgCl_2$ concentration, and annealing temperature. The final concentrations used for PCR with these primers was 1.5 mM $MgCl_2$, 0.2 μ M for each primer, 200 μ M for each dNTP, and 0.8 U of *Taq* DNA polymerase in a 25- μ l reaction. The program for thermal cycling was denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1 min for 40 cycles, using 3-min denaturation and extension steps for the first three cycles and a final extension for 10 min at 72°C.

DNA extraction. DNA from fungal cultures, spores, or infected leaf tissue was obtained using a modification of a plant tissue extraction (7). Samples were ground in 400 μ l of extraction buffer, vortexed, and placed at -70°C for 20 min before incubating at room temperature for 40 min. After addition of isopropanol (1:1 vol/vol) to 300 μ l of the extract, the samples were placed at -70°C for 5 min or -20°C for greater than 25 min and then centrifuged at 16,000 $\times g$ for 20 min. The DNA pellet was dried and suspended in 100 μ l of sterile, double-distilled water for use in subsequent PCR reactions. For each extraction, a negative control was included that contained only extraction buffer to assure that the buffers and reagents were not contaminated.

Determination of sensitivity and specificity. The sensitivity of the procedure to detect *U. hordei* was determined by testing a dilution series of sporidia. Sporidia were shaken at 250 rpm overnight at room temperature in HCM broth. The cells were centrifuged for 5 min at 4,500 $\times g$ and washed with sterile distilled water, and the concentration was determined by counting the cells with a hemocytometer. The cells were adjusted to a concentration of 1×10^8 cells per ml, and a serial 10-fold dilution was performed down to 1×10^4 cells per ml. Each dilution (10 μ l) was extracted as described above. Thus, an initial concentration of 1×10^8 cells per ml was equivalent to extracting 1×10^6 sporidia. DNA from 300 μ l,

or 75% of the total amount of DNA, was precipitated with isopropanol (1:1 vol/vol) and dissolved in 100 μ l of sterile double-distilled water. Therefore, the resulting 100 μ l of DNA was extracted from the equivalent of 750,000 sporidia. The DNA was used at a rate of 5 μ l per 25- μ l PCR reaction, so that a PCR reaction of the extraction of 1×10^6 sporidia would be the equivalent of amplifying the DNA from 37,500 haploid sporidia. A similar extraction of the same dilution series of sporidia was done that included a 1-cm segment of uninfected barley leaf tissue. Control DNA extractions were also done with no sporidia, with and without uninfected barley leaf tissue. This experiment was performed twice with identical results.

The specificity of this assay was determined by performing PCR amplification using primers Uh1 and Uh4 with DNA from uninoculated barley plant tissue, other common barley leaf pathogens, and other members of the family Ustilaginales. DNA was extracted as described above from teliospores of *Ustilago* spp. and *Tilletia* spp.; from conidia of *C. sativus*, *Pyrenophora teres*, and *R. secalis*; from uredia of *Puccinia striiformis*; and from leaf samples infected with *E. graminis*. PCR reactions were performed as stated above with the Uh1 and Uh4 primers. Assays yielding positive results were repeated twice, while samples yielding negative results were tested three times. To ensure that the quality of the extracted DNA was adequate for amplification, samples were assayed using universal ITS primers (32).

Inoculation and greenhouse experiments. The barley differential cultivars for covered smut (28) including Odessa (C.I. 934), Himalaya (C.I. 1312), Lion (C.I. 923), Trebi (C.I. 936), Pannier (C.I. 1330), Excelsior (C.I. 1248), and Hannchen (C.I. 531) were provided by W. Pedersen. Teliospores of *U. hordei* race 8, which is avirulent on all of the cultivars except the universally susceptible 'Odessa', were used for plant inoculations (28). A modification of the vacuum inoculation method was used (29). Seeds of each barley cultivar were dehulled and surface-sterilized for 5 min in 5.25% sodium hypochlorite. A total of 30 seeds were inoculated by adding approximately 25 mg of race 8 teliospores to the seeds with 0.5 mg of carboxymethylcellulose and 1 ml of sterile water containing 0.001% Tween 20. The seeds were vacuum-infiltrated three times for 10 min, dried, and planted in damp vermiculite at a depth of 3 cm. After the seeds were incubated at 16°C for 7 days, 15 seedlings of each variety being tested were transplanted into 6-inch pots containing a steam-sterilized greenhouse mix of soil/sand/peat (1:1:1 vol/vol/vol) with three plants per pot. The plants were grown in a glasshouse with 16-h days, weekly fertilization, and a night-day temperature of 18 to 21°C.

Ten plants from each cultivar were tagged, and leaf tissue was collected from the primary tiller. Samples were taken from each leaf after two higher leaves had emerged. For example, the first leaf was collected when the plant had a total of three leaves. The penultimate leaf was collected when the flag leaf had emerged, and the flag leaf was collected when the head emerged. Preliminary experiments indicated that the distribution of *U. hordei* in an individual leaf was not uniform; therefore, three samples per leaf were collected to maximize the likelihood for obtaining infected tissue. Each sample consisted of a disc obtained using a sterile microcentrifuge tube cap to punch leaf samples directly into the tube from 2.5 cm below the leaf tip, the center of the leaf, and 2.5 cm above the base of the leaf. Leaf samples were stored in the microcentrifuge tubes at -70°C until the DNA was extracted. The presence of disease symptoms in each sampled tiller was also recorded.

RESULTS

Primers and ITS sequence. DNA extracted from *U. hordei* race 8 teliospores was amplified using ITS1 and ITS4 primers and ITS3 and ITS4 primers, resulting in 791- and 486-bp fragments, respectively (Fig. 1A). The sequence of the ITS regions, including the 5.8S rDNA and primer regions, is shown in Figure 1B (Gen-

Bank accession #AF105224). Primers Uh1 and Uh4 were constructed from the ITS regions of this sequence and resulted in the amplification of a 574-bp fragment when used for PCR on *U. hordei* DNA (Fig. 1A and B).

Sensitivity of PCR detection assay. DNA extracted from a 10-fold dilution series of sporidia was used for PCR with Uh1 and Uh4 primers. DNA fragments, amplified using 5 µl of each extraction per 25-µl PCR reaction, were seen down to 375 sporidia, while weak bands were still apparent with as few as 4 to 38 sporidia (Fig. 2). The inclusion of barley leaf tissue in the DNA extraction increased the intensity of the PCR product. This was most obvious in the amplification of 38 sporidia. Four sporidia were also detectable in the presence of barley leaf tissue DNA (Fig. 2). Amplification of control extractions without DNA and of uninoculated barley leaf tissue were negative.

Specificity of Uh1 and Uh4 primers. Primers Uh1 and Uh4 specifically amplified a DNA fragment of similar size from all *Ustilago* spp. using an annealing temperature of 57°C. DNA from *Tilletia* spp., uninoculated barley, and other potential barley leaf contaminants did not give a PCR product when amplified with these primers at this annealing temperature (Table 1). DNA from *U. bulbata* was not amplified with an annealing temperature of 63°C.

Analysis of leaf tissue from inoculated barley varieties. To test whether the PCR assay could reproducibly detect *U. hordei* in leaf tissue, susceptible 'Odessa' and resistant 'Excelsior' were inoculated and assayed throughout the growth of the plant. An example of typical results (Fig. 3) shows the PCR products from the second leaves of 'Excelsior'. Positive reactions included both strong and weak products. The complete analysis, each with 10 plants, was performed three times for 'Odessa' and twice with 'Excelsior'. The data was essentially the same for each repetition; therefore, complete results from one representative experiment is shown in Table 2. In each case, the control was a plant of the same cultivar that was not inoculated. Only strong positives were counted when

calculating percent positive for each leaf. Controls included for each set of PCR reactions consisted of sterile, double-distilled water in place of DNA and a mock extraction performed without tissue.

'Odessa' produced an unusually high number of leaves when grown under our experimental conditions (Table 2) (18). All of the inoculated plants of 'Odessa' that survived had smutted heads, and each plant yielded positive PCR products at multiple collection points. The number and position of positive leaves varied for each plant. For example, plant 5 had a strong positive PCR product for each leaf of the plant except the twelfth leaf, which was weakly positive. Plant 3, on the other hand, only yielded strong PCR products for three of the first four leaves; subsequent leaves were negative or weakly positive. Leaves from the uninoculated 'Odessa' control were consistently negative.

'Excelsior' was resistant to race 8 of *U. hordei*, and none of the inoculated 'Excelsior' plants developed symptoms in either experiment. However, *U. hordei* was detected in the first three leaves of several plants of 'Excelsior', indicating that it had entered the resistant plant but was unable to grow throughout the plant (Table 2).

Having tested the utility and reproducibility of the PCR assay with 'Odessa' and 'Excelsior', the other differential cultivars were tested once. All of these cultivars (Hannchen, Himalaya, Lion, Trebi, and Pannier) have been described as resistant to race 8 of *U. hordei* (28). Like 'Excelsior', 'Himalaya', 'Lion', and 'Trebi' had no smutted heads and yielded positive PCR products in only the bottom three to four leaves. Inoculated plants of 'Hannchen', on the other hand, were not completely resistant. Four of the nine tillers tested had smutted heads (Table 2). Therefore, 'Hannchen' was moderately susceptible to race 8 of *U. hordei*. While PCR products were consistently found in the higher leaves of plants of 'Hannchen' that had symptoms, the fungus was only detected in leaves 1 to 4 of plants lacking symptoms (Table 2).

None of the 10 plants of 'Pannier' yielded positive PCR products, even in the lower leaves, but one plant was smutted. 'Pannier' was

Fig. 1. Development of oligonucleotide primers from the internal transcribed spacer (ITS) regions of the ribosomal DNA (rDNA) of *Ustilago hordei*. **A**, rDNA-specific primers ITS1, ITS3, and ITS4 (32) were used to amplify the internal transcribed spacer regions using polymerase chain reaction, resulting in 791- and 486-bp fragments. Primers Uh1 and Uh4 amplified a 574-bp fragment. The arrowheads indicate the 3' end of each primer. **B**, Sequence of the ITS region of *U. hordei* spanning the ITS1 and ITS4 primer sites. The ITS regions are in bold, separated by the 5.8S rDNA gene (3). Primer sequences are underlined.

previously described as resistant to race 8 of *U. hordei* (27), but 10% infection would be considered intermediate resistance (27).

DISCUSSION

The ability to detect *U. hordei* in barley tissue provides a method to study fungal infection of the host and compare differences in infection for different avirulence gene-resistance gene pairs. The pathway of infection of *U. hordei* in barley tissue has previously been examined microscopically (19). Staining infected plant tissue preparations showed that the fungus was concentrated in the growing point of the plant. Fungal mycelium was found in the flag leaf, but other leaves were not examined (19). In addition to the low sensitivity of microscopic evaluation, the procedure is also destructive, so it was not possible to monitor symptom development in the plants that were examined. It was known that teliospores from aggressive races of *U. hordei* can form on the upper leaves of susceptible plants (8,10). We have shown that *U. hordei* is present in the leaves of infected barley plants, even when there are no outward signs of its presence. Leaves were collected when three upper leaves had emerged in order to monitor fungal growth in a less invasive way. This allowed the plant to survive so that fungal detection could be compared with symptom development.

PCR amplification of fungal DNA in a crude DNA extraction from leaf tissue allowed for the detection of the presumably small amounts of the fungus. Amplification with primers Uh1 and Uh4

could detect four *U. hordei* sporidia in a DNA preparation that included barley DNA. This would be equivalent to two diploid teliospores or dikaryotic cells. The phenomenon of additional sensitivity noted in samples containing host DNA has been attributed to heterologous DNA stabilizing the target DNA and making it more available (21). The sensitivity of PCR does require extra precautions to avoid contamination. Control amplifications of uninoculated plant tissue and extraction buffer lacking DNA were always included to monitor contamination.

In a preliminary experiment, *U. hordei* was more easily detected in younger leaves. For example, at the four-leaf stage, the top two leaves were positive and the bottom two were negative. This result might have been due to senescence of the older leaves, making the DNA harder to extract. This result posed a problem for collecting leaves without interfering with the growth of the plant. We chose to do our greenhouse experiments by collecting the leaves at a slightly older stage, as opposed to collecting the youngest leaves and interfering with plant growth. This procedure may more accurately detect the fungus if the leaves are collected at an earlier stage but with fewer leaves taken from the plant.

The specificity of this detection method would be important when working with plants infected with *U. hordei* in the greenhouse or field. Common field pathogens tested negative with this method. While contamination by other *Ustilago* spp. is improbable in greenhouse experiments, these primers will not distinguish between *Ustilago* spp. in field-grown plants. Comparison of the primer sequences Uh1 and Uh4 to the equivalent sequences in the other *Ustilago* spp. (1) showed that, while the sequence for Uh1 varied from most of

Fig. 2. Amplification of DNA extracted from a sporidia dilution series. Lanes 1 and 8 were loaded with 1 µg of ϕ x174 *Hae*III-digested DNA standard. Lanes 2 to 6 show the 574-bp polymerase chain reaction (PCR) products from 37,500, 3,750, 375, 38, and 4 sporidia, respectively. Lanes 9 to 13 are the same as lanes 2 to 6, except that uninfected barley leaf tissue was added to the sporidia before DNA extraction. Lane 7 shows the PCR product of a control extraction with no sporidia. Lane 14 shows an extraction of barley leaf tissue alone.

Fig. 3. Polymerase chain reaction (PCR) amplification products from DNA extracted from the second leaves of inoculated plants of 'Excelsior'. The lane number corresponds to the plant number. The first lane was loaded with the ϕ x174 *Hae*III DNA standard. Three control PCR reactions were included with each experiment. First, leaf tissue from an uninoculated plant from the same cultivar at the same stage was extracted (lane C). The other controls consisted of sterile, double-distilled water in place of DNA (lane W) and PCR of a mock extraction performed without tissue (lane E). Examples of strong positive, weak positive, and negative PCR products are shown in lanes 7, 6, and 5, respectively.

TABLE 1. Polymerase chain reactions (PCRs) of barley, barley fungal leaf pathogens, and members of the family Ustilaginales using Uh1 and Uh4 primers

Plant pathogen	Common name	Source	PCR product ^a	
			57°C	63°C
<i>Ustilago hordei</i>	Covered smut of barley	Teliospores	+	+
<i>U. avenae</i>	Loose smut of oats	Teliospores	+	+
<i>U. bullata</i>	Loose smut of crested wheat grass	Teliospores	+	–
<i>U. kolleri</i>	Covered smut of oats	Teliospores	+	+
<i>U. nuda</i>	Loose smut of barley	Teliospores	+	+
<i>U. tritici</i>	Loose smut of wheat	Teliospores	+	+
<i>Tilletia controversa</i>	Dwarf bunt of wheat	Teliospores	–	ND
<i>T. laevis</i>	Common bunt of wheat	Teliospores	–	ND
<i>Hordeum vulgare</i>	Barley	Leaf tissue	–	ND
<i>Cochliobolus sativus</i>	Barley spot blotch	Conidia	–	ND
<i>Pyrenophora teres</i>	Barley net blotch	Conidia	–	ND
<i>Rhynchosporium secalis</i>	Barley scald	Conidia	–	ND
<i>Erysiphe graminis</i>	Powdery mildew	Infected tissue	–	ND
<i>Puccinia striiformis</i>	Barley stripe rust	Uredia	–	ND

^a + = a positive PCR product using Uh1 and Uh4 primers; – = no PCR product was present; and ND = PCR not done.

the other species, Uh4 was very similar. *U. kolleri* and *U. avenae* have the same sequence as Uh1, while *U. tritici*, *U. nuda*, and *U. bullata* sequences differed from Uh1 by 4, 7, and 10 bases, respectively. Uh4 has the same sequence for all the species except *U. avenae* in which there was one base difference. Only DNA from *U. bullata*, which is only 50% identical to Uh1 in sequence with a different 3' clamp, was not amplified at an elevated annealing temperature. It may be possible to design a different primer pair or vary conditions so that the primers are more specific, but the low MgCl₂ concentration, low primer concentration, and high annealing temperature used here would have optimized specificity with primers Uh1 and Uh4. The assay was specific for *U. hordei* using an annealing temperature of 65°C, but the band was extremely weak and would not be useful for detecting the low levels of *U. hordei* found in leaves. The ITS sequences of *U. kolleri* and *U. avenae* (1) are so similar to that of *U. hordei* that it may not be possible to differentiate these species.

Detection of *U. hordei* in leaf tissue allowed the study of the presence of this pathogen in inoculated differential barley varieties. Detection of the fungus in different leaves of individual plants was variable, even in plants with smutted heads. The number of positive reactions for each leaf in a smutted plant ranged from 14 to 93%. Taken together with evidence that the fungus is present in leaves at low levels and that growth within a leaf is not uniform,

the variability in detection of *U. hordei* is most likely due to heterogeneous growth in the plant. Distribution of the pathogen among tillers in a single plant is known to be variable, because not all tillers become smutted (11). Upper leaves of infected plants often resulted in no PCR product. This could be due to a failure in amplification common when an excess of target DNA is used in the PCR reaction (9). Extraction from leaves containing teliospores consistently gave negative results when used for PCR at standard quantities (data not shown).

Groth and Person (11) concluded that *U. hordei* mycelium is present for at least a short period in resistant plants. Our experiments supported their conclusion. Several highly resistant cultivars were shown to contain *U. hordei* DNA in the first leaves, but not in later leaves. Thus, although the fungus could infect the resistant plants, growth was restricted and the plants remained symptomless. It may be that in some host-race combinations, the fungus may be totally excluded from entering beyond the pericarp (11). It would be useful to determine if a symptomless plant has escaped infection, resisted infection, or resisted disease development following a successful infection (12). 'Pannier' may be an example of a plant that normally resists infection but is susceptible if the fungus breaches the initial defense response. It was reported that penetrating mycelia were stopped within the first or second cell in the coleoptiles of 'Pannier' (17). Our experiments did not detect the fungus

TABLE 2. Detection of *Ustilago hordei* by polymerase chain reaction in three covered smut differential cultivars

Plant no.	Leaf number															Symptoms ^a
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
'Odessa' - susceptible																
0	○ ^b	○	●	●	○	●	○	○	●	◐	●	●	○*	...	...	+
1	○	◐	●	●	○	○	○	○	Died	...	...	...	...	...	...	...
2	○	○	○	●	○	◐	○	○	●	◐	◐	○	◐	◐*	...	+
3	●	●	○	●	○	○	◐	○	◐	◐	◐	○	○	○	○*	+
4	●	●	○	●	○	○	○	○	●	●	◐	○	●	◐*	...	+
5	●	●	●	●	●	●	●	●	●	●	●	◐	●	●*	...	+
6	○	●	●	●	○	○	●	●	●	●	●	●	●	●	●*	+
7	○	●	●	●	○	○	○	●	●	●	●	●	●	●*	...	+
8	●	●	●	●	○	○	○	●	○	●	◐	◐	◐	◐*	...	+
9	○	●	●	●	●	○	●	●	●	○	◐	○	○	●	◐*	+
10	○	○	○	●	●	○	●	○	●	●	◐	○	●	◐*	...	+
Control ^c	○	○	○	○	○	○	○	○	○	○	○	○	○*	...	...	—
Positive (%) ^d	36	64	64	100	27	18	36	45	80	60	40	30	50	44	33	100
'Excelsior' - resistant																
0	○	○	○	○	○	○	○	○	○	○	○*	...	...	...	...	—
1	●	○	○	○	○	○	○	○	○	○	○	○*	...	...	...	—
2	○	●	○	○	○	○	○	○	○	○	○*	...	...	...	...	—
3	○	○	○	○	○	◐	○	○	○	○	○	○*	...	...	...	—
4	○	○	○	○	○	○	○	○	○	○	○	○*	...	...	...	—
5	○	○	○	○	○	○	○	○	○	○	○	○*	...	...	...	—
6	◐	●	●	○	○	○	○	○	○	○	○	○*	...	...	...	—
7	●	●	●	○	○	○	○	○	○	○	◐	○*	...	...	...	—
8	●	○	◐	○	○	○	○	○	○	○	○	○*	...	...	...	—
9	○	○	○	○	○	○	○	○	○	○	○	○*	...	...	...	—
Control	○	○	○	○	○	○	○	○	○	○	○	○	○*	...	...	—
Positive (%)	30	30	20	0	0	0	0	0	0	0	0	0	0	...	...	0
'Hannchen' - moderately susceptible																
0	◐	○	●	Died	...	...	...	...	...	...	...	...	...	...	...	...
1	●	○	●	○	○	○	●	◐	●	●	◐	◐	●*	...	...	+
2	●	○	●	●	○	○	○	○	○	○	○	○*	...	...	...	—
3	●	○	●	○	○	○	○	○	○	○	○	○*	...	...	...	—
4	●	○	◐	○	●	●	●	◐	○	●	●	○	○*	...	...	+
5	●	○	●	○	○	○	●	◐	○	●	●	○	○*	...	...	+
6	●	○	○	○	○	○	○	○	○	○	○	○*	...	...	...	—
7	●	○	●	●	○	◐	●	○	○	●	◐	○*	...	...	...	+
8	○	○	○	○	○	○	○	○	○	○	○	○*	...	...	...	—
9	●	○	○	○	○	○	○	○	○	○	○	○	○*	...	...	—
Control	○	○	○	○	○	○	○	○	○	○	○	○	○*	...	...	—
Positive (%)	80	0	60	22	11	11	44	0	11	44	22	11	20	...	...	44

^a + = a smutted head on the tiller collected, and - = the head was not smutted.

^b ○ = no product, ● = strong product, ○ = weak product, * = flag leaf, and ... = no data.

^c Control was an uninoculated plant of the same variety.

^d Only strong positives (●) were used to calculate percent positive.

even in the early leaves of 'Pannier'. Kiesling (17) obtained no smutted plants of 'Pannier' out of 115 checks. Having 1 smutted plant out of 10 in our experiment suggests that, if infection occurs, disease can develop. The negative PCR results in the smutted plant may indicate that growth of the fungus in this variety is restricted to the growing points and not present at detectable levels in the leaves. Another possibility is that the infected plant was not actually 'Pannier' but a seed contaminant. However, 'Pannier' has unique morphological characteristics and all of the plants looked identical, suggesting that the infected plant was truly 'Pannier'.

'Hannchen' has been reported to be resistant to race 8 (24,28). Our result, in which 'Hannchen' proved to be only moderately resistant, may be a result of inbreeding of race 8. Progeny of a teliospore obtained from an infection of 'Odessa' gained virulence to 'Hannchen' (24). The increased number of infected plants of 'Hannchen' may also be due to the inoculation procedure. Susceptibility to covered smut was originally defined as having over 36% infection, while 6 to 35% infection was intermediate and 0 to 5% infection was resistant (27). Many methods have been attempted to improve the efficiency of the artificial inoculation procedure for covered smut (26). Our inoculation procedure, which has consistently resulted in 90 to 100% smutted heads in surviving plants of 'Odessa', may have also increased infection in varieties normally resistant to race 8.

There are several characteristics of covered smut disease that have deterred the incorporation of genetic resistance into commercial barley varieties. The time required before symptom development, inefficient inoculation methods, and confusion in the literature on the identity of resistance genes (31) make breeding for covered smut resistance a difficult exercise. Detection of *U. hordei* in plant tissue prior to heading should assist these efforts. If total exclusion of the pathogen were deemed important, the resistance gene or genes found in 'Pannier' might be desirable. Because of the uneven distribution of the pathogen in an infected plant, it may be necessary to improve the accuracy and consistency of the procedure described here. Collecting leaves as they emerge might improve the accuracy, as might collecting leaves at specific stages. For example, in the resistant cultivars tested, no pathogen was detected after the fourth leaf. Thus, sampling the fifth or sixth leaf immediately after emergence might be most reliable for identifying resistant plants. Experiments examining different races of *U. hordei* on the differential barley cultivars would help characterize the available covered smut resistance genes and determine the mechanisms by which host resistance occurs.

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