Techniques

Identifying Wheat Genotypes Resistant to Eyespot Disease with a β-Glucuronidase–Transformed Strain of *Pseudocercosporella herpotrichoides*

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ABSTRACT

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Resistance to *Pseudocercosporella herpotrichoides* in wheat was determined by inoculating 2-wk-old seedlings with a transformed strain of the pathogen that constitutively expresses β-glucuronidase (GUS) and measuring disease development with a GUS enzyme assay, visual disease scoring, and an enzyme-linked immunosorbent assay (ELISA). Differences among resistant and susceptible genotypes were apparent 2 wk after inoculation; however, the greatest differences among resistant and susceptible genotypes occurred 4- to 6-wk and 6- to 8-wk after inoculation for the GUS assay and visual disease ratings, respectively. ELISA did not differentiate among resistant genotypes at any sample date in this study. Values obtained with the GUS system provided the largest differences among and between resistant and susceptible genotypes. In addition, the GUS system has the advantage of being objective, faster, and more sensitive than visual disease ratings.

Additional keywords: disease resistance, plant breeding, reporter genes, *Triticum aestivum*.

Eyespot, caused by *Pseudocercosporella herpotrichoides* (Fron) Deighton (teleomorph = *Tapesia yallundae* Wallwork & Spooner) is a chronic, yield-limiting disease of winter wheat (*Triticum aestivum* L.) in the Pacific Northwest (PNW) region of the United States and other parts of the world (20,29). Eyespot has been effectively managed with benzimidazole fungicides in the PNW since 1978. However, the discovery of *P. herpotrichoides* isolates

resistant to benzimidazoles (18) has made disease-resistant cultivars the most economical and desirable method of controlling eyespot. Currently, Madsen and Hyak are the only winter wheat cultivars adapted to the PNW and highly resistant to *P. herpotrichoides* (1,2,28).

Wheat breeders have worked to increase eyespot resistance in commercial cultivars adapted to the PNW since 1974 (22), but the currently available methods of screening for resistance are slow or not highly discriminating. Allan and Roberts (3) used a lesion index that is correlated with lodging incidence and visual disease scores to select lines for eyespot resistance. However, visual assessment does not effectively differentiate between highly resistant and resistant genotypes (19,28), and plants must be grown to maturity for resistance to be evaluated accurately. McMillin et al (15) reported a close association between the gene for eyespot resistance, *Pch1*, derived from *Aegilops ventricosa* Tausch, and an endopeptidase locus, *Ep-d1*. When used as a selection marker, the isozyme associated with *Ep-d1* allows screening of plants at an early age but is useful only for resistance derived from *A. ventricosa*. Lind (13) showed that quantitative differences in eyespot resistance among wheat cultivars could be measured more accurately with an enzyme-linked immunosorbent assay (ELISA) than with visual disease scoring. Although *P. herpotrichoides* can be detected with ELISA in presymptomatic wheat stems as early as tillering stage (Zadok's growth stages 20–29) (26,27), consistent differentiation of eyespot resistance among genotypes was observed only from anthesis to maturity (13).

Resistance to *P. herpotrichoides* in wheat is established early in the infection process (5,19,24). Penetration and colonization of resistant and susceptible genotypes involves the same processes; however, penetration frequency is greater and growth is faster in susceptible than resistant genotypes, resulting in more extensive colonization (5,14,19,24). Thus, a seedling test for resistance to *P. herpotrichoides* should be possible. Seedling tests provide the advantages of being quicker and less variable than field tests (28). Although seedling tests correlate well with field resistance, highly resistant and resistant genotypes cannot be differentiated (4,14, 19,24). Methods for enhancing disease development, reducing environmental variation, and increasing the ability to detect the pathogen are needed to improve seedling tests for resistance.

The β-glucuronidase (GUS) gene-fusion system has been used extensively in plant molecular biology studies for the analysis of gene expression (10). The advantages of this reporter gene system for studying disease development (i.e., colonization) include the low endogenous background activity of GUS in plants and fungi and the ease and sensitivity of detection of GUS activity (9,23). Bunkers (6) transformed *P. herpotrichoides* with a plasmid containing the *Escherichia coli gusA* gene attached to the constitutive glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter fragment from *Aspergillus nidulans*. GUS transformants were mitotically stable and exhibited unaltered growth rate, morphology, and pathogenicity (6). Thus, the GUS reporter gene allows detection and quantification of the pathogen in the plant, because GUS activity is proportional to growth of *P. herpotrichoides*. Recently, Oliver et al (21) reported the use of β-glucuronidase activity of GUS-transformed *Cladosporium fulvum* to quantify fungal biomass in tomato lines containing different resistance genes.

This study was undertaken to evaluate the utility of the GUS reporter gene system for identifying resistance to *P. herpotrichoides* in wheat seedlings. The resistance of six wheat genotypes with known resistance reactions to *P. herpotrichoides* was investigated by GUS assay, ELISA, and visual disease ratings. These methods were compared for their ability to differentiate among genotypes with different levels of resistance to *P. herpotrichoides*. A preliminary report was published (7).

MATERIALS AND METHODS

Relationship between fungal growth and GUS activity in vitro. A GUS-transformed strain of *P. herpotrichoides* (P84-8, courtesy of G. Bunkers, Monsanto Co., St. Louis) was used throughout the study (6). *P. herpotrichoides* P84-8 was originally isolated from a wheat plant with eyespot collected in eastern Washington. Cultural characteristics and growth rate are typical of wheat-type isolates of this pathogen (25), which are predominant in Washington. GUS activity was assessed from both agar and broth cultures. Five-millimeter-diameter blocks of mycelium from stock cultures of P84-8 were placed on Difco potato-dextrose agar (PDA) (Difco Laboratories, Detroit) and incubated at 20 C in the dark. Broth cultures were prepared by seeding 250 ml of Difco potato-dextrose broth (PDB) with 200 μl of a 1×10^6 conidia per milliliter suspension and were incubated with shaking at room temperature. Radial growth based on colony diameter and GUS activity were measured at 0, 3, 7, 10, 14, 17, and 21 days after inoculation. Fresh weight of P84-8 grown on PDB and corresponding GUS activity were measured only until 14 days after inoculation.

GUS was extracted from agar cultures by removing the mycelia and agar from the petri dish and soaking them overnight at 4 C in 5 ml of extraction buffer (50 mM $NaHPO_4$, pH 7.0, 5 mM dithiothreitol, 10 mM Na_2EDTA, 0.1% sodium lauryl sarcosine, and 0.1% Triton X-100) (11). Fungal mycelium from each broth culture was isolated by filtration and soaked in 2 ml of extraction buffer overnight at 4 C. After soaking, samples from agar and broth cultures were centrifuged at 13,800 *g* for 10 min at 4 C, and the supernatants were collected and tested for GUS activity. GUS activity was determined fluorimetrically by measuring the conversion of 4-methylumbelliferyl glucuronide (MUG) to 4-methylumbelliferone (MU) using the procedure of Jefferson et al (11) with slight modification. Ten microliters of the supernatant was mixed with 200 μl of MUG substrate in a 1.5-ml microfuge tube and incubated at 37 C for 1 h. The reaction was stopped by adding 1,290 μl of 0.2 M Na_2CO_3. GUS activity was determined with a Hoefer TKO 100 fluorimeter (Hoefer Science Instruments, San Francisco) and expressed as nanomoles of MU produced per sample. Uninoculated PDA and PDB were used as controls.

Evaluation of eyespot resistance. *Wheat germ plasm and cultural practices.* Five wheat genotypes were selected on the basis of their field resistance to *P. herpotrichoides* (18,19): VPM(1124-R25-1), highly resistant; Cappelle Desprez(PI 262223) and Cerco(CI 15922), resistant; Daws(CI 17419) and Selection 101(CI 13438), susceptible. Chinese Spring, which is susceptible, was included because it is widely used in genetic studies. Five seeds per genotype were sown in each 10.6-cm-diameter plastic pot in a commercial potting mix (55-35-10 peat-pumice-sand [w/w]) and covered with 2 cm of vermiculite. Plants were grown in a growth chamber at 12 C with 12 h of light of about 400 μE and 95–100% relative humidity. Plants were watered by placing pots in pans with approximately 2 cm of standing water. Plants were fertilized at 3 wk after inoculation with Nutriculture at 300 mg/L (20-20-20 N-P-K, Plant Marvel Laboratories, Chicago) applied to the water.

Inoculum preparation and inoculation. Conidia were produced by growing P84-8 on 1.5% water agar (Bacto-agar, Difco) for 2 wk at 16 C under continuous near-UV light (17). Conidia were washed from the agar with sterile distilled water. In the first experiment, conidia (1×10^5 conidia per milliliter) were sprayed (5 ml per pot) onto 2-wk-old seedlings (16). In the second experiment, 250 μl of a suspension of water agar and conidia was pipeted into 1.5-cm-long plastic drinking straws (6-mm-diameter) placed around the stems of 2-wk-old seedlings at the soil surface. Inoculum was placed in straws to improve the uniformity of contact with the entire stem base. Agar was included with the inoculum to increase its viscosity and prevent immediate absorption by the soil. The agar-conidia suspension was prepared by blending 20 ml of solidified 1.5% water agar in 40 ml of sterile distilled water and conidia. Conidia were counted and adjusted to 1×10^5 ml of suspension. Plants inoculated with water only were maintained as controls.

Evaluation of resistance. One pot each of inoculated and control plants per genotype in each replicate were sampled at 2-wk intervals after inoculation for 6 and 8 wk in experiments 1 and 2, respectively. Plants were washed free of soil, and the roots

and shoots were removed to obtain 4-cm-long basal stem samples. Plants were kept on ice or at −20 C until further use. Disease progress was assessed by visually rating plants for disease severity, with the Du Pont Advisor, a cereal eyespot antigen ELISA kit (Du Pont Company, Wilmington, DE) (26,27) and with the GUS enzyme assay. All the plants in each pot at each sample date were rated visually by a 1–5 scale, where 1 = a lesion on the first leaf sheath only; 2 = a lesion on the first leaf sheath and a small lesion or speck on the second leaf sheath; 3 = a lesion on the first leaf sheath and a lesion covering one-third of the second leaf sheath; 4 = a lesion on the first leaf sheath and a lesion covering two-thirds of the second leaf sheath; and 5 = a lesion covering the entire first and second leaf sheaths.

Two and three plants per pot were tested with ELISA and for GUS activity in experiments 1 and 2, respectively. Plants were frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. Ground samples were transferred to 1.5-ml microfuge tubes, and 400 (experiment 1) or 600 μl (experiment 2) of extraction buffer was added and mixed with a vortex mixer. Samples were centrifuged at 29,200 *g* for 15 min at 4 C, and the supernatants were retained for GUS assay and ELISA. Samples for ELISA were prepared by diluting 50 μl of the supernatant into 450 μl of the ELISA buffer supplied with the kit and adding 100 μl of the diluted extract to precoated wells of a microtiter plate. Plates were developed according to the directions; optical density was measured in a Molecular Devices Emax microplate reader (Molecular Devices, Menlo Park, CA). Results were expressed as antigen units per milliliter, where one antigen unit is defined as 2.26 ng of cultured *P. herpotrichoides* (26,27). GUS activity was determined fluorimetrically by the modified procedure of Jefferson et al (11) described above. GUS activity was expressed as nanomoles of MU produced per sample. Uninoculated plants were tested to determine background GUS activity.

Comparison of inoculation methods. The effect of inoculation method on disease severity and uniformity was further studied with Cappelle Desprez (resistant) and Chinese Spring (susceptible). Seed was sown and inoculated either by spraying with a suspension of conidia (500 μl per plant) or by pipeting a suspension of water agar and conidia into a 1.5-cm-long plastic straw placed around the basal part of the stem (described above). Ten plants per treatment (two plants per replicate) were inoculated 14 days after planting and incubated as previously described. Eyespot severity, based on GUS activity per plant, was determined (described above) 5 wk after inoculation.

Experimental design and statistical analyses. Time-course experiments to evaluate resistance were arranged in a randomized complete block, split-plot design, where genotype was the main plot factor and sample date was the subplot factor. A total of 180 and 240 pots were used in experiments 1 and 2, respectively, consisting of six genotypes, uninoculated controls, three or four sample dates, and five replicates. Treatments in all other experiments were arranged in a randomized complete block design with five replicates. All experiments were repeated at least once. Analyses of variance and correlations were conducted with SAS statistical analysis software (SAS Institute, Cary, NC). GUS assay and ELISA data were transformed by a logarithmic transformation before analysis due to nonindependence of mean and variance. Means were differentiated by Duncan's multiple range test ($P = 0.05$); however, untransformed means are presented. Fisher's least significant difference ($P = 0.05$) was calculated to compare means between inoculation methods.

RESULTS

Relationship between fungal growth and GUS activity. GUS activity increased in direct proportion to radial growth of P84-8 during the 21-day sample period (Fig. 1). The correlation coefficient for radial growth and GUS activity was highly significant ($r = 0.99$, $P = 0.001$). GUS activity was detected as early as 3 days after seeding the culture medium and increased 583-fold after 21 days. The GUS activity per unit area of fungal mycelium remained constant from 10 to 21 days after inoculation (data not shown). A similar relationship was observed between GUS activity and fresh weight of P84-8 mycelium. After 7, 10, and 14 days, the weight of mycelium was 0.4, 0.8, and 1.3 g, respec-

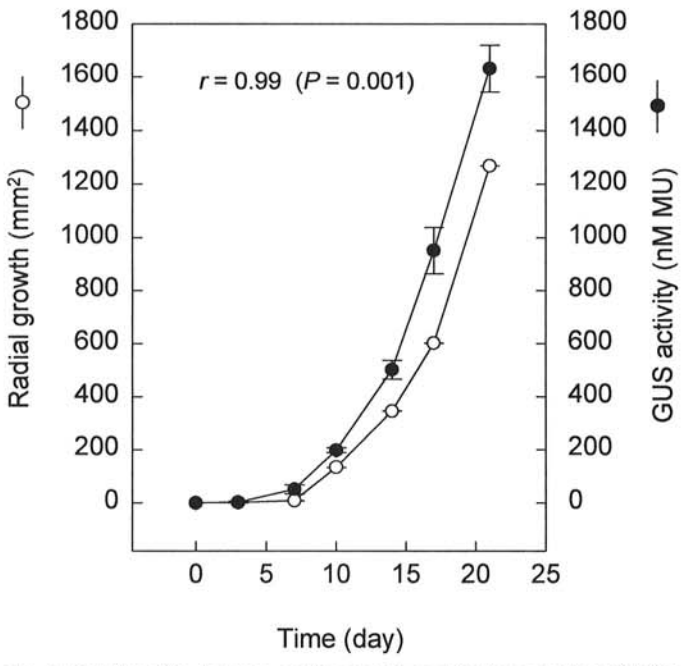

Fig. 1. Relationship between radial growth and β-glucuronidase (GUS) activity of isolate P84-8 of *Pseudocercosporella herpotrichoides*, which was transformed with the *Escherichia coli gusA* gene. The pathogen was grown on potato-dextrose agar at 20 C in the dark. GUS activity was measured based on the amount of 4-methylumbelliferone (MU) produced by the hydrolysis of 4-methyl umbelliferyl glucuronide substrate. Bars represent the standard error of the mean, ranging from 0.6 to 1.2 mm² and 6.0 to 176.9 nM MU for radial growth and GUS activity, respectively.

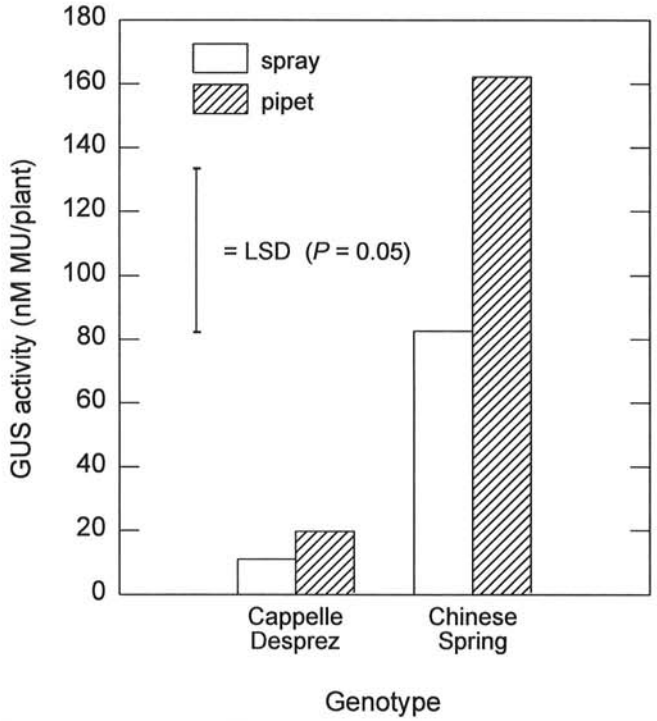

Fig. 2. β-glucuronidase (GUS) activity of Cappelle Desprez (resistant) and Chinese Spring (susceptible) wheat genotypes 5 wk after inoculation with a GUS-transformed isolate (P84-8) of *Pseudocercosporella herpotrichoides*. Two-week-old seedlings were inoculated by pipeting an agar suspension of conidia into straw-collared stem bases or by spraying plants with a suspension of conidia. MU = 4-methylumbelliferone.

tively, and GUS activity was 730, 3,165, and 5,890 nM MU, respectively. There was a significant positive correlation ($r = 0.99$, $P = 0.001$) between GUS activity and mycelial weight. Similar results were obtained when these experiments were repeated.

Effect of inoculation method on eyespot severity. Placing inoculum in plastic straws resulted in nearly doubled GUS activity compared to spray-inoculated plants (162 versus 83 nM MU per plant, respectively) (Fig. 2). A nearly significant interaction ($P = 0.07$) between genotype and inoculation method occurred as a result of significantly higher GUS activity in the susceptible genotype Chinese Spring when inoculum was placed in plastic straws around stem bases than when conidia were sprayed onto plants (Fig. 2). No significant difference in GUS activity between the two methods was observed in the resistant genotype Cappelle Desprez.

Eyespot resistance in wheat genotypes. Differences among genotypes, sample dates, and the genotype-sample date interaction were significant ($P \leq 0.05$) in both experiments for all methods of disease assessment. Disease intensity was greater and more uniform in the second experiment (Fig. 3B, D, and F) in which plants were inoculated with the agar-conidia inoculum placed in straw collars than in the first experiment (Fig. 3A, C, and E) in which plants were sprayed with a conidial suspension.

The relative resistance reaction of genotypes did not differ in these experiments; therefore, only data for individual sample dates from the second experiment are presented (Table 1). Growth of *P. herpotrichoides* in wheat seedlings was detected as early as 2 wk after inoculation, and significant differences among resistant and susceptible genotypes were observed at 6 wk after inoculation with all methods. The genotypes VPM, Cappelle Desprez, and Cerco were resistant, whereas Chinese Spring, Daws, and Selection 101 were susceptible based on the three disease evaluation methods. The highly resistant, resistant, and susceptible genotypes were distinguished 4- to 6-wk after inoculation with the GUS assay and 6- to 8-wk after inoculation with visual disease ratings. Differences among highly resistant, resistant, and susceptible genotypes were not consistently distinguished with ELISA. Chinese Spring was the most susceptible genotype tested based on the GUS assay and ELISA, but this was not detected with visual scoring.

There was a significant ($P \leq 0.05$) positive correlation between visual disease ratings and GUS activity and ELISA values at all sample dates (range of r value = 0.45–0.76). However, correlation coefficients between visual disease ratings and the GUS assay were numerically larger ($r = 0.59$–0.76) than between visual disease ratings and ELISA ($r = 0.45$–0.66).

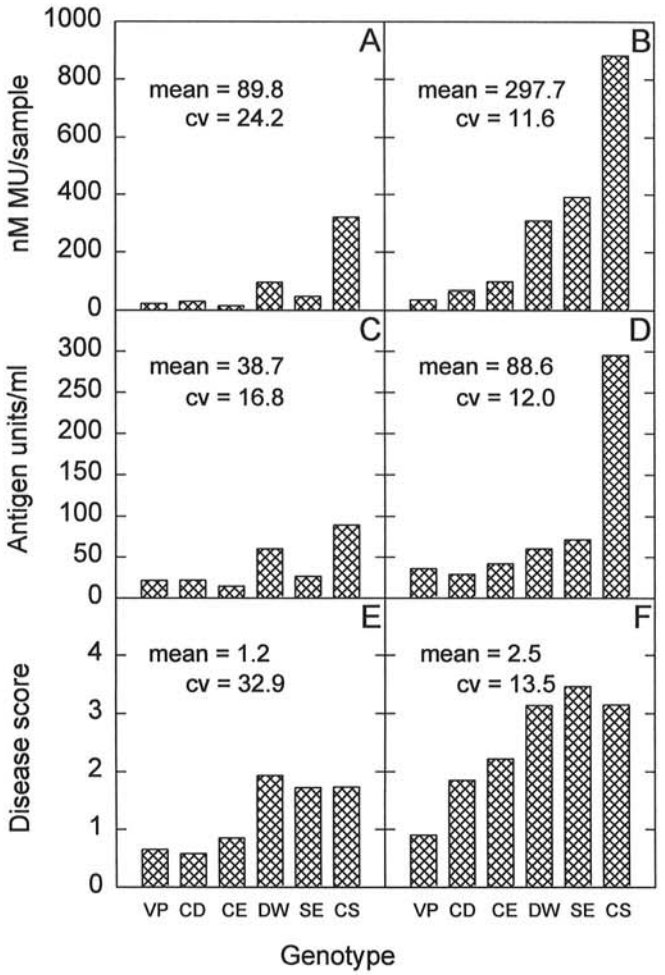

Fig. 3. A and B, β-glucuronidase (GUS) activity, **C and D,** enzyme-linked immunosorbent assay (ELISA) value, and **E and F,** visual disease scores of six wheat genotypes inoculated by spraying 2-wk-old seedlings with a suspension of conidia (**A, C, and E**) or by pipeting comminuted water agar and conidia into straw-collared stems (**B, D, and F**) with a GUS-transformed strain (P84-8) of *Pseudocercosporella herpotrichoides*. **A–F** represent the average of four sample dates and five replicates. Genotypes are listed in order of decreasing resistance to *P. herpotrichoides*: CD = Cappelle Desprez, CE = Cerco, CS = Chinese Spring, DW = Daws, SE = Selection 101, and VP = VPM. MU = 4-methylumbelliferone.

DISCUSSION

An effective selection method is a fundamental component of a successful breeding program. The GUS reporter gene method

TABLE 1. Reaction of six wheat genotypes to inoculation with a β-glucuronidase (GUS)-transformed strain (P84-8) of *Pseudocercosporella herpotrichoides* with three disease rating systems at four sample dates

		Rating system[y,z]		
Time[x]	Genotype	GUS	ELISA	Visual
2	VPM	1.1 c	1.5 d	0.2 c
	Capelle Desprez	2.2 c	1.7 d	0.2 bc
	Cerco	5.5 b	4.1 bc	0.4 a–c
	Daws	12.3 ab	6.3 ab	0.8 a
	Selection 101	19.1 a	7.1 a	0.7 a
	Chinese Spring	12.9 a	3.4 c	0.6 ab
4	VPM	37.3 c	18.6 c	1.2 d
	Capelle Desprez	90.8 b	22.5 bc	2.2 c
	Cerco	97.7 b	27.0 a–c	2.8 b
	Daws	127.0 b	20.6 bc	3.8 a
	Selection 101	264.9 a	32.0 ab	3.8 a
	Chinese Spring	223.0 a	40.5 a	3.1 b
6	VPM	36.6 d	19.4 c	1.0 d
	Capelle Desprez	84.0 c	20.9 c	2.6 c
	Cerco	106.8 c	21.0 c	2.8 c
	Daws	560.2 b	76.4 b	4.0 b
	Selection 101	754.4 b	111.2 b	4.7 a
	Chinese Spring	1,523.4 a	217.1 a	4.2 ab
8	VPM	66.4 d	89.8 bc	1.3 e
	Capelle Desprez	98.9 cd	69.4 c	2.3 d
	Cerco	166.6 c	98.9 bc	2.8 c
	Daws	535.2 b	135.0 b	4.0 b
	Selection 101	524.0 b	134.0 b	4.7 a
	Chinese Spring	1,645.9 a	753.3 a	4.7 a

[x] Weeks after inoculation.

[y] GUS = GUS activity (nanomoles 4-methylumbelliferone per sample); enzyme-linked immunosorbent assay (ELISA) = antigen units per milliliter; Visual = visual disease rating: 1 = a lesion on the first leaf sheath only; 2 = a lesion on the first leaf sheath and a small lesion or speck on the second leaf sheath; 3 = a lesion on the first leaf sheath and a lesion covering one-third of the second leaf sheath; 4 = a lesion on the first leaf sheath and a lesion covering two-thirds of the second leaf sheath; and 5 = a lesion on the entire second leaf sheath.

[z] Figures represent the mean of five replicates. Duncan's new multiple range test ($P \leq 0.05$). Means within a column and sample date followed by the same letter are not significantly different. Analysis of variance and mean separation for GUS and ELISA were conducted on log-transformed data; however, untransformed means are presented.

described here represents a new approach to screening for disease resistance and has proven to be a powerful tool for evaluating resistance to *P. herpotrichoides* in the early stages of wheat growth. Results of in vitro studies demonstrated a direct relationship between growth of *P. herpotrichoides* (strain P84-8) and GUS activity. Thus, we were able to determine which genotypes restricted pathogen growth (resistant) and which allowed the pathogen to grow relatively unimpeded (susceptible). At the same time, plant-to-plant variation in disease development was reduced by inoculating seedlings with agar-conidia inoculum placed in straw collars around stem bases. This method is a modification of a technique described by Macer (14) that allows more precise control of inoculum rate but retains the uniformity of inoculum application to each stem. Use of the GUS reporter gene system to evaluate wheat for resistance to *P. herpotrichoides* has several advantages over visual disease assessment, including speed, quantitative ability, and, most importantly, the elimination of errors due to subjectivity. It also allows flexibility such that plant materials can be frozen (−20 C) without affecting GUS detection. In subsequent experiments, similar results were obtained when infected stems were soaked without grinding in extraction buffer overnight at 4 C (T. D. Murray, *unpublished data*). The inability to use transformed pathogens under field conditions due to governmental regulations is a major limitation of this system. Also, similar to other eyespot evaluation methods, destructive plant sampling is done to assess eyespot infection using the GUS assay.

The GUS reporter gene enabled differentiation among highly resistant, resistant, and susceptible cultivars. Although differences among resistant and susceptible genotypes in GUS activity were observed 2 wk after inoculation, the greatest differentiation occurred 4 and 6 wk after inoculation, when three and four phenotypic classes were distinguished, respectively. Based on the resistance genes known to be carried in these lines (8,12,22), three or four phenotypic classes were expected.

Visual disease ratings allowed differentiation of resistant and susceptible genotypes at 6 and 8 wk after inoculation into four and five phenotypic classes, respectively. However, the application of this method to breeding materials is limited by the rating scale, which is too narrow (1–5) to accurately classify large numbers of genotypes. Moreover, the method is labor intensive and subjective. The GUS reporter gene system allows greater differentiation of resistance because the GUS readings are proportional to pathogen growth and, thus, not limited by an artificial scale. For example, GUS activity in the susceptible genotype Chinese Spring was 41-fold greater than the highly resistant VPM at 6 wk after inoculation, compared to fourfold greater visual disease ratings at the same stage. This suggests that subtle differences in resistance based on visual disease ratings can be amplified by the GUS system.

The Du Pont Advisor ELISA is also a quantitative assay that can rapidly and accurately identify *P. herpotrichoides* presymptomatically in wheat plants at early stages of growth (26,27). In this study, however, ELISA was unable to differentiate among resistant genotypes at any stage. It is possible that sample dates were too early for ELISA to clearly differentiate genotypes. Lind (13) reported that ELISA could not differentiate genotypes until after anthesis. The inability to distinguish genotypes with ELISA at earlier growth stages was attributed to the sensitivity of the assay (13); differences among genotypes were not apparent until the quantity of fungal antigen exceeded the limit of detection. For this ELISA, the necessary quantity apparently did not occur until near anthesis.

Reducing environmental influences on phenotypic expression of resistance is important to improve differentiation of genotypes. Greater uniformity of infection and disease severity was obtained when plants were inoculated with a suspension of comminuted water agar and conidia placed in plastic collars around stem bases (Fig. 3B, D, and F) than when conidia were sprayed directly on plants (Fig. 3A, C, and E). The experiment on inoculation methods (Fig. 2) confirmed this observation. With this method, a significant increase in disease severity was detected only in the susceptible genotype Chinese Spring, not in the resistant genotype Cappelle Desprez, thereby expanding the range of GUS measurements. Strausbaugh and Murray (28) reported that coleoptile orientation relative to the first leaf sheath influenced eyespot development when conidia were sprayed on seedlings. This method also eliminates the effect of coleoptile orientation on eyespot development, because inoculum contacts the entire stem base uniformly.

Use of the GUS reporter gene to evaluate resistance in wheat to *P. herpotrichoides* is a novel method that efficiently differentiates genotypes with varying levels of resistance. The ability to distinguish resistant genotypes as seedlings based on the amount of fungal growth makes this method a powerful tool for screening and breeding eyespot resistant cultivars. This method also should be applicable to other pathogen-suscept systems in which resistance is associated with reduced pathogen colonization.

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