Elucidation of *Septoria tritici* × Wheat Interactions Using GUS-Expressing Isolates

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ABSTRACT

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Isolate ISR398 of *Septoria tritici* (which produces none to few pycnidia on the wheat cv. Seri 82 and high coverage on cv. Shafir) and isolate ISR8036 (which is virulent on both cultivars) were genetically cotransformed using the selectable marker gene *hph*, which confers resistance to hygromycin B (*hygB*), and the reporter gene *uidA*, encoding β -glucuronidase (GUS). Most of the genetically transformed isolates (98.8%) produced similar pycnidial coverage on seedlings of 'Seri 82' and 'Shafir' as the two wild-type isolates. Southern analysis of 25 randomly selected *hygB*^RGUS⁺ transformants probed with the *uidA* sequence revealed multiple insertion sites. GUS activity was determined fluorimetrically by measuring the conversion of 4-methylumbelliferyl β -D-glucuronide (MUG) to 4-methylumbelliferone (MU). The high GUS-expressing transformants 398D97 and 8036E27 were used to elucidate fungal development within inoculated leaf tissue by using GUS activity to estimate the fungal proteins content in planta. Increase in fungal biomass was recorded in 'Shafir' in-

oculated with the GUS-expressing transformants 398D97 and 8036E27 following a 12-day latent period. A 15-day latent period was recorded in 'Seri 82' inoculated with 8036E27, whereas an 18-day latent period was recorded on 'Seri 82' inoculated with 398D97 and the two mixtures 398D97 + ISR8036 and ISR398 + 8036E27. The rate of fungal development and the estimated level of fungal proteins at the pycnidia maturation stage was high in leaves of 'Shafir' and moderate to low on 'Seri 82', even in cases in which no significant differences were recorded in pycnidial coverage. An endogenous capacity to hydrolyze β -1,4-D-glucuronidase was recorded in leaves inoculated with wild-type isolates. The latent periods in MU production of the *uidA*-expressing transformants mimicked those recorded for the wild-type isolates. However, at all stages, the levels of MU produced in wheat inoculated with wild-type isolates were markedly lower than those produced by GUS-expressing transformants. The mode of interaction (compatible or incompatible) determined the onset of the induction, rate, and level of enzyme production.

Additional keywords: common wheat, genetic transformation, *Mycosphaerella graminicola*, *Septoria tritici* blotch of wheat, *Triticum aestivum*.

Septoria tritici blotch (STB) of wheat incited by *Mycosphaerella graminicola* (Fuckel) J. Schrot. in Cohn (anamorph *Septoria tritici* Roberge in Desmaz.) can attain epidemic proportions and cause significant reductions in yield (12,33). In recent years, STB has become a major wheat disease in northern European countries (e.g., Germany and the United Kingdom) in addition to countries with a Mediterranean-type climate. Physiological specialization was reported in *S. tritici* both in seedlings and under field conditions (7,9,10,12,19,20), but the magnitude of specificity in both pathogen and host and the impact on breeding for disease resistance has not been clearly determined. Several assumptions were raised in order to explain the specific interaction between virulence and resistance loci, including a gene-for-gene interpretation (9,18,19). Resistance is defined as fungal incapability to form pycnidia on leaves, although it has not been clear whether or not the mycelia are equally spread in leaf tissues of susceptible and resistant hosts.

Cohen and Eyal (3) reported that mycelia could be histologically detected in the intercellular spaces of mesophyll cells of the resistant wheat cv. Kavkaz/K4500 L.6.A.4 inoculated with *S. tritici* isolate ISR398^(AvSer), which is avirulent on this host. Kema et al. (21) reported that mycelial mass in this resistant cultivar and in the susceptible cv. Shafir was low and comparable until 8 days post-inoculation, and diverged thereafter exponentially, as measured histologically and immunologically.

Deviations from the expected host response were recorded following inoculation with avirulent and virulent isolates, either mixed together (45) or used in challenge inoculation (15). Marked suppressions in pycnidial coverage were observed on seedlings of wheat cv. Seri 82 inoculated with the avirulent isolate ISR398 (denoted here as Seri 82^(R398) and ISR398^(AvSer), respectively) followed 2, 5, or 10 days later with the virulent isolate ISR8036 (15). Significant reductions were also recorded on 'Shafir', which is susceptible to both isolates. The induced resistance of the 'Seri 82'^(R398) seedling, triggered by the avirulent isolate, was assigned to a mechanism or mechanisms associated with pycnidial production (15).

The timed events associated with the infection of wheat genotypes by single, mixed, or sequential inoculations with *S. tritici* isolates are difficult to elucidate due to lack of specific fungal markers with a quantitative reporting capability. Genetic transformation has been established in many phytopathogenic fungi (4–6,8,17,26,29,34,42,43) and recently, also, for *S. tritici* (27,28,32). Unique marker genes have been introduced into the fungal nuclear genome and employed for studying penetration and colonization of plant host tissues by the pathogen (8,22,28,37,41) and for qualitative and quantitative assessments of host × pathogen interactions (35,41,42). Tagged *S. tritici* mutants (resulting from genetic transformation), which carry distinct reporter genes, may provide the measures needed to elucidate biological processes and fungal genes associated with host × pathogen interaction.

The objective of this study was to develop a reporting system that allows quantitative assessment of postinoculation events and pycnidial production in the *S. tritici*-wheat pathosystem by using transgenic isolates with known virulence that carry the reporter gene

uidA, encoding β -glucuronidase (GUS), and the *hph* selectable marker gene.

MATERIALS AND METHODS

Wheat cultivars and *S. tritici* cultures. Wheat cvs. Seri 82 (Kavkaz/BUHO“S”//KAL/BB; CM33027-, VEERY 5, CIMMYT, Mexico, D.F., Mexico), designated ‘Seri 82’^(R398) (i.e., resistant to the isolate ISR398), and Shafir (Sonora 64/Tezanos Pinto Precoc//Nainari 60/3/Florence Aurore; Hazera Seed Co., Mivhor, M.P. Sde Gat, Israel) were used for pathogenicity analyses of wild-type isolates and transformants.

Isolates ISR398 (ATCC 48507), designated ISR398^(AvSer) (i.e., avirulent on ‘Seri 82’), and ISR8036 of *S. tritici* were used in all experiments. Culturing of isolates followed that of Zelikovitch and Eyal (45). For long storage, conidia of *S. tritici* isolates grown in liquid sucrose-yeast medium (LS) and suspended in glycerol (1:1 vol/vol conidia in media/glycerol to a final concentration of 50% glycerol) were distributed into a series of 1.5-ml Eppendorf vials and stored at -70°C . Transformed isolates were reisolated from single pycnidia following inoculation of seedling leaves of ‘Seri 82’ or ‘Shafir’ and cultured on malt agar without or with hygromycin B (hygB; 50 $\mu\text{g/ml}$).

Plasmids. Two closed circular plasmids, pHR (*hph*) and pGR (*uidA*), were separately or cotransformed into protoplasts of *S. tritici*. pHR is a modified pAN7-1 (34) vector carrying the *Escherichia coli hph* gene that encodes the enzyme hygromycin phosphotransferase conferring resistance to hygB. The *hph* gene is controlled by the constitutive glyceraldehyde-3-phosphate dehydrogenase promoter (*Pgpd*) and the *trpC* terminator (polyadenylation signal; *TtrpC*) from *Aspergillus nidulans*. pGR, a modified pNOM102 (26),

includes the *uidA* reporter gene from *E. coli* encoding GUS, linked to the same promoter and terminator sequences. Both plasmids carry the 570-base pair (bp) *S. tritici Bgl*-genomic fragment (Sr) consisting of 15 short tandem repeated sequences cloned in the *Hind*III site downstream to the terminator (30,32) (Fig. 1C).

Transformation of *S. tritici*. Conidia (1×10^8) were incubated in 100 ml of LS at 28°C by shaking at 200 rpm. Conidia were harvested after 18 h, pretreated as described by TeBeest and Weidemann (40), and washed with 1.2 M MgSO_4 . Protoplasts were produced according to Cooley et al. (4) with 25 mg of Novozyme 234 (InterSpex Products, Inc., Foster City, CA) per ml and 1.2 M MgSO_4 as an osmotic stabilizer. Pelleted protoplasts (about 10^6 to 10^7) were suspended in 100 μl of STC (1.2 M sorbitol; 10 mM Tris-HCl, pH 7.5; and 50 mM CaCl_2) and incubated with 10 μg of circular plasmid on ice for 30 min. Polyethylene glycol (PEG) solution (30% PEG 6000, 50 mM CaCl_2 , and 10 mM Tris, pH 7.0) was added twice (100 μl followed by 1 ml), with gentle mixing in between. The protoplasts solution was transferred to 10 ml of LSS (10 g of yeast extract per liter and 1 M sucrose). Following incubation for 72 h at 28°C on a gyratory shaker (70 rpm), the germinating cells were then transferred to malt agar with 50 μg of hygB per ml as a selective medium, whereas nontransformed protoplasts served as controls.

The designation of each transformant included the parental isolate (e.g., 398^(AvSer) or 8036), a letter assigned to the transformation series (e.g., D or E), and a number of the colony (e.g., 398D97^(AvSer) or 8036E27). AvSer indicates avirulence on ‘Seri 82’. This symbol was omitted in the figures for simplicity.

GUS assays. Colonies resistant to hygB were tested for GUS activity to confirm cotransformation of *hph* and *uidA* genes. Single colonies were transferred to 200 μl of LS in microtiter plates. Fifty

Fig. 1. Southern analyses of total DNA from **A**, *Septoria tritici* wild-type isolates ISR398^(AvSer) and **B**, ISR8036 and their derived transformants, resulting from cotransformation with plasmids carrying the *hph* and *uidA* genes. Total DNA (5 μg) was digested with *Bgl*II (a unique restriction site in both plasmids) and probed with *uidA*. Stringent hybridization and washing conditions (65°C , 0.1 SSC [1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% sodium dodecyl sulfate) were used. The positions of size markers (λ *Bst*EII) in kilobases are shown on the left. Arrows on the right indicate the size of linear pGR plasmid. **C**, Schematic drawing of transformation vectors pGR and pHR. Restriction enzymes: B = *Bgl*II, BH = *Bam*HI, H = *Hind*III, NC = *Nco*I, RI = *Eco*RI, SI = *Sac*I, and SII = *Sac*II.

microliters of X-Gluc solution (500 µg of 5-bromo-4-chloro-3-indolyl glucuronide per ml; 100 mM sodium phosphate, pH 7.0; 100 µM potassium ferrocyanide; and 100 µM potassium ferricyanide) were added; thereafter, the plates were incubated at 37°C for 24 h.

GUS activity *in vitro* and *in planta* was monitored by measuring the conversion of 4-methyl-umbelliferyl β-D-glucuronide (MUG) to 4-methylumbelliferone (MU) (16). About 50 mg of conidia was harvested from malt agar plates and ground in 200 µl of GUS extraction buffer (0.1 M Na-PO₄ buffer, pH 7.0; 10 mM EDTA; 0.1% Triton X-100; 0.1% N-sodium lauroyl sarcosine; and 10 mM β-mercaptoethanol). After centrifugation at 12,000 × g for 15 min, the supernatant protein concentration was determined using the Bradford method (1). GUS activity was monitored according to Jefferson (16), by incubating increasing concentrations of fungal total proteins with 2 mM MUG dissolved in GUS extraction buffer in a final volume of 200 µl at 37°C for 10 min. The reaction was stopped by adding 50 µl of stop buffer (1 M Na₂CO₃).

GUS activity in inoculated seedlings was assayed similarly. Excised tip segments of primary leaves (4 cm long) were washed in H₂O for 10 min to discard spores from the leaf surface and then kept at -70°C. Each treatment included 12 leaf segments in four replicates of three segments. The three segments were ground in a microfuge tube with 300 µl of extraction buffer at 4°C. Following centrifugation and determination of protein concentration, as described above, 30 µl of crude extract was incubated with 2 mM MUG in GUS extraction buffer in a final volume of 200 µl for 150 min at 37°C. MU fluorescence was monitored by using a FL500 fluorescent plate recorder (Bio-Tec Instruments, Winooski, VT) with excitation wavelength of 360 nm and emission wavelength of 460 nm, in parallel to 0 to 20 µM MU standard samples.

DNA procedures. Plasmid propagation and handling were performed according to Sambrook et al. (36). Total DNA was isolated from conidia of *S. tritici* according to procedures of Halperin et al. (15). Southern blot analysis was carried out according to Sambrook et al. (36). The DNA was blotted onto a Hybond-N⁺ membrane (Amersham International, Amersham, United Kingdom). DNA probes were labeled with (α-³²P)dCTP by random priming (13) and hybridized as described in Halperin et al. (15).

Mitotic stability of hygB resistance *in vitro*. Single colonies of 12 hygB-resistant (hygB^R) or hygB^RGUS⁺ transformants were cultured in 30 ml of LS without selection at 18°C in a reciprocal shaker at 150 rpm. Subculturing using a 10⁻² dilution was repeated five times at 7-day intervals. Forty-eight colonies generated from each transformant were then transferred to malt agar with or without 50 µg of hygB per ml to test the stability of the *hph* expression.

Mitotic stability of hygB resistance and GUS activity following propagation in plants. Pycnidiospores were isolated from individual pycnidia that were developed on seedling leaves of wheat cvs. Seri 82^(R398) and Shafir, which had been sprayed with 25 different transformed isolates (hygB^R or hygB^RGUS⁺). The single-pycnidia isolates were first cultured on nonselective medium and thereafter transferred to malt agar medium containing 50 µg of hygB per ml to test the maintenance of *hph* expression. For checking the stability of *uidA* expression, single-pycnidia isolates originated from two GUS-expressing transformants (hygB^RGUS⁺) were assayed for GUS activity using X-Gluc as a substrate.

Plant inoculation. Seedlings (10 days old, two-leaf stage) of spring wheat cultivars (*Triticum aestivum* L.) were used for inoculation with *S. tritici* isolates (wild-type parental isolates, transformants, or 1:1 isolate mixtures), according to procedures described by Zelikovitch and Eyal (45). Conidia cultures, stored at -70°C, were used for routine inoculations, thus providing homogenous cultures for each experiment. This strategy also avoids possible attenuation of virulence, due to continuous subculturing, and the need for periodical reisolations (every 3 months) from pycnidia on infected leaves. Twenty plants per cultivar in two replications were inoculated with each isolate in all of the pathogenicity trials.

Plants were inoculated with 30 ml of conidial suspension (10⁷ spores per ml) per isolate of 4- to 5-day-old *S. tritici* cultures and then incubated in a humidity chamber for 48 h. Plants were then kept in the same growth chamber with daily illumination of 12 h of cool white fluorescent light at an intensity of 150 µE m⁻² s⁻¹ at a constant temperature of 18°C. Disease severity (estimation of percent pycnidial coverage) on primary leaves was visually assessed 21 days after inoculation with the aid of standard drawings (11).

Monitoring postinoculation in planta events. Transformants 398D97^(AvSer) and 8036E27 (derivatives of ISR398^(AvSer) and ISR8036, respectively) expressing high GUS levels were used to elucidate postinoculation events in planta. The isolates were sprayed onto seedlings of 'Seri 82'^(R398) and 'Shafir' singly or in isolate mixtures (398D97^(AvSer) + ISR8036 and 8036E27 + ISR398^(AvSer)). Leaf segments were harvested on days 2, 6, 12, 15, 18, and 21 after inoculation, homogenized, and assayed for GUS activity with MUG substrate. Percent pycnidial coverage was estimated 21 days after inoculation. Uninfected seedlings and those inoculated with wild-type isolates served as controls for MU background.

The net difference in MU level between GUS assays of plants inoculated with GUS transformants and those inoculated with the appropriate wild-type isolates was calculated at each time point. The value of fungal biomass, or fungal protein content, was calculated based on the linear correlation between MU production and fungal protein content in the GUS assays (Fig. 2).

Statistical analyses. The significance of differences between pycnidia coverage of the wild-type isolates and the different transformants on 'Seri 82'^(R398) and 'Shafir' was performed with the Levene's test for variances following the analysis of variance, using the 1990 BMDP (BMDP Statistical Software, Inc., Los Angeles).

RESULTS

Transformation. Isolates ISR398^(AvSer) and ISR8036 were transformed with the plasmid pHR, containing the *hph* selectable marker gene, or cotransformed with pHR and pGR, carrying the *uidA* reporter gene. Transformation with pHR alone or with pGR was obtained at an average frequency of four hygB^R colonies per µg of plasmid on 50 µg of hygB per ml in malt agar. The growth of the wild-type isolates was completely inhibited by 20 µg of the anti-

Fig. 2. Correlation between β-glucuronidase (GUS) activity and fungal protein content in cultured 398D97^(AvSer) transformant and the inhibitory effect of plant extract. GUS activity was measured by the conversion of 4-methylumbelliferyl β-D-glucuronide (MUG) to 4-methylumbelliferone (MU) in the absence (◆) and presence of 'Shafir' (○) or 'Seri 82'^(R398) (△) leaf extract in the reaction. Linear regression analysis showed *r*² values of 0.98 and 0.96, respectively.

biotic per ml. The majority of the *hygB*^R transformants grew well on malt medium containing 200 µg of *hygB* per ml. About 90% of *S. tritici* colonies, resulting from cotransformation and selected on *hygB* medium, expressed GUS activity.

Pathogenicity analyses of transformants. Pathogenicity analyses on seedlings of wheat cvs. Seri 82^(R398) and Shafir were conducted with 263 *S. tritici* independent ISR398^(AvSer) and ISR8036 transformants, carrying the *hph* gene either alone or together with *uidA*. The wild-type isolates were used as controls. Pycnidial coverage of less than 5% was recorded on 'Seri 82'^(R398) and of more than 20% on 'Shafir' (susceptible to both isolates) inoculated with ISR398^(AvSer), whereas high pycnidial coverage (>20%) was recorded on 'Shafir' and 'Seri 82'^(R398) inoculated with ISR8036 (virulent). The majority of the ISR398^(AvSer)-derived (162 out of 164) and ISR8036-derived (99 out of 99) transformants were statistically similar in their pycnidial coverage to the parental isolates. Two ISR398^(AvSer) transformants (0.8%) expressed significant phenotypic deviation from the wild-type isolate: 398D25 initiated high pycnidial coverage on 'Seri 82'^(R398) and 398D46 showed almost no symptoms on 'Shafir'.

Phenotypic stability of transformants. The mitotic stability of *hygB* resistance in the fungal genome was tested in culture. Transformants were grown for six cycles on nonselective LS medium, after which 48 colonies of each transformant were separately transferred to malt agar containing 50 µg of *hygB* per ml. All 576 colonies originating from 12 *hygB*^R transformants maintained their resistance to *hygB*. The mitotic stability of *hygB* resistance and GUS activity were also tested after completing a life cycle on wheat seedlings. A total of 275 subisolates obtained from pycnidia of 22 out of 25 (88%) *hygB*^R transformants (7 to 16 for each) survived on the selective medium. The 45 subisolates derived from the remaining three transformants segregated in their response to the antibiotic; eight did not grow on *hygB*. All 181 subisolates obtained from pycnidia of the two GUS-expressing transformants (104 from 8036E27 and 77 from 398D97^(AvSer)) maintained *hygB* resistance and expressed GUS activity in the X-Gluc assay.

Fig. 3. Pycnidial coverage (%) on seedlings of wheat cvs. Seri 82^(R398) and Shafir inoculated with the *Septoria tritici* wild-type isolates ISR398^(AvSer) and ISR8036, β-glucuronidase (GUS)-expressing transformants 398D97^(AvSer) and 8036E27, and transformant + wild-type isolate mixtures. Histograms with the same letter are not significantly different ($P = 0.05$). For the sake of simplicity, the (AvSer) code for ISR398 (i.e., ISR398^(AvSer)) and its derivatives are not included.

Molecular analyses of transformants. Twenty-five cotransformants, exhibiting the same pathogenicity as the wild-type isolates ISR398^(AvSer) and ISR8036, were further analyzed by Southern blot hybridization. Total DNA was isolated from conidia of ISR398^(AvSer), ISR8036, and the cotransformants and digested with *Bgl*II, which has a unique restriction site in the *gpd* promoter region of both plasmids (Fig. 1). Most of these transformants showed the presence of several fragments when probed with the *uidA* (Fig. 1) or *hph* sequence (data not shown), suggesting that multiple integration events occurred in these transformants. A strong hybridization band of 7.7 kilobase pairs (kbp), which corresponds to the size of the linear pGR plasmid (Fig. 1, arrow on the right), was observed in DNA of many transformants when probed with the *uidA* sequence. This suggests that different numbers of pGR copies were integrated and arranged in tandem repeats in various sites of the genome. Additional hybridization bands of different sizes that were also recognized by the *hph* probe (data not shown) indicate that mutual recombination events probably occurred between the two types of vectors prior to the integration into the fungal genome. The weak bands in the 398D21, 398D57, 8036E8003, and 8036E8013 lanes (Fig. 1) represent insertion events with a single copy of the *uidA* sequence. Hybridization with the 570-bp *S. tritici* repetitive sequence present in both vectors (data not shown) indicated that homologous recombination events were targeted in none of the checked transformants.

GUS expression. The level of GUS expression in the different transformants was determined by the MUG assay. Different levels of expression, which varied from 0.2 to 116 pmol of MU per µg of total protein per min, were observed. No GUS activity (less than 0.1 pmol of MU per µg of total protein per min) was detected in the wild-type isolates. Transformants 398D97, 8036E24, 398D98, and 8036E27 were categorized as high expressors, with GUS activity of 116, 63, 41, and 39 pmol of MU per µg of total protein per min, respectively. There were also transformants (398D13, 398D57, 8036E3, 8036E44, and 8036E51) that expressed less than 1 pmol of MU per µg of total protein per min. In general, transformants with a very low number of *uidA* inserts displayed lower GUS activity, but no linear correlation was found between the number of *uidA* copies (quantified by the NIH Image 1.62b7 computer program following scanning of specific bands; NIH, Bethesda, MD) and the level of GUS expression. Some transformants with a high number of copies (8036E100 and 8036E21) showed moderate or low GUS activity (23.4 and 10.1 pmol of MU per µg of total protein per min, respectively).

Transformants with high GUS expression were used to evaluate correlation between GUS activity and total protein content. Increasing amounts of fungal proteins, extracted from transformants 398D97^(AvSer) and 8036E27 that were cultivated in vitro, were tested for GUS activity by MUG assays. A linear correlation ($r^2 = 0.98$) between fungal protein content and GUS activity was observed (Fig. 2). However, when crude extracts of healthy wheat seedlings of 'Shafir' or 'Seri 82'^(R398) were added to the reactions, the level of MU fluorescence was reduced, although the linear correlation between this fluorescence and fungal protein content was still maintained (Fig. 2). Similar values were obtained for the two tested cultivars.

Monitoring postinoculation events in planta. Transformants 398D97^(AvSer) and 8036E27 were not statistically different in pycnidial coverage from the corresponding wild-type isolates ISR398^(AvSer) and ISR8036 (Fig. 3). High pycnidial coverage (>30%) was detected on 'Shafir' inoculated with the wild-type isolates ISR398^(AvSer) and ISR8036 or their derived transformants. An isolate-specific differential response was recorded on 'Seri 82'^(R398) (Fig. 3). The wild-type isolate ISR8036 and its derived transformant 8036E27 both incited high pycnidial coverage (>30%) on 'Seri 82'^(R398), whereas none to few pycnidia (<2%) were produced on 'Seri 82'^(R398) inoculated with the wild-type isolate ISR398^(AvSer) and its derived transformant 398D97^(AvSer).

Inoculations with isolate mixtures of ISR8036 or 8036E27 and ISR398^(R398) or its derived transformant, 398D97^(AvSer), always yielded a significantly lower pycnidial coverage on 'Seri 82'^(R398) in comparison to ISR8036 or 8036E27 alone (Fig. 3).

The linear correlation between MU fluorescence and fungal protein content (Fig. 2) enabled the evaluation of changes in fungal biomass within the infected wheat tissues. GUS activity in seedling leaves inoculated with transformants 398D97^(AvSer) and 8036E27 was determined, and the net MU fluorescence was calculated (Fig. 4). According to the MU fluorescence, very low levels of estimated fungal biomass were detected in leaves at 2, 6, and 12 days after inoculation with all of the compatible interactions (398D97^(AvSer)- 'Shafir', 8036E27-'Shafir', and 8036E27-'Seri 82'^(R398)) and the incompatible interaction (398D97^(AvSer)- 'Seri 82'^(R398)) (Fig. 4A). A fast increase in the rate of fungal biomass accumulation in leaves was recorded in 'Shafir' after 12 days postinoculation with 8036E27 or 398D97^(AvSer), reaching a maximum of 7.4 and 6.4 µg of total fungal protein per mm of leaf with 51 and 41% pycnidial coverage on day 21 postinoculation, respectively. The estimated fungal biomass detected in tissue of 'Seri 82'^(R398) inoculated with either one of the isolates was 2.9 to 3.3 times lower than were the corresponding values recorded in 'Shafir'. In 'Seri 82'^(R398) inoculated with the virulent transformant 8036E27, a delay of 3 days in fungal biomass increase (from 12 to 15 days after inoculation), a slower rate of biomass development, a maximal fungal total proteins value of 2.2 µg, and a pycnidial coverage of 33% were recorded. A longer latent period of 18 days after inoculation, a maximal protein of 2.2 µg per mm of leaf, and a 1% pycnidial coverage were recorded on leaves of 'Seri 82'^(R398) inoculated with the avirulent transformant 398D97^(AvSer). The significant nonspecific reduction in fungal biomass in both compatible and incompatible interactions in 'Seri 82'^(R398) might be attributed to factors associated with that wheat cultivar. The reduction in pycnidial coverage observed in 'Seri 82'^(R398) inoculated with the avirulent transformant 398D97^(AvSer) was not matched with a similar reduction in fungal biomass, indicating that, although 398D97^(AvSer) mycelia were developed in 'Seri 82'^(R398), almost no pycnidia were produced (Fig. 3).

Inoculation with a GUS-expressing transformant mixed together with a wild-type isolate allowed the estimation of fungal biomass of individual isolates within leaf tissue. The latent period in the 8036E27 development in the presence of ISR398^(AvSer) and, vice

versa, 398D97^(AvSer) + ISR8036, on 'Shafir' was extended to 15 days rather than the 12 days in 'Shafir' inoculated with each of the transformants singly (Fig. 4B). The rate of development of 8036E27 in the presence of ISR398^(AvSer) on 'Shafir' was reduced, reaching a maximal level of 4.1 µg of protein per mm (approximately 50% of 8036E27 alone) and a pycnidial coverage of 43%. A fungal protein level of 4.9 µg/mm by day 21 (77% of 398D97^(AvSer) alone) was recorded in 'Shafir' inoculated with 398D97^(AvSer) mixed with ISR8036. No reduction in pycnidial coverage was observed (Fig. 3). In 'Seri 82'^(R398) inoculated with the isolate mixtures 8036E27 + ISR398^(AvSer) or 398D97^(AvSer) + ISR8036, the latent period extended to 18 days (Fig. 4C). The maximal protein levels, 1.4 µg/mm of leaf for 8036E27 and 1.4 for 398D97^(AvSer), were not different statistically from those determined when each transformant sprayed singly (71 and 64%, respectively) (Fig. 4C). However, pycnidial coverage on 'Seri 82'^(R398) inoculated with each of the isolate mixtures was statistically different from 'Seri 82'^(R398) inoculated with either ISR8036 or 8036E27 singly (Fig. 3). These findings indicate that a defense response, which correlates with the reduction in pycnidial formation, was triggered only in 'Seri 82'^(R398) by the presence of the wild-type ISR398^(AvSer) or its derived transformant 398D97^(AvSer).

Endogenous MUG hydrolyzing capacity was gradually developed in wheat leaves following inoculation with wild-type *S. tritici* isolates. This hydrolyzing capacity was not detected in GUS assays performed with conidiospores in culture or with uninoculated wheat leaves. The levels of MU fluorescence obtained in the assays were significantly lower ($P < 0.05$) than those recorded in wheat leaf tissue inoculated with the GUS-expressing transformants. The MU values obtained on day 21 in 'Shafir' inoculated with the *uidA*-carrying transformants 8036E27 and 398D97^(AvSer) were 3.4- and 3.6-fold higher than those obtained in leaves inoculated with the respective wild-type isolates.

The time course of changes in the level of MUG hydrolyzing capacity in leaves inoculated with wild-type isolates (Fig. 5) was obtained in a separate experiments, in which 'Seri 82' and 'Shafir' were inoculated with single or mixed wild-type isolates. The increase in MUG hydrolyzing capacity in 'Shafir' inoculated with ISR398^(AvSer) was faster, reaching a maximal MU level on day 18 after inoculation (1.16 pmol of MU per mm of wheat leaf per min). This value was significantly higher than the corresponding values

Fig. 4. The progression in planta of fungal biomass assessed by the conversion of 4-methylumbelliferyl β-D-glucuronide (MUG) to 4-methylumbelliferone (MU) in seedlings of wheat cvs. Seri 82^(R398) and Shafir inoculated with β-glucuronidase (GUS)-expressing transformants of *Septoria tritici*. **A**, Fungal biomass in leaves inoculated with single transformants, 398D97^(AvSer) (●) and 8036E27 (▲). **B**, Fungal biomass in 'Shafir' leaves inoculated with a mixture of isolates, 398D97^(AvSer) + ISR8036 (○) and 8036E27 + ISR398^(AvSer) (△). **C**, Fungal biomass in leaves of 'Seri 82'^(R398) inoculated with the same isolate mixture combinations. Values of fungal proteins per mm of leaf segment on day 21 after inoculation with a common letter are not significantly different ($P = 0.05$). Estimation of fungal proteins in leaves is based on MU net fluorescence as described in text.

recorded for the other two treatments on 'Shafir', whereas statistically similar pycnidial coverage values were recorded on 'Shafir' inoculated with ISR398^(AvSer) (60.5%), ISR8036 (62.9%), and the isolate mixture ISR398^(AvSer) + ISR8036 (60.5%). Similar to the 8036E27 pattern of development (Fig. 4C), the latent period of the endogenous MUG hydrolyzing activity in 'Seri 82'^(R398) inoculated with ISR8036 was extended to 15 days, reaching a maximal level of 0.69 pmol of MU per mm of wheat leaf per min on day 18 (Fig. 5) and pycnidial coverage of 53.4% on day 21 after inoculation.

A longer latent period of 18 days after inoculation in MU production was recorded in leaves of 'Seri 82'^(R398) inoculated with ISR398^(AvSer) (with a maximal level of 0.1 pmol of MU per mm of wheat leaf per min) and with the isolate mixture ISR398^(AvSer) + ISR8036 (a maximal level of 0.07). Pycnidial coverage values of 10.9 and 9.6% were recorded on 'Seri 82'^(R398) inoculated with these two treatments, respectively. Linear correlation ($r^2 = 0.892$) between the level of MU fluorescence on day 18 after inoculation and pycnidial coverage on day 21 was obtained. The time course of the appearance of the endogenous, as-yet-unidentified, β -1,4-glucuronidase in the two wheat cultivars inoculated with wild-type isolates resembled the estimated fungal biomass development in plants inoculated with the corresponding GUS-expressing transformants (Fig. 4).

The levels of MU fluorescence in wheat tissue of 'Shafir' inoculated with the transformed isolates expressing low GUS activity (8036E8, 8036E62, 398D35, and 398D65) were not statistically different from the recorded levels in wheat tissue inoculated with the wild-type isolates (data not shown).

DISCUSSION

Genetic transformation of *S. tritici* isolates was reported for the first time by transforming protoplasts derived from conidia with circular plasmid vectors bearing the *hph* and *uidA* genes driven by the *Aspergillus nidulans* *gpd* promoter (32). The *gpd* promoter has been used and efficiently expressed in other fungal transformation systems (29,37). A transformation frequency of about four hygB^R colonies per μ g of plasmid DNA was obtained. Similar transfor-

mation frequencies were recorded in *Magnaporthe grisea* (38,44) and other filamentous fungi (8). In this work, cotransformation of pHR and pGR sharing similar pUC19, *Pgpd*, *TtrpC* sequences, and a *S. tritici* repetitive DNA sequence of 570 bp was obtained at high frequency (>90%). A high level of cotransformation (80%) was also reported for *Gaeumannomyces graminis* transformed with pAN8-1 and pBT3 (29), whereas only 50% of the hygB^R *Trichoderma harzianum* transformants expressed the cointroduced *uidA* gene (41).

Almost all hygB^R transformants were stable throughout several culturing cycles on nonselective media. Some subisolates (1.9%) lost hygB resistance after passage through leaf tissue. In all analyzed transformants, the plasmids carrying the *hph* and *uidA* genes were integrated or cointegrated randomly in one or more sites of the *S. tritici* genome, in single or multiple copies. The homologous *S. tritici* 570-bp genomic fragment could have affected recombination and influenced transformation efficiency. However, the molecular analyses suggest that the integration events had occurred randomly, despite the presence of the *S. tritici* 570-bp genomic sequence in the two plasmids. Thus, it is plausible to assume that the 570-bp sequence is too short to serve as a targeting sequence. Shiotani and Tsuge (39) showed that homologous recombination of circular plasmids into the genome of *Alternaria alternata* was directly correlated with the size of the homologous sequence. An increase in homologous recombination frequency was observed when linear plasmids, digested within the homologous sequence, were introduced.

Most of the transformants (99.2%) tested were similar in virulence to the parental isolates. Two transformants out of 164 (1.2%) of isolate ISR398^(AvSer) (avirulent on 'Seri 82'^(R398) and virulent on 'Shafir') have shown a shift in virulence: a shift from avirulent to virulent on 'Seri 82'^(R398) (398D25, hygB^RGUS⁺) or a shift from virulent to avirulent on 'Shafir' (398D46, hygB^R) (31). In general, the phenotypic stability of virulence of *S. tritici* is rather high, and specificity in isolate $\times$ cultivar interaction is highly repeatable. The isolates ISR398^(AvSer) and ISR8036, which have been subcultured and reisolated from pycnidia for more than 18 years, consistently express stability in virulence patterns on differential cultivars (9). These two isolates also showed stability in DNA fingerprinting following repetitive subculturing on media and reisolation from pycnidia (30). McDonald et al. (24) and Chen et al. (2) suggested that the structure of *Mycosphaerella graminicola* populations may be very stable with regard to the assayed DNA fingerprints and 10 restriction fragment length polymorphism loci.

The *uidA* (GUS) gene has been widely used as a transgenic reporter in many systems. The expression of GUS in conidia, pycnidia, and mycelium of *S. tritici* can be easily monitored by using X-Gluc or MUG as substrates. However, the use of the GUS reporter gene to follow postinoculation events in planta required indirect quantitative assessment of fungal biomass, based on fluorescence of MU product, since too-low penetration of the X-Gluc substrate across the wheat leaf tissues was observed. MU evaluation showed that GUS activity in the transformants, cultured in vitro, is linearly correlated with total fungal protein content. Inoculation of wheat cultivars with different GUS-expressing isolates showed that inoculation with transformants with high GUS expression was essential for obtaining significant GUS activity. The lack of linear correlation between GUS expression and *uidA* copy number required early selection of transformants on the basis of expression rather than on copy number. When such transformants were used, it was possible to estimate the fungal biomass in inoculated seedlings, based on a calibration curve of MU level as a function of total fungal protein content, in the presence of plant crude extract (Fig. 2). Green fluorescing protein (GFP) is another reporter gene that has been used in some fungi, including *S. tritici* (23,28,42). Fluorescent hyphae were observed within maize leaves inoculated with *Ustilago maydis* or *Cochliobolus heterostrophus* isolates expressing GFP. When a *S. tritici* isolate carrying the GFP gene was inoculated on

Fig. 5. The capacity to hydrolyze 4-methylumbelliferyl β -D-glucuronide (MUG) to 4-methylumbelliferone (MU) developed in seedling leaves of wheat cvs. Seri 82^(R398) and Shafir after inoculation with wild-type *Septoria tritici* isolates ISR398^(AvSer) and ISR8036 alone or with isolate mixtures. All isolates were devoid of the *uidA* gene. ● = ISR398^(AvSer), ▲ = ISR8036, □ = ISR398^(AvSer) + ISR8036, and * = uninoculated control. Values of MU per mm of leaf per min on day 18 after inoculation with common letter are not significantly different ($P = 0.05$). Estimation of fungal proteins in leaves is based on MU net fluorescence as described in text.

wheat leaves, the expression of GFP was only observed in spores and hyphae located on the external leaf surfaces, but not in hyphae within the leaf (28). It is likely that the direct use of GFP as a fungal reporter gene in planta would also require an adaptation of an indirect quantitative approach to correctly assess fungal development events.

Monitoring the time course of GUS activity in leaves inoculated with *S. tritici* transformants differing in their virulence on the two differential wheat cultivars elucidated variations in the length of the initial latent period of fungal growth and in the rate of mycelial development thereafter, both corresponding to the incompatible-compatible interactions. Moreover, it provided information on pathogenicity capabilities of the different isolates and the contribution of the wheat genotypes to the interaction.

The lag period and the consecutive increase in fungal biomass represent a multiphase process in fungal colonization of wheat leaf tissue. A lag period of 12 days was observed for both isolates in 'Shafir' and a longer lag in 'Seri 82'^(R398), 15 days following inoculation with isolate 8036E27 (virulent) and 18 days for isolate 398D97^(AvSer) (avirulent). Significant differences in fungal development were observed among the different compatible interactions. 'Seri 82'^(R398) inoculated with the virulent isolate 8036E27 resulted in high pycnidial coverage but a longer latent period (15 days) and a lower fungal biomass (3.3 fold) than that of 'Shafir' inoculated similarly with 8036E27. Whereas 'Seri 82'^(R398) inoculated with 398D97^(AvSer) produced none to few pycnidia and a low level of fungal biomass. These differences were observed in several repeated trials. Fungal biomass evaluations, determined in enzyme-linked immunosorbent assay by Kema et al. (21), revealed significant differences between susceptible ('Shafir') and resistant (Kavkaz/K4500 L.6.A.4) cultivars inoculated with a differentiating *S. tritici* isolate. The differences were attributed to specific incompatible and compatible interactions. The current study pointed out growth retardation of virulent and avirulent isolates on 'Seri 82'^(R398) compared with 'Shafir'. It may indicate the existence of nonspecific plant-inhibiting mechanisms (structural, chemical, and so on) attributed to specific cultivar genotypes.

Suppression of pycnidial coverage on 'Seri 82'^(R398) inoculated with a mixture of avirulent and virulent *S. tritici* isolates was reported in previous studies (15,45). However, the use of GUS-expressing isolates has provided a means to distinguish in planta between the individual components of the isolate mixture. Inoculation of 'Seri 82'^(R398) and 'Shafir' with combinations of a GUS-tagged and wild-type isolates unrevealed the particular contribution of each pathotype in the isolate mixture, from 2 days after inoculation until pycnidia maturation on day 21. On 'Seri 82'^(R398), isolate mixtures of 398D97^(AvSer) (GUS expresser) + ISR8036 (wild type) or, vice versa, ISR398^(AvSer) (wild-type) + 8036E27 (GUS expresser) revealed a prolonged latent period and not statistically different lower values of fungal biomass of the virulent 8036E27 and the avirulent 398D97^(AvSer) isolates, although strong suppressions in pycnidial coverage were recorded. It seems that pycnidial induction in the *S. tritici* × wheat pathosystem is dictated by the mode of the interaction (compatible-incompatible) and is not linearly correlated with mycelial content within the wheat leaves. It may be indicative that the two developmental phases, fungal biomass and pycnidia production, are differentially regulated. The findings strongly support the hypothesis that isolate ISR398^(AvSer) and its derived transformants induce resistance in 'Seri 82'^(R398) (VEERY 5). It is proposed that isolate ISR398^(AvSer) expresses a certain avirulent gene product that is being recognized by a locus conferring resistance in VEERY lines (Negev [Hazera Seed Co.] and PFAU/VEE#9//URES [CIMMYT]; data not shown) leading to an induced resistance-like response.

The MUG hydrolyzing capacity detected in plants of the two cultivars inoculated with the wild-type isolates, ISR398^(AvSer) or ISR8036, mimics the pattern of ectopic GUS activity in the transformants × wheat cultivars interacting systems, though with statistical differ-

ences in rate and maximal production. In the *S. tritici*-wheat pathosystem, the mode of the interaction (incompatible-compatible) determines the onset of induction, rate, and level of enzyme production. Plant-derived β -1,4-glucuronidase was reported to inactivate a toxic compound composed of polymers of glucuronic acid produced by *Pyrenophora graminea* (14). The authors suggested that this toxin produced by *P. graminea* is a virulence rather than a pathogenicity factor. High endogenous GUS activity in *Cercospora beticola* was used to localize the fungus in infected *Beta vulgaris* tissue (25). The similarities between endogenous GUS activity and the pattern of fungal development in planta indicate that *S. tritici* most likely produces this enzyme in the course of colonization of wheat leaf tissue. The role of this enzyme in pathogenesis remains to be elucidated.

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