Transformation of *Bipolaris sorokiniana* with the GUS gene and Use for Studying Fungal Colonization of Barley Roots

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ABSTRACT

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Bipolaris sorokiniana, a fungal pathogen of cereals, was transformed with the GUS (β-glucuronidase) gene, using a plasmid (pGUS5) containing GUS A with the constitutive promotor GPD-1 and a hygromycin-resistance gene (hph) under the control of the same promotor. Hygromycin resistance was used as the selectable marker. Colonies growing on medium with hygromycin were obtained at a frequency of 6.5×10^{-7} per viable fungal protoplast. Nineteen out of 20 tested hygromycin-resistant colonies were also GUS positive. Transformants that showed a stable expression of GUS activity and hygromycin resistance after several conidiation cycles and several months of growth on nonselective medium could be selected. However, some transformants lost their GUS activity and hygromycin resistance after two conidiation cycles on nonselective medium. The stable transformants did not differ from the wild-type strain in growth rate or pathogenicity. A significant, positive correlation was found between GUS activity and ergosterol content and between GUS activity and lesion size in barley roots infected with a transformed strain. GUS activity could be detected in the root tissue before disease symptoms appeared, and only negligible background GUS activity was found in roots infected with the wild-type fungus. These results indicate that GUS-transformed strains of *B. sorokiniana* can be used for studying fungal colonization and growth in plant tissue.

Additional keywords: *Cochliobolus sativus*.

Bipolaris sorokiniana (Sacc. in Sorok.) Shoemaker, (teleomorph: *Cochliobolus sativus* (Ito & Kuribayashi) Drechs. ex Dastur) is a fungal pathogen of cereal crops. The fungus can infect leaves, seeds, and roots and causes serious plant diseases in several countries. Cultivars of barley and other cereals differ in resistance to the pathogen, but no complete resistance exists. Knowledge of the physiological aspects of infection of barley by this fungus is accumulating; for instance, the involvement of phytotoxin production in the infection process (16) and ultrastructural aspects of infection of root and above-ground plant parts (4,10) have been studied in our laboratory and elsewhere. The development of the disease varies widely with environmental conditions, and *B. sorokiniana* is known to be sensitive to competition from other microorganisms present in the rhizosphere (6). In general, more knowledge of the ecology of necrotrophic fungi like *B. sorokiniana*, particularly in the rhizosphere, is necessary to better manage disease problems. Therefore, there is a need for methods, besides rating of disease symptoms, to detect and quantify fungal growth in soil, rhizosphere, and plant tissue. One possibility is to use biological markers. Most fungi, but no other organisms, contain ergosterol, and this compound has been used to quantify fungal biomass in plant tissue (7,8,15). However, to monitor a particular fungus under natural conditions, a more specific marker is necessary.

During the last few years, methods to transform fungi have become available (17,18). Through transformation, marker genes can be introduced into the fungal genome and can later be monitored. A prerequisite is that characteristics such as competitive ability and pathogenicity are not changed by the transformation. The GUS (β-glucuronidase) gene from *Escherichia coli*, used as a reporter gene in plant molecular biology, has several advantages. There are very low background activities in most plant and fungal systems investigated, enhancing the sensitivity of detection. The enzyme is very stable and can easily be assayed by different methods (11). Several fungi have been GUS transformed (3,5,14,18) without loss of pathogenicity, but no data have been published on the use of GUS transformants in fungal population studies.

The objectives of this study were to transform *B. sorokiniana* with the GUS gene and investigate the stability of expression and potential for use in ecological and epidemiological studies. Transformation of *B. sorokiniana*, to our knowledge, has not been reported before.

MATERIALS AND METHODS

Fungus and transformation. An isolate of *B. sorokiniana* (teleomorph: *C. sativus*) was used in all experiments. The fungus was obtained from diseased barley and maintained on potato-dextrose agar (Difco Laboratories, Detroit, MI) at 4 C. To generate protoplasts, 100 ml of potato-dextrose broth was inoculated with 10^5 conidia of *B. sorokiniana*. The mycelium was harvested on nylon gauze after 2 days incubation on a shaker at 200 rpm at 28 C. Protoplasts were produced according to the method of Harling et al (9) with Novozym 234 (Novo Industri AS, Copenhagen) at 7.5 mg ml^{-1} and 1 M $MgSO_4$ as an osmotic stabilizer. About 10^7 protoplasts were consistently obtained from 25 mg (fresh weight) of mycelium, and 20–30% of the protoplasts could be regenerated and produced colonies on regeneration media. The plasmid used was pGUS5 containing the GUS gene (GUS A) with the constitutive promotor glyceraldehyde 3-phosphate dehydrogenase gene (GPD-1) of *C. heterostrophus* (21) and a hygromycin-resistance gene (hph) under the control of the same promotor. The GPD-1 gene was from O. C. Yoder, Department of Plant Pathology, Cornell University, Ithaca, NY. The promotor region was cloned in front of hph and GUS by Mönke and Schäfer (14).

About 10^6–10^7 pelleted protoplasts were suspended in 100 μl of STC (1.2 M sorbitol, 10 mM Tris, pH 7, 50 mM $CaCl_2$),

and 20 μg of plasmid DNA was added. The solution was mixed gently, and 2 volumes of PEG solution (60% PEG [polyethylene glycol] 4000, 50 mM $CaCl_2$, 10 mM Tris, pH 7) was added and gently mixed. Before plating on defined regeneration media (9), 1 ml of STC was added to obtain a larger volume, and the solution was spread on 10 plates of regeneration medium (15 ml per plate). The plates were incubated overnight at 28 C, and the next day an overlayer containing 10 ml of water agar with hygromycin B was made. The final hygromycin concentration over the total 25 ml was 40 μg ml^{-1}. Untransformed protoplasts were spread on five plates as controls. The procedure was repeated several times, and in total, 100 regeneration plates were used.

Colonies that grew on the regeneration medium with hygromycin B were transferred to new regeneration medium containing hygromycin B to obtain single-spore cultures of the transformants. Because the fungus failed to sporulate on regeneration medium, Czapek Dox medium was used instead with hygromycin B at 40 μg ml^{-1}. After incubation at 25 C overnight, single germinated conidia were picked out under a binocular microscope and placed on new Czapek Dox medium with 40 μg of hygromycin B per milliliter.

Selection among the transformants. Transformants were compared with the wild-type strain in radial growth rate, GUS activity, and ability to produce disease symptoms on barley roots. Petri dishes with Czapek Dox agar were inoculated with the different transformants. Their radial growth rates were monitored by measuring the diameter of the colony at different time intervals. Three replicate Petri dishes were used for each strain.

Sabouraud dextrose broth (200 μl) in Eppendorf tubes was inoculated with *B. sorokiniana* and incubated overnight at 25 C. The next day, 100 μl of 0.1% X-gluc solution (0.5% Triton X-100, 50 mM sodium-phosphate buffer, pH 7.5, 5% *N,N*-dimethylformamide) was added. GUS-positive isolates were stained blue. For quantitative determination of GUS activity, 5 ml of Czapek Dox liquid medium (Oxoid) was inoculated with 5,000 spores and incubated on a shaker (200 rpm) for 48 h. Three replicates were used for each transformant. The mycelium was harvested by centrifugation, and 3 ml of extraction buffer was added (11). GUS activity was evaluated fluorimetrically (11). The total protein content of the samples was determined with the method of Bradford (2).

Seeds of barley (cv. Tellus; W. Weibull AB, Landskrona, Sweden) were allowed to germinate on moist filter paper. When the roots were about 2 mm long, seeds were transferred to cellulose filter-paper sheets (400 × 220 mm). Another paper sheet with a plastic layer was placed on top, and the resulting sandwich was rolled together, with the lower end of the roll placed in distilled water (Fig. 1). *B. sorokiniana* inoculum was prepared by adding 10^4 spores to 10 ml of Czapek Dox (Oxoid) agar in a petri dish. After 2 days at 25 C, 5-mm agar disks were cut with a cork borer and used as inoculum. When the roots were about 15 cm long, the paper rolls were opened, and the fungal inocula were placed on the roots 5 cm below the seed. Ten plants were used for each fungal strain. The extent of the disease symptoms, indicated by a brown discoloration, was measured as millimeters of infected root 3, 6, and 9 days after inoculation. Infected root pieces from each transformant were sampled after 10 days and placed in GUS extraction buffer (11), sonicated with six short bursts (3 s each), and the GUS activity of the infected roots was determined fluorimetrically (11).

Southern blotting and hybridization. DNA was isolated using the method of Turgeon et al (20). Southern analysis was carried out both with uncut DNA and with DNA cut with *XbaI* (one restriction site in pGUS5) after fractionation by electrophoresis on 1% agarose gel. Southern blotting and hybridization were carried out with a nonradioactive method (12), and pGUS5 was used as a probe.

Test of mitotic stability. Transformed isolates were grown on Czapek Dox medium (without hygromycin), and single-spore cultures were made by spreading spores on new Czapek Dox medium, picking out a single germinated conidium, and placing it on new Czapek Dox medium. Two weeks of incubation at 25 C followed, and new conidia were collected from the new colony, and the procedure was repeated. After six conidiation cycles, a new Southern analysis was carried out.

Time study of GUS activity in pure culture. Five milliliters of Czapek Dox liquid medium (Oxoid) was inoculated with 5,000 spores of transformant T5. The tubes were incubated on a rotary shaker at 25 C. The mycelium from three replicate tubes was harvested after 3, 22, 50, 72.5, and 96 h by filtration on a Millipore membrane filter (0.2 μm; Millipore Corporation, Bedford, MA) and washed with distilled water. The filter with the mycelium was put into a tube, and 3 ml of GUS extraction buffer was added. GUS activity was determined as described above.

Correlation between GUS activity and ergosterol content of infected roots. Barley roots were inoculated with *B. sorokiniana* as described above. Both the wild-type and transformant T5 strains were used. Samples for determination of GUS activity and ergosterol content were taken from the roots 2, 3, and 4 days after inoculation. Each sample consisted of four roots taken from one plant. Two-centimeter root pieces, both above and below the inoculation disk (which was not included), were taken from six to 12 replicate plants for T5 and from four plants for the wild-type strain. At sampling, the size of the lesion was measured and expressed as the percentage of the sampled root length. The sample was divided into two parts: one for GUS activity and one for determination of ergosterol content. Ergosterol content was determined with a modification (I. Almgren, *personal com-*

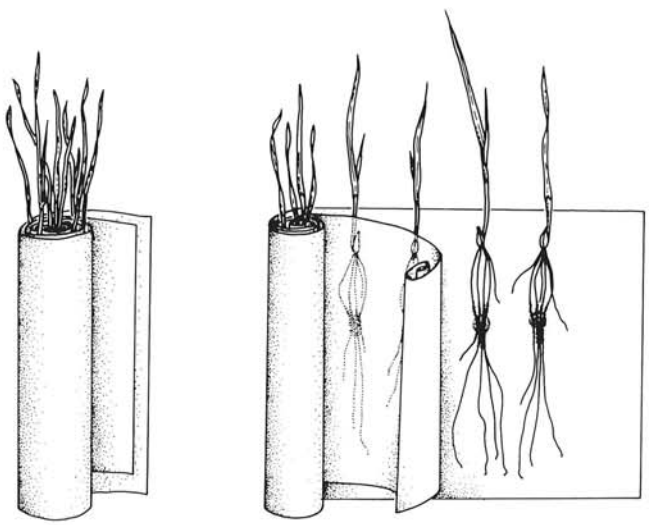

Fig. 1. Paper rolls used for inoculation of barley-seedling roots with *Bipolaris sorokiniana*.

TABLE 1. Characteristics of single-spore cultures of five GUS transformants of *Bipolaris sorokiniana* in comparison with the wild-type strain

Strain	Radial growth rate[w]	Lesion size[x]	GUS pure culture[y]	GUS roots[z]
Wild-type	0.59 ± 0.02	33 ± 1.9	<0.1	<0.001
T1	0.63 ± 0.04	35 ± 1.0	57 ± 4.4a	0.18
T2	0.29 ± 0.03***	34 ± 1.4	58 ± 4.6a	0.23
T3	0.57 ± 0.03	35 ± 0.9	57 ± 6.4a	0.19
T4	0.52 ± 0.05	40 ± 1.5*	41 ± 4.8a	0.17
T5	0.55 ± 0.04	31 ± 2.0	54 ± 5.2a	0.15
Mean coefficient of variation (%):	12	17	17	

[w] Millimeters per hour (mean ± SE, three replications. *** = significantly different from the wild-type at $P < 0.001$.

[x] Nine days after inoculation (in millimeters, mean ± SE, 10 plants). * = significantly different from the wild-type at $P < 0.05$.

[y] Nanomoles of 4-methylumbelliferone (MU) per minute per milligram of protein (mean ± SE, three replications). Values followed by the same letter do not differ significantly. Duncan's multiple range test.

[z] Nanomoles of MU per minute per centimeter of diseased root (pooled samples).

munication) of the method developed by Newell et al (15). Briefly, the samples were extracted (80 C) for 2 h in methanol. Hydrolysis of sterol esters was effected by adding a 4% solution of KOH in methanol at 80 C for 30 min. Two phases were formed by adding water and *n*-hexane. The organic phase was withdrawn and pooled with another two *n*-hexane–washings of the methanol/water phase. The solvent was evaporated under N_2, dissolved in acetonitrile, and injected into a Varian model 5000 HPLC (Walnut Creek, CA) equipped with a model UV100 detector and a 7.5-cm Novapak C18 column (3.9 mm diameter; Waters, Milford, MA). The mobile phase was acetonitrile/hexane/2-propanol (90:5:5, v/v/v).

Statistical analysis. Statistical analysis of the data was carried out with SAS Statistical Programs, release 6.06 (SAS Institute, Inc., Cary, NC). The significance of differences between the wild-type and different transformant strains was investigated with Student's *t* test. Correlation between different variables was tested with the Pearson correlation coefficient. Effects of time and fungal isolate on lesion size and ergosterol content were investigated with analysis of variance, using the GLM procedure. Multiple comparisons were made with Duncan's multiple range test.

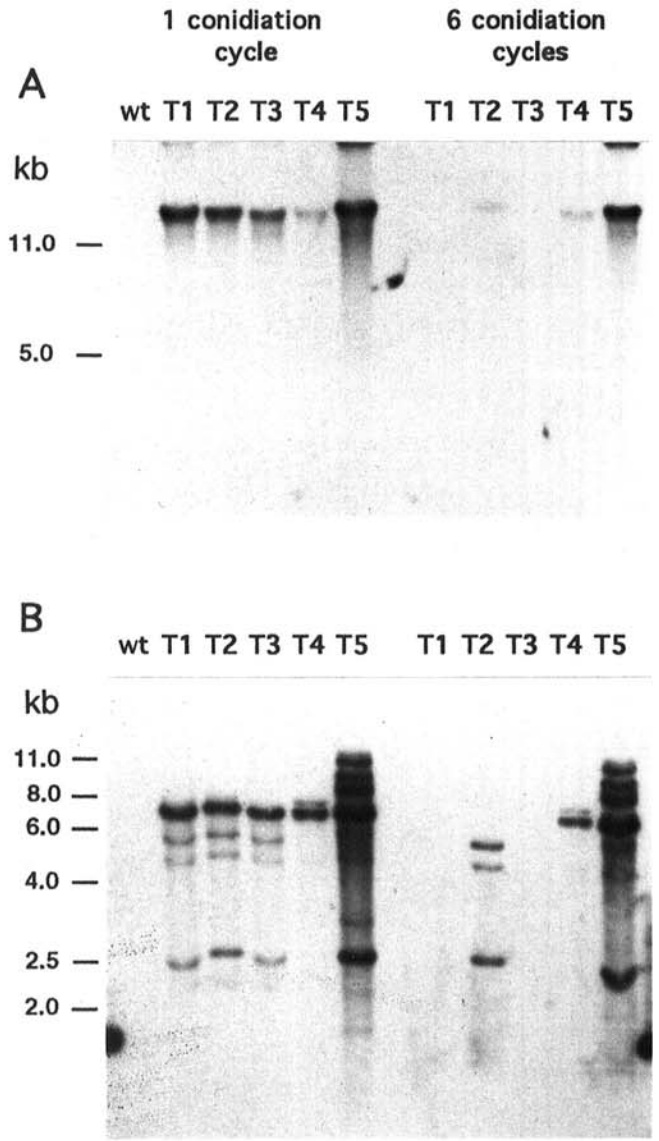

Fig. 2. Southern blot of DNA from the wild-type strain and single-spore cultures of five transformants of *Bipolaris sorokiniana* after one and six conidiation cycles. The whole plasmid, pGUS5, was used as a probe. **A,** Uncut DNA. **B,** DNA cut with *Xba*I (one restriction site in pGUS5).

RESULTS

Transformation. Colonies started to appear on the selective medium 3 days after transformation. The transformation frequency varied in the different trials between 7.8×10^{-5} and 1.3×10^{-4} per viable protoplast. However, more than 99% of those colonies stopped growing after a few days or failed to grow after transfer to new selective medium. In total, 30 colonies continued to grow after transfer to new medium, corresponding to a transformation frequency of 6.5×10^{-7} per viable protoplast. The phenotypes of these transformants were more or less similar to the wild-type strain. No colonies appeared on plates with the untransformed control because hygromycin B at 40 $\mu g\ ml^{-1}$ completely inhibited growth of the wild-type strain. Twenty transformants were selected for characterization of GUS activity, radial growth rate on Czapek Dox agar media, and pathogenicity tests. All except one were GUS positive, and 15 had a radial growth rate not significantly different from the wild-type strain. All except four of the transformants produced disease symptoms on barley roots similar to those produced by the wild-type, i.e., lesion size did not significantly differ from the wild-type strain 3, 6, or 9 days after inoculation. The other transformants produced smaller lesions than the wild-type. Diseased root segments from the pathogenicity test were sampled for quantification of GUS activity in the roots. The GUS activity, expressed as nanomoles of 4-methylumbelliferone (MU) per minute per centimeter of root, varied between 0.023 and 0.22 nmol among the transformants. No correlation was found between pathogenicity and GUS activity. Uninoculated roots and roots infected with the wild-type strain showed no or negligible GUS activity. Five transformants with the highest GUS activity were selected for further tests (Table 1).

Southern analysis. DNA was isolated from the five transformants and from the wild-type strain, and a Southern analysis was made. The filter was hybridized with pGUS5 as a probe. The probe hybridized to uncut DNA from all five transformants but not to DNA from the wild-type strain. Several bands appeared after cutting the fungal DNA with *Xba*I (one restriction site in pGUS5). T1, T2, T3, and T5 had more than three bands, indicating that integration took place at different sites in the fungal genome (Fig. 2). No further effort was made to determine copy number or number of integration sites.

Mitotic stability. To test the mitotic stability of the transformant strains, single-spore cultures were made in successive cycles. Afterward, the transformants were tested again for GUS activity and hygromycin resistance. Two of the five transformants lost their GUS activity and hygromycin resistance during this process. The other transformants continued to show GUS activity and hygromycin resistance (Table 2). A Southern analysis also was made after six conidiation cycles. Isolates that lost their GUS activity apparently also lost their plasmid DNA (Fig. 2)

Development of GUS activity in pure culture. The GUS activity of transformant T5 was determined at different time intervals after inoculation of liquid media. GUS activity increased exponentially ($r^2 = 0.93$) with time up to 72.5 h (Fig. 3).

Development of GUS activity in infected roots and correlation with ergosterol content and lesion size. After 1 day no clear disease

TABLE 2. GUS activity (in nanomoles of 4-methylumbelliferone per minute per milligram of protein of transformants of *Bipolaris sorokiniana*) after different numbers of conidiation cycles. Each value represents the mean of three replicates ± SE

Transformant	Conidiation cycles[z]		
	2	4	6
T1	<0.1	<0.1	<0.1
T2	133 ± 9a	92 ± 25a	24 ± 2b
T3	<0.1	<0.1	<0.1
T4	23 ± 4a	29 ± 12a	55 ± 24a
T5	785 ± 77a	601 ± 8a	631 ± 125a

[z] Values followed by the same letter did not differ significantly (only for horizontal comparisons) according to Duncan's multiple range test.

symptoms had developed. However, GUS activity already could be measured easily after 15 h. The GUS activity at that time was at least 20 times higher than the background measured with uninoculated roots or roots inoculated with the wild-type strain. The GUS activity measured in infected roots had a mean coefficient of variation of about 50%. The mean value increased with time in accordance with the lesion size (Table 3). There was a significant, positive correlation between ergosterol content of the roots and GUS activity in root extracts ($r = 0.58$, $n = 25$, $P = 0.002$; Fig. 4). There was also a significant correlation between GUS activity and lesion size ($r = 0.62$, $n = 25$, $P = 0.001$) and between ergosterol content and lesion size ($r = 0.74$, $n = 35$, $P = 0.0001$)

DISCUSSION

B. sorokiniana was successfully transformed, expressing both GUS activity and hygromycin resistance. The plasmid DNA was incorporated into the fungal genome as indicated by Southern analysis (Fig. 2). Some of the transformant strains were stable and had a more or less constant level of GUS expression after different numbers of conidiation cycles, but no or negligible GUS activity was found in the wild-type strain. However, many colonies that first appeared on the selective medium after transformation lost their hygromycin resistance and stopped growing. Probably, the plasmid DNA was never integrated into the genome of those transformants. Mönke and Schäfer (14) studied transient expression of the GUS gene in *C. heterostrophus* and found in Southern analysis that, initially, GUS-positive transformants had no vector DNA.

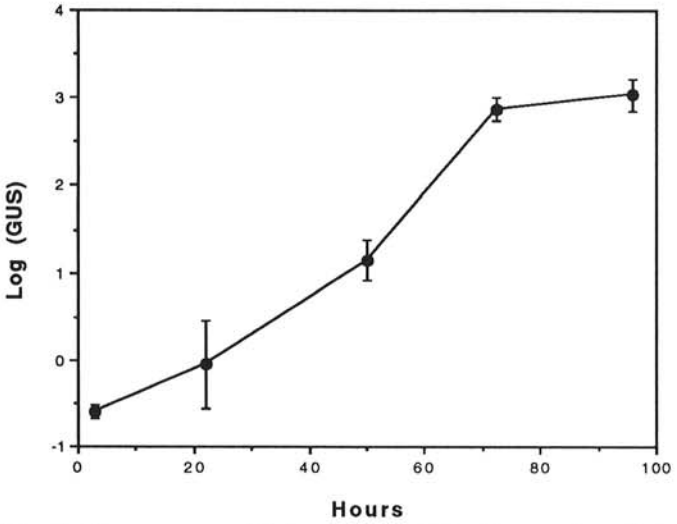

Fig. 3. Time course of total GUS activity (nanomoles of 4-methylumbelliferone per minute) in shake cultures of *Bipolaris sorokiniana* transformant T5. Vertical bars represent standard deviations. Five milliliters of Czapek Dox liquid medium was inoculated with 5,000 spores of transformant T5 and incubated on a shaker at 25 C. Three replicate tubes were harvested after different time intervals, and the total GUS activity in the sample was determined.

The level of expression varied among the different transformant strains between 24 and 631 nmol of MU per minute per milligram of protein after six conidiation cycles. Transformant T5 had the highest specific GUS activity and also gave the most intense signal in the Southern analysis. Bunkers (3) GUS-transformed *Pseudocercosporella herpotrichoides* and showed values about 10 times higher, but these values also varied between different transformants. Roberts et al (18) reported both higher and lower values depending on the fungal species transformed. In the present study, none of the transformants showed reduced pathogenicity, measured as lesion size on inoculated roots (Table 1), although in the second experiment (Table 3) somewhat smaller lesions were formed by the transformant T5 than by the wild-type strain. However, because the ergosterol content of the roots infected by the wild-type and transformant T5 strains did not differ significantly, it can be assumed that the fungal growth in the root tissue did not differ.

Ergosterol content frequently has been used as a measurement of total fungal biomass in plant tissue (7,8,15) because most fungi contain ergosterol. We found a positive correlation between GUS activity and ergosterol content in roots infected with transformed *B. sorokiniana* (Fig. 4). Our result indicates that the transformed strain can be used for quantification of the fungus in plant tissue. We found only negligible background GUS activity in plant tissue and in the wild-type strain. Most bacterial, fungal, and plant systems tested had only negligible background GUS activity (11,22). This makes it possible to study fungal population biology in complex systems, perhaps also in soil, even though the background activity may vary due to the presence of bacteria with intrinsic GUS activity.

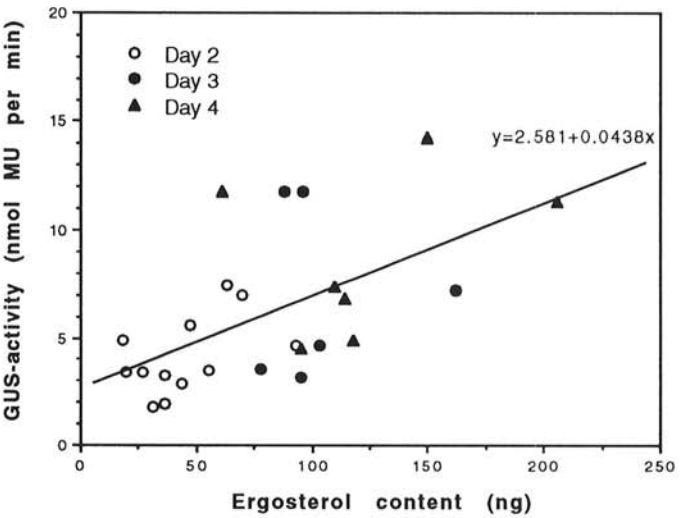

Fig. 4. Relationship between ergosterol content and GUS activity in barley roots inoculated with *Bipolaris sorokiniana* transformant T5. Roots of barley plants were sampled 2, 3, and 4 days after inoculation with transformant T5. Each sample was divided into two parts: one for GUS assay and the other for determination of ergosterol content.

TABLE 3. Lesion size, ergosterol content, and GUS activity of barley roots measured after different time intervals of infection with *Bipolaris sorokiniana* wild-type (wt) and transformant T5 strains (means ± SE)

	Lesion size (% root)		Ergosterol content (ng)		GUS activity (nmoles of MU min^{-1})[z]	
Day	wt	T5	wt	T5	wt	T5
2	20.6 ± 3.0	17.3 ± 1.0	61 ± 21	45 ± 6	<0.02	4.2 ± 0.5
3	30.6 ± 4.7	27.5 ± 2.8	101 ± 22	104 ± 12	<0.02	7.0 ± 1.6
4	55.6 ± 3.2	42.9 ± 1.9	161 ± 39	128 ± 17	<0.02	8.7 ± 1.4
ANOVA (*P* values)						
Day	0.0001		0.0001		0.012	
Strain	0.0039		0.2149		...	
Interaction	0.1042		0.5404		...	

[z] MU = 4-methylumbelliferone.

In *B. sorokiniana*, many aspects of growth and survival could be investigated with help of GUS-transformed strains. One example is the influence on growth of competition from other rhizosphere- and root-colonizing fungi and other organisms, which is difficult to investigate without a specific marker. Resistance to *B. sorokiniana* varies among cultivars, but no complete resistance exists (13). In particular, the plant resistance to fungal growth in root and shoot tissue would be interesting to study. GUS-transformed strains could be used to investigate the growth rate of *B. sorokiniana* fungus in plant tissue of varieties with different levels of resistance and perhaps be used in screening for disease resistance in plant breeding at certain levels, depending on the possibility of automating the sampling and analysis.

An important application of this technique would be to monitor growth and survival of biocontrol fungi in a manner more precise than the counts of colony-forming units that are most often reported. Alabouvette et al (1) reported successful GUS transformation of nonpathogenic *Fusarium* spp. used in biological control and indicated the possibilities of studying competition between pathogenic and nonpathogenic *Fusarium* spp. in the rhizosphere. Couteaudier et al (5) transformed *F. oxysporum* with the GUS gene and suggested that this method could be a useful tool for studying fungal growth in plants because they could easily detect GUS activity in infected flax roots. Other authors also have stressed the possibilities of using this technique for studying fungal populations (3,18). However, to our knowledge, no data on fungal population development with GUS-transformed fungal strains and correlations with other methods have been published before.

GUS activity could be detected very early in the infection process, before disease symptoms appeared. The lowest level of GUS activity measured in the samples of the experiment presented in Table 3 was about 150 times higher than the background activity, whereas the lowest ergosterol content (about 20 ng) approached the detection limit for ergosterol (about 5 ng) in plant tissue. This shows that GUS assay of transformed fungi is a very sensitive method for measuring the presence of fungi. This also was indicated in a pilot experiment in which conidial spores were extracted and assayed for GUS activity. Only about 200 spores were necessary for detection of GUS because they repeatedly gave higher values than the background, measured with spores from the wild-type strain (E. Liljeroth, H.-B. Jansson, and W. Schäfer, *unpublished data*). This makes it possible to study fungi in plant tissue quantitatively at a very early phase of colonization, which would otherwise be difficult.

Quantitative measurements of fungal biomass should always be interpreted with care because the content of the biomarker can vary under different conditions. Culture age influenced the ergosterol content in fungal mycelium in a study by Torres et al (19). The ergosterol content of the fungus increased with culture age. The expression of GUS could also, for example, vary with the age of the mycelium or with environmental conditions. In our study, there was an exponential increase in GUS activity with time in shaker cultures of transformant T5 (Fig. 3) in accordance with what could be expected on the basis of accumulated fungal biomass, and there was a significant correlation between GUS activity and ergosterol content. However, the ratio between GUS activity and ergosterol content tended to be higher in samples with lower amounts of ergosterol (Fig. 5). This may be explained by a lower ergosterol content in younger mycelia, as found by Torres et al (19) but could also be due to variations in the specific activity of GUS. To evaluate the relationship between GUS activity and fungal biomass more carefully, experiments need to be performed in which GUS activity, ergosterol content, and hyphal weights are compared at different stages of development of fungal hyphae under different environmental conditions.

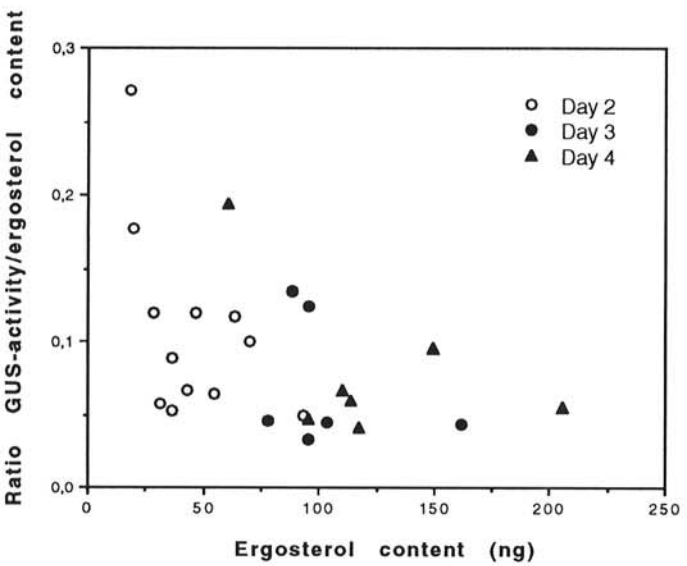

Fig. 5. Relationship between ergosterol content and the GUS activity/ergosterol ratio in barley roots inoculated with *Bipolaris sorokiniana* transformant T5.

Large experimental variation is often a problem when determining fungal biomass in soil or plant tissue. Even though the coefficient of variation was around 50%, the variation was not higher for GUS activity than for ergosterol content. The variation among replicates was much lower in pure-culture measurements (17%).

In conclusion, we believe that this technique offers interesting possibilities for studying fungal population biology, because it is specific and apparently very sensitive. Another important advantage is that the procedure of extraction and assaying GUS activity is very simple and straightforward. Extracted samples can be stored for months without loss of activity (11), and with a system for automatic sampling in the GUS assay, many samples could be run in a short time, which is essential for population studies. However, the method has to be carefully calibrated with other methods under different environmental conditions.

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