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Short communication

Identification of resistance to barley leaf stripe using a *Pyrenophora graminea* transformant expressing β-glucuronidase

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Abstract

A *Pyrenophora graminea* strain expressing the β-glucuronidase gene (GUS) was obtained via genetic transformation, and used to follow the penetration of the pathogen inside barley germinating seeds and the colonization of host tissues. Significant differences between resistant and susceptible barley cultivars were observed in the colonization of artificially-infected embryos by the fungus. These results suggest that the GUS transgenic strain of *P. graminea* will be useful for the early screening of barley cultivars for resistance to leaf stripe disease.

Abbreviations: MUG – 4-methylumbelliferyl-β-D-glucuronide; PEG – polyethylene glycol; X-Gluc – 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid.

Pyrenophora graminea [anamorph *Drechslera graminea* (Rabenh. ex Schlecht.) Shoemaker] is the agent of barley leaf stripe, a disease occurring in almost all barley growing areas. It is a seed-borne pathogen which infects the seedling through the coleorhiza (Skoropad and Arny, 1956) from where it grows intercellularly in the parenchyma layers of the scutellar node up to the coleoptile. Colonization proceeds into the leaves and the stems and the plant becomes systemically infected (Platenkamp, 1976). Symptoms can appear at the first-leaf stage, but more often they become evident at the 4–5 leaf stage and the plants are often unable to produce ears.

Skoropad and Arny (1956) have observed that, in resistant barley varieties, hyphae seem to degenerate in the basal part of the coleorhiza and in the scutellar node, as in a sort of hypersensitive reaction. Many questions regarding the growth and behaviour of the fungus during the early stages of infection remain unanswered because of the difficulty of monitoring the growth of the fungus inside the pericarp first and into the plantlet later.

A large variability exists both in *P. graminea* isolates and in barley genotypes, the latter ranging from highly susceptible to highly resistant (Knudsen, 1986; Delogu et al., 1989; Gatti et al., 1992). Quantitative or general resistance is partial and polygenic, and postulated to be effective against almost all strains of the pathogen. In this way, cultivars which possess general resistance should be less easily attacked by new virulent races of the pathogen which might arise. For this reason, current breeding strategies focus on general resistance rather than specific resistance. Regarding quantitative resistance to barley leaf stripe, a major gene was mapped on the chromosome 1 of a resistant barley cultivar, being responsible for 58.5% of the variation in disease resistance (Pecchioni et al., 1996). The use of resistant cultivars still remains the most suitable means of disease control.

Reliable methods for the evaluation of resistance of barley genotypes are, therefore, necessary. The development of a laboratory assay to screen for resistance to leaf stripe would be of great advantage. Here we describe a resistance test using a *P. graminea*

transformant expressing the β-glucuronidase gene (GUS).

With the development of transformation systems for several pathogenic fungi, the GUS gene has been used as a selective marker for transformants (Bailey et al., 1993; Roberts et al., 1989; Mönke and Schäfer, 1993; Bunkers, 1991; Richard et al., 1992). Transgenic isolates expressing β-glucuronidase gene have been used for studying fungal growth and hyphal biomass in infected plant tissue (Oliver et al., 1993; Liljeroth et al., 1993; Couteaudier et al., 1993) and in analysing plant–pathogen interactions (de la Peña and Murray, 1994; Kamoun et al., 1998).

Here we present the co-transformation of a virulent *P. graminea* isolate using the phleomycin-resistant plasmid pAN8-1 (Mattern et al., 1988) and the β-glucuronidase carrying vector pNOM102 (Roberts et al., 1989). Because of the low level of GUS activity in barley plants and the availability of a sensitive histochemical detection system, we used one of the GUS expressing transformants obtained, to study infection and to explore the possibility to discriminate between susceptible and resistant cultivars.

P. graminea does not possess endogenous β-glucuronidase activity so that we were able to select the transformants for β-glucuronidase production after the first selection based on phleomycin resistance (Aragona and Porta-Puglia, 1993). Stable co-transformants were recovered at a frequency of 80% by a rapid qualitative MUG assay on mycelium. Briefly, the mycelium of each colony was homogenized in eppendorfs with extraction buffer (50 mM $NaPO_4$ pH 7.0, 10 mM beta-mercaptoethanol, 10 mM Na_2EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100) (1 ml g^{-1} fresh weight). After centrifugation at 12,000 rpm for 10 min at 4 °C, 10 µl extract were mixed with 250 µl assay buffer (1 mM MUG in extraction buffer) and incubated at 37 °C (Jefferson, 1987). At time zero and at 10 min intervals for 40 min, 50 µl aliquots were taken and stopped by addition of 25 µl 1 M Na_2CO_3. Blue fluorescence could be observed for GUS producing isolates using a long-wave UV light lamp. Two GUS-expressing transformants which showed unaltered virulence, growth rate and morphology, as compared with the non-transformed strain, were selected. Southern analysis and hybridization using the GUS gene as probe showed that in both transformants integration of transforming vector (pNOM102) had occurred in one single locus in tandem copies (data not shown). GUS activity in the extracts of mycelium was estimated by fluorometry (Jefferson, 1987). The protein content of the samples was determined (Bradford, 1976) and 3 µg of protein were used for each sample. The amount of 4-methylumbelliferone (MU) produced was 9 and 13 nmol min^{-1} mg $protein^{-1}$ for the two GUS transformants, while in the non-transformed isolate GUS activity was <0.05 nmol MU min^{-1} mg $protein^{-1}$. The reported values are the average of three replicates per sample and the experiment was repeated three times with similar results.

To investigate the site of infection in barley germinating seeds, artificial inoculation was performed using the GUS^+ *P. graminea* transformant producing 13 nmol MU min^{-1} mg $protein^{-1}$ producing strain). With this aim, two barley cultivars, susceptible and resistant to leaf stripe respectively, were infected by the 'sandwich method'. Briefly, surface sterilized barley seeds, were placed in a Petri dish between two layers of PDA colonized by the GUS^+ isolate and incubated at 6–8 °C for 2 weeks. The germinated seeds were removed from the 'sandwich' and the plantlets excised from the seed-coat. The roots were cut away and the plantlets were cut in half along the longitudinal axis and stained overnight in an X-Gluc solution (1 mM X-Gluc, 0.1 M $NaPO_4$, 0.005 M K^+ ferrycyanide, 0.005 M K^+ ferrocyanide, 0.1% Triton X-100, 10 mM Na_2EDTA). After destaining in a graded ethanol series (25%, 50%, 70%, 95% and two or more times in 100%), the plantlets of the susceptible barley cultivar appeared uniformly deep-blue stained, while those of the resistant cultivar showed a weak-blue zone confined to their basal part, where the coleorhiza was disrupted and the fungus had penetrated the seedling (Figure 1A and B). Figure 1C shows a longitudinal section of an infected susceptible plantlet obtained by a kryotome (Leitz, 1720): the blue stained fungus has clearly spread from the coleorhiza up to the coleoptile. Cross sections of the stained embryos were obtained by a vibratome (Technical Products International, series 1000). In the layers of the apical meristem of shoot, the blue stained hyphae were numerous in the susceptible cultivar and they have colonized all the host tissue (Figure 1D), while in the resistant one very few hyphae were found in the host tissues (Figure 1E) after two weeks of incubation. These observations are in full agreement with the histological observations of Skoropad and Arny (1956) and Platenkamp (1976). The unstained half plantlet was incubated on sterile moist paper in a Petri dish, to verify that no fungus other than *P. graminea* was present.

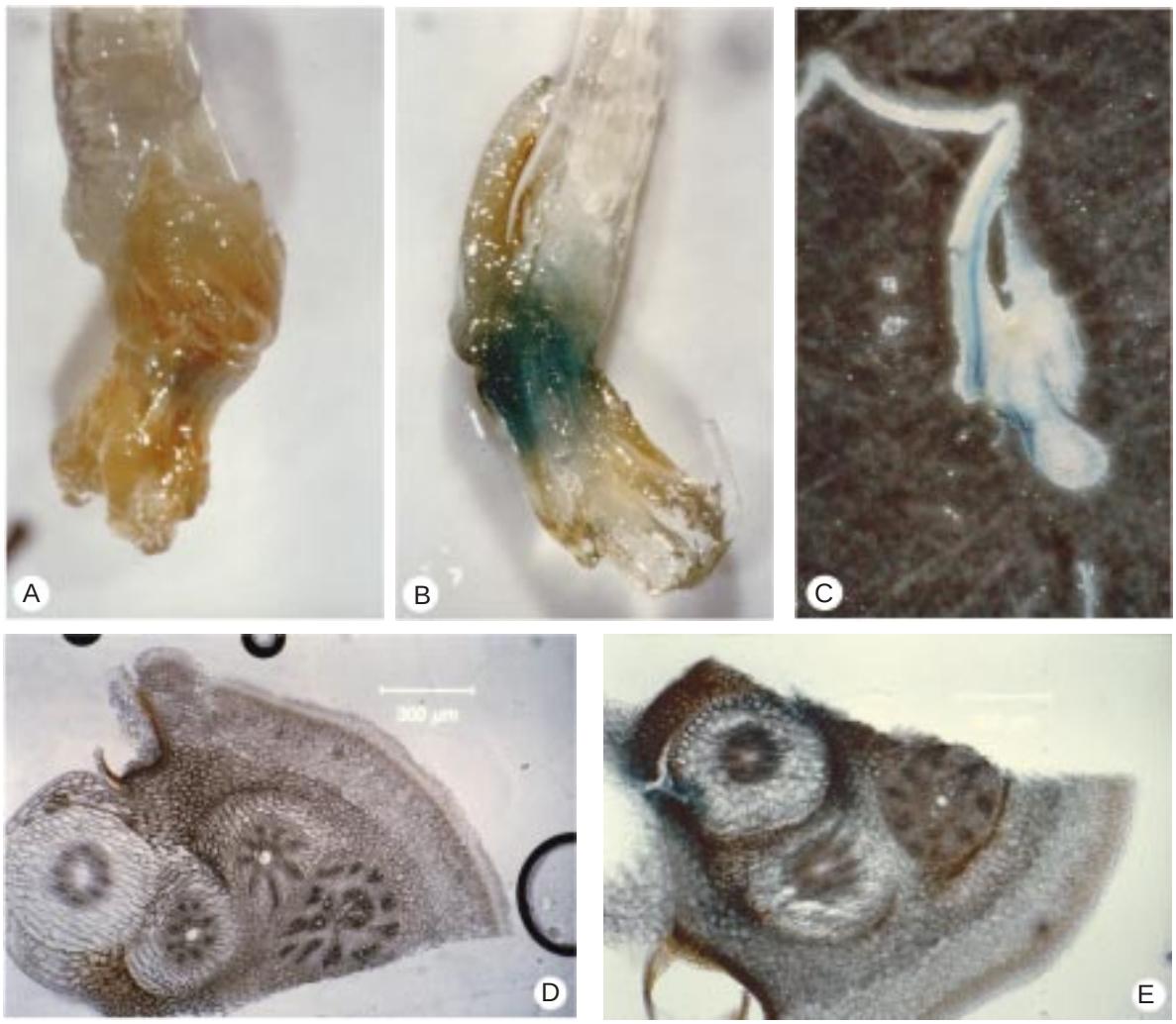

Figure 1. Histochemical localization of a GUS-expressing isolate of *Pyrenophora graminea* in artificially-infected plantlets, after staining with X-gluc for 16 h at 37 °C. The plantlet of a resistant barley cultivar (A) is compared with one from a susceptible variety (B). A longitudinal section of a susceptible embryo (C) cut by using a kryotome is also illustrated. Plant material was first incubated in PEG overnight at 50 °C to prepare the samples for the kryotome. Alternatively, after staining with X-gluc, plantlets were stored in 0.1 M sodium phosphate buffer at 4 °C and then embedded in 4% agar for cutting with the vibratome. Cross sections (100 µm thick) of a susceptible (D) and a resistant (E) barley embryo, viewed in the plane of the apical meristem of shoot, are illustrated.

GUS assays were also performed on the stems and the leaves of plants artificially-infected with the GUS *P. graminea* transformant. At the stage of second leaf, still with no symptoms, the leaves from one resistant and one susceptible cultivar were detached, cut into small pieces and vacuum infiltrated with the X-Gluc solution with the addition of 10% DMSO. After infiltration, the leaves were incubated in the same solution for 48 h at 37 °C. After destaining, the microscopic analysis showed the presence of the blue stained fungal hyphae in the stems and leaves of the susceptible cultivar but not in the resistant one, nor in the controls which consisted of the same cultivars infected by the wild type isolate (data not shown). The experiments were repeated at least three times and the results were always similar.

To summarize, a *P. graminea* strain, constitutively expressing the GUS gene without affecting

pathogenicity, was obtained and an effective and reliable method for evaluation of general resistance of barley to *P. graminea* has been developed. The ability to distinguish between compatible and incompatible barley–*P. graminea* interactions at the very early stages of infection represents an important tool in screening for resistance. Until now screening was performed by sowing the artificially infected germinating seeds under greenhouse conditions and waiting for a month to evaluate the infected plants. The use of the GUS assay described is time saving and quantitative, allowing resistance to be correlated with the pathogen growth. Moreover, staining for β-glucuronidase production being specific for *P. graminea*, the described technique allows rapid location of the fungus within the plant tissue eliminating any confusion with other fungi frequently present on barley seeds.

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