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Short communication

Quantification of late blight resistance of potato using transgenic *Phytophthora infestans* expressing ß-glucuronidase

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Abstract

A transgenic *Phytophthora infestans* strain that constitutively produces and secretes ß-glucuronidase (GUS) was used in detached leaf assays to quantify the levels of resistance to late blight in potato cultivars Surprise, Irene, Pimpernel, Alpha and Bintje. Four days after inoculation levels of GUS activity were determined in infected leaves. Significant differences between the various cultivars were observed. Discrimination between resistant and susceptible cultivars was possible based solely on levels of GUS activity. Regression analysis revealed a positive correlation between *in planta* GUS levels and field resistance expressed as Area under Disease Progress Curve (ADPC).

Phytophthora infestans (Mont.) de Bary causes late blight, a major disease of potato and tomato. Breeding for resistance of potato to late blight has been extensive in this century. Two types of resistance were identified (Umaerus and Umaerus, 1994; Wastie, 1991): (i) specific resistance which is generally monogenic (*R*-gene mediated) and is only effective against a subset of strains of the pathogen (races), and (ii) general or quantitative resistance which is partial and polygenic, and thought to be effective against all strains of the pathogen. Potato cultivars containing *R*-genes proved ineffective in the field as new virulent races of the pathogen quickly evolved. In contrast, cultivars that possess general resistance proved durable (Colon et al., 1995; Turkensteen, 1993). Based on these observations, current breeding strategies focus on general resistance.

Due to its quantitative nature, general resistance of potato to late blight cannot be as easily evaluated as specific resistance. Reliable phenotypic evaluation of general resistance is important for breeding programs, but is especially crucial for modern genetic analyses such as the detection and mapping of quantitative trait loci (QTLs) (Leonards-Schippers et al., 1994). The development of easily scored molecular markers linked to QTLs is expected to greatly facilitate breeding for quantitative traits such as general resistance. However, precise and quantitative methods for the estimation of resistance of particular genotypes are necessary. In addition to field trials, laboratory tests to screen for general resistance to late blight have been developed (Hodgson, 1961; Umaerus and Lihnell, 1976; Beckman et al., 1994; Harrison et al., 1990). The objective of this study was to analyse the suitability of an additional quantitative resistance test exploiting a transgenic *P.infestans* strain that expresses the reporter gene ß-glucuronidase (GUS).

Since the development of DNA transformation procedures for plant pathogenic organisms, transformants expressing reporter genes have been used to monitor disease progression *in planta* and consequently to evaluate disease resistance. One particularly remarkable example is the expression of the *Vibrio fischeri* luciferase (*lux*) operon in the plant pathogenic

bacterium *Xanthomonas campestris*, which allows for continuous and non-destructive monitoring of bacterial growth *in planta* (Dane and Shaw, 1993; Shaw and Kado, 1986). In plant pathogenic fungi, the GUS reporter gene has been used to visualize infecting hyphae, to measure fungal biomass, to identify resistant plant genotypes, and to study promoter activity (Ashby and Johnstone, 1993; Bunkers, 1991; De la Pena and Murray, 1994; Kohler et al., 1995; Oliver et al., 1989; Roberts et al., 1989; Van den Ackerveken et al., 1995; Wubben et al., 1994). In recent studies we used the GUS reporter gene for analysing promoter activity in the oomycetous plant pathogen *P. infestans* (Van West et al., 1998). Here we demonstrate the usefulness of GUS as a marker gene to measure fungal biomass of *P. infestans* and to estimate levels of general resistance of potato to late blight.

DNA transformation of *P. infestans* strain 88069 with plasmid pHAMT35G containing the GUS coding *uidA* gene driven by the *Bremia lactucae ham34* promoter (Judelson et al., 1991), and selection and characterization of strain EY6 have been described previously (Van West et al., 1998). The *ham34* promoter acts as a constitutive promoter in *P. infestans* and EY6 appeared to produce high levels of GUS, much of it being secreted. The mechanism through which EY6 secretes GUS is not known. In infection assays, EY6 showed unaltered virulence on potato and tomato and therefore was selected for this study. To determine the relationship between fungal biomass and GUS activity, EY6 was grown for three, four, five, six, seven, ten, thirteen and sixteen days in 10 ml liquid modified Plich medium (containing per liter 0.5 g KH_2PO_4, 0.25 g $MgSO_4.7H_2O$, 1 g asparagine, 1 mg thiamine, 0.5 g yeast extract, 10 mg ß-sitosterol and 25 g glucose) without shaking at 18 °C in the dark. Mycelia were collected by filtration and fresh weight was determined. Mycelia and culture filtrates (four replicas for each time point) were stored at −80 °C and GUS activity was determined by fluorometry as described (Van West et al., 1998). Up to thirteen days the fresh mycelial weight increased after which confluent growth was reached (Figure 1A). EY6 secretes GUS and we found that GUS activity in the mycelium did not change during growth (data not shown). In contrast, GUS activity in the filtrates of these cultures increased gradually, especially during the period of exponential growth between days six and thirteen (Figure 1A). However, when growth ceased after day thirteen, a significant drop in GUS activity was noted suggesting that increases in GUS activity occur only during the growth phase. Indeed, using simple regression analysis, positive correlation was found between the extracellular GUS levels and the fresh weight of EY6 mycelium collected up to day thirteen (Figure 1B; N= 28, r^2=0.82, p=0.0001). These results show that the GUS activity produced by

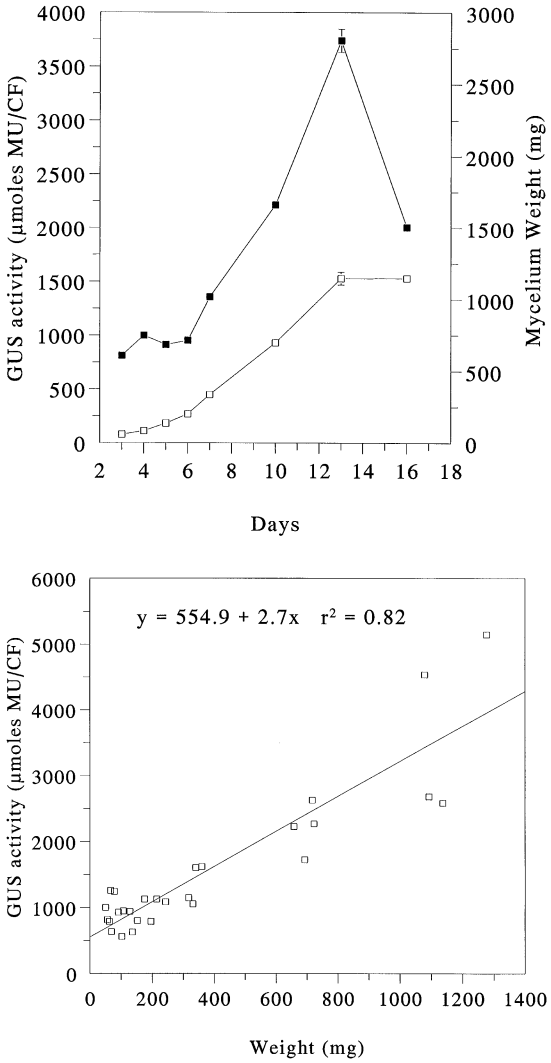

Figure 1. Relationship between fungal biomass and extracellular ß-glucuronidase (GUS) levels of *Phytophthora infestans* EY6. A). Time course of fungal growth *in vitro* expressed as fresh mycelium weight in mg (open squares, right scale) and total extracellular GUS activity expressed in μmoles of 4-methylumbelliferone (MU) per culture filtrate (closed squares, left scale). Bars represent standard errors of the mean (N=4). B). Correlation of extracellular GUS activity with fresh mycelium weight between days two and fourteen determined by regression analysis (p=0.0001, N=28). Regression analysis (Little and Hills, 1978) was performed using the software SlideWrite Plus (Advanced Graphics Software Inc.).

EY6 can be used to estimate fungal biomass during the growth phase of the fungus.

High levels of general resistance of potato to *P. infestans* are expected to correspond to slow increases in fungal biomass over time (Pieterse et al., 1993). To determine whether GUS activity of EY6 *in planta* reflects levels of general resistance, late blight assays with *P. infestans* EY6 were performed on certified Dutch potato cultivars with varying levels of resistance. Cultivars Bintje and Alpha are susceptible to late blight and exhibit no or very low levels of general resistance. In contrast, cultivars Pimpernel, Irene, and Surprise have significant levels of general resistance (Colon et al., 1995). All cultivars were grown in the greenhouse under standardized conditions. The third, fourth, or fifth leaf (counting from the top) of six to eight-week-old plants were collected and placed in Petri dishes containing 15 ml of 15% water agar. Each leaflet was inoculated on the lower side with one 10 μl droplet containing 10^3zoospores of EY6 (Van West et al., 1998). The detached leaves were incubated in a growth chamber (18 °C, 16 h photoperiod) and were checked daily for lesion development. Whole infected leaflets were collected at days two, three, and four after inoculation and stored at −80 °C. Frozen leaflets were ground in a mortar and the fine powder was transferred to 1.5-ml microfuge tubes. One ml of extraction buffer (0.5 M sodium phosphate pH 7, 0.1 M Na_2EDTA, 10% Triton X-100, 10% N-Lauroylsarcosine) was added to the powder and mixed by vortexing. The mixture was centrifuged for 2 min at 13,000 rpm and the supernatant was stored at −80 °C. GUS activity was measured by fluorometry as described (Van West et al., 1998). An increase in both lesion size and GUS activity was noted for all five cultivars during the first three days after inoculation (data not shown), confirming that the level of GUS activity correlates with EY6 biomass. At day four, the levels obtained for the susceptible cultivars Bintje and Alpha were significantly higher than those obtained for the resistant cultivars Pimpernel, Irene, and Surprise (Figure 2A, Student's t-test, $p<0.05$).

The relationship between GUS levels in infected leaves and field resistance of the tested cultivars was examined using regression analysis. Positive correlation was found between the GUS levels determined by fluorometry in extracts of infected leaves four days after inoculation and the ADPC (Area under Disease Progress Curve) values obtained for the same cultivars in field tests conducted between 1991 and 1993 at CPRO-DLO, Wageningen, NL (Colon et al., 1995)

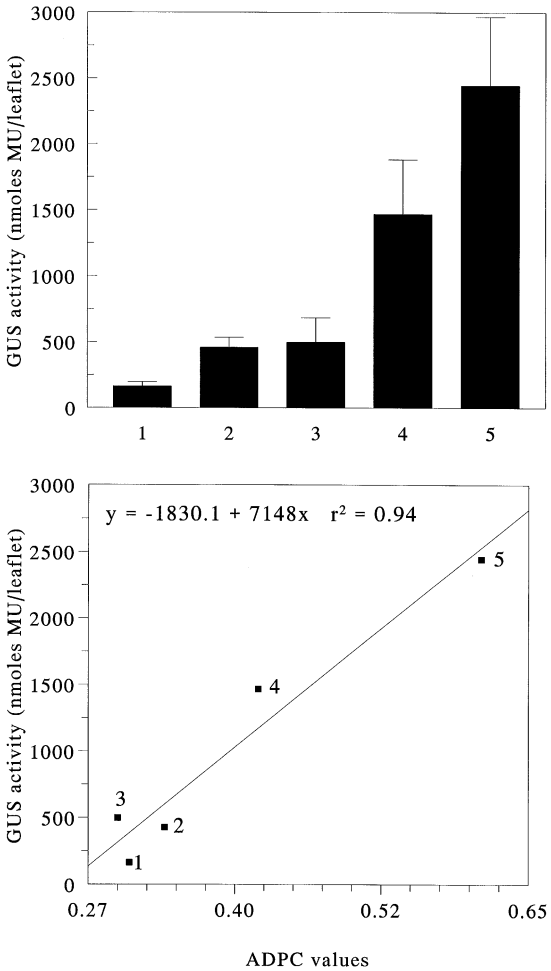

Figure 2. Relationship between ß-glucuronidase (GUS) levels and field resistance in potato cultivars Surprise [1], Irene [2], Pimpernel [3], Alpha [4], and Bintje [5]. A). Levels of ß-glucuronidase (GUS) activity *in planta* in leaflets infected by *Phytophthora infestans* EY6 four days after inoculation. Levels of GUS activity were determined by fluorometry and expressed in nmoles of 4-methylumbelliferone (MU) per leaflet. Bars indicate standard errors of the mean (N=4). B). Correlation of GUS activity levels in the various cultivars four days after inoculation with *Phytophthora infestans* EY6 with the Area Under Disease Progress Curve (ADPC) values determined by regression analysis (p=0.006, N=5; performed as described in Figure 1). The ADPC values were obtained from Colon et al. (1995).

(Figure 2B; N = 5, $r^2 = 0.94$, p = 0.006). These results indicate that the level of GUS activity measured in detached leaf assays can be used as a marker for field resistance levels.

So far, three methods for evaluation of general resistance of potato to *P. infestans* have been reported: (i) a leaf disc bioassay (Hodgson, 1961), (ii) measurement of lesion size and lesion growth over time (Umaerus and Lihnell, 1976), and (iii) ELISA-based

serological quantification of fungal biomass (Beckman et al., 1994; Harrison et al., 1990). In this study we present a fourth method, based on measurements of GUS activity from a transgenic *P. infestans* strain. Our results show that differences in levels of GUS activity reflect changes in *P. infestans* biomass, and that these GUS activity levels significantly correlate with levels of field resistance of five potato cultivars, Surprise, Irene, Pimpernel, Alpha and Bintje.

When comparing the feasibility of the four methods it is obvious that the leaf disc and lesion size bioassays are less time consuming than the ELISA and GUS assays. Moreover, these methods require no specific equipment such as a spectrophotometer (ELISA) or a fluorometer (GUS assay) nor a containment facility for recombinant organisms (GUS assay). Another drawback of the GUS assay is the necessity to transform the GUS construct to each *P. infestans* isolate one wants to use in the resistance tests. When determining the relative levels of general resistance in cultivars that do not contain major resistance genes the availability of one GUS expressing *P. infestans* strain is in principle sufficient. However, when using the GUS assay for measuring resistance levels in R-gene containing potato lines, GUS expressing strains representing different races of *P. infestans* have to be obtained. A disadvantage of the leaf disc bioassay is that it is poorly quantitative, as the infected area of the disc is estimated visually by comparing it to an evaluation chart. Sequential measurements of lesion size are more quantitative than leaf disc bioassays but less objective than the ELISA and GUS assays. In conclusion, the most covenient semi-quantitative approach to determine field resistance in detached leaves is to measure lesion growth over time. For more quantitative measurements and for confirmation of the results, lesion measurements can be complemented by either ELISA or GUS assays.

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