

Use of Fungal Transformants Expressing β -Glucuronidase Activity to Detect Infection and Measure Hyphal Biomass in Infected Plant Tissues

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Strains of the tomato pathogen *Cladosporium fulvum* and the Brassica pathogen *Leptosphaeria maculans* constitutively expressing β -glucuronidase were produced by cotransformation of a hygromycin-encoding vector pAN7-1 and a *GUS* encoding vector pNOM102. Their β -glucuronidase activity was used to detect histochemically the presence of fungal hyphae in host plant tissue. In addition, the β -glucuronidase activity of *C. fulvum* was used to quantify fungal biomass in the cotyledons of near-isogenic lines of tomato containing either no *Cf* resistance gene, or *Cf-3*, *Cf-5*, or *Cf-9* resistance genes. β -Glucuronidase activity was significantly reduced in incompatible interactions on *Cf3*, *Cf5*, and *Cf9* plants as compared to the compatible interaction on *Cf0*. Histochemical staining could also differentiate these interactions. These results demonstrate that the production of β -glucuronidase-expressing strains of fungi provides a facile means to detect infection and quantify biomass. Applications of this technique are discussed.

A key requirement in the study of fungal-plant interactions is the ability to detect the pathogen within plant tissue and to quantify fungal biomass. The latter is particularly important when attempting to assess a fungal isolate's pathogenicity, virulence, or aggressiveness and when determining the host/nonhost status of a plant species, the relative strengths of different resistance genes, the influence of genetic background on resistance, or the level of tolerance particular genotypes impart. There are numerous methods of detecting fungal hyphae within plant tissue and to quantify fungal biomass (e.g., Bruzzese and Hasan 1983; Plassard *et al.* 1982; Bonfante-Fasolo *et al.* 1990; Newall *et al.* 1987; Joosten *et al.* 1990). However, they all suffer from excessive time consumption, insensitivity, need for special equipment, and/or the destruction of the

sample. We describe here a method of fungal detection and biomass quantification that is rapid and sensitive and requires only standard laboratory equipment.

The method involves marking the fungal strains with chimeric genes expressing the β -glucuronidase of *Escherichia coli* (*GUS*) (Jefferson *et al.* 1986, 1987; Jefferson 1987). β -Glucuronidase has many advantages as a reporter gene. The enzyme is stable and its activity is easily quantified by fluorometric assays using the substrate 4-methylumbelliferyl β -D-glucuronide (MUG). It can also be detected histochemically using 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-Gluc) as the substrate. The enzyme is essentially absent from all fungi and plants studied.

Our strategy has been to construct strains of pathogenic fungi constitutively expressing β -glucuronidase. We have previously described the isolation and characterization of *GUS*-expressing strains of *Cladosporium fulvum* (Cooke) (*syn. Fulvia fulva* (Ciferri)), the tomato leaf mold organism (Roberts *et al.* 1989). Here we describe the production by cotransformation of *Leptosphaeria maculans* (Desm.) Ces de Not. strains expressing *GUS*. The transformed strains were used to demonstrate the feasibility of detection and quantification of fungal biomass using simple assay methods. As a very wide range of pathogenic and saprophytic fungi have proved to be easily transformed, at least at low frequency (Fincham 1989), this method should prove to have wide applicability.

Fungal transformation and subsequent stability.

Hygromycin-resistant colonies of *L. maculans* were obtained following cotransformation with plasmids pNOM102 and pAN7-1, at a frequency of 72 colonies per milligram of DNA, using methods based on Roberts *et al.* 1989, Punt *et al.* 1987, and Farman and Oliver 1989, 1992. Cotransformants were detected by first plating onto hygromycin selection plates ($50 \mu\text{g ml}^{-1}$) and then transferring the resistant colonies to Czapek Dox agar plates supplemented with $50 \mu\text{g ml}^{-1}$ X-Gluc. Blue halos were visible around some transferred colonies after only 15 min at 25° C. After 48 hr, 26 of the 72 transferred colonies (36%) were dark blue. The rest of the colonies exhibited small blue areas in the agar at the center of the colony. This may represent transient expression of the *GUS* gene in

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the absence of integration. The GUS activity of six transformants was measured in extracts of mycelium and was found to vary from 8 to 40 nmol/min/mg protein. Wild-type isolates of *L. maculans* exhibit GUS activity <0.1 nmol/min/mg protein. Southern hybridization analysis of DNA revealed a variety of plasmid copy numbers and hybridization patterns, but there was no obvious correlation between the hybridization pattern and GUS activity.

The GUS activity of the *L. maculans* transformants was stable; colonies still expressed GUS after at least six subcultures.

Plant infection studies.

The six cotransformants and the wild-type *L. maculans* isolate were individually inoculated onto the susceptible oilseed rape line CrGC5 (Table 1). Disease symptoms were

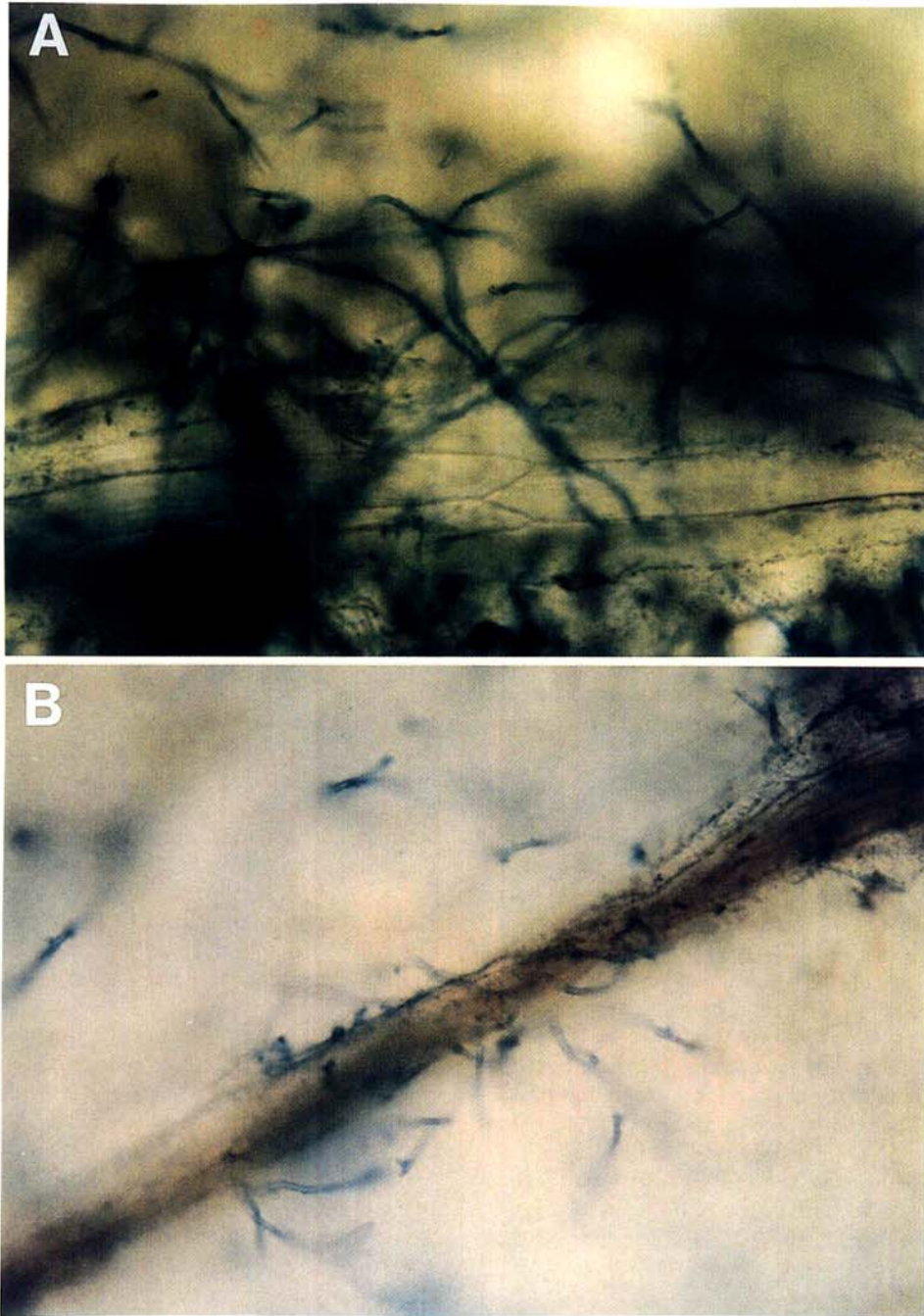


Fig. 1. Photomicrographs of hand sections of oilseed rape leaves cut 7 days after inoculation with a β -glucuronidase-expressing isolate of *Leptosphaeria maculans* and stained with X-Gluc. **A**, Within the necrotic lesion; **B**, within the asymptomatic area 5 mm outside the lesion border. *Brassica napus* plants were cultivated in a growth chamber maintained at 23° C during the day and 18° C at night and were infected as described by Hammond and Lewis (1987). Tissue sections were stained with 80 μ g ml⁻¹ X-Gluc in a 50 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA, and 0.05% (v/v) Triton X-100.

visible on all plants after 7 days, indicating that the transformation procedure had not greatly affected pathogenicity. Sectioning of the leaves and infiltration with X-Gluc revealed the presence of blue hyphae within the necrotic lesion (Fig. 1A) and in the asymptomatic areas outside the lesion (Fig. 1B). These observations confirm earlier results indicating that pioneer hyphae of *L. maculans* escape the necrotic lesion formed by leaf inoculations and colonize living tissue by a biotrophic mode of parasitism (Hammond and Lewis 1987).

To test the ability of the GUS system to distinguish compatible and incompatible reactions, similar experiments were performed using the previously described *GUS*-expressing *C. fulvum* strain 9 (Roberts *et al.* 1989) and near-isogenic tomato lines of the cultivar Moneymaker carrying resistance genes *Cf-3*, *Cf-5*, *Cf-9*, or no resistance gene (*Cf0*) (Table 1). Total GUS activity of infected cotyledons was determined over a time course 2–17 days postinfection (Fig. 2A). The *C. fulvum* strain used was derived from race 4 (i.e., it expresses avirulence genes 3, 5, and 9). Thus, the strain is fully compatible with *Cf0* and *Cf4* but incompatible with *Cf3*, *Cf5*, and *Cf9* plants. The GUS activity was significantly higher in the compatible interaction than in the three incompatible interactions. The activity with *Cf3* is intermediate between *Cf0* and *Cf5*, *Cf9*. The *Cf-3* resistance gene is described as a weak race-specific resistance gene (Lazarovits and Higgins 1976), whereas *Cf-5* and *Cf-9* genes each condition strong race-specific resistances (Higgins and deWit 1985). Statistical analysis indicated a clear differentiation of the compatible interaction on *Cf0* from the three incompatible interactions from day 8 onwards. Similar results were obtained when the GUS assays were performed *in planta* on excised cotyledon disks, where only minimal sample preparation was used (Fig. 2B). In these experiments, the tissue disks excised from infected plants were vacuum infiltrated in a 1-ml aliquot of MUG assay buffer and after incubation for 16 hr at 37°C, methylumbelliferone (MU) levels in the buffer surrounding the disk were quantified. The extracellular mode of parasitism of *C. fulvum* permitted this minimal preparation type of GUS activity quantification.

Inoculation of resistant lines is accompanied by the development of superficial hyphae but very little leaf penetration (deWit 1977). This was found in infections of *Cf5* and *Cf9* plants. Macroscopic and microscopic examination (Fig. 3) revealed blue-stained hyphae on the surface but only short lengths of distorted hyphae within the substomatal space and a few neighboring lower mesophyll cells. In contrast, microscopic examination of the compatible interaction on *Cf0* reveals extensive intercellular ramification by the blue-stained hyphae from day 8 onwards. With *Cf3* there was extensive intercellular mycelium but only sparse conidiophore formation. No differences were observed in the intensity of staining at the advancing hyphal front whether located on the cotyledon surface or throughout compatible and incompatible infection development *in planta*. However, in compatible interactions from 12 days onwards mycelium within the center of lesions tended to stain less well, presumably due to the partial transfer of their cytoplasmic contents to the

developing conidiophores and conidia.

The results of this investigation indicate that the use of *GUS*-expressing pathogenic fungi is a practical method of detecting and quantifying fungal biomass within host tissue. In the studies with *C. fulvum* and tomato, statis-

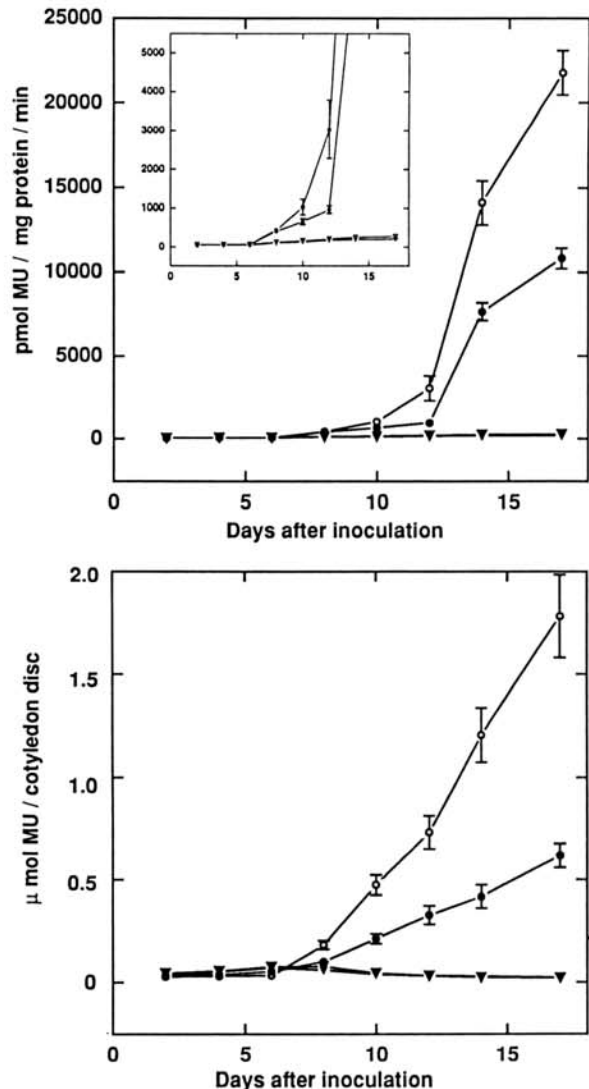


Fig. 2. Tomato cotyledons infected with a transformed race 4 of *Cladosporium fulvum* constitutively expressing the *GUS* gene. Time course of changes in GUS enzyme activity assayed *in vitro* (A), and *in planta* (B); ○, *Cf0*; ●, *Cf3*; ▼, *Cf5* and *Cf9*. The insert in A is an expansion of the y-axis and each bar represents twice the standard error of the sample mean. Twelve-day-old *Lycopersicon esculentum* plants grown in a chamber at 24°C during the 16-hr day and 18°C at night with light supplied at a photon flux density of 500 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ were inoculated with a 2×10^5 conidia per milliliter suspension (de Wit 1977). Inoculated plants were kept for 3 days in plastic propagators to maintain humidity near saturation, then the relative humidity was reduced to approximately 80%. Each experiment was done three times using a minimum of four plants per genotype per time point. Fluorometric GUS assays were performed essentially as described by Jefferson *et al.* (1986). Protein concentrations were estimated by dye-binding assays (Bradford 1976). *In planta* MUG assays were achieved by infiltrating an 8-mm-diameter cotyledon disk with MUG buffer in a 1-ml volume, incubating the floating disks for 16 hr at 37°C, and then determining methylumbelliferone (MU) levels in the buffer. One tissue disk was taken from the middle of each infected cotyledon.

tically significant results were gathered with a few hours hands-on experimental time. The *in planta* type of GUS assay is particularly labor saving and is probably applicable to all extracellularly colonizing pathogens. This approach has other useful applications. In plant breeding studies involving large numbers of plants, a technique to screen

plants for resistance both rapidly and quantitatively would be valuable. Often the host's genetic background influences either the effectiveness of resistance genes or the plant's ability to support fungal infection. Also many resistance genes appear to show incomplete dominance. This makes a visual plus/minus scoring of disease in segregating populations difficult. Similarly, the effect of fungicides or chemicals on pathogens could be assessed using *GUS* transformants. This could apply to both initial screens of chemi-

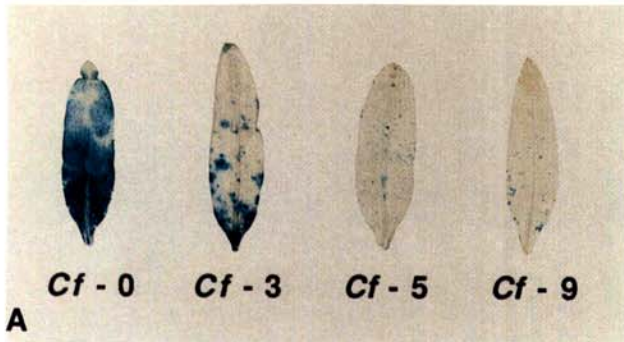


Fig. 3. Appearance of cotyledons of different tomato *Cf* genotypes 14 days after inoculation with a *GUS* expressing race of *C. fulvum*. Whole mounts were stained for 16 hr with 0.5 mg ml^{-1} X-Gluc (in the buffer described in Fig. 1 with 1 mM potassium ferricyanide/ferrocyanide added) and then cleared in 70% ethanol; **A**, macroscopic appearance, **B**, viewed in the plane of the lower mesophyll (bar = $20 \mu\text{M}$). Microscopic observations were made on a Zeiss Universal instrument under phase contrast and photomicrographs were prepared with Kodak Ektachrome 160 Tungsten film. The results presented are based on a minimum of 150 observations of penetration sites per interaction per time point.

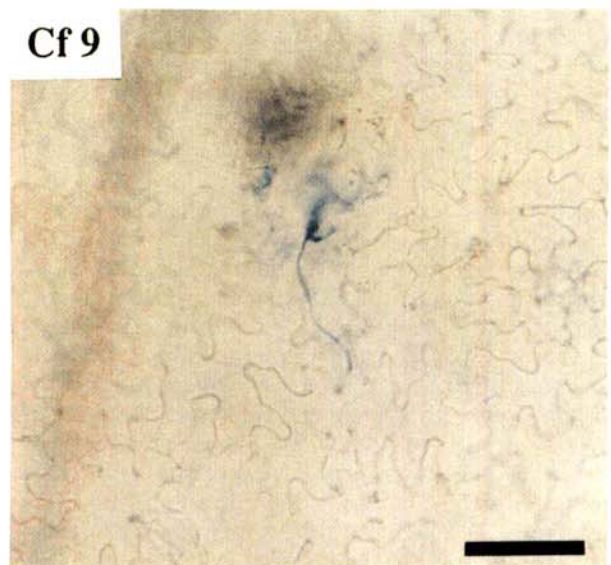
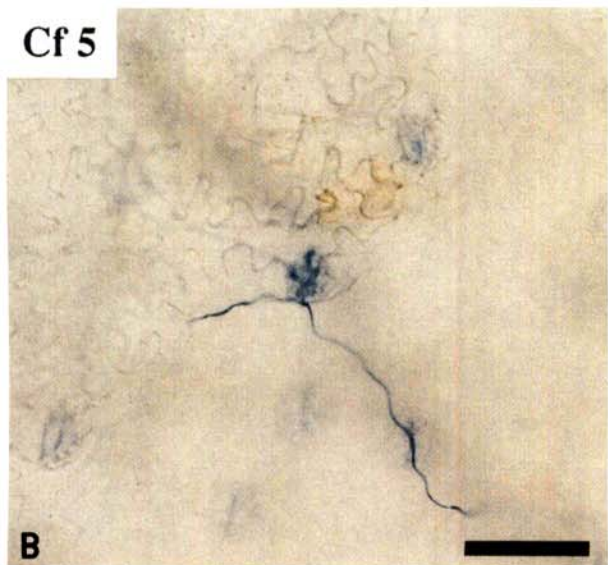
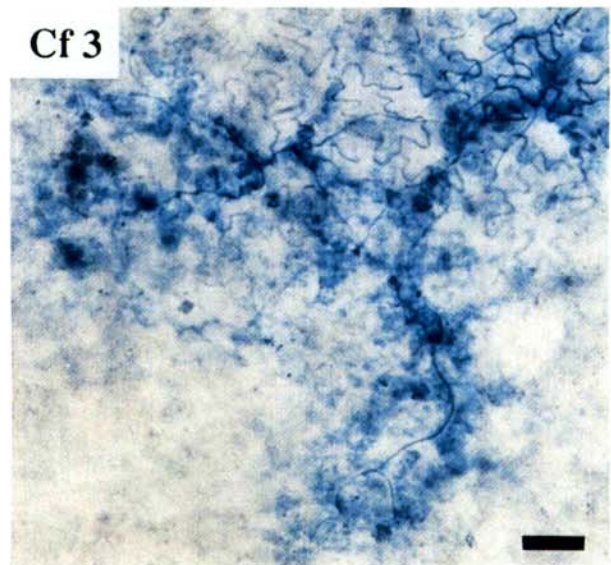
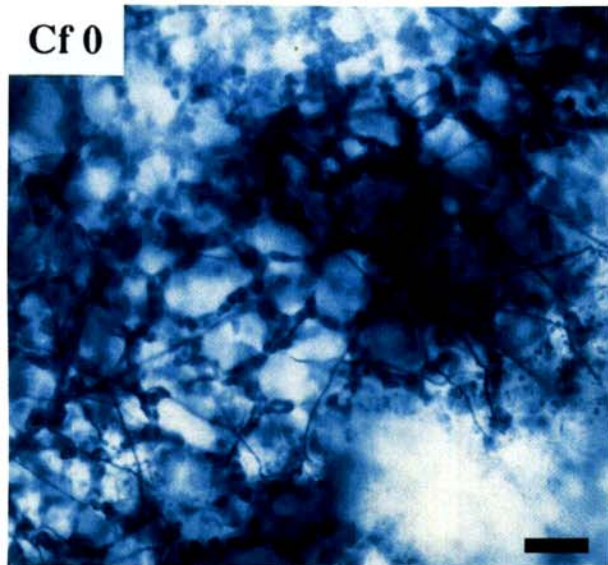


Table 1. Fungi and plants used in this study

Species	Genotype	Description	Reference/source
Fungi			
<i>Cladosporium fulvum</i>	Race 4::GUS ⁺ tr9	Avr 3,5,9 but avr 4	Roberts <i>et al.</i> 1989
<i>Leptosphaeria maculans</i>	Isolate 558A	Virulent on line CrGC5	Farman and Oliver 1988
Plants			
<i>Lycopersicon esculentum</i>	Near-isogenic lines of the cv. Moneymaker		W. Gerlach ^a
	Cf0	Susceptible to all <i>C. fulvum</i> races	
	Cf3	Susceptible to races lacking Avr 3	
	Cf5	Susceptible to races lacking Avr 5	
	Cf9	Susceptible to races lacking Avr 9	
<i>Brassica napus</i>	CrGC5	Rapid cycling line	P. Williams ^b

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^b P. Williams, Crucifer Genetics Cooperative, Madison, WI.

cal compounds and in the formulation of regimes for these chemical treatments. Such techniques would be applicable to foliar and root pathogens. *GUS*-expressing fungi could also be used in assessment of the risk of releasing genetically modified organisms.

Three major disadvantages present themselves. Firstly, it is necessary to have a functioning transformation system in order to obtain the *GUS*-expressing strains. This is not a serious problem for many nonobligate pathogens. However, obligate pathogens are recalcitrant to transformation at present. Secondly, it is important to use a vector construct that gives constitutive expression of the *GUS* gene throughout the phase of the life cycle of interest without affecting pathogenicity. This has been achieved with the pNOM102 construct. The third problem is the need to obtain authorization for the use of genetically engineered organisms. Some of the applications discussed above may not be easily compatible with strict containment conditions. However, the ability to distinguish compatible from an incompatible tomato-*C. fulvum* interaction before sporulation does minimize the restrictions required when using a transformed race as a screening tool.

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Standard genetic nomenclature should be used (Demerec *et al.* Genetics 54:61-76, 1966). Follow genetic terminology recommended by Rieger *et al.* in the most recent edition of the "Glossary of Genetics and Cytogenetics: Classical and Molecular" (Springer-Verlag, New

York). Follow usage of plasmid symbols proposed by Novick *et al.* (Bacteriol. Rev. 40:168-169, 1976).

Use the term "cultivar" for recognized agronomic and horticultural plant varieties and "lines" for other variants of a species. Identify the source of cultivars and include CI and PI numbers when appropriate. Enclose the name of a cultivar in single quotation marks only when it immediately follows the botanical name.

Names of unusual proprietary materials and special apparatus should be followed by the manufacturer's name and city in parentheses. List bacteriocides and fungicides by their approved common or generic names. Use the chemical name if a common name is not available.

Tables and Figures

Each table and figure, with its footnotes and legend, respectively, should be self-explanatory to a well-informed reader, without reference to the text but possibly with reference to other tables or figures. Tables and figures should be numbered serially with Arabic numbers, according to their order of citation in the text. If only a few values are to be presented, they should be in the text, rather than in a table or figure. Data in tables or figures should not be repeated in the text.

The title of a table should summarize the information in the table without repeating any subheading from the table. Subheadings should be brief. Abbreviations are acceptable, but each nonstandard abbreviation should be explained in a footnote of the first table (or caption of the first figure) in which it is used, even if previously defined in the text.

Each table should be typed with three lines extending the full width of the table, one just under the title and one each immediately above and immediately below the data entries. Footnotes are designated with superscript lowercase letters. Do not use ditto marks.

All graphs and line drawings should be submitted as original artwork or as crisp black-and-white reproductions (PMTs or photostats). Computer-generated, laser-printed materials will be accepted if they have smooth, reproducible lines.

The final sizes of figures in MPMI are 8.3 cm wide for one-column and 17.4 cm wide for two-column figures. The maximum height of figures is 23.5 cm, including captions and headings. Numbers and lettering (use upper- and lowercase) should be about 3.5 mm high (e.g., 10 point Universe 55 or other sans serif type). Panels in figures should be labeled A, B, C, etc. and should be 6 mm high (e.g., 18 point). All figures should be prepared to these measurements.

If line drawings or graphs are to be published as a composite figure, the parts of the composite should be mounted on cardboard in appropriate positions when the manuscript is submitted.

Complex formulas, metabolic and genetic schemes, and similar illustrative material should in almost all cases be presented as figures. Nucleotide and amino acid sequences are to be submitted as figures. The lettering for sequences should be no less than 3 mm high, with no more than 100 characters per line; a figure presenting a sequence should be no more than a single printed page in the journal. Sequences should also be submitted to GenBank; guidelines will be supplied.

For graphs, affix index (tick) marks to ordinates and abscissae. Photographs should be submitted as clear, high-quality prints. Photographs of poor quality will not be accepted. Composite prints and prints with extraneous labeling should be mounted on cardboard. Sizes of figures and labeling should be as shown above. Prints should be cropped at right angles to show only essential details. Insert a scale bar where necessary to indicate magnification. For composite figures, match photographs for similarity of contrast, background density, and subject content.

Figure captions should describe the contents of figures so that they are understandable when considered apart from the text.

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