Technical Advances

Use of Monoclonal Antibodies to Determine Biomass of *Cladosporium fulvum* in Infected Tomato Leaves

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A monoclonal antibody, OX-CH1, was raised against surface washings of *Cladosporium herbarum*. This antibody recognizes an epitope that is found in various fungi belonging to the genus *Cladosporium*, including *C. fulvum*, the causal agent of tomato leaf mold. The epitope is present at comparable levels in two different races of *C. fulvum* and in transgenic isolates derived from them. The epitope is heat- and protease-resistant but sensitive to oxidation with periodate and it is constitutively expressed in *C. fulvum* grown in pure culture and on the plant. *C. fulvum* can be detected in infected tissues at levels starting from around 1 mg fresh weight of fungus per g fresh weight of leaf tissue. Noninfected tomato leaves do not cross-react with OX-CH1. We have developed an enzyme-linked immunosorbent assay (ELISA) for fungal biomass in tomato leaves and compared it with the assay based on measurements of β -glucuronidase (GUS) activity in tissues infected with a transgenic isolate of *C. fulvum* race 4 carrying a *uidA* gene; the two assays give similar results.

Additional keywords: PTA-ELISA, quantitative.

There is a need in plant pathology to be able to quantify, at all stages of infection, the extent of vegetative growth of specific fungal pathogens within host tissues. However, few satisfactory methods of determining the biomass of fungi in vivo are available (Dewey et al. 1997) and almost no attempt has been made to compare and validate such methods.

Counting colonies on leaf surfaces or the measurement of the surface area producing spores have been used as indicators of the level of infection of phylloplane pathogens such as *Erysiphe* and *Botrytis* (Newton and Reglinski 1993; Elad et al. 1994; Köhl et al. 1995) but such methods do not give quantitative information about the early stages of infection or the extent of growth in vivo. Measurements of vegetative fungal growth in vivo have mostly relied on quantification of constituents, such as chitin and ergosterol (Ride and Drysdale 1972; Hicks and Newell 1983; Lumsden et al. 1990). Such methods are not very sensitive and are limited because specific fungal populations cannot be selectively measured (Lumsden et al. 1990). Furthermore, some markers have been shown to relate poorly to the biomass of the fungus in ques-

tion (Bermingham et al. 1995b). Alternative techniques include molecular methods based on the use of nucleic acid primers with internal control standards (Nicholson et al. 1996; Hu et al. 1993; Robb et al. 1994), measurement of β -glucuronidase (GUS) activity in GUS transformants (Oliver et al. 1993), and immunological methods.

Nucleic-acid-based methods of detection have the advantage of being extremely sensitive and specific but, while they have proved very useful in epidemiological studies on the spread of specific isolates, their use for quantitative studies of pathogens in plant tissues is often difficult; interference from nonfungal substances present in host plant extracts, such as phenols, is a common problem (Johanson 1994; Miller 1996). In contrast, measurement of the levels of GUS activity in plant tissues is straightforward but limited to studies with isolates that carry a *uidA* transgene. Immunoassays are relatively robust, easy to perform and to replicate, and have proved useful in the detection and quantification of fungi grown in axenic culture but their use in the detection and quantification of fungal pathogens in plants and mixed microbial populations has been limited by the difficulty of producing antisera (i.e., polyclonal antibodies, PABs) that are specific to the pathogen. Antisera raised to mycelial fragments or culture fluids frequently cross-react with related and unrelated fungi and with extracts from host tissues (Dewey and Thornton 1995; Miller 1996). In some cases this can be reduced by cross-adsorption against host and other fungal tissues but the resulting titer is often greatly reduced and the specificity is generally not reproducible (Mohan 1989). Nevertheless, successful enzyme-linked immunosorbent assays (ELISAs) employing antisera have been reported by Ricker et al. (1991) for *Botrytis cinerea* in grape juice, by Harrison et al. (1990) and Beckman et al. (1994) for *Phytophthora infestans* in potato leaves, and by Newton and Reglinski (1993) for *Erysiphe graminis* in barley leaves. Development of quantitative assays employing genus- or species-specific monoclonal antibodies (MAbs) holds considerably more promise. Kessel et al. (1997) used a near-genus-specific MAb to quantify, by digital image analysis, the extent of growth of *B. cinerea* in diseased lily leaves in the presence of the biocontrol agent *Ulocladium atrum*. Genus- or species-specific MAbs have also been employed in ELISAs to quantify *Humicola lanuginosa* in rice grains (Dewey et al. 1992), the eyespot pathogen *Pseudocercospora herpotrichoides* in winter cereals (Priestley and Dewey 1993), *Rhizoctonia solani* and *Trichoderma harzianum* in soils

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(Thornton et al. 1993; Thornton and Dewey 1996), aquatic hyphomycetes growing on decaying leaves in streams (Bermingham et al. 1995a), and *Verticillium dahliae* in potato stems (Plasencia et al. 1996).

In an unrelated study, in which we were raising MABs to *Cladosporium herbarum*, we obtained a number of genus-specific MABs, one of which, OX-CH1, has proved useful in the quantification of *C. fulvum* in tomato leaves. This paper discusses the suitability of OX-CH1, in plate trapped antigen (PTA)-ELISAs, to measure the biomass of *C. fulvum* in infected tomato leaves at different stages after infection, and validation of this method by comparison with biomass measurements with a *C. fulvum* strain expressing a *uidA* gene.

RESULTS

Production and screening of hybridoma cell lines.

Three fusions were carried out with splenocytes from mice immunized with either germinating or nongerminating spores of *C. herbarum*. Very few cell lines that secreted MABs that recognized the immunogen were obtained from these fusions and, of these, none were specific. However, three more fusions were carried out with splenocytes from mice immunized with surface washings of cultures of *C. herbarum*. From these fusions, nine cell lines that recognized the immunogen *C. herbarum* were obtained, and of these five recognized antigens from both *C. herbarum* and *C. fulvum*. The four cell lines with the highest absorbance values in PTA-ELISAs against surface antigens of *C. fulvum* race 4—OX-CH1, OX-CH8, OX-CH10,

and OX-CH11—were selected for further study. Supernatants from these cell lines were tested against surface antigens from a range of related and unrelated fungi and all were genus-specific. The results obtained with OX-CH1 are shown in Figure 1. Of the four cell lines selected for further study, supernatants from the cell line OX-CH1 gave the highest absorbance values and the greatest difference between extracts from infected and healthy leaves. This cell line was therefore chosen for the development of an immunological biomass assay.

Development of biomass assay.

Phosphate-buffered saline (PBS) was found to be the best buffer for extracting mycelial antigens from infected leaves and freeze-dried mycelium. An extract of 1/5 (wt/vol) from infected tomato leaves gave the highest difference and the least cross-reactivity between extracts from healthy and infected tomato leaves. However, with heavily infected leaves we found that it was necessary to use higher dilutions. The final assay system adopted was one in which a 1/5 (wt/vol) extract was prepared from frozen ground leaves and further diluted in PBS to give 1/10, 1/20, 1/40, and 1/80 extracts. Infected leaves were tested in triplicate at the same time as a set of mycelial antigen reference standards. For reference standards a cell-free PBS extract from a stock of freeze-dried mycelium of *C. fulvum* (1 mg ml⁻¹) diluted into PBS was used. This dilution series was found to give a very satisfactory standard calibration curve with absorbance values in each assay being proportional to the concentration of antigens in the extract over the range of 1 to 62.5 µg ml⁻¹ (Fig. 2). The biomass

Fig. 1. Plate trapped antigen–enzyme-linked immunosorbent assay of monoclonal antibody OX-CH1 against surface washings of selected fungi.

of mycelium in infected leaves was estimated by inverse prediction from the calibration curve. It is necessary to run a set of reference standards with each set of assays because the absolute values for absorbance vary from day to day (cf. Sutula 1996).

Determination of antibody immunoglobulin class and characterization of antigen.

All four chosen antibodies (OX-CH1, OX-CH8, OX-CH10, and OX-CH11) were immunotyped and identified as IgM MAbs. The antigens recognized by these MAbs were characterized by heat, protease, and periodate treatment and by chemical deglycosylation. The epitopes recognized by these MAbs were only slightly sensitive to boiling, autoclaving, freeze/thawing, and prolonged protease treatment. In PTA-ELISAs when the antigens were pre-treated with periodate the absorbance values for all four antibodies were reduced by around 60% and were almost completely eliminated by chemical deglycosylation with the Oxford GlycoSciences kit. The results indicate that OX-CH1, OX-CH8, OX-CH10, and OX-CH11 all recognize carbohydrate epitopes, on either a carbohydrate or glycoprotein molecule.

Effect of age and differences between isolates on antigen production.

In vitro cultures of *C. fulvum* race 4 of different ages had comparable quantities of the OX-CH1 antigen. The abundance of the OX-CH1 antigen in different races of *C. fulvum*, and transgenic strains derived from these, was compared. Equivalent signals were obtained in all four isolates tested (Fig. 2).

Fig. 2. Calibration curve with monoclonal antibody OX-CH1 tested by plate trapped antigen-enzyme-linked immunosorbent assay against four-fold dilutions (100 µl per well) of a phosphate-buffered saline extract of lyophilized mycelium of *Cladosporium fulvum* race 4 (dotted squares), race 4 transformed with the *uidA* gene (filled-in squares), race 4 strain 85 (filled-in diamonds), and race 5 (open diamonds).

Comparison of GUS-based and immuno-biomass assays (PTA-ELISAs) in quantification of mycelium in leaves at different stages post-infection.

The PTA-ELISA was directly compared with a GUS-based assay by measuring fungal biomass in tomato leaves infected with a *C. fulvum* isolate carrying a transgenic *uidA* gene. Both the PTA-ELISA and the GUS assay could detect the fungus at very low levels ($<1 \text{ mg g}^{-1}$) in infected leaves immediately after inoculation (Fig. 3). No GUS activity or OX-CH1 antigen could be detected in uninoculated plants (not shown). After a lag phase of about 7 days the fungal colonization of the leaf followed a period of exponential growth. The course of infection, as determined by the two methods, was equivalent except for the later stages of infection when the GUS activity increased between 14 and 18 days after inoculation, reaching 21 mg g^{-1} fresh weight, whereas fungal biomass measured by PTA-ELISA remained constant at approximately 15 mg g^{-1} . This experiment was performed 3 times and the data presented in Figure 3 are representative of the results obtained.

DISCUSSION

This study has shown that the progress of infection of *C. fulvum* in infected tomato leaves can be measured by determining fungal biomass with the genus-specific MAb OX-CH1 in a PTA-ELISA.

The genus-specific hybridoma cell line secreting the MAb, OX-CH1, used for the development of the biomass assay was produced from splenocytes of mice immunized with saline surface washings of *C. herbarum*. The fact that nine poten-

Fig. 3. Biomass of the *Cladosporium fulvum* race 4 carrying the *uidA* gene, in infected tomato leaves determined by plate trapped antigen-enzyme-linked immunosorbent assay (PTA-ELISA) and by measuring β -glucuronidase (GUS) activity. Tomato plants were inoculated and the leaves sampled immediately after inoculation (0) and at 4, 7, 11, 14, and 18 days thereafter. GUS assays, dotted squares. PTA-ELISAs, filled-in diamonds.

tially useful cell lines were raised with surface washings as the immunogen whereas none were raised from splenocytes of mice immunized with a suspension of spores or germinating spores confirms that surface washings of fungi are a good source of immunogens for producing specific MAbs (Bermingham et al. 1995a, 1995b; Dewey and Thornton 1995). The lack of immunogenicity of germinating spores of *C. herbarum* is surprising because this fungus is well known to be allergenic (Aukhurst and Borch 1984; Breitenbach et al. 1997). It is interesting that the four MAbs that were genus-specific all belonged to the immunoglobulin class IgM and recognized heat-stable, periodate-sensitive, carbohydrate epitopes. In previous studies we have also found that the genus-specific MAbs, almost without exception, belong to the immunoglobulin class IgM and recognize heat-stable, carbohydrate epitopes (Dewey and Thornton 1995; Dewey et al. 1997).

The use of MAbs to determine biomass of fungal material in infected plants has been reported before but in only a few cases has any attempt been made to validate these measurements by alternative methods (Dewey et al. 1992, 1997). A transgenic strain of *C. fulvum* expressing GUS activity has been used successfully to determine the biomass of this strain in infected leaf tissues (Oliver et al. 1993), to compare the activity of *C. fulvum* resistance genes in tomato, and to identify other genes required for full expression of *C. fulvum* gene resistance (Hammond-Kosack et al. 1994). We therefore chose to compare directly the biomass assay by PTA-ELISA with the GUS-based assay by measuring OX-CH1 activity in tomato leaves infected with a *C. fulvum* strain carrying a GUS reporter gene. We found that the two methods gave remarkably similar results with regard to both sensitivity and course of infection. The only difference is that whereas the GUS activity in infected leaves still increased in the very last stages of infection (14 to 18 days), in the PTA-ELISAs there appears to be a stabilization of the signal. This difference was found to occur reproducibly. It could reflect a difference in the nature of the two assays and it is difficult to tell which of the two parameters best reflects fungal biomass at this stage. One possibility is that at the later stages of the infection cycle a large proportion of the biomass is represented by spores and that OX-CH1 does not recognize spores. This, however, has not proved to be the case, as we have found that the antibody does recognize *C. fulvum* spores quantitatively (results not shown). Alternatively, because GUS activity is very stable in infected leaves, in spite of stasis in fungal growth, the total activity of the enzyme being produced could increase and could accumulate even in the absence of an increase in fungal mass. It is also possible that the fungal antigen recognized by OX-CH1 is subject to degradation by plant hydrolytic enzymes known to be produced during the later stages of infection (Joosten and de Wit 1989). Our data cannot distinguish between the last two possibilities. The GUS assay is sensitive and easy to carry out. It is, however, limited to those isolates that have been artificially transformed with the bacterial *uidA* gene. In this respect the PTA-ELISA is of greater general applicability as it can be employed for a variety of different isolates of *C. fulvum* and does not require the use of transgenic organisms; hence, the PTA-ELISA is the method of choice if such limitations are of importance.

It is necessary to run a set of reference standards with each set of PTA-ELISAs because of the inherent day-to-day variation in all ELISAs (Sutula 1996; Dewey et al. 1997). Reference standards can easily be prepared from a stock of freeze-dried mycelium. It is worth noting that we found that a dilution series of the mycelial antigens in PBS alone gave almost the same absorbance values as mycelial antigens plus extracts from healthy tomato leaves, and so for all comparative purposes a simple dilution series of the mycelial antigens in PBS is sufficient.

The amount of antigen produced by each isolate remained almost constant at all stages of growth of cultures in vitro. This suggests that the epitope recognized by OX-CH1 is continuously expressed and is a reliable indicator of fungal biomass, confirming the comparative data obtained in vivo with isolates expressing GUS activity.

In conclusion, we have raised MAbs and developed an ELISA-based method for the detection and quantification of *C. fulvum* in infected tomato leaves. The assay is robust and simple to use. We believe this assay will be of great help, for example, in the analysis of the effects of directed mutations on the pathogenicity of the fungus, in identifying new pathogenicity genes in *C. fulvum*, and for comparing quantitatively the course of infection in the interaction between different races of the pathogen in tomato plants expressing different resistance genes.

MATERIALS AND METHODS

Tomato plants and inoculation.

Tomato seeds (cv. Money Maker) were sown in peat-based compost (Astro, Goundrey & Son, Oxford, UK) and placed in the greenhouse. Three weeks after sowing, the seedlings were transplanted into individual pots (10 cm diameter) in peat-based compost. Natural light was supplemented with artificial illumination to produce a 16-h day and 8-h night. The tomato plants were inoculated 4 to 5 weeks after sowing. Prior to inoculation, the plants were decapitated, leaving 3 or 4 fully expanded leaves. Spore suspensions (approximately 5×10^6 spores ml⁻¹ in water) of *C. fulvum* were sprayed onto the underside of the fully expanded leaflets. The plants were then placed for 24 h in closed, humidified chambers in the greenhouse. The chambers were subsequently opened. Samples of six leaflets from different plants were taken and frozen at -80°C.

Fungal cultures.

MAbs were raised against a strain of *Cladosporium herbarum* (IMI 299105). Cultures of *C. fulvum* races 4 and 5 and the transgenic derivatives of wild-type race 4 strain 85 and *uidA* were obtained from the collection of P. Spanu. *C. fulvum* race 4 was used for all experiments unless otherwise stated. Cultures of *C. fulvum* were maintained on potato dextrose agar (Oxoid) and liquid cultures were grown in B5 medium (Gamborg et al. 1968) for 2 days. For details of isolates of other fungi used for screening hybridoma supernatants and methods of culture see Table 1.

Preparation of PBS extracts from freeze-dried mycelium.

Mycelium from liquid cultures was ground in liquid nitrogen, freeze-dried, and extracted with PBS (0.8% NaCl; 0.02% KCl; 0.115% Na₂HPO₄; 0.02% KH₂PO₄; pH 7.2) (1 mg ml⁻¹)

for 30 min. For the set of reference standards the cell-free supernatant from this extract was used to coat microtiter wells by fourfold dilutions into further PBS.

Preparation of immunogen and immunization regime.

Ten- to 14-day-old cultures of *C. herbarum* in petri dishes were washed with 5 ml of PBS. The surface washings were filtered through a 70-µm pore size sterile nylon cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ) to remove mycelial fragments, and then centrifuged at $12,000 \times g$ to remove spores. The supernatant that was used as the immunogen was aliquoted and stored at -20°C prior to use. Three 6-week-old BALB/c female mice were each given three intraperitoneal injections (300 µl per injection) of the immunogen, at 2-week intervals, to which Quil A adjuvant (Superfos, Vedbaek, Denmark) had been added to give a final concentration of 0.01%.

Table 1. List of fungi used for screening antibodies, source, and conditions of growth

Fungal cultures	Isolate	Source ^a	Medium ^b	Temp.
<i>Alternaria alternata</i>	317	IPO	OA	20°C
<i>Alternaria alternata</i>	319	IPO	OA	20°C
<i>Alternaria alternata</i>	1H	NKT	PDA	20°C
<i>Alternaria</i> sp.	3	SW	PDA	20°C
<i>Aspergillus flavus</i>	10-21	FMD	PDA	25°C
<i>Aspergillus flavus</i>	10-1	FMD	PDA	25°C
<i>Aspergillus niger</i>	D	FMD	PDA	25°C
<i>Aspergillus niger</i>	M5	FMD	PDA	25°C
<i>Botrytis cinerea</i>	P69	FMD	PDA	18°C
<i>Botrytis cinerea</i>	700	IPO	PDA	18°C
<i>Cladosporium cladosporioides</i>	549	IPO	OA	20°C
<i>Cladosporium cladosporioides</i>	564	IPO	OA	20°C
<i>Cladosporium cucumerinum</i>	55	SW	PDA	20°C
<i>Cladosporium fulvum</i>	race 4	PS	PDA	20°C
<i>Cladosporium fulvum</i>	race 4	PS	PDA	20°C
<i>Cladosporium fulvum</i>	strain 85			
<i>Cladosporium fulvum</i>	race 4	PS	PDA	20°C
<i>Cladosporium fulvum</i>	TuidA			
<i>Cladosporium fulvum</i>	race 5	PS	PDA	20°C
<i>Cladosporium herbarum</i>	299105	IMI	PDA	20°C
<i>Cladosporium herbarum</i>	587	IPO	OA	20°C
<i>Cladosporium herbarum</i>	593	IPO	OA	20°C
<i>Cladosporium macrocarpum</i>	557	IPO	OA	20°C
<i>Cladosporium macrocarpum</i>	565	IPO	OA	20°C
<i>Cladosporium sphaerospermum</i>	1N	NKT	PDA	20°C
<i>Epicoccum nigrum</i>	506	IPO	OA	20°C
<i>Epicoccum nigrum</i>	518	IPO	OA	20°C
<i>Penicillium chrysogenum</i>	20-24	SW	PDA	20°C
<i>Penicillium expansum</i>	300562	IMI	PDA	20°C
<i>Scopulariopsis brevicaulis</i>	S.S1	FMD	PDA	20°C
<i>Stemphylium</i> sp.	391	IPO	OA	20°C
<i>Stemphylium</i> sp.	432	IPO	OA	20°C
<i>Ulocladium atrum</i>	385	IPO	OA	20°C
<i>Ulocladium chartarum</i>	356	IPO	OA	20°C
<i>Ulocladium chartarum</i>	362	IPO	OA	20°C
<i>Ulocladium cucurbitae</i>	738	IPO	OA	20°C
<i>Ulocladium</i> sp.	739	IPO	OA	20°C

^a FMD: F. M. Dewey; SW: S. Watkinson; PS: P. Spanu; and NKT: N. Karpovich-Tate, Department of Plant Sciences, University of Oxford, England. IMI: International Mycological Institute, Egham, England; IPO: Instituut voor Plantenziekten, Onderzoek, Wageningen, the Netherlands.

^b OA: oat agar (20 g of milled oat, 15 g of agar, and 1 liter of tap water); PDA: potato dextrose agar (Oxoid).

Production and screening of hybridomas for antibody specificity.

Hybridoma cells were produced by the method described by Dewey et al. (1989), and the supernatants screened by PTA-ELISA against surface washings and germinated spores of *C. herbarum* immobilized in microtiter wells. Wells were arranged in strips and coated with 50 µl of either cell-free PBS surface washings from 10- to 14-day-old potato dextrose agar (PDA, Oxoid, CM149) or oat agar (OA, see Table 1 for details) plate cultures of fungal isolates diluted fourfold in PBS or spore suspensions in 2% glucose (5×10^4 spores ml⁻¹). Supernatants from positive clones were further tested by PTA-ELISA against surface washings and germinating spores of other fungi.

ELISA formats.

Screening of hybridoma supernatants was performed by PTA-ELISA with microtiter wells (Lab Systems, Helsinki, Finland, 9502037) coated with either fourfold dilutions of PBS surface washings from 10-day-old plate culture of *C. herbarum* IMI or germinated spores in 2% glucose (5×10^6 spores ml⁻¹). Wells with surface washings were incubated at 4°C overnight, whereas wells with spore suspension were incubated overnight at 24°C or 37°C (*Aspergillus* spp.) to allow germination of the spores. Coated wells were washed four times with PBS and air dried at 24°C in a laminar flow hood. The strips were either stored in sealed polythene bags at 4°C or used directly. Washed wells containing immobilized antigen were incubated successively with hybridoma supernatant, goat anti-mouse polyvalent immunoglobulin (classes IgG, IgA, and IgM) peroxidase conjugate (Sigma, Poole, Dorset, UK, A-0412), and the substrate tetramethyl benzidine with washings with PBST (PBS plus 0.05% Tween 20) between incubations as given in Bossi and Dewey (1992). Absorbance values were read on an ELISA automated microplate reader (MRX, Dynatech, Billingham, Sussex, UK). Working volumes for the hybridoma screening assays were 50 µl per well and all incubations were performed at room temperature.

The PTA-ELISAs for biomass were essentially the same as above except that all working volumes were 100 and 200 µl for the blocking step. Wells were coated in triplicate with PBS extracts from frozen tomato leaf powder (1/5, wt/vol) that had been centrifuged at $12,000 \times g$ for 15 min. After incubation overnight at 4°C wells were washed 4 times in PBS and then blocked with 0.3% (wt/vol) casein in PBS. Then the wells were incubated successively with the MAb for 1 h and with goat anti-mouse polyvalent peroxidase conjugate (Sigma, A-0412) for another hour. Bound antibody was visualized by incubating wells with tetramethyl benzidine substrate solution for 30 min. Reaction was stopped by adding 100 µl of 3 M H₂SO₄, and absorbance values were read on an ELISA automated microplate reader (Dynatech MRX). Between the incubations, wells were rinsed 4 times with PBST. Assays of test samples were always done in triplicate. With each set of assays a dilution series of mycelial antigen standards was always included. A standard curve of absorbance against mycelial antigen concentration was plotted and from this the antigen values of the test samples determined accordingly.

Determination of Ig subclass and cloning procedure.

The Ig subclass of MAbs from selected cell lines was determined with a commercial MAb isotyping kit according to manufacturer's instructions (Sigma, ISO-1). Hybridoma cell lines were cloned by limiting dilution, and selected cell lines were grown in bulk in a nonselective medium, preserved by slowly freezing in fetal bovine serum/dimethyl sulfoxide (92:8 [vol/vol]), and maintained in liquid nitrogen.

Antigen characterization.

Protease digestion of immobilized antigen was carried out by PTA-ELISA on wells coated with doubling dilutions of surface washings of *C. fulvum* race 4. After the coated wells were washed with PBS, they were incubated with pronase (Sigma, P-5147), 0.25 U per well, at 37°C for different periods of time ranging from 5 min to 24 h, then washed three times for 5 min with PBST, blocked with casein (0.3% [wt/vol], in PBS) for 30 min, and assayed by PTA-ELISA with OX-CH1 MAb. Control wells were treated with PBS.

The effect of heat treatment on antigenic material from the cell-free surface washings, diluted 1 in 4 in PBS, from 10-day-old PDA plate culture of *C. fulvum* race 4, was determined by boiling for 5, 30, and 60 min or autoclaving at 121°C for 20 min, cooling, centrifuging at 12,000 × *g* for 5 min, and then double diluting into PBS (50 µl per well). After immobilization overnight at 4°C, wells were washed, blocked with casein (0.3% [wt/vol], in PBS) for 30 min, and assayed by PTA-ELISA with OX-CH1. Nontreated surface washings served as a positive control.

The effect of periodate oxidation on immobilized antigens was determined by incubating wells coated with doubling dilutions of surface washings of *C. fulvum* race 4 (50 µl per well) with 50 µl of sodium metaperiodate (20 mM NaIO₄ in 50 mM sodium acetate buffer, pH 4.5) (Sigma, S-1878) for different periods of time ranging from 5 min to 24 h, in darkness at 4°C; control wells were incubated with buffer alone. Wells were then given three 5-min washes with PBST, blocked with casein (0.3% [wt/vol], in PBS) for 30 min, and then assayed by PTA-ELISA with OX-CH1.

Deglycosylation of antigens was carried out on cell-free surface washings of *C. fulvum* race 4 diluted fourfold with PBS and initially dialyzed overnight at 4°C against 0.1% (vol/vol) trifluoroacetic acid and then transferred to the bottom of a glass reaction vial and lyophilized. Deglycosylation was performed with the GlycoFree Deglycosylation Kit (Oxford GlycoSciences, Abingdon, Oxon, UK, K-500) according to the manufacturer's instruction. Deglycosylated surface washings were again dialyzed overnight at 4°C against ammonium bicarbonate buffer, plated into microtiter wells (50 µl per well) by the method of doubling dilutions as described earlier for immobilization overnight at 4°C, blocked with casein (0.3% [wt/vol], in PBS) for 30 min, and processed for PTA-ELISA. Nontreated surface washings and surface washings dialyzed overnight at 4°C against 0.1% (vol/vol) trifluoroacetic acid were used as controls.

GUS assays.

GUS activity in the leaves infected with an isolate of *C. fulvum* race 4 transformed with *uidA* gene was determined with the fluorometric assay with methylumbelliferone as a sub-

strate (Jefferson 1987). Standards were measured with extracts from the GUS-expressing *C. fulvum* grown in vitro.

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