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Expression of a Plant Protein by *Neurospora crassa*

SHELLY J. RASMUSSEN-WILSON,* JEFF S. PALAS, VERONICA J. WOLF, CATHY S. TAFT, AND CLAUDE P. SELITRENNIKOFF

MycoTox, Inc., Denver, Colorado 80262†

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Heterologous expression of plant genes may serve as an important alternative for producing plant proteins. We have investigated the ability of the fungus *Neurospora crassa* to secrete zeamatin, a protein produced by *Zea mays*. Zeamatin was induced after being fused to glucoamylase, an extracellular hydrolase produced by *N. crassa*. Glucoamylase induction and other culture parameters were monitored in untransformed *N. crassa* grown in shaken liquid culture. A DNA plasmid, pGEZ, was constructed by inserting zeamatin-encoding cDNA into an expression cassette containing the promoter, a truncated open reading frame, and the terminator sequence of the *N. crassa* glucoamylase gene. Zeamatin-encoding cDNA was modified at the N terminus to include a *kex-2* protease site, allowing cleavage of the chimeric product in the secretory pathway. Strains containing the chimeric gene construct were grown in liquid culture and induced for glucoamylase and zeamatin production. Zeamatin antibody detected a protein in a Western blot of concentrated culture supernatants that comigrated with authentic zeamatin. Secreted zeamatin was active in inhibiting the growth of *Candida albicans* in an agar diffusion assay, indicating that zeamatin had been correctly synthesized, processed, and secreted by *N. crassa*.

Plants represent a largely untapped reservoir of useful agents, but unfortunately many plants are noncrop species. Traditional methods of plant extraction may not be feasible for plants that cannot be grown in abundance or are rare or endangered. Such problems in acquiring plant proteins in large quantities make heterologous expression of plant genes by fungi attractive.

Glucoamylase (GLA; exo-1,4-α-D-glucan glucohydrolase; EC 3.2.1.3), an enzyme which cleaves glucose units from maltose and amylose, is secreted by numerous fungi, including *Aspergillus* spp., *Penicillium oxalicum*, *Saccharomyces* spp., *Rhizopus oryzae*, and *Neurospora crassa* (11, 12, 26, 31, 32). Production of GLA has been especially well characterized in *Aspergillus* species, and GLA is used commercially for the production of food and beverage syrups (7). More recently, the GLA gene has been cloned and its promoter has been used to drive heterologous gene expression by *Aspergillus* species (1, 2, 26, 29, 30).

The filamentous fungus *N. crassa* has potential as a host for heterologous protein production for several important reasons. *N. crassa* has extremely well-characterized transmission genetics (19) and is easily cultivated in simple media. Additionally, the *N. crassa* GLA gene (*gla-1*), its promoter (*gla-1p*), and its terminator sequences are known (25).

The model plant protein used in this study was zeamatin, an antifungal protein produced by *Zea mays* (20, 27). Zeamatin has multiple disulfide bridges that are required for activity, necessitating the use of a eukaryotic system for expression to avoid the possibility of improper folding. *N. crassa* has 17 of the 20 preferred codons used by maize (5, 18), while another possible fungal host, *Saccharomyces cerevisiae*, has only 3 of the 20 and *Pichia pastoris* and *Schizosaccharomyces pombe* have only 2 of the 20 (9). Zeamatin has been purified to apparent homogeneity, and the gene has been cloned and sequenced (20, 21, 27). Zeamatin is reversibly inactivated in the presence of >100 mM salts (27) and thus in Vogel's medium N (3), which is routinely used to culture *N. crassa*.

A modified zeamatin-encoding cDNA was introduced into *N. crassa* to test the feasibility of this fungus expressing and secreting a plant protein in its active form. A chimeric DNA construct encoding zeamatin and GLA was used to transform *N. crassa*. We determined conditions for GLA production in submerged liquid shaken culture to induce expression and secretion of zeamatin by transformants. This report also describes the characterization of one of these strains, which was tested for zeamatin secretion by antizeamatin antibody recognition and inhibition of *Candida albicans* growth in an agar diffusion assay.

MATERIALS AND METHODS

Fungal strain and culture conditions. Wild-type *N. crassa* (74-OR8-1a), obtained from the Fungal Genetics Stock Center (Kansas City, Kans.), was grown on 5- and 50-ml 1.5% (wt/vol) agar-solidified cultures containing Vogel's medium N (3) and 1.5% (wt/vol) sucrose. Cultures were grown for 5 to 7 days at 30°C and stored at −20°C. Macroconidial suspensions were prepared by flooding 50-ml agar cultures with sterile water and filtering the resulting suspensions through sterile glass wool.

Cultures of *N. crassa* were grown in 250-ml baffled flasks containing 50 ml of Vogel's medium N supplemented with 0.6, 1.0, or 1.4% (wt/vol) glucose and 0.1% (vol/vol) Tween 20. Cultures were inoculated to 10^6 cells/ml (final concentration) from frozen macroconidial stocks. The initial pH of the culture medium was 5.5. Cultures were incubated for 6 days at 25°C on a rotary shaker at 100 rpm. Duplicate cultures were sampled every 12 h; 2 to 4 ml of culture fluid was cleared of particulates by using 0.45-μm-pore-size syringe filters (MSI) and stored at −20°C for subsequent analyses. Transformed *N. crassa* strains were grown similarly in 100-ml shaken cultures without Tween 20. Hyphae were harvested on day 4, and culture supernatants were collected by filtration through Whatman no. 1 filter paper and stored at −20°C. Culture supernatants were concentrated in pressure cells equipped with 10-kDa-cutoff filters (Amicon). Concentrated supernatants were filter sterilized with 0.45-μm-pore-size syringe filters prior to analysis.

GLA purification. GLA was partially purified, as described previously (24), from the pooled supernatants of *N. crassa* cultures grown in Vogel's medium N supplemented with 1% (wt/vol) glucose. Briefly, after 6 days of growth, 50-ml liquid cultures were harvested through eight layers of cheesecloth and solid ammonium sulfate (360 g/liter) was added to pooled supernatants (300 ml). Precipitates were collected by centrifugation at 7,000 × *g* for 10 min, dissolved in 50 mM sodium acetate (pH 5.0), and dialyzed overnight against the same buffer. The protein concentration of the partially purified preparation (0.18 mg/ml) was determined by the Bradford method (Bio-Rad) with bovine serum albumin (BSA) fraction V as a standard. Partially purified GLA served as a standard for GLA detection and quantitation as described below.

* Corresponding author. Mailing address: MycoTox, Inc., 4200 E. Ninth Ave., Box B-111, Denver, CO 80262. Phone: (303) 315-5237. Fax: (303) 315-4024. E-mail: swilso@mycotox.abti.com.

† Subsidiary of Alpha-Beta Technology, Worcester, MA 01605.

GLA detection assay using immunoblots. Proteins from a sample of partially purified GLA preparation were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (14) on a 7.5% (wt/vol) acrylamide gel. Gels were silver stained, a kit from Boehringer Mannheim, or blotted onto nitrocellulose, using a semidry transfer cell (Bio-Rad), for Western blot analysis.

Using Tris-buffered saline with 0.1% (vol/vol) Tween 20 (TBS-T; 8 g of NaCl, 200 mg of KCl, and 3 g of Tris base per liter; pH 7.6), blots were blocked with 5% (wt/vol) nonfat dry milk in TBS-T overnight at room temperature and then incubated for 1 h with polyclonal anti-GLA antibody diluted 1:5,000 in TBS-T. Blots were rinsed repeatedly with TBS-T and then incubated for 1 h with polyclonal goat anti-rabbit antibody linked to horseradish peroxidase (HRP; Jackson Immunoresearch Laboratories) diluted 1:2,000 in TBS-T. After repeated rinses with TBS-T, blots were incubated with enhanced chemiluminescence (ECL) solutions from a kit (Amersham) according to the manufacturer's instructions. Western blots of the partially purified GLA preparation revealed a single band which corresponded to a silver-stained protein of approximately 78 kDa. Individual lanes of separated, silver-stained proteins were visualized as a histogram by using an IS1000 digital imaging system (Alpha Inotech Corp., San Leandro, Calif.). GLA represented approximately 17% of the total protein in the partially purified GLA preparation, which contained 0.18 mg of protein/ml.

GLA present in culture samples was quantified by a dot blot procedure with polyclonal anti-GLA antibody, as described above for Western blotting. Extracellular culture fluid (100 μl) was boiled to inactivate proteases and then blotted onto nitrocellulose by using a Milliblot-D filtration apparatus (Millipore). Blots were blocked and treated with anti-GLA antibody as described above. The amount of GLA in a sample, visible as dark circles on film, was quantified by using an IS1000 PhotoImager. Based on the amount of GLA in the partially purified GLA standard (0.03 mg of GLA/mg of protein), pixel density values per milligram of GLA were determined for the standards on each blot. Pixel values for each sample, compared to the standards, were used to calculate the amount of GLA protein (in milligrams per liter) present in culture supernatants.

Miscellaneous procedures. GLA activity of culture samples was determined as described previously (24), using 0.2 ml of filtered culture medium and 0.8 ml of 50 mM sodium acetate buffer, pH 5.0, containing 1.0% (wt/vol) amylose as the substrate. The amount of glucose hydrolyzed from amylose was measured with a diagnostic kit (Sigma Chemical Co.) which utilizes HRP, glucose oxidase, and *o*-dianisidine. Flask culture glucose levels were also measured with the diagnostic kit. Flask culture pH values were measured with colorpHast strips (EM Reagents).

Biomass measurements were determined by harvesting the contents of 50-ml cultures over tared filters in a Buchner funnel. Hyphal mats were washed with 2 volumes of water and dried for 24 h at 55°C. Extracellular protein was measured by the Lowry method (16) with sucrose-free Vogel's medium N added to standards containing BSA fraction V. Protease activity was determined by the addition of 1.0 ml of flask culture supernatant to 5 mg of hide powder azure (Sigma). Reaction mixtures were incubated for 18 h at 37°C, and reactions were terminated by boiling the mixtures 3 to 5 min. For determination of released azure, the absorbance (A_{600}) of each sample was compared to that of a medium-only control.

Bacterial strains and plasmids. *Escherichia coli* DH5α cells (Gibco BRL) were used in the subcloning of plasmids according to procedures described by Sambrook et al. (22). Restriction enzymes were purchased from New England BioLabs and Promega and used according to the manufacturer's recommendations. Plasmid pMP6 contained a hygromycin resistance gene. Plasmid pZL1 contained the full-length zeamatin-encoding cDNA (Ciba-Geigy). Plasmid pGE with elements of the *N. crassa* GLA gene was obtained under license from Neugenesis Corporation (Honolulu, Hawaii).

PCR site-directed mutagenesis. Plasmid pZL1 containing the full-length zeamatin-encoding cDNA was used as the template DNA. Oligonucleotide primers were designed to integrate a 5′ *kex-2* site and restriction sites at both ends of the amplified zeamatin-encoding DNA fragment for chimeric cloning. A 655-bp DNA fragment was amplified with a forward primer containing the *Eco*RI and *kex-2* sites (5′-GCGAATTCCGGCAAGCGCGCTGTGTTC-3′) and a reverse primer containing an *Xba*I site (5′-GTTCATCTAGATCACGGGCAGAAGA CGA-3′).

Approximately 100 ng of template DNA was amplified in 50-μl reaction mixtures containing 200 μM deoxynucleoside triphosphates, 0.6 μM (each) forward and reverse primers, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$, 10% (wt/vol) dimethyl sulfoxide, and 2.5 U of *Taq* DNA polymerase. Amplifications were performed in a GeneAmp 2400 thermal cycler (Perkin-Elmer) with an initial cycle of 94°C for 3 min and 30 subsequent cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 2 min. The amplification products were visualized on 1.0% (wt/vol) agarose gels in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA) stained with ethidium bromide.

Construction of the chimeric *gla-1*:*zeamatin* plasmid. The plasmid pGE, containing the *gla-1* promoter, a truncated open reading frame (*gla-1* ORF), a multiple cloning site, and the *gla-1* terminator sequence, was used to form the chimeric *gla-1*:*zeamatin* construct (pGEZ). The *gla-1* ORF was truncated at bp 376 of the GLA sequence reported by Stone et al. (25). The amplified zeamatin cDNA fragment and the plasmid pGE were digested with *Eco*RI and *Xba*I restriction enzymes and were isolated by agarose gel electrophoresis. The purified zeamatin DNA was ligated into pGE and transformed into *Escherichia coli* DH5α cells, and transformants were selected for ampicillin resistance as described in reference 22

The nucleotide sequence of the resulting plasmid (pGEZ) was confirmed by restriction analyses and DNA sequencing from both ends of the *gla-1*:*zeamatin* construct. Primers were designed to sequence across the chimeric zeamatin cDNA from the flanking ends of the *gla-1* ORF and *gla-1* terminator. DNA sequencing was performed at Cornell University's DNA Services Facility (Ithaca, N.Y.).

Transformation of *N. crassa* with pGEZ. Competent *N. crassa* cells were prepared according to a protocol described by Vollmer and Yanofsky (28) that was modified by Selitrennikoff and Sachs (23). Protoplasts were cotransformed with the *gla-1*:*zeamatin* construct (pGEZ) and pMP6 (to confer hygromycin resistance) at a ratio of 5 to 1 by a lipofectin-modified procedure described by Selitrennikoff and Sachs (23). Transformed conidia were germinated on plating medium containing 2% sorbose (3) and hygromycin (200 μg/ml).

Screening of transformed *N. crassa*. Primary transformants were transferred to agar slants containing Vogel's medium N with 1.5% (wt/vol) sucrose and hygromycin (200 μg/ml) and incubated at 25°C for 5 to 7 days. Genomic DNA was extracted from primary transformants (8) and screened by PCR with primers specific for the *gla-1* ORF-*zeamatin* cDNA fusion. A 798-bp product was amplified with the forward *gla-1p*-specific primer (5′-GGAAGCTGAGGTTTGCCG AACTTAGACGACC-3′) and the *zeamatin*-specific reverse primer (5′-GAACT TGCACCCCGTGCGCGCCCAGATGC-3′). Amplifications were done in 10-μl reaction mixtures containing 100 ng of genomic DNA, 200 μM deoxynucleoside triphosphates, primers (0.5 μM each), 50 mM Tris (pH 8.3), 250 μg of BSA/ml, 3.0 mM $MgCl_2$, 10% (vol/vol) dimethyl sulfoxide, and 1.5 U of *Taq* DNA polymerase. PCR was performed on a Rapidcycler (Idaho Technology) with an initial cycle of 94°C for 30 s and 30 subsequent cycles of 94°C for 0 s, 50°C for 0 s, and 72°C for 15 s.

Isolation of homokaryons. Microconidium (homokaryon) induction and isolation were performed as described previously by Ebbole and Sachs (4). Microconidia were germinated at 34°C on plating medium as described above for primary transformants. Colonies appearing after 3 to 7 days were transferred to agar slants containing hygromycin and Vogel's medium N with 1.5% (wt/vol) sucrose and hygromycin and, after 5 to 7 days of growth, were screened by PCR with *zeamatin* insert-specific primers.

Western blotting. Concentrated supernatants from transformed and untransformed shaken liquid cultures of *N. crassa* were separated on 12% (wt/vol) acrylamide gels (14). Samples were boiled (95°C) in sample buffer without a reducing agent (27) for 5 min. The blots were transferred to nitrocellulose, blocked with 5% (wt/vol) nonfat dry milk for 1 h, and incubated overnight with a 1:250 dilution of antizeamatin primary antibody in TBS-T. The blots were rinsed repeatedly and then incubated with goat anti-rabbit HRP for 1 h. Finally, the blots were rinsed with TBS-T and treated with ECL solutions as directed by the manufacturer.

***C. albicans* inhibition assay.** Inhibition of growth of *C. albicans* (strain B366; ATCC 56884) by culture supernatants of transformed strains of *N. crassa* was determined as described previously (20, 27). Concentrated *N. crassa* supernatants were placed onto sterile paper disks arranged on the surface of solidified agar containing *C. albicans*. The plates were incubated overnight at 37°C and monitored for the appearance of zones of inhibition of growth.

RESULTS

GLA production in liquid shaken culture. Figure 1 summarizes the parameters measured in *N. crassa* cultures grown on various concentrations of glucose. Glucose levels remained at the initial concentrations for the first 12 h (Fig. 1A) but fell rapidly to undetectable levels after 24 h for 0.6% (wt/vol) glucose cultures and after 36 h for 1.0 and 1.4% (wt/vol) glucose cultures. GLA protein levels (Fig. 1A) increased in cultures and plateaued after 96 to 120 h of incubation. Concentrations of GLA protein in flask cultures on day 6 were 2.5 mg/liter for 0.6% glucose cultures, 3.0 mg/liter for 1.0% glucose cultures, and 3.2 mg/liter for 1.4% glucose cultures.

GLA activity was not detected until 48 h after cultures were initiated (Fig. 1B). The specific activity of GLA in supernatants of 0.6% glucose cultures after 48 h of growth was 0.79 U/mg. In contrast, the specific activities for 1.0 and 1.4% glucose cultures were 0.32 and 0.44 U/mg, respectively. These results agree with and extend reports that GLA activity is present only after glucose is depleted from the medium (24). With regard to *N. crassa* cultured on other carbohydrates, Koh-Luar et al. reported GLA activities as high as 1.5 U/ml in starch-containing cultures and as low as 0.2 U/ml for cultures grown in the presence of cellulose (13).

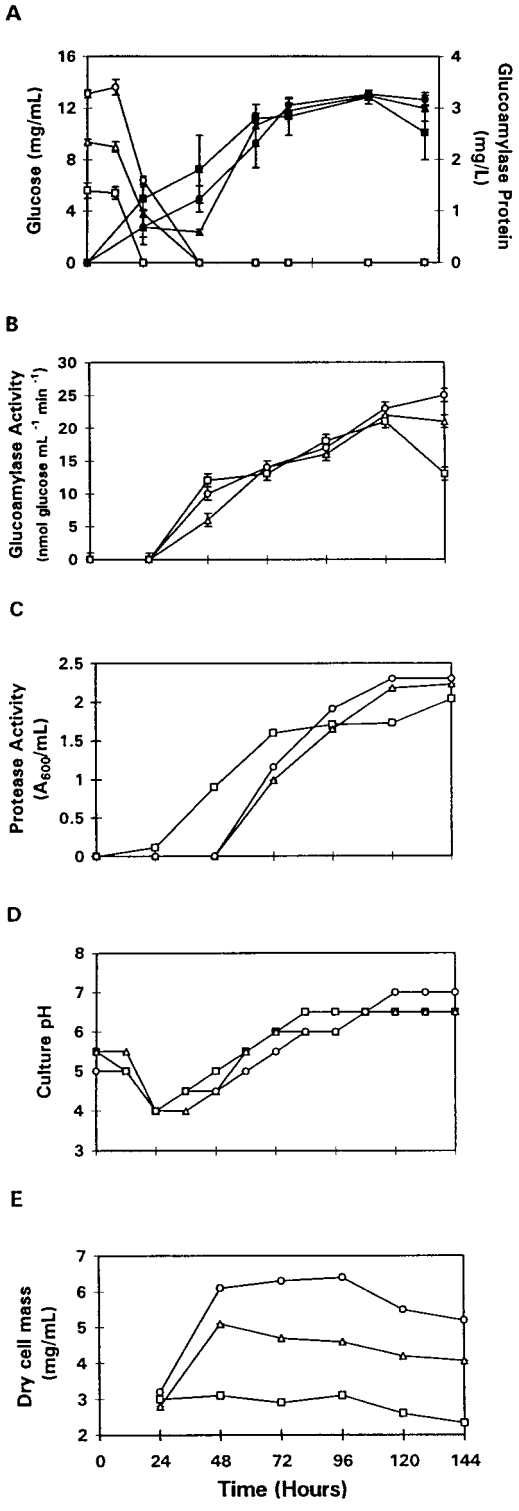

FIG. 1. Characterization of *N. crassa* liquid shaken cultures. Media contained 0.6% (wt/vol) (□), 1.0% (wt/vol) (△), or 1.4% (wt/vol) (○) glucose in 50-ml cultures in 250-ml baffled flasks. Culture and assay conditions are described in Materials and Methods. (A) Extracellular glucose concentration (open symbols) and GLA protein production (closed symbols). (B) GLA activity in culture supernatants as measured by evolution of glucose from amylose. Data points and bars in panels A and B represent the averages and standard deviations, respectively, of assays in duplicate from duplicate flasks. (C) Determination of extracellular protease activity in culture supernatants. Each point and its bars represent the average and the standard deviation from the mean, respectively, of duplicate culture samples. (D and E) Culture pH and dry cell mass.

Since proteases are of obvious concern in the development of a heterologous gene expression system, we monitored extracellular protease levels in cultures. Protease activity (Fig. 1C), expressed as absorbance (A_{600}) units per milliliter, was first detected at day 2 for 0.6% (wt/vol) glucose cultures and at day 3 for 1.0 and 1.4% (wt/vol) glucose cultures. Extracellular protease levels continued to increase throughout the 6-day incubation period, with final measured values of 2.0, 2.2, and 2.3 absorbance units/ml for 0.6, 1.0, and 1.4% glucose cultures, respectively.

Culture pH (Fig. 1D) initially dropped from 5.5 to 4.0 at 24 h and then increased to pH 6.5 for 0.6% glucose cultures at 84 h of incubation and pH 7.0 for 1.0 and 1.4% glucose cultures at 120 h. Biomass (Fig. 1E) peaked on day 2 for 0.6% glucose cultures at 3.3 mg/ml, on day 3 for 1.0% glucose cultures at 4.7 mg/ml, and on day 4 for 1.4% glucose cultures at 6.4 mg of dry cell mass/ml.

Extracellular protein levels in liquid shaken flask cultures grown on glucose were also determined. Flask cultures containing 0.6% (wt/vol) initial glucose concentrations had a maximum extracellular protein concentration of 100 μg/ml on day 5, and 1.0 and 1.4% glucose cultures had maximum extracellular protein levels of 80 and 130 μg/ml, respectively, on day 6 (data not shown). In contrast, *N. crassa* cultures grown on starch had only 20 μg of extracellular protein/ml after 2 days of incubation (13).

Together, these results indicate that GLA is produced in liquid shaken cultures with glucose as a carbon source. GLA activity was not detected until the extracellular glucose had been depleted (Fig. 1A), as has been reported previously (13, 24), but GLA protein was detected as early as 24 h of incubation, when glucose was still present in the culture supernatants of 1.0 and 1.4% (wt/vol) glucose cultures (Fig. 1A).

Formation of a *gla-1*:*zeamatin* DNA construct. For transformation into *N. crassa*, a plasmid (pGE) was modified to contain the cDNA encoding zeamatin within the truncated *gla-1* gene of *N. crassa*. The expression cassette pGE contained the *gla-1* promoter, a truncated *gla-1* ORF, and the *gla-1* terminator sequence (Fig. 2).

Zeamatin-encoding cDNA was amplified and modified by PCR site-directed mutagenesis to add unique restriction sites for ligation and an in-frame *kex-2* protease site to the 5′ end of the zeamatin-encoding cDNA so that the fusion protein is cleaved as it is translated into the endoplasmic reticulum (Fig. 2). A PCR product of 655 bp was amplified from the zeamatin-encoding cDNA (data not shown) and directionally inserted at the 3′ end of the *gla-1* ORF of pGE to form pGEZ (Fig. 2). The insertion, orientation, and sequence of pGEZ were confirmed as described in Materials and Methods.

Transformation of *N. crassa* with the *gla-1*:*zeamatin* construct. Upon successful construction of the chimeric *gla-1*:*zeamatin* plasmid (pGEZ), we transformed *N. crassa*. Competent protoplasts of *N. crassa* were cotransformed with pGEZ and pMP6. DNA was extracted from primary transformants and amplified by PCR to identify strains which contained the chimeric DNA. These results are shown in Fig. 3A. Thirty transformants containing the *gla-1*:*zeamatin* DNA were identified from ~300 primary transformants.

Induction and isolation of microconidia. Protoplasts used for transformation are typically multinucleate, as are the re-

Culture pH was determined from single measurements, and each dry cell mass point and its bars represent the average and the standard deviation from the mean, respectively, for duplicate culture samples.

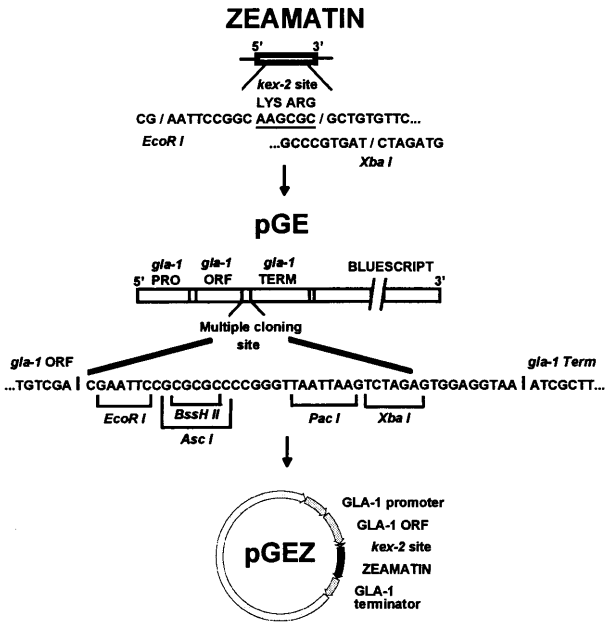

FIG. 2. Schematic of the *gla-1*:*zeamatin* construct. PCR primers were designed to amplify the ORF of zeamatin-encoding cDNA by adding an *Eco*RI endonuclease site and a *kex-2* protease site at the 5′ end and an endonuclease site (*Xba*I site shown) at the 3′ end after the first stop codon. The recombinant zeamatin-encoding cDNA fragment was ligated directionally into the multiple cloning site of pGE to form pGEZ for transformation into *N. crassa*. Sequences of pGE are illustrated with the permission of Neugenesis Corporation.

sulting hyphae and macroconidia. Additionally, exogenous DNA integrates into each nucleus randomly, resulting in transformants that are heterokaryotic. To study individual transformants for zeamatin secretion, we transferred 30 primary transformants to iodoacetate-containing medium and isolated uninucleate microconidia. These microconidia were plated on sorbose-containing medium to form individual colonies, and the colonies were screened by PCR, as described in Materials and Methods, for the presence of the *gla-1*:*zeamatin* chimeric DNA. (Fig. 3B). From 30 positive colonies, 10 transformants were selected at random and transferred to agar-solidified medium to provide macroconidia for use in liquid cultures to test for the production of zeamatin.

Expression of zeamatin by transformants. Transformed and untransformed *N. crassa* isolates were grown in liquid shaken medium as described in Materials and Methods. Cultures were harvested on day 4 of growth, and the media were concentrated. Although some hyphal lysis was apparent, levels of GLA protein present in transformant culture supernatants were similar to levels observed in supernatants of untransformed *N. crassa* cultures (data not shown). Concentrated culture supernatants from transformed and untransformed strains were tested for the presence of zeamatin.

The results presented in Fig. 4 show that the concentrated culture supernatant of one transformant containing pGEZ, *N. crassa* 406-1, contained a secreted protein that was recognized by antizeamatin serum and comigrated with purified zeamatin from corn samples. These results indicated that *N. crassa* synthesized and secreted zeamatin.

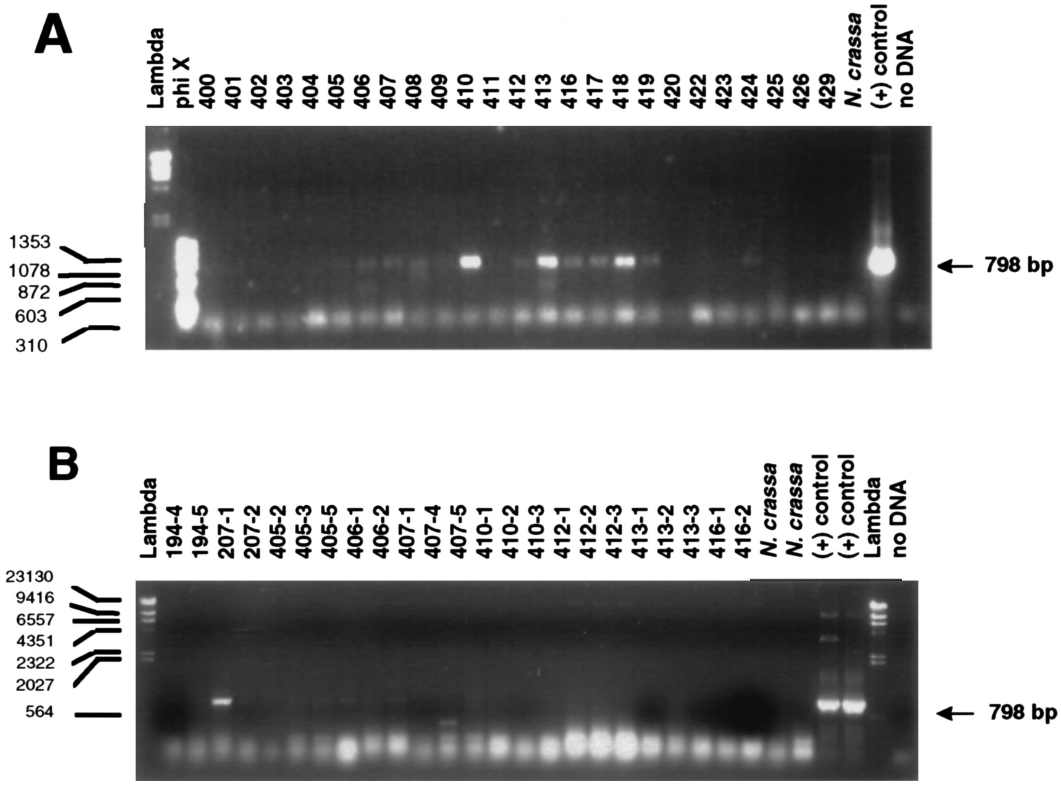

FIG. 3. Chimeric insert amplified from *N. crassa* transformants by using *gla-1*:*zeamatin*-specific primers. Products were amplified from the genomic DNAs of several primary transformants (A) and isolated homokaryons (B). Arrows indicate the position of the 798-bp amplified product. Conditions for *N. crassa* DNA extraction, PCR, and electrophoresis are described in Materials and Methods. *N. crassa*, untransformed genomic DNA negative control; (+) control, pGEZ-positive control. Lambda DNA-*Hin*dIII and PhiX174-*Hae*III markers were used on ethidium bromide-stained 1% (wt/vol) agarose gels.

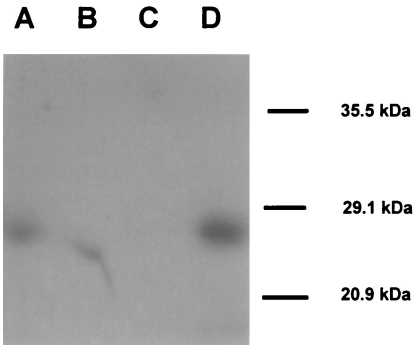

FIG. 4. Western blotting of culture supernatant. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose as described in Materials and Methods. Nitrocellulose blots were incubated with antizeamatin antibody (1:250) overnight. Zeamatin protein was visualized by using secondary antibody (goat anti-rabbit HRP) and ECL as described in Materials and Methods. Lanes: A, zeamatin from corn, 100 ng; B, zeamatin from corn, 50 ng; C, untransformed *N. crassa*, 40 μg of protein; D, 406-1 (zeamatin-transformed *N. crassa*), 20 μg of protein.

Zeamatin secreted by *N. crassa* is active. The results presented in Fig. 4 showed that zeamatin was secreted by transformant 406-1. To determine whether the secreted zeamatin was active, we tested the effect of recombinant zeamatin on the growth of *C. albicans*. The results presented in Fig. 5 show the effect of the concentrated culture supernatants of transformant 406-1 (Fig. 5A) and an untransformed control (Fig. 5B) on *C. albicans* growth. Note that the culture supernatant of an untransformed control had no effect on *C. albicans* growth (Fig. 5B). In sharp contrast, only the culture supernatant of the transformant containing the chimeric *gla-1*:*zeamatin* DNA had antifungal activity (Fig. 5A). The enhanced growth of *C. albicans* around the zone of inhibition in Fig. 5A and around the disk of Fig. 5B likely resulted from the concentrated proteins of *N. crassa* present in the concentrated culture supernatants. No enhanced growth or inhibition was observed in medium-only controls (data not shown). We interpret these results to indicate that the secreted zeamatin was active.

DISCUSSION

The aim of the present study was to determine the feasibility of using *N. crassa* as a plant gene expression host. Plant proteins similar to zeamatin have been expressed in other organisms. For example, thaumatin, an intensely sweet African shrub protein with sequence homology to zeamatin, was expressed in the filamentous fungus *Aspergillus oryzae*, using a yeast promoter and the thaumatin signal sequence for direction to the secretory pathway (10); however, it is not known whether the recombinant thaumatin was active. Synthetic thaumatin genes were used for expression in the yeast *S. cerevisiae* (15) and bacteria (6). Zeamatin-like protein, a protein very similar to zeamatin, has been reported to be expressed in insect cells (17), but it is not known whether this protein was active.

The expression of zeamatin under the control of *N. crassa* GLA promoter required the analysis of GLA production and various other parameters in shaken liquid cultures. These pilot data could then be used for scale-up to fermentors for commercial production of recombinant zeamatin and other plant proteins.

Rates of GLA induction were reported to increase 100-fold when washed hyphae were shifted from glucose-containing medium to medium without glucose (24). GLA was then rapidly secreted, a process monitored by the immunoprecipitation of radiolabeled GLA with anti-GLA antibody (24). GLA induction was determined by both enzymatic hydrolase activity and immunoassay. The use of an anti-GLA antibody as a means of quantifying GLA protein production has not been previously reported for *N. crassa*, but an immunoassay has been used for GLA protein determination in *Aspergillus* species (29). GLA activity was present only in the absence of glucose. However, GLA protein was detected prior to GLA activity in shaken flask cultures, and this may reflect the greater sensitivity of the immunoassay.

Biomass values for *N. crassa* liquid shaken cultures grown on glucose were considerably lower than those reported by Koh-Luar et al. (13) for growth of *N. crassa* under stationary conditions on 1.0% (wt/vol) sucrose (30 mg/ml), 1.0% (wt/vol) cellobiose (23 mg/ml), or 1.0% (wt/vol) starch (32 mg/ml) but higher than that for growth on 1.0% (wt/vol) cellulose (1 mg/ml). Protease activity was present in cultures throughout the incubation period. Zeamatin is highly resistant to protease digestion (27); however, the use of protease-deficient strains of *N. crassa* might be necessary to increase yields of other plant proteins that are less resistant than zeamatin.

N. crassa was transformed with the *gla–1*:*zeamatin* DNA construct. Resulting transformants containing pGEZ were identified, induced to form homokaryons, and grown in liquid culture for secretion of zeamatin. A protein that was recognized by the antizeamatin antibody and comigrated with authentic zeamatin from corn was detected in culture supernatants, indicating that zeamatin had been synthesized and secreted by *N. crassa*.

Critically, the secreted zeamatin was active. We infer from these results that zeamatin did not kill *N. crassa* because of the high salt concentration in the production medium. Importantly, this work shows that *N. crassa* synthesized and processed zeamatin, including the formation of eight disulfide bonds, correctly. It is not known whether zeamatin has other posttranslational modifications, such as glycosylation; thus, the ability of *N. crassa* to carry out these functions on heterologous proteins cannot be addressed by our results.

The observed levels of secreted zeamatin in culture supernatants were low (~10 μg/liter) in contrast to those of other hosts. However, the availability of other promoters for maximal expression of heterologous proteins should dramatically improve yields. Taken together, our results show that *N. crassa* has significant potential for secretion of plant proteins that are either rare or difficult to extract from plant hosts.

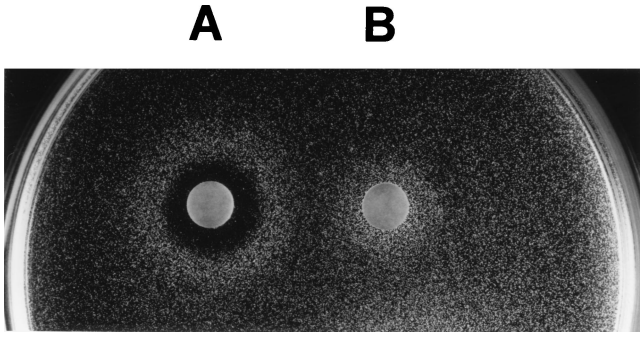

FIG. 5. *C. albicans* agar diffusion assay. Sterile concentrated supernatants (50 μl) from *N. crassa* transformant 406-1 and an untransformed control were loaded onto sterile paper disks. (A) *N. crassa* transformant 406-1; (B) untransformed control. Details of the assay are provided in Materials and Methods.

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