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Silencing of the Aspergillopepsin B (*pepB*) Gene of *Aspergillus awamori* by Antisense RNA Expression or Protease Removal by Gene Disruption Results in a Large Increase in Thaumatin Production

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Aspergillopepsin B was identified in culture broths of *Aspergillus awamori* by in situ detection of its proteolytic activity and by immunodetection with anti-aspergillopepsin B antibodies. Severe thaumatin degradation was observed after in vitro treatment of thaumatin with purified aspergillopepsin B. The *pepB* gene encoding aspergillopepsin B of *A. awamori* was cloned and characterized. It is located in chromosome IV of *A. awamori*, as shown by pulsed-field gel electrophoresis, and encodes a protein of 282 amino acids with high similarity to the aspergillopepsin B of *Aspergillus niger* var. *macrosporus*. The *pepB* gene is expressed at high rates as a monocistronic 1.0-kb transcript in media with casein at acidic pH values. An antisense cassette constructed by inserting the *pepB* gene in the antisense orientation downstream from the *gpdA* promoter resulted in a good level of antisense mRNA, as shown by reverse transcription-PCR. Partial silencing of the *pepB* gene by the antisense mRNA resulted in a 31% increase in thaumatin yield. However, significant residual degradation of thaumatin still occurred. To completely remove aspergillopepsin B, the *pepB* gene was deleted by double crossover. Two of the selected transformants lacked the endogenous *pepB* gene and did not form aspergillopepsin B. Thaumatin yields increased by between 45% in transformant APB 7/25 and 125% in transformant 7/36 with respect to the parental strain. Reduction of proteolytic degradation by gene silencing with antisense mRNA or total removal of the aspergillopepsin B by directed gene deletion was a very useful method for improving thaumatin production in *A. awamori*.

Filamentous fungi, particularly *Aspergillus niger*, *Aspergillus awamori*, and *Trichoderma reesei*, are excellent host systems for secretion of homologous and heterologous proteins (4). Proteolytic degradation is considered one of the major problems during protein production in *Aspergillus* species (3, 7, 29, 37). Proteolytic degradation affects mainly heterologous proteins and can be explained, in part, by the presence in these proteins of recognition sites for proteases (e.g., the KEX2 system) in greater numbers than in the homologous proteins (9).

Thaumatin is a sweet-tasting protein (14, 15, 38) used as a sweetener in human food or as a feed additive (13). Overproduction of thaumatin in *A. awamori* has been difficult to achieve because of proteolytic degradation.

We have reported the obtaining of thaumatin-producing strains by transformation with an expression cassette containing a synthetic thaumatin gene (with an optimized codon usage) (28). Thaumatin degradation by aspergillopepsin A has been determined, and the inactivation of this protease resulted in a significant increase of extracellular thaumatin (29). Even when an aspergillopepsin A-deficient mutant, *lpr66*, was used as the host strain (29), some thaumatin degradation still occurred, mainly at late stages of fermentation, indicating the presence of other proteases different from aspergillopepsin A in culture broths of this strain.

Targeted disruption of specific genes is difficult in many filamentous fungi, because recombination is ectopic in most cases (11, 12, 17). There is, indeed, very little information on targeted gene disruption in *A. awamori*. In *Penicillium chrysogenum*, a more efficient targeted gene replacement has been obtained by the double-marker selection procedure (25).

An alternative method for reduction of expression of a particular gene is the use of antisense RNA. Although the technique is simple, the effectiveness of the method is influenced by many factors (1). This technique has been successfully used to silence the *creA* gene in *Aspergillus nidulans* (6). It was, therefore, of interest to try to silence the *pepB* gene in *A. awamori* by the antisense RNA technique, as a first approach to elimination of the negative effect of the presence of aspergillopepsin B on thaumatin accumulation.

We report in this article that significant amounts of antisense RNA of the *pepB* gene are formed by using a strong fungal promoter but that the aspergillopepsin B is not completely removed from the broths. On the other hand, *pepB* gene disruption by replacement using the double-marker selection procedure led to the complete loss of aspergillopepsin B and to a 100% increase in thaumatin accumulation under optimal fermentation conditions.

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MATERIALS AND METHODS

Microbial strains. The wild-type *A. awamori* NRRL 3112 strain was used for transcriptional analysis of the *pepB* gene. *A. awamori lpr66*, a mutant defective in aspergillopepsin A (29), was used as host for thaumatin production. The thaumatin-producing strains TB2b1-44 and TGDTh-4, derived from *A. awamori lpr66* (28), and strain TB2b1-44-*pyrG45*, a *pyrG* mutant obtained by UV mutagenesis from TB2b1-44, were used for antisense RNA and *pepB* gene disruption studies.

Escherichia coli DH5α served as host for plasmid amplification and purification, and *E. coli* LE392 was used for isolation of bacteriophages from an *A. awamori* genomic library.

Media and growth conditions. *A. awamori* strains were maintained on solid Power sporulation medium (16) (supplemented with 140 μg of uridine/ml when required) at 30°C for 3 days. Seed cultures of thaumatin-producing strains in CM medium (5 g of malt extract, 5 g of yeast extract, and 5 g of glucose per liter) were inoculated with 10^6 spores/ml and grown at 30°C in a rotary G10 incubator (New Brunswick Scientific, New Brunswick, N.J.) for 48 h. For thaumatin production studies or protease detection, *A. awamori* strains were grown in MDFA medium (34), inoculated with an 18% seed culture, and grown at 30°C for 96 to 120 h in a rotary shaker. For transcriptional analysis, wild-type *A. awamori* was grown in CAC medium (20 g of wheat flour, 5 g of peanut flour, 2 g of citric acid, and 2 g of trisodium citrate per liter) or SSM medium (7), both of which were inoculated with a 15% seed culture.

Transformation of *A. awamori*. Protoplasts of *A. awamori* strains were transformed by the polyethylene glycol method (39). Phleomycin- or hygromycin-resistant transformants were selected in tryptic soy agar (Difco, Detroit, Mich.), with 1 M sorbitol as osmotic stabilizer supplemented with 25 μg of phleomycin or 150 μg of hygromycin per ml, respectively. In transformations with vectors containing the *A. niger pyrG* gene, transformants were selected in Czapek minimal medium, with 1 M sorbitol as osmotic stabilizer.

DNA and RNA isolation. Total DNAs of *A. awamori* strains were obtained by a modification of the Ausubel method (5) as follows. The fungi were grown in CM medium for 48 h at 30°C and harvested by filtration on Nytal 30 filters. The mycelium, frozen in liquid nitrogen, was ground to a fine powder in a mortar. The frozen powdered mycelium (100 mg) was mixed with 500 μl of neutral phenol, 500 μl of chloroform-isoamyl alcohol (CIA; 24:1), and 500 μl of lysis buffer (200 mM Tris-HCl [pH 8.2], 100 mM EDTA, 1% sodium dodecyl sulfate [SDS]). The mixture was held at 50°C for 20 min and centrifuged at 16,000 × *g* for 20 min. The upper aqueous layer was collected and extracted with 1 volume of phenol-CIA and then with 1 volume of CIA. The DNA was precipitated with a 1/10 volume of 3 M sodium acetate (pH 7.0) and 2.5 volumes of ethanol, recovered by centrifugation at 16,000 × *g* for 30 min, and resuspended in Tris-EDTA buffer.

Total RNAs of *A. awamori* strains were obtained from mycelium grown in CAC or SSM medium by the phenol-SDS method (5).

Pulsed-field gel electrophoresis. Resolution of the *A. awamori* chromosomes was performed by pulsed-field gel electrophoresis (PFGE), as described previously (10), using a CHEF-DRII Gene Pulser (Bio-Rad). Total electrophoresis time was 180 h, the pulse time ramp was from 45 min to 90 min, and the voltage was 40 V.

Southern analysis. Total DNA from *A. awamori* strains was digested with restriction endonucleases, electrophoresed in 0.8% agarose, and blotted by standard techniques (33) on a Hybond XL nylon membrane (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom). The probe used to clone the *A. awamori pepB* gene was a 2.7-kb PCR-amplified fragment (see below). To analyze transformants overexpressing the *pepB* gene, a 2.6-kb *Xba*I fragment containing the complete *pepB* gene was used as probe. The disruption of *pepB* was analyzed by hybridization with a 1,869-bp fragment (*Xho*I-*Eco*72I) containing this gene as probe. All probes were labeled with digoxigenin by the random-priming method, except that used to clone the *pepB* gene, which was labeled by nick translation with [^{32}P]dCTP.

The nylon filter was hybridized overnight at 42°C in a buffer containing 40% formamide, 5× SSC, 0.1× sarcosine, 0.02% SDS, and 2% blocking reagent, washed once with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 42°C for 15 min, once with 0.1× SSC–0.1% SDS at 42°C for 15 min, and once more with 0.1× SSC–0.1% SDS at 65°C. Digoxigenin detection was carried out as described by the manufacturer (Amersham Pharmacia Biotech) using disodium 3-{4-methoxyspiro[1,2-dioxetane-3,2′-(5′-chloro)tricyclo [3.3.1.1^{3,7}]decan]-4-yl}phenyl phosphate (CSPD) as substrate.

Northern analysis. Total RNA (5 μg) was run on a 1.2% agarose–formaldehyde gel. The gel was blotted onto a nylon filter (Nytran 0.45; Schleicher and Schuell) by standard methods, and the RNA was fixed by UV irradiation with a UV-Stratalinker 2400 lamp (Stratagene, La Jolla, Calif.). The filters were prehybridized for 3 h at 42°C in a mixture of 50% formamide, 5× Denhardt's solution, 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA, pH 7.7), 0.1% SDS, and 500 μg of denatured salmon sperm DNA per ml and were then hybridized at 42°C for 18 h in the same buffer containing 100 μg of denatured salmon sperm DNA per ml. The probes used were a 1,050-bp *Xho*I-*Eco*RV fragment containing the *pepB* gene and an 830-bp *Kpn*I-*Nco*I fragment corresponding to the β-actin gene of *A. nidulans*. The filters were washed once in 2× SSC–0.1% SDS at 42°C for 15 min, once in 0.1× SSC–0.1% SDS at 42°C for 15 min, once more in 0.1× SSC–0.1% SDS at 65°C for 15 min, and exposed to Amersham X-ray film.

PCR and reverse transcription (RT)-PCR amplification. The *pepB* gene was amplified from genomic DNA of *A. awamori* by using the primers pepb1 (5′-TTTTGGATCCAACAATGAGGGGAAAAGG-3′) and pepb2 (5′-TTTTGGATCCCATCGTCCCTGCATTGGC-3′), which were designed according to the sequence of the *pepB* gene of *A. niger* var. *macrosporus* (20). The PCR was carried out with *Pfu* DNA polymerase and consisted of one cycle at 96°C for 2 min and 30 cycles at 96°C for 1 min, 55°C for 45 s, 72°C for 2 min, and then 72°C for 10 min. Following the PCR, amplified DNA was digested with *Bam*HI and cloned into a pBluescript KS(+) vector.

To detect antisense transcripts of the *pepB* gene, RT-PCR was performed with the SuperScript One Step System (Gibco-BRL, Carlsbad, Calif.). Total RNA of transformants asH5 and TGDTh-4 grown in CAC medium for 24 h was used as template. The primers used were as follows: (i) for amplification of a 601-bp fragment corresponding to a *pepB* sense transcript, primers apbS (5′-CTCAAGCTGAACGGCACCTCCAAC-3′) and apbAS (5′-GGGCCGACAGTGGAACCGTCGC-3′); (ii) for amplification of a 721-bp fragment corresponding to the antisense *pepB* mRNA, primers apbAS and cyc1 (5′-AAGGAAAAGGGGGACGGA-3′); and (iii) for amplification of a 335-bp fragment corresponding to the endogenous *gdhA* gene, which was used as a control for the reaction, primers In1 (5′-ATGTCTAACCTTCCTCAC-3′) and In2 (5′-CACCCTTACCACCACCCA-3′). The program used for cDNA amplification consisted of 50°C for 30 min and then 94°C for 2 min. The coupled PCR consisted of 40 cycles at 94°C for 15 s, 55°C for 30 s and 72°C for 1 min, and a final extension at 72°C for 10 min.

DNA sequencing. DNA fragments containing the *pepB* gene region were subcloned into pBluescript KS(+) or pUC19 and sequenced using the GeneAmp PCR 2400 system coupled to the ABI-PRISM 310 automatic sequencer (Perkin-Elmer, Norwalk, Conn.). Computer analyses of nucleotide and amino acid sequences were made with the DNASTAR programs (DNASTAR, Inc., London, United Kingdom).

Immunoblotting. Immunoblot analysis of *A. awamori* culture supernatants was performed using electrophoresis after protein resolution on an SDS–12% polyacrylamide gel. The culture supernatant proteins were concentrated 10-fold by precipitation with 10% (wt/vol) trichloroacetic acid. After electrophoresis, the proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore) in a Minitransblot electroblotting system (Bio-Rad). The membranes were treated with anti-aspergillopepsin B polyclonal antibodies (serum dilution, 1:2,500) in 50 mM Tris-HCl (pH 8.0)–150 mM sodium chloride containing Tween 20 at 0.2% (vol/vol) for 1 h and then for 30 min with an anti-rabbit alkaline phosphatase conjugate (1:10,000; Sigma Aldrich) in the same buffer. The membranes were treated with a BCIP-NBT (5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium) substrate for alkaline phosphatase (Sigma Aldrich) until the color was developed.

Quantification of aspergillopepsin B and thaumatin by ELISA. Aspergillopepsin B present in culture media of *A. awamori* transformants was quantified by an enzyme-linked immunosorbent assay (ELISA). The wells of plates (Nunc Immunoplates) were coated with dilutions of the supernatant samples (1:10 to 1:1,280) overnight at 4°C. The plates were washed three times with phosphate-buffered saline (PBS) plus 0.1% Tween 20 (PBS-T), blocked with PBS-T containing 5% dry milk for 1 h, and washed three times with PBS-T. The aspergillopepsin B was prepared for measurement by the addition of rabbit anti-aspergillopepsin B antiserum (1:1,000 dilution) for 1 h, followed by a 1:5,000 dilution of a goat anti-rabbit commercial alkaline phosphatase conjugate (Sigma Aldrich) for 30 min. The antigen-antibody complexes were quantified using a stabilized substrate solution for alkaline phosphatase (Sigma Aldrich) at 405 and 620 nm in a Scanning Autoreader and Microplate Workstation (CERES 900C; Bio-Tek Instruments). Decreasing concentrations of aspergillopepsin B were used as standards.

Thaumatin was quantified as described previously (28).

Zymogen detection of acid proteases. A continuous electrophoretic system was used to detect acid proteases, thus avoiding their pH-related denaturation (35). Acrylamide gels were made in 50 mM sodium acetate (pH 4.5) containing 0.2% (wt/vol) gelatin, 0.1% SDS, and 2 mM sodium sulfite. The samples were electrophoresed in 200 mM sodium acetate (pH 4.5) as electrode buffer for 2 h at 150 V at 4°C, and then gels were soaked twice for 15 min in 10 volumes of 2.5%

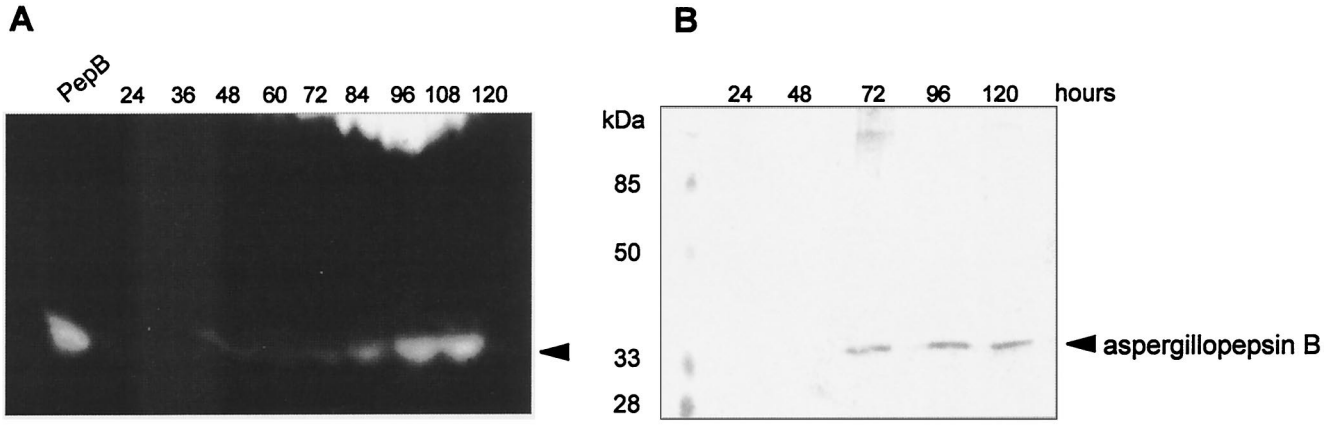

FIG. 1. (A) Zymogram analysis showing the presence of aspergillopepsin B (arrowheads, panels A and B) in culture filtrates from *A. awamori lpr66* grown in MDFA medium. Culture broth supernatants (20 μl) sampled at different time points were loaded in each lane. As a control, 75 ng of purified aspergillopepsin B was loaded (first lane). (B) Immunoblot analysis of aspergillopepsin B in culture filtrates of *lpr66* strain grown in MDFA medium. Size markers (in kDa) are indicated at the left of panel B.

(vol/vol) Triton X-100 to remove SDS and incubated for 12 h at 30°C in 50 mM sodium acetate (pH 4.0)–0.1% Triton X-100–10 mM calcium chloride. The gels were stained with Coomassie R-250 for 1 h and destained with H_2O-methanol-acetic acid (73:20:7). The acid proteases were detected as white bands on a blue background.

Proteolytic activity. The extracellular proteolytic activity of *A. awamori* strains was determined as described by Anson (2), using denatured hemoglobin as substrate.

One unit of proteolytic activity was defined, in accordance with the definition used by M. L. Anson (2), as the amount of enzyme that releases trichloroacetic soluble peptides equivalent to an absorbance of 1.0 at a wavelength of 280 nm.

RESULTS

Aspergillopepsin B in culture broths of *A. awamori*. Zymogram analysis of the culture broths in MDFA medium (routinely used to produce thaumatin in cultures of *A. awamori*) showed the presence of four protease bands after gel electrophoresis. The presence of aspergillopepsin B was detected by in situ detection of its activity (Fig. 1A) and confirmed by immunodetection with anti-aspergillopepsin B antibodies in samples taken at between 72 and 120 h of cultivation in MDFA medium (Fig. 1B). Other proteolytic activities of very high molecular weight (probably protein complexes) were also detected in this strain (Fig. 1A).

Thaumatin is degraded by aspergillopepsin B. The accumulation of aspergillopepsin B was in agreement with the observed decrease in thaumatin production in *A. awamori* cultures after 72 h. To determine if the thaumatin production decrease is due to proteolysis by aspergillopepsin B, a thaumatin degradation study using purified aspergillopepsin B was performed. After incubation of pure thaumatin with aspergillopepsin B (35:1, wt/wt) at 30°C for 1 h, severe proteolytic degradation of thaumatin was observed, and after 2 h, thaumatin was completely degraded (Fig. 2). However, control incubations of pure thaumatin without the protease at 30°C for 2 h did not result in significant degradation. This result suggests that aspergillopepsin B may decrease the in vivo yield of thaumatin in *A. awamori* cultures and encouraged us to clone and disrupt the *pepB* gene encoding aspergillopepsin B.

The *pepB* gene of *A. awamori* encodes aspergillopepsin B and

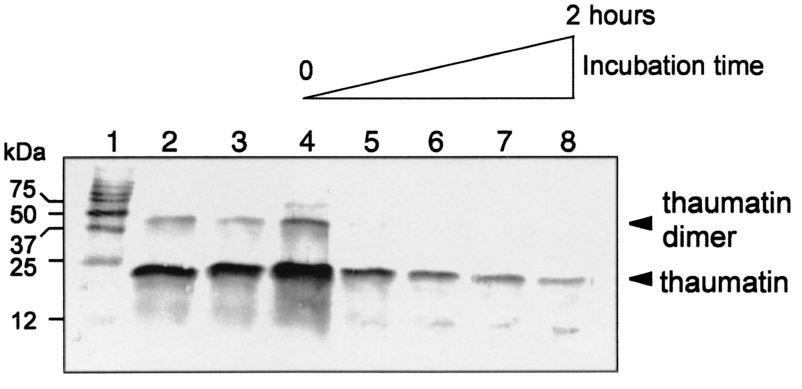

FIG. 2. Thaumatin degradation by aspergillopepsin B. Thaumatin (200 ng) was incubated with 5 ng of pure aspergillopepsin B at 30°C in 50 mM sodium acetate buffer, pH 4.0. Samples were taken after increasing incubation times, subjected to SDS-polyacrylamide gel electrophoresis, and analyzed with anti-thaumatin antibodies. Lane 1, size markers; lane 2, thaumatin after incubation for 2 h at 30°C in 50 mM sodium acetate buffer without aspergillopepsin B; lane 3, thaumatin after incubation for 2 h with boiled enzyme; lanes 4 to 8, thaumatin incubated with aspergillopepsin B for increasing periods of time (0, 0.5, 1, 1.5, and 2.0 h, respectively).

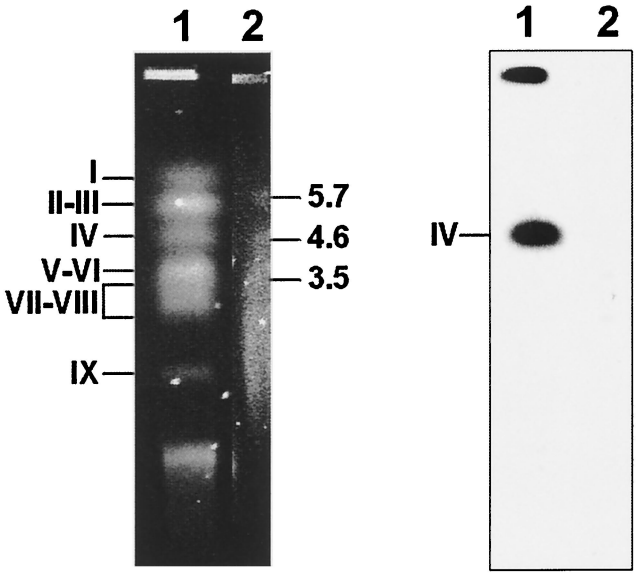

FIG. 3. Separation of the chromosomes of *A. awamori* by PFGE and location of the *pepB* gene. Left panel: resolution of chromosomes of *A. awamori* (lane 1). Chromosomes from *Schizosaccharomyces pombe* (lane 2) were used as molecular weight markers to calculate the sizes of the *A. awamori* chromosomes. Right panel: location of the *pepB* gene by hybridization with a 2.6-kb *Xba*I probe containing the *pepB* gene; the hybridization signal appears on chromosome IV. Electrophoresis conditions were as indicated in Materials and Methods.

is located in chromosome IV. A 2.7-kb fragment was amplified from genomic *A. awamori* DNA by PCR with primers pepb1 and pepb2 (see Materials and Methods) based on the *A. niger* var. *macrosporus pepB* gene sequence (20). This fragment was partially sequenced and shown to correspond to the *pepB* gene of *A. awamori* and was therefore used as a probe to isolate the *A. awamori pepB* gene from a λGEM12 genomic library.

Under stringent hybridization conditions, 57 positive phage plaques were identified; 6 of them were analyzed by Southern blotting, and all of them contained the *pepB* gene. A 3.7-kb *Bam*HI fragment carrying the complete *pepB* gene with flanking sequences was cloned in a pBluescript KS(−) vector, resulting in plasmid pAPB-4B. The DNA fragment sequenced showed a 97.1% identity at the nucleotide level with the *A. niger* var. *macrosporus pepB* gene and encodes a protein 97% identical to aspergillopepsin B.

Using Southern analysis after resolution by PFGE of the chromosomal bands of *A. awamori*, the *pepB* gene was located in the 4.6-Mb chromosome IV (Fig. 3).

Transcription of the *pepB* gene is higher at acid pH. To analyze *pepB* gene expression, *A. awamori* was grown in two protein-rich media, CAC and SSM, which show different pH patterns. In CAC medium, *A. awamori* growth resulted in a pH rise from 4 to 7 with 60 h of incubation, whereas in SSM medium, there was a pH reduction from 7 to 4 in the same period of time. Northern analysis revealed a single 1.0-kb *pepB* transcript (Fig. 4). The transcript level was higher when acid pH prevailed, i.e., at early time points in CAC medium and at late time points in SSM medium. Clearly, therefore, the expression of *pepB* appears to be regulated by the pH of the culture (see Discussion).

An increase of the *pepB* gene copy number leads to high levels of aspergillopepsin B. To determine any potential transcriptional limitation in the regulation of the *pepB* gene, a 2.6-kb fragment containing the *pepB* gene and 1.2 kb of its promoter region were subcloned in pIBRC43 vector containing the *ble* gene as selective marker (10), resulting in plasmid pAPB-ble. Wild-type *A. awamori* was transformed with this plasmid, and transformants containing multiple copies were selected. Six of them, chosen at random, were grown in CAC medium for 72 h, and their culture supernatants were analyzed for aspergillopepsin B protein and total proteolytic activity.

As shown in Fig. 5, levels of aspergillopepsin B increased in all transformants, ranging from a 2.5-fold increase in transformant TB-28 to a 4.8-fold increase in TB-19. The increase of aspergillopepsin B was observed at early time points in the cultures, which correlates with the early expression of the *pepB* gene observed in CAC medium. The proteolytic activity increased 1.8 to 3.4 times with respect to that of the wild type, in parallel to that of the aspergillopepsin protein in these transformants in the cultures at early time points (Fig. 5A).

These results indicate that transcriptional factors are not limiting for expression of an increased number of *pepB* gene copies in *A. awamori*.

Expression of a *pepB* antisense RNA cassette in a thaumatin-producing strain. To improve thaumatin production in *A. awamori*, it was necessary to remove the proteolytic activity due to the presence of aspergillopepsin B. For this purpose, a *pepB*-antisense RNA cassette was constructed by inserting the complete *pepB* gene in antisense position between the promoter of the *gpdA* gene from *A. nidulans* and the terminator of the *cyc1* gene from *Saccharomyces cerevisiae*. This cassette was introduced in the pAN7-1 vector, and the resulting plasmid (named pAPBas2) (Fig. 6A) was used to transform the thaumatin-producing strain *A. awamori* TGDTh-4. Five transformants were randomly selected, grown in CAC medium, and assayed for aspergillopepsin B production. A reduction of the level of this protein, ranging from 10% in transformant asX4 to 70% in transformant asH5, was observed at 16 h of fermentation (Fig. 6B). A similar reduction of the level of aspergillopepsin B was also observed at 26 h of culture. No significant differences in growth or ability to sporulate were observed between transformants and the host strain.

The presence of antisense RNA in transformant asH5 and the parental strain TGDTh-4, grown for 24 h in CAC medium, was studied by RT-PCR, using as primers the oligonucleotides described in the Fig. 6 legend (for a control, the transcript of the endogenous *gdhA* gene was also amplified). An amplified 601-bp fragment corresponding to the transcript of the endogenous *pepB* gene and another 335-bp fragment corresponding to the *gdhA* gene were observed in both strains. In addition, a 721-bp fragment, corresponding to the expected size of the antisense *pep*B RNA, was observed only in transformant asH5 (Fig. 6C), confirming the functionality of the antisense construct.

The amount of antisense RNA as quantified by gel electrophoresis (Fig. 6C) may be insufficient to titrate out completely the sense mRNA (see Discussion).

Antisense downregulation of *pepB* results in a small increase in thaumatin production. The thaumatin production of transformant asH5 was analyzed in MDFA medium in which

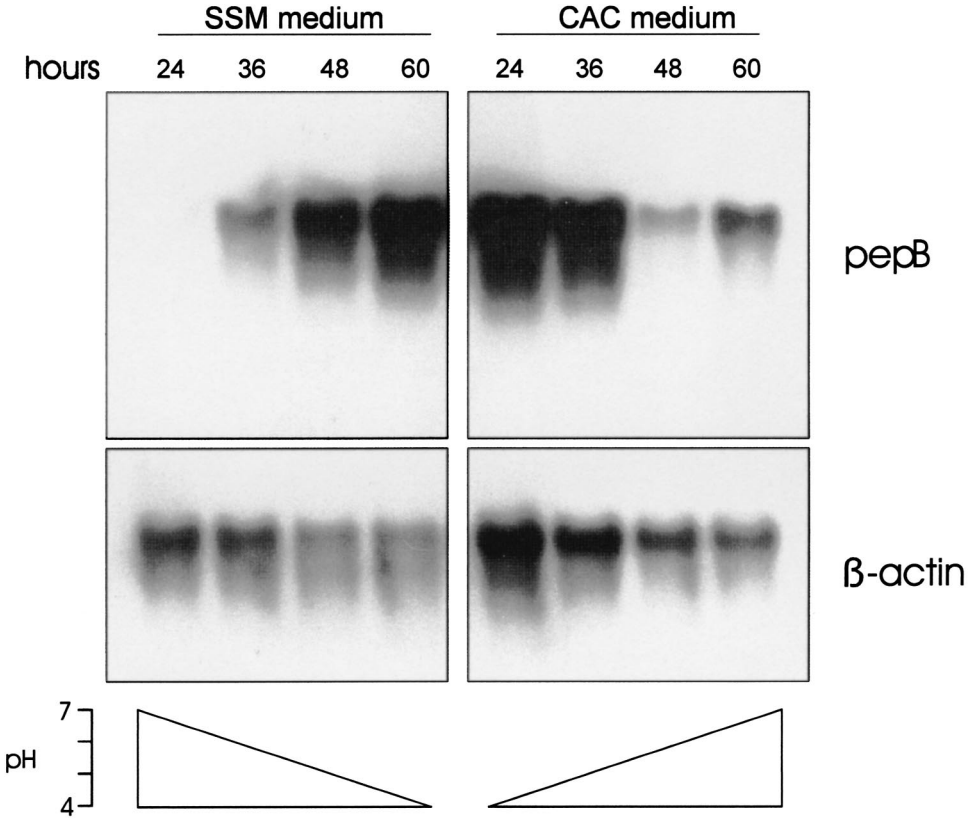

FIG. 4. Northern analysis of *pepB* gene expression. *A. awamori* wild type was grown in SSM and CAC media for 60 h, and RNA was extracted from samples taken at 24, 36, 48, and 60 h. After blotting, the filters were hybridized with probes for the *pepB* gene of *A. awamori* (upper panels) or the β-actin gene of *A. nidulans* (lower panels). The pattern of pH values during fermentation in each medium is indicated at the bottom.

the $SO_4(NH_4)_2$ was replaced by 1% casein (hereafter designated MDFAC medium). In these conditions, the thaumatin *tha* gene is expressed but the *pepB* gene is also strongly transcribed. A 31% (±4%) average increase of the presence of thaumatin, from 8 mg/liter in strain TGDTh-4 to 10.5 mg/liter in transformant asH5, was observed in three different experiments at 48 h of fermentation. After this time, however, there was a decrease of the accumulated thaumatin in transformant asH5, indicating that thaumatin was still being degraded despite the formation of the antisense mRNA, probably because antisense mRNA has only a partial silencing effect on *pepB* gene expression.

Deletion of *pepB* removes aspergillopepsin B and produces a significant increase in thaumatin production. Since the *pepB* antisense strategy did not result in a large increase in thaumatin production, disruption of *pepB* was performed using a gene replacement strategy (double crossover) with plasmids containing two selectable markers (hygromycin resistance and *pyrG* auxotrophy). For this purpose, plasmid pAPB-XI was constructed by replacing a 478-bp fragment of the *pepB* gene with the hygromycin resistance cassette. This plasmid contains a 2.6-kb region upstream of the *pepB* gene and 5.2 kb of homologous fungal DNA downstream of the *pepB* gene for recombination (Fig. 7). As a control, plasmid pAPB-X, which has no homologous sequences at its ends, was also used.

After digestion with *Not*I, these constructions were transformed into the strain TB2b1-44-*pyrG45* (a *pyrG* derivative of the thaumatin-producing strain) and transformants with a Hyg^R Ura^- phenotype (formed by homologous double recombination in the *pepB* locus) were selected. A total of 829 hygromycin-resistant transformants were obtained in seven transformation experiments; 39 of them also showed an Ura^- phenotype, indicating that 4.7% of the single integrations had also recombined for the second marker (*pyrG* gene). The number of Hyg^R Ura^- transformants obtained was higher with plasmid pAPB-XI (9.8%) than with plasmid pAPB-X (1.7%), suggesting that the presence of homologous regions at the ends of the linearized plasmid is important for the improvement of transformation. In a Southern hybridization, two of these transformants, named APB 7/25 and APB 7/36, showed the expected pattern for *pepB* gene disruption (Fig. 7).

As determined by immunoblotting, the transformants APB 7/25 and APB 7/36 did not show the aspergillopepsin B protein band in culture filtrates of CAC medium, therefore allowing us to determine unequivocally the effect of aspergillopepsin B on thaumatin degradation. These two aspergillopepsin B-deficient strains were grown in MDFAC medium to induce maximal *pepB* gene expression (23) and in MDFAC medium in which sucrose was replaced with glycerol (a nonrepressible carbon source for the *pepB* gene) (hereafter designated MDFACG medium).

Thaumatin production in MDFA medium was 8.5 mg/liter in

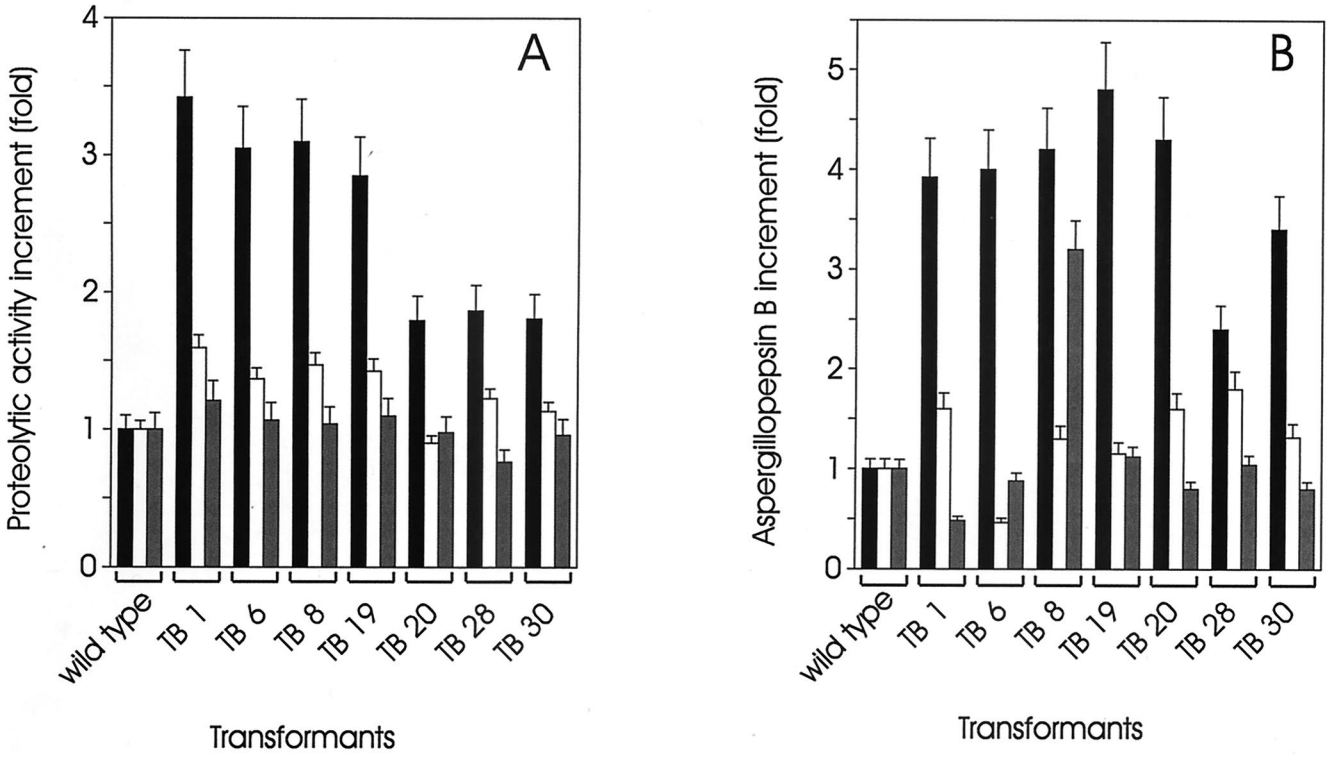

FIG. 5. Amplification of the *pepB* gene of *A. awamori.* (A) Total proteolytic activity at 24 h (black column), 48 h (white), and 72 h (gray) in transformants with multiple copies of the *pepB* gene compared to that of the wild type. (B) Aspergillopepsin B produced by transformants with multiple copies of the *pepB* gene.

the parental strain TB2b1-44-*pyrG45* and 11 and 13 mg/liter in transformants APB 7/25 and APB 7/36, respectively (Fig. 8A). In the casein-containing MDFAC medium, the control strain produced less thaumatin, due to aspergillopepsin B induction (about 5.5 mg/liter), but the aspergillopepsin B-deficient strain showed higher increases of thaumatin production (a 45% increase in transformant APB 7/25 and a 90% increase in strain APB 7/36 in 72-h cultures) (Fig. 8B). Similarly, in MDFACG medium, the production of thaumatin in transformant APB 7/36 was 125% higher than that obtained in the control strain (Fig. 8C). A comparison of the two *pepB*-disrupted clones indicated that in MDFA and MDFAC media there were no significant differences up to 48 h, but after that time point, strain 7/36 consistently accumulated higher thaumatin levels than strain 7/25. Since both lack aspergillopepsin B, it seems likely that the differences are due to alterations of other genetic loci affecting thaumatin secretion which were caused during protoplast transformation. In summary, disruption of the *pepB* gene results in a large increase of thaumatin production, particularly in media that favor aspergillopepsin B formation in the parental strain.

DISCUSSION

Four different proteases were found in culture filtrates of thaumatin-producing *A. awamori* strains. As reported in this article, one of them was identified by zymogram and immunological analysis as aspergillopepsin B. Purified aspergillopepsin B degraded thaumatin in vitro.

The cloned *A. awamori pepB* gene showed a high homology with the homologous gene of *A. niger* var. *macrosporus*. The deduced amino acid sequence of the protein correlates perfectly with those of the two subunits described previously for the *A. niger* acid proteinase A (36), an 11-amino acid spacer sequence and a 40-amino acid propeptide. At the end of the spacer sequence there is a Lys-Arg amino acid pair, suggesting that the maturation of subunits in vivo may be mediated by the KexA protease (which recognizes this pair of basic amino acids) (21).

Aspergillopepsin B showed significant homology with three other fungal proteins, scytalidopepsin B of *Scytalidium lignicolum* (26) (55% identity) and endopeptidases B and C of *Cryphonectria parasitica* (22) (42% identity). The homology was found mainly in the major subunit, indicating that the proteolytic center is located in that subunit. This hypothesis is supported by the conservation in the major subunit of a glutamic acid (position 219) at the *A. awamori* enzyme and of an aspartic acid residue (position 123) corresponding to the catalytic site of aspergillopepsin B of *A. niger* (19).

The *pepB* gene is highly expressed in media with proteins as the carbon source, in agreement with previous data for *A. niger* (23). Expression was higher at acidic pH values than at neutral ones. These results suggest that the *pepB* gene is probably controlled by the pH regulator PacC.

The aim of this work was to analyze the effect on thaumatin degradation of silencing *pepB* gene expression. A good level of antisense *pepB* RNA was obtained by using the promoter re-

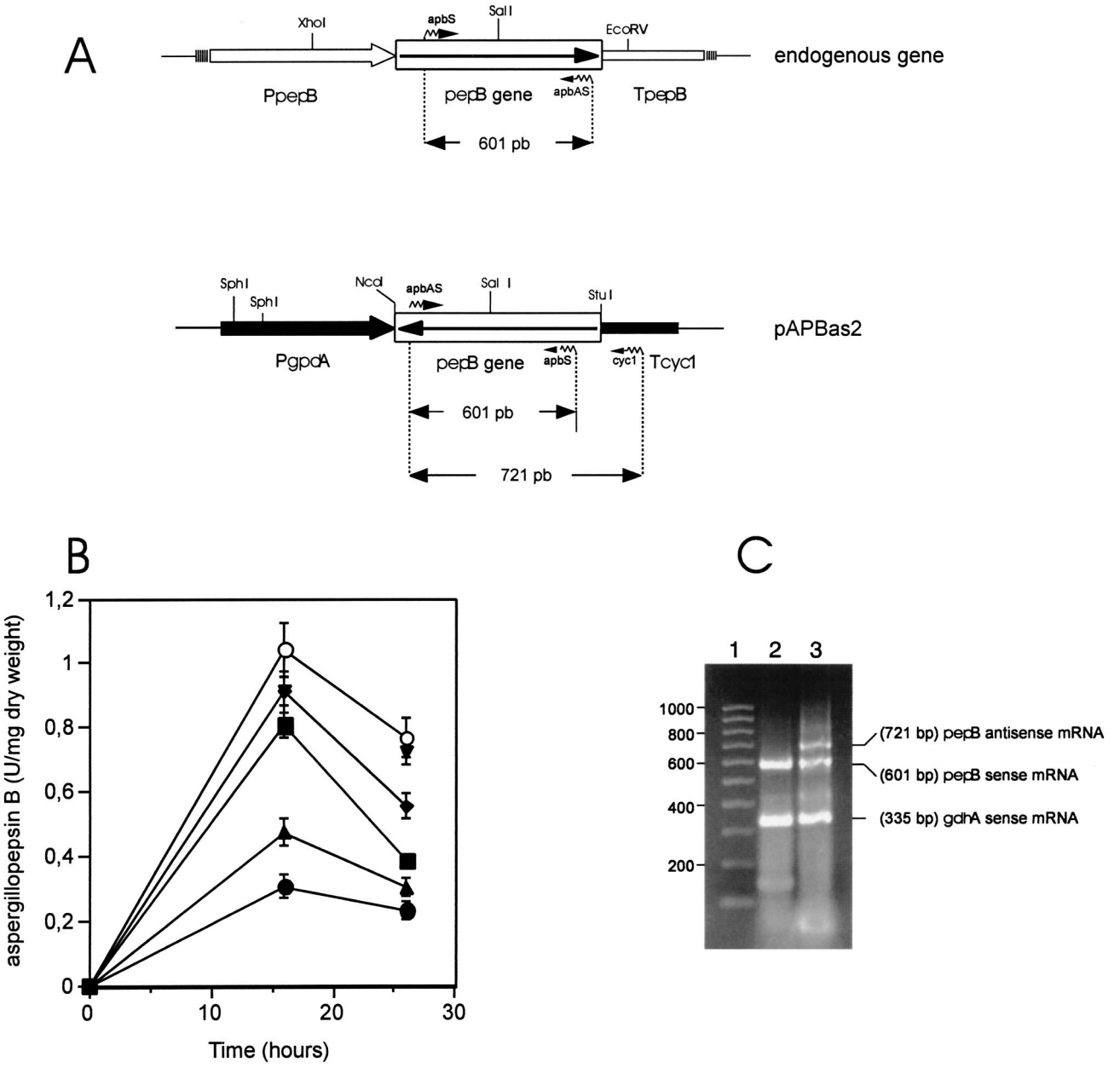

FIG. 6. Scheme of the antisense strategy developed for *pepB* gene attenuation. (A) Map of plasmid pAPBas2, containing the *pepB* gene in antisense orientation. *PgpdA* corresponds to the promoter of the glyceraldehyde-3-phosphate dehydrogenase of *A. nidulans*. This plasmid also contains the *hph* gene of *E. coli*, which confers hygromycin resistance. The apbS, apbAS, and cyc1 primers are shown by thin wavy lines with arrows. (B) Production of aspergillopepsin B (relative to the parental strain TGDTh-4) in several transformants containing the *pepB* gene antisense cassette. (○), transformant TGDTh-4; (●), asH5; (▼), asH7; (▲), asX1; (◆), asX4; (■), asX7. (C) Detection of *pepB* antisense transcript by RT-PCR analysis, using the primers apbS, apbAS, and cyc1 described above. Lane 1, molecular weight markers; lane 2, parental strain TGDTh-4; lane 3, transformant asH5. The aspergillopepsin B unit was defined with regard to proteolytic activities in Materials and Methods.

gion of the *gpdA* gene of *A. nidulans* with glucose as the carbon source (30, 32). These conditions are also adequate for low expression of the sense *pepB* gene. However, the level of antisense RNA may not have been sufficient to titrate out completely the *pepB* mRNA, even when the antisense RNA was expressed from the strong *gpdA* fungal promoter (see Fig. 6C). Expression of the antisense *pepB* led to a reduction in the presence of aspergillopepsin B ranging from 10% in transformant asX4 to 70% in transformant asH5, values similar to those obtained in *Aspergillus* with other antisense strategies (6, 24, 31, 40). However, the small increment in the increase of thaumatin production observed after antisense RNA formation indicated that the remaining aspergillopepsin B still degraded thaumatin; therefore, it was necessary to disrupt the *pepB* gene in the thaumatin-producing strain TB2b1-44-*pyrG45* to remove completely aspergillopepsin B from culture broths.

An important factor in directed integration was the presence of homologous sequences at the ends of the disruption cas-

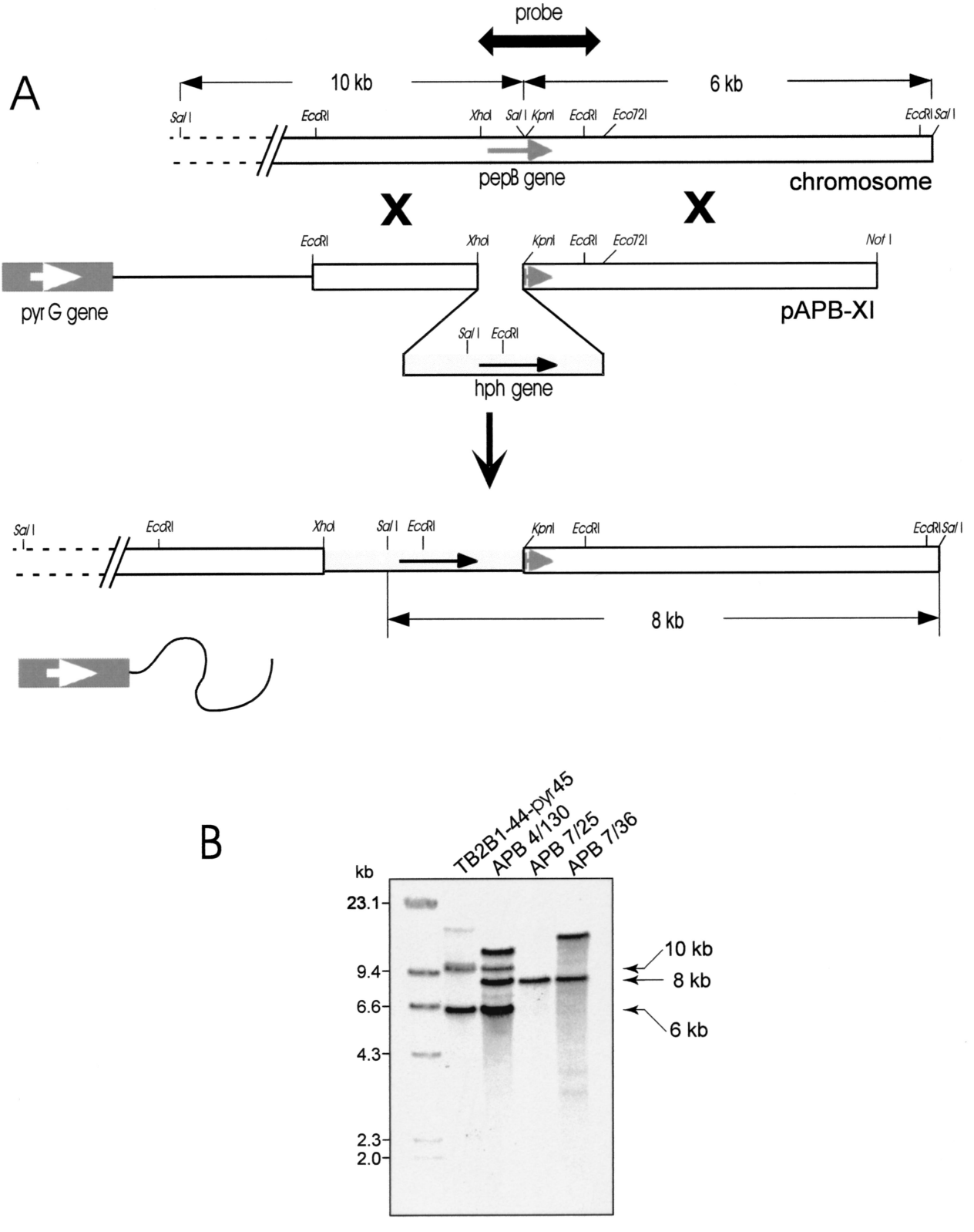

FIG. 7. (A) Disruption strategy for the *pepB* gene of *A. awamori*. The expected pattern of hybridizing fragments is indicated for the parental strain (10.0 and 6.0 kb) and for the disrupted transformants (8.0 kb) using the indicated *pepB* probe (1,050-bp *Xho*I-*Eco*RV fragment). (B) Southern analysis of *pepB* disruption of the parental strain (TB2b1-44-*pyrG45*), a transformant with ectopic integration (APB 4/130), and two disrupted transformants (APB 7/25 and APB 7/36). The transformant APB 7/36 also contained an ectopic integration of the disruption cassette.

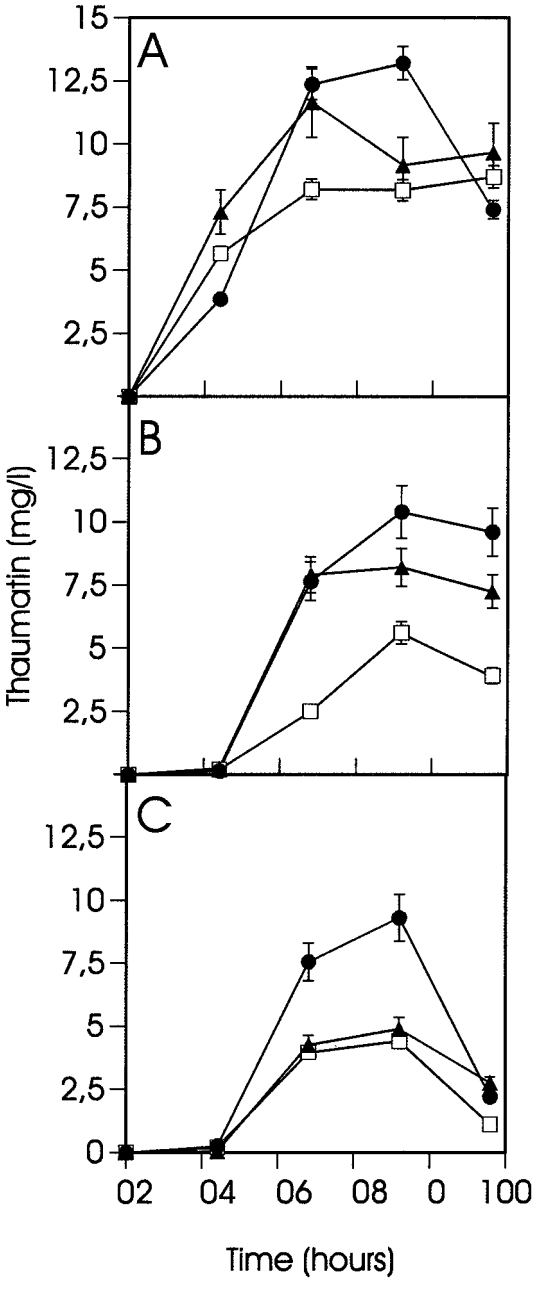

FIG. 8. Analysis of thaumatin production by transformants APB 7/25 (▲) and APB 7/36 (●), with the *pepB* gene disrupted, compared to the parental strain TB2b1-44-*pyrG45* (□). All strains were grown in MDFA medium and in modified MDFA with different carbon and nitrogen sources to induce or repress *pepB* expression (see Results). (A) Production in MDFA medium. (B) Production in MDFAC medium. (C) Production in MDFACG medium. Datum points are the averages of duplicate determinations from three separate cultures. Vertical bars indicate standard deviations of the mean values.

sette. Plasmid pAPB-XI (with a region of homology at the ends of the linearized plasmid) gave a 5.7-fold higher number of Hyg^R Ura^- transformants than plasmid pAPB-X (without homology at both ends). This result emphasizes the importance of the initial interaction between the ends of the plasmid and a homologous region in the chromosome for directed integration. The level of clean *pepB* gene replacement obtained by double crossover was 0.24% of those of the transformants, that is, significantly lower than the values obtained with similar homologous DNA fragments in *A. nidulans* and other species of *Aspergillus* (7, 8, 18, 27).

Thaumatin production in the *pepB*-disrupted transformant APB 7/36 increased between 45% and 125% with respect to the parental strain, depending on the culture medium. The largest increase was observed in MDFACG medium, where the level of expression of the *pepB* gene in the parental strain was higher, confirming the effect of aspergillopepsin B on thaumatin degradation in vivo.

The relative contribution of aspergillopepsin B to the total proteolytic activity of the wild-type *A. awamori* can be inferred from the results of the *pepB* disruption and amplification studies. The introduction of multiple copies of the *pepB* gene in wild-type *A. awamori* resulted in a significant increase in the presence of aspergillopepsin B, but this increase was not proportional to the copy number introduced (data not shown). Our data indicate that a fivefold increment of aspergillopepsin B results in a threefold increment of total proteolytic activity, indicating that the presence of aspergillopepsin B is responsible for at least 30 to 50% of extracellular proteolytic activity.

In summary, removal of proteolytic degradation due to the presence of aspergillopepsin B is a useful method for increasing thaumatin production in *A. awamori*. This strategy, in association with the removal of aspergillopepsin A by classical mutagenesis (29) and the physiological optimization of culture medium, has resulted in a large increment of increase in thaumatin production.

However, residual proteolytic activities (other than those of aspergillopepsin A and aspergillopepsin B) remain in the *pepB*-disrupted strain. The roles of these proteases and their contribution to thaumatin degradation are unclear at this time. Some of them might be intracellular proteolytic activities required for adequate protein processing and thus might be indispensable.

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