Modulation of *Aspergillus awamori* Thaumatin Secretion by Modification of *bipA* Gene Expression

Marta Lombraña,[1] Francisco J. Moralejo,[1] Rosa Pinto,[1] and Juan F. Martín[1,2]*

Instituto de Biotecnología de León (INBIOTEC)[1] and Área de Microbiología, Facultad de Ciencias Biológicas y Ambientales, Universidad de León,[2] León, Spain

Two different strains, *Aspergillus awamori* TGDTh-4 and *A. awamori* TGP-3 overexpressing a synthetic gene encoding the plant sweet protein thaumatin, showed an unfolded protein response. To facilitate protein secretion, the chaperone BiPA gene was expressed in *A. awamori* under control of the strong constitutive promoter of the *gpdA* gene. A good correlation was observed between the level of the *bipA* transcript in different strains and the amount of thaumatin secreted. Thaumatin secretion was increased 2- to 2.5-fold in transformants overexpressing the *bipA* gene compared with the parental strain. Secretion of the homologous proteins α-amylase and glucoamylase was not affected by the *bipA* gene overexpression. The requirement for BiPA for secretion of thaumatin was confirmed by attenuation of the endogenous *bipA* gene expression with an antisense RNA cassette. The decrease in *bipA* expression reduced the amount of secreted thaumatin up to 80% without affecting the secretion of the homologous α-amylase and glucoamylase proteins. The BiPA protein is, therefore, very important for secretion of some heterologous proteins, such as thaumatin in *A. awamori*.

One of the main bottlenecks in the production of heterologous proteins in filamentous fungi (11) is the secretion of the proteins. Protein traffic through the secretory pathway depends upon the levels of chaperones and foldases that play an important role in protein folding during secretion (1, 5). It has been observed previously that bottlenecks in the secretory pathway of *Aspergillus awamori* result in partial intracellular retention of heterologous proteins (4, 18). However, the molecular mechanism underlying these bottlenecks remains largely unknown.

The BiPA protein is a molecular chaperone that contains an HDEL retention signal and is present in the endoplasmic reticulum (2, 24). BiPA plays an important role in the protein traffic process by binding to the newly synthesized polypeptides and promoting their proper folding. It also binds to aberrant proteins, preventing them from leaving the endoplasmic reticulum to continue through the secretory pathway (26). Indeed, the BiPA protein appears to have a quality control function, discriminating between properly folded proteins to be exported (secretion competent) and inadequately folded proteins (12). The *bipA* gene of *A. awamori* was cloned (14) in order to study its role in protein secretion.

Thaumatin is an extremely sweet protein derived from the tropical plant *Thaumatococcus daniellii* (40). Its large-scale production is of great interest because of its use as a human food sweetener and as an animal feed supplement (8). The 22-kDa thaumatin protein contains eight disulfide bonds, and proper folding is required for the sweet taste. A synthetic thaumatin gene (*tha*) has been expressed previously in *A. awamori* (18, 19), but part of the thaumatin is retained intracellularly, indicating that it does not proceed adequately through the protein secretory pathway. Overexpression of heterologous proteins in filamentous fungi frequently results in an unfolded protein response (UPR) that leads to increased synthesis of chaperones (23, 36).

It has been observed previously that a defined level of the foldase protein disulfide isomerase (PDI) is required for optimal secretion of thaumatin by *A. awamori* (21). However, an excess of PDI in the cell reduced thaumatin production. It is likely that foldases and other chaperones have distinct roles in folding and secretion of different proteins.

In order to understand the role of BiPA in homologous and heterologous protein secretion, it was of interest to study the effect of different BiPA levels in *A. awamori*. These different levels could be obtained by overexpression and attenuation of the *bipA* gene.

In this paper we show that overexpression of the *bipA* gene in *A. awamori* causes an increase in the secreted levels of the heterologous protein thaumatin and that there is an optimal level of *bipA* expression, above which the secreted levels of thaumatin decrease. On the other hand, attenuation of the *bipA* gene causes a decrease in the secreted levels. Homologous proteins, such as α-amylase and glucoamylase, are not affected in any case.

MATERIALS AND METHODS

Strains. *A. awamori* TGDTh-4, containing the *tha* gene under control of the nitrogen-regulated *gdhA* (glutamate dehydrogenase) promoter (18), was used as a host strain for *bipA* gene overexpression studies. *A. awamori* strain TGP-3, which produces thaumatin under control of the constitutive *gpdA* (glyceraldehyde-3-phosphate dehydrogenase) promoter, was used as a host strain for *bipA* gene downregulation studies. Both strains were derived from the mutant *A. awamori lpr*66, which is defective in aspergillopepsin A (19). *Escherichia coli* DH5α served as a host for plasmid amplification and purification.

Media and growth conditions. All *A. awamori* strains were grown on solid Power sporulation medium (9) at 30°C for 3 days. For isolation of total DNA, *A. awamori* spores were inoculated into CM medium (5 g of malt extract per liter, 5 g of yeast extract per liter, 5 g of glucose per liter), and the cultures were grown at 30°C for 48 h in a rotary shaker at 300 rpm.

For thaumatin, α-amylase, or glucoamylase production studies, *A. awamori* seed cultures in CM medium were used to inoculate (18%, vol/vol) 100 ml of

* Corresponding author. Mailing address: Área de Microbiología, Faculad de Ciencias Biológicas y Ambientales, Universidad de León, Campus de Vegazana s/n, 24071 León, Spain. Phone: 34 987 291 505. Fax: 34 987 291 506. E-mail: degjmm@unileon.es.
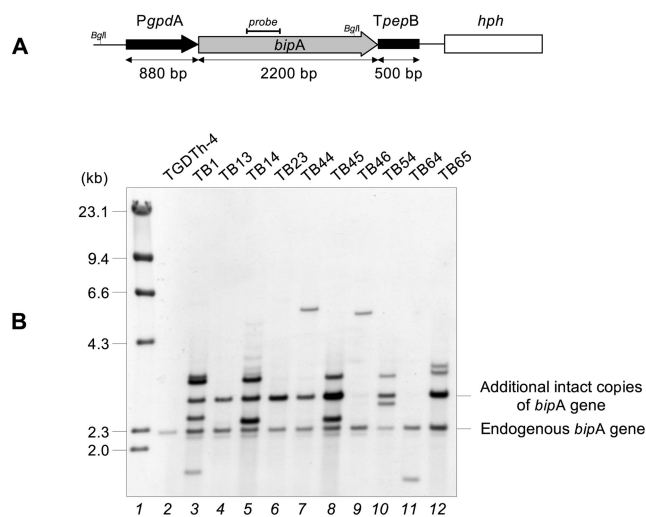
FIG. 1. Overexpression cassette of the *bipA* gene of *A. awamori* and Southern blot analysis of its expression. (A) The *bipA* gene (gray arrow) is under control of the *A. nidulans gpd* promoter (P*gpd*A) (black arrow) and the *A. awamori* aspergillopepsin B transcriptional terminator (T*pep*B) (black box). The *hph* gene (open box) from *E. coli* was used as a selectable marker. (B) Total DNA was digested with BglI and hybridized with a 400-bp NcoI fragment close to the 5′ end of the *bipA* gene as a probe (solid line in panel A). Lane 1, size markers (λ HindIII fragments); lane 2, control strain TGDTh-4; lanes 3 to 12, *A. awamori* transformants expressing the *bipA* gene.

MDFA medium (18, 19) in 500-ml flasks and grown at 30°C for 60 h in a rotary shaker at 300 rpm. Culture samples for transcriptional analysis were collected after 48 h of incubation in MDFA medium.

Plasmids and plasmid construction. Plasmid pGpd-BiP-H (containing the expression cassette shown in Fig. 1A) was used for overexpression of the *bipA* gene. This plasmid contains the *A. awamori bipA* gene under control of the *Aspergillus nidulans gpdA* promoter (P*gpd*A) and the *pepB* gene transcriptional terminator of *A. awamori* (T*pep*B). The hygromycin resistance gene (*hph*) from *E. coli* (under control of the *A. nidulans gpdA* promoter [P*gpd*A] and the *trpC* gene transcriptional terminator [T*trp*C] of the same organism) was used as a selectable marker. A 2.2-kb fragment of genomic DNA corresponding to the open reading frame of the *bipA* gene was amplified from plasmid pB4S5-SK+ (14) by PCR by using the following oligonucleotides as primers: ObipA (5′-TT TTCCATGGCGCGCATCTCGCATCAG-3′), which corresponded to positions 1 to 21 of the *bipA* gene and introduced an NcoI site into its 5′ end; and ObipB (5′-TTACAGTTCATCATGCCCGCT-3′), which corresponded to positions 2168 to 2189 of the gene.

The PCR-amplified fragment was digested with the restriction enzymes StuI and NcoI and was cloned in plasmid pIBFM4 (20). The plasmid obtained, designated pGpd-BiP, was digested with the ApaI and XbaI restriction enzymes to obtain the *bipA* overexpression cassette, which was subcloned in vector pAN7-1Z (20) carrying the hygromycin resistance gene to obtain plasmid pGpd-BiP-H.

For the *bipA* gene attenuation studies, plasmid pGdhBiPAS3 (containing the antisense expression cassette shown below [see Fig. 5A]) was constructed. This plasmid contained a fragment of the *bipA* gene of *A. awamori* in the antisense position under control of the *A. awamori* glutamate dehydrogenase promoter (P*gdh*A) and the transcriptional terminator region of the *cyc1* gene of *Saccharomyces cerevisiae*. The hygromycin resistance cassette was used as a selectable marker.

The *bipA* gene used in the antisense construction included a 1,526-bp fragment of cDNA corresponding to the *bipA* gene except for the first 490 bp of the 5′ end. This fragment was amplified by PCR by using plasmid pMar (which contained the *bipA* gene in a pBluescript vector [Lombraña and Martín, unpublished data]) as the template and oligonucleotides ObipAS1 (5′-GTTTCCGCCCGGGGTTCT TGGT-3′), corresponding to positions 605 to 626 of the *bipA* gene, and ObipAS2 (5′-CAGTTCACCATGGCCGCTGG-3′), corresponding to positions 2166 to 2186 of the gene, as primers. The latter primer introduced an NcoI site into the 3′ end of the amplified fragment.

The cloned PCR product was used to replace an NcoI-StuI fragment containing the *ble* gene of plasmid pIBRC43 (3) to create pGdhBiPAS2, which carried the *bipA* gene in the antisense position. The antisense cassette was subcloned from pGdhBiPAS2 into pAN71 (27) (digested with Eco147I-XhoI) to obtain pGdhBiPAS3.

Transformation of *A. awamori*. Protoplasts of *A. awamori* strains were transformed by the polyethylene glycol method (41). Hygromycin-resistant transformants were selected in tryptic soy agar containing 1 M sorbitol as an osmotic stabilizer supplemented with 200 μg of hygromycin per ml.

DNA purification and Southern hybridization. Extraction and purification of total *A. awamori* DNA were performed as described previously (18, 19).

Southern analysis was performed by standard techniques (33). To analyze transformants overexpressing the *bipA* gene, a 400-bp NcoI fragment close to the amino-terminal end of *bipA* was used as a probe, and the total DNA of the different transformant and control strains were digested with BglI (Fig. 1A). Transformants containing the antisense *bipA* gene were probed with a 1.1-kb NcoI fragment of the *bipA* antisense construct; total DNA was digested with NcoI (see Fig. 5A). All probes were labeled with digoxigenin by the random priming method by following the instructions of the manufacturer (Roche Diagnostics GmbH, Mannheim, Germany).

The nylon filter was hybridized overnight at 42°C in a buffer containing 40% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sarcosine, 0.02% sodium dodecyl sulfate (SDS), and 2% blocking reagent. The filter was washed once with 2× SSC–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 for 15 min. Digoxigenin detection was performed as described by the manufacturer (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, United Kingdom).

RNA isolation and purification. For RNA isolation, mycelia of *A. awamori* strains, frozen in liquid nitrogen, were ground to a fine powder in a mortar. The frozen powdered mycelium (50 mg for each sample) was mixed with 1.3 ml of EFA buffer (1 volume of extraction buffer, 1 volume of phenol, and 7 μl of β-mercaptoethanol per ml) and mixed for 1 min with a vortex mixer. The extraction buffer contained 4 M guanidinium isothiocyanate and 0.5% (wt/vol) *N*-laurylsarcosine in 25 mM sodium citrate buffer (pH 7.0). The mixture was centrifuged at 16,000 × *g* for 5 min at 4°C. The supernatant was collected, and 130 μl of chloroform-isoamyl alcohol (24:1) was added to each tube. After shaking, the tubes were centrifuged again at 16,000 × *g* at 4°C for 5 min. The upper layer was collected and extracted with 1 volume of phenol-chloroform and then with 1 volume of chloroform.

The RNA was precipitated with 0.75 volume of 8 M LiCl for 30 min at 4°C, and the precipitate was collected by centrifugation at 16,000 × *g* and 4°C for 15 min. After two washes with 70% (vol/vol) ethanol, the RNA was redissolved in water treated with diethyl pyrocarbonate. The mRNA was purified from the total RNA preparation by using an Oligotex mRNA kit (QIAGEN, Crawley, United Kingdom).

Northern analysis. Purified mRNA (5 μg) was electrophoresed on a 1.2% (wt/vol) agarose formaldehyde gel at 87 V for 4 h. The gel was blotted onto a nylon filter (Zeta probe GT membrane; Bio-Rad, Richmond, Calif.) by standard methods, and the RNA was fixed by UV irradiation with a UV-Stratalinker 2400 lamp (Stratagene, La Jolla, Calif.). The filter was prehybridized for 4 h at 50°C in high-SDS buffer (7% SDS, 50% formamide, 5× SSC, 2% blocking reagent, 50 mM sodium phosphate [pH 7.0], 0.1% *N*-laurylsarcosine) and then hybridized at 50°C for 14 h in the same buffer containing the labeled probe. A 1.9-kb BamHI fragment corresponding to the complete cDNA of the *bipA* gene was used as the probe. A 0.83-kb NcoI-KpnI fragment of the *A. nidulans* β-actin gene was used as the probe in control hybridizations.

After hybridization, the filters were washed twice in 2× SSC–0.1% SDS for 30 min, once in 1× SSC–0.1% SDS for 30 min, and once in 0.2× SSC–0.1% SDS at 55°C for 45 min.

RT-PCR amplification. To detect antisense transcripts of the *bipA* gene, reverse transcription (RT)-PCR was performed with the Superscript One Step system (Invitrogen, Carlsbad, Calif.). Purified mRNA of transformants and control strain TGP-3 grown in MDFA medium for 48 h were used as templates. The primers used are shown in Table 1. The program used for cDNA amplification included a reverse transcription step at 45°C for 40 min and 94°C for 2 min, followed by a PCR consisting of 40 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 1.4 min and a final extension step at 72°C for 10 min.

Extraction of intracellular proteins. To isolate the intracellular proteins, washed mycelia of *A. awamori* strains were frozen in liquid nitrogen and ground in a mortar. The frozen powdered mycelium (100 mg for each sample) was mixed with extraction buffer (625 mM Tris buffer [pH 6.5] containing 1 mM dithiothre-

TABLE 1. Primers used for amplification of sense and antisense *bipA* RNA

Fragment amplified	Oligonucleotide	Sequence	Position in gene
bipA transcript (986 bp)	ORNABiPAS1	5′-TTCCGCCATGGTTCTTG-3′	608–621
	ORNABiPAS2	5′-TTGGTCAGGGAACGCTCA-3′	1631–1648
Antisense *bipA* RNA	ORNABiPAS1	5′-TTCCGCCATGGTTCTTG-3′	608–621
	Op*gdh*[a]	5′-TTCCTGTTTTCCTAGATACCCCA-3′	

[a] The Op*gdh* oligonucleotide corresponds to the nucleotide sequence between the CTATA and CAAT boxes of the *gdhA* promoter.

itol, 1 μM phenylmethylsulfonyl fluoride, and 8 μl protease inhibitor cocktail [Sigma, St. Louis, Mo.] per ml).

Proteases in the protein mixtures were inactivated at 100°C for 15 min (to avoid proteolytic processing of BiPA) and then were centrifuged at 16,000 × *g* and 4°C for 20 min. The supernatants containing the intracellular proteins were collected and kept at −20°C until they were used.

Immunoblot analysis of *A. awamori* culture supernatants and intracellular proteins. After electrophoresis of proteins on SDS-polyacrylamide gels, the proteins were transferred to a polyvinylidene difluoride membrane (Inmobilon-P; Millipore Corp. Bedford, Mass.) by using a Minitransblot electroblotting system (Bio-Rad). The membranes were treated with (i) anti-thaumatin polyclonal antibodies (serum dilution, 1:15,000), (ii) anti-glucoamylase polyclonal antibodies (serum dilution, 1:30,000), (iii) anti-PDI polyclonal antibodies (serum dilution, 1:10,000), or (iv) anti-BiP polyclonal antibodies (serum dilution, 1:100,000) in 50 mM Tris-HCl (pH 8.0)–150 mM sodium chloride containing Tween 20 at a concentration of 0.2% (vol/vol) for 1 h and then for 30 min with anti-rabbit alkaline phosphatase conjugate (1:10,000; Sigma) in the same buffer. The membranes were treated with a BCIP (5-bromo-4-chloro-3-indolylphosphate)—nitroblue tetrazolium substrate for alkaline phosphatase until the color was developed.

Anti-PDI and anti-glucoamylase antibodies were provided by D. Archer and D. Jeenes. Anti-BiP and anti-thaumatin antibodies were developed in our laboratory.

Quantification of α-amylase activity and thaumatin in *A. awamori* culture supernatants. α-Amylase activity was measured by the method of García-González et al. (10). One unit of activity was defined as the amount of enzyme that released 1 nmol of maltose per s at 37°C and pH 7.2, and the specific activity was expressed in units per milligram of protein. Thaumatin was quantified by an enzyme-linked immunosorbent assay (ELISA) method as described previously (18).

Densitometric analysis. *bipA* expression levels and thaumatin secretion levels were measured by densitometry of the secreted thaumatin and *bipA* gene expression signals by using the software Science Lab 2003 (Fuji Photo Film Co., Ltd., Tokyo, Japan).

RESULTS

Overexpression of the *bipA* gene in *A. awamori*. To test the effect of the BiPA protein on secretion of heterologous and homologous proteins in *A. awamori*, an expression cassette containing the *bipA* gene under control of the constitutive promoter P*gpdA* of *A. nidulans* (Fig. 1A) was introduced into the thaumatin-producing strain *A. awamori* TGDTh-4 (18).

Ten transformants were randomly selected for Southern analysis (Fig. 1B). This analysis revealed the presence of a 2.3-kb fragment corresponding to the endogenous gene which was observed in both transformant and control strains. Another band at 3.0 kb, corresponding to the intact additional copies of the gene, was observed in most of the transformants.

The intensity of the 3.0-kb hybridization fragment in different transformants, compared with the intensity of the endogenous *bipA* gene, indicated that there was a different copy number of the gene in each transformant. Other bands corresponding to noncharacterized integration events were also observed.

The transformants that exhibited different Southern hybridization patterns were selected for transcriptional analysis of the *bipA* gene. Northern blot analysis (Fig. 2) showed that there was an increase in the *bipA* mRNA level in some of the transformants (particularly strains TB13, TB23, and TB65) compared with the *bipA* transcript levels observed in the TGDTh-4 and *lpr*66 control strains; *lpr*66 did not contain the *tha* gene (it was a nonproducing strain) (Fig. 3).

Transformant TB64, which did not contain any additional intact copies of the *bipA* gene, did not exhibit an increase in the *bipA* transcript level compared to parental strain TGDTh-4.

An interesting observation was made when we compared the levels of *bipA* mRNA in the TGDTh-4 and *lpr*66 control strains. As shown in Fig. 2A, expression of the thaumatin-encoding gene in strain TGDTh-4 resulted in levels of *bipA* mRNA that were 30% higher than those in parental strain *lpr*66, presumably due to the UPR that is known to enhance the chaperone levels in response to accumulation of unfolded exogenous proteins.

Effect of *bipA* gene overexpression on secretion of thaumatin. Transformants TB13, TB14, and TB23 (Fig. 3A) showed higher levels of secreted thaumatin than the control TGDTh-4 strain; nevertheless, transformants TB64 and TB65 showed levels that were even lower than the level in this control strain, and strain *lpr*66 showed no thaumatin signal, as expected. There was a nonlinear correlation with *bipA* gene expression intensity since these transformants showed high levels of the *bipA* transcript (Fig. 2). Transformant TB64, which did not produce the band corresponding to the intact additional copies of the *bipA* gene in the Southern analysis, showed a very faint thaumatin signal in the Western analysis. This fact could be explained by the partial or total integration of the *bipA* expression vector at the site of the thaumatin expression cassette, which could have caused a rearrangement that decreased the thaumatin production in this transformant strain. Alterna-
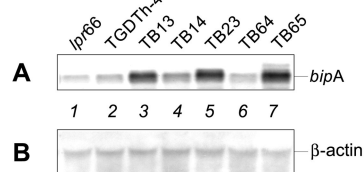

FIG. 2. (A) Northern blot analysis of transformants overexpressing the *bipA* gene. Hybridization was performed with a 1,900-bp BamHI probe of the *bipA* gene. Lane 1, control strain *lpr*66 (untransformed); lane 2, control strain TGDTh-4, containing the *tha* expression cassette; lanes 3 to 7, *A. awamori* transformants overexpressing the *bipA* gene. (B) Control Northern blot analysis of the same RNA samples performed with a 0.83-kb NcoI-KpnI fragment of the constitutive *A. nidulans* β-actin gene as the probe.
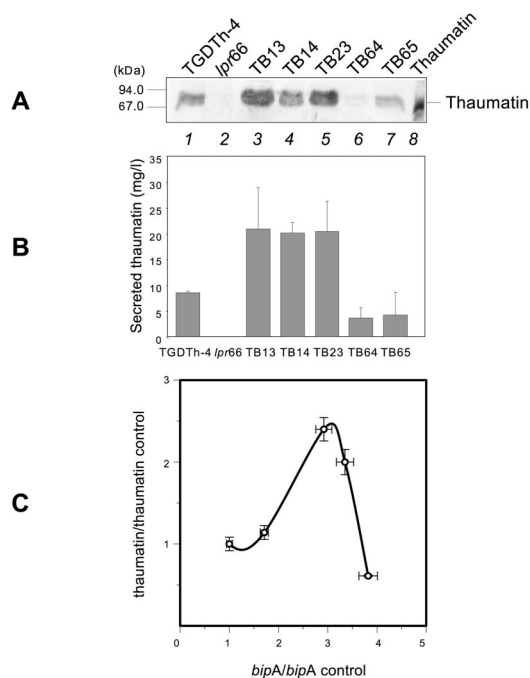
FIG. 3. Secreted thaumatin in control strains and *A. awamori* transformants overexpressing the *bipA* gene. (A) Immunoblots with anti-thaumatin antibodies (1:15,000). Lane 1, control strain TGDTh-4, containing the *tha* expression cassette; lane 2, control strain *lpr*66 (untransformed); lanes 3 to 7, *A. awamori* TB transformants containing the *bipA* overexpression cassette; lane 8, purified 22-kDa thaumatin from *T. daniellii* (50 ng). The molecular masses of size markers are indicated on the left. (B) Levels of extracellular thaumatin measured by ELISA in the *A. awamori* control strain TGDTh-4 and in transformants overexpressing the *bipA* gene. The error bars indicate standard deviations for three measurements. (C) Correlation between thaumatin secretion and *bipA* expression levels in the control strain and different *A. awamori* transformants overexpressing the *bipA* gene. The data are expressed in units relative to the control strain levels. From left to right the data points correspond to the following strains: TGDTh-4, TB14, TB13, TB23, and TB65. Note that the thaumatin secretion levels depend strongly on the *bipA* expression levels.

tively, an alteration or a disruption of the thaumatin expression cassette could have been caused by integration of the *bipA* expression vector, which also would have caused a reduction in thaumatin secretion.

Results similar to the results obtained in the Western blot analysis were observed when thaumatin was quantified by an ELISA method (Fig. 3B). Transformants TB13, TB14, and TB23 showed a maximal twofold increase in the extracellular thaumatin level compared to parental strain TGDTh-4.

The quantitative correlation between *bipA* gene expression and thaumatin secretion is shown in Fig. 3C. As described previously for other foldases (21), a defined level of *bipA* expression is required for optimal secretion of thaumatin, and lower and higher levels of *bipA* expression cause decreases in secreted thaumatin levels. Transformant TB64 did not follow the same trend as the rest of transformants, presumably because of its different integration pattern, which could have caused rearrangements or even disruption at the site of the thaumatin expression cassette, as explained above.

Intracellular BiPA and PDI protein levels. The levels of intracellular BiPA protein in transformants and control strains were analyzed with anti-BiP antibodies. Surprisingly, the BiPA protein was extremely unstable following cell disruption, and several immunoreactive bands were observed in the Western blot analysis (data not shown), suggesting that there was rapid proteolytic degradation of BiPA. Transformants TB13, TB14, and TB23 showed higher intensity of the immunoreactive bands, but, due to the degradation, it was difficult to quantify the real BiPA levels in the different strains.

In order to study whether overexpression of the *bipA* gene had any effect on the PDI chaperone, intracellular protein samples were analyzed by Western blotting with anti-PDI antibodies (data not shown). There were no differences between transformant strains and the control TGDTh-4 strain. Nevertheless, the PDI levels in strain *lpr*66 (which does not produce thaumatin) were much lower than those in the TGDTh-4 strain. These results indicate again that there was an UPR effect on PDI levels when the heterologous protein thaumatin was overexpressed in the TGDTh-4 strain.

Effect of *bipA* gene overexpression on secretion of homologous proteins. To test the effect of *bipA* gene overexpression on secretion of homologous proteins, the α-amylase activity was measured in the culture supernatants of the different *A. awamori* strains, and this protein was also identified by SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 4). No apparent differences were found in the α-amylase activity values (Fig. 4A) between transformants overexpressing the *bipA* gene and control strains or in the α-amylase protein band intensities in SDS-PAGE gels (Fig. 4B). The α-amylase band was the major protein band in *A. awamori* culture supernatants and was identified in the gels by using pure α-amylase. Similar results were obtained when the secreted glucoamylase, another homologous protein, was visualized by Western blot analysis (Fig. 4C) in control and transformant strains.

Attenuation of the *bipA* gene in *A. awamori*: some transformants show very high levels of the antisense RNA. To determine if low BiPA levels might be limiting for thaumatin secretion, downregulation of the *bipA* gene was carried out by using an antisense RNA strategy. An expression cassette containing a truncated *bipA* gene in the antisense position under control of the P*gdhA* promoter (Fig. 5A) was introduced into the thaumatin-producing strain *A. awamori* TGP-3 (18).

Southern analysis of 10 randomly isolated transformants revealed the existence of several clones with different numbers of copies of the antisense *bipA* gene (Fig. 5B). A 3.3-kb band corresponding to the endogenous *bipA* gene was found in both transformant and control strains. Another band at 1.1 kb corresponding to the intact antisense construct was observed only in the transformants. In some strains other bands corresponding to noncanonical integration events were also observed.

The six transformants that contained only intact copies of the antisense cassette were selected for further study. Northern blot analysis showed that there was a 1,596-bp signal corresponding to antisense *bipA* RNA in some of the transformants, compared to the 2,178-bp *bipA* RNA signal observed in both transformants and the TGP-3 and *lpr*66 control strains (Fig. 6). Higher levels of expression of *bipA* antisense RNA were observed in transformants BiPAS8, BiPAS9, and BiPAS16. In these transformants, the antisense transcript levels were similar to or higher than (e.g., transformants BiPAS9 and BiPAS16) those of the endogenous *bipA* gene.
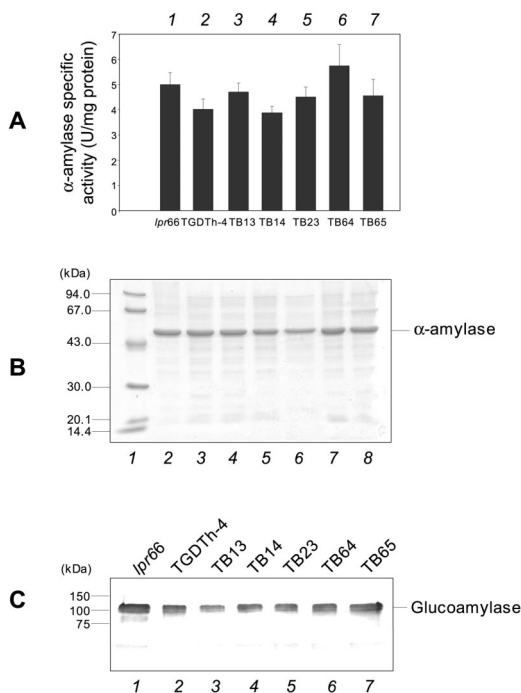
FIG. 4. Levels of homologous extracellular enzymes in *A. awamori* transformants. (A) Extracellular α-amylase activities of control strains *lpr*66 and TGDTh-4 (bars 1 and 2, respectively) and transformants overexpressing the *bipA* gene (bars 3 to 7). The error bars indicate standard deviations for three measurements. (B) SDS-PAGE of extracellular culture supernatants. Total proteins (Coomassie blue stained) are shown. Note that α-amylase was directly observed in Coomassie blue-stained gels as it was the most abundant protein. Lane 1, molecular mass markers (molecular masses are indicated on the left); lanes 2 and 3, *A. awamori* control strains *lpr*66 and TGDTh-4, respectively; lanes 4 to 8, transformants overexpressing the *bipA* gene. (C) Immunoblot of secreted glucoamylase levels obtained with anti-glucoamylase antibodies (1:30,000). Lanes 1 and 2, *A. awamori* control strains *lpr*66 and TGDTh-4, respectively; lanes 3 to 7, transformants overexpressing the *bipA* gene. The molecular masses of size markers are indicated on the left.

By comparing of the levels of *bipA* RNA in the TGP-3 and *lpr*66 control strains (Fig. 6), we concluded that overproduction of thaumatin in the TGP-3 strain produced a UPR that resulted in overexpression of the *bipA* chaperone gene in this strain (which showed levels of *bipA* expression that were 10% higher than those of the *lpr*66 control strain), as observed also in the TGDTh-4 strain (see above).

RT-PCR amplification confirmed that there were high levels of the antisense RNA. The presence of antisense RNA in transformant strains was also studied by RT-PCR (Fig. 7A). By using purified mRNA as the template, an amplified 986-bp fragment corresponding to the sense transcript of the endogenous *bipA* gene was observed in both transformant and control strains when ORNABiPAS1 and ORNABiPAS2 were used as the primers. In addition, another amplified fragment, a 1,570-bp fragment corresponding to the antisense *bipA* RNA, was observed in most of the transformants analyzed but not in the control strains (Fig. 7B). These results confirmed the functionality of the antisense construct.

***bipA* gene downregulation limits thaumatin secretion: correlation of antisense RNA levels with secreted thaumatin.** Cul-
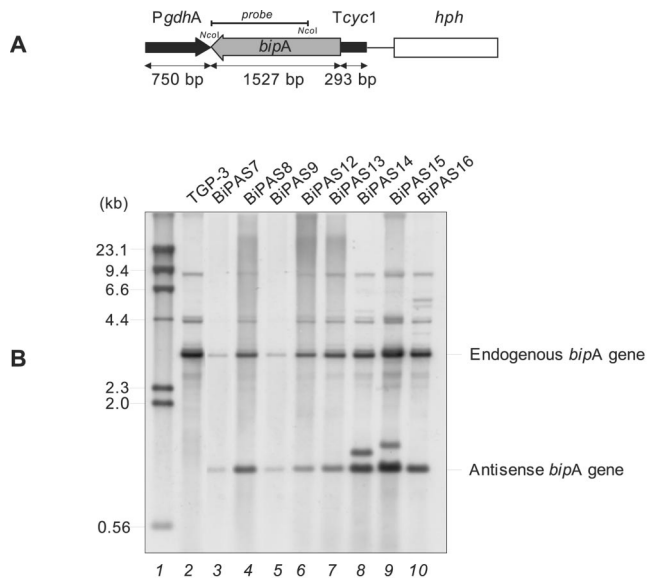

FIG. 5. Downregulation of the *bipA* gene of *A. awamori*. (A) A fragment of the *bipA* gene (gray arrow), in the antisense position, is under control of the *A. awamori gdh* promoter (P*gdhA*) (black arrow) and the *S. cerevisiae* transcriptional terminator of the *cyc1* gene (T*cyc1*) (black box). The *hph* gene (open box) from *E. coli* was used as a selectable marker. (B) Southern blot analysis of transformants containing the *bipA* antisense cassette. Total DNA was digested with NcoI and hybridized with a 1,100-bp NcoI fragment of the *bipA* gene as a probe (solid line in panel A). Lane 1, size markers (λ HindIII fragments) (sizes are indicated on the left); lane 2, *A. awamori* control strain TGP-3, containing a *tha* expression cassette; lanes 3 to 10, *A. awamori bipA* antisense transformants.

ture supernatants from transformants containing the antisense RNA cassette were analyzed with anti-thaumatin antibodies (Fig. 8A). The transformants overexpressing the antisense RNA, particularly transformants BiPAS8 and BiPAS9, showed levels of secreted thaumatin that were up to 80% lower than those of control strain TGP-3 (Fig. 8B). On the other hand, transformants BiPAS7 and BiPAS12, which produced low levels of antisense RNA or no antisense RNA, showed minor differences in thaumatin secretion compared to the control strain.
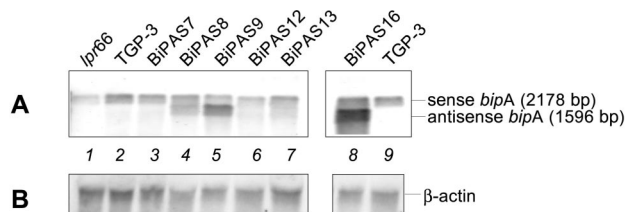

FIG. 6. (A) Northern blot analysis of transformants containing the *bipA* antisense cassette. Hybridization was performed with a 1,900-bp BamHI fragment of the *bipA* gene as the probe. Lane 1, control strain *lpr*66 (untransformed); lane 2, control strain TGP-3, containing a *tha* expression cassette; lanes 3 to 9, *A. awamori* antisense *bipA* transformants. The upper hybridizing band corresponds to the *bipA* transcript (2,178 bp). The lower band corresponds to the *bipA* antisense RNA (1,596 bp). (B) Northern blot analysis of the same samples. A 0.83-kb NcoI-KpnI fragment of the constitutive *A. nidulans* β-actin gene was used as the probe in control hybridizations.
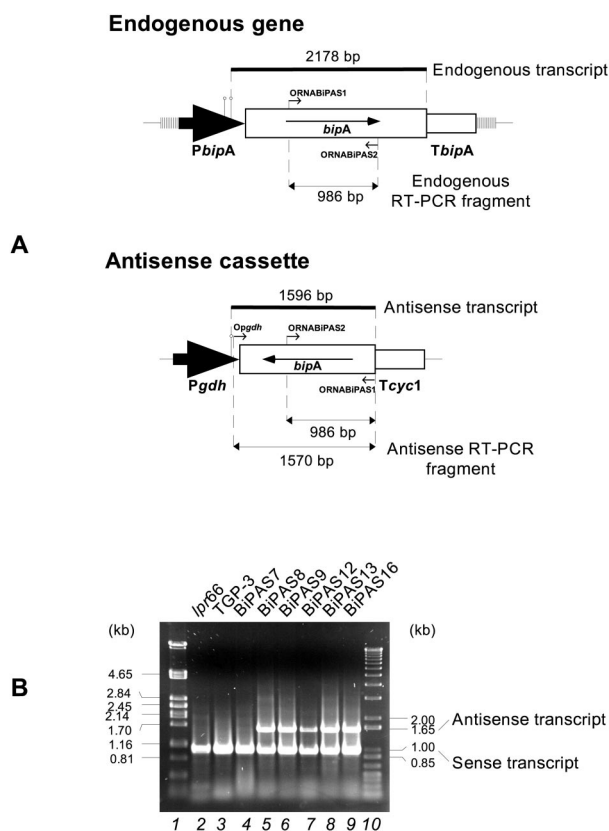
Endogenous gene

Antisense cassette

FIG. 7. RT-PCR analysis of *bipA* transcripts in transformants containing the antisense construction. (A) Diagram of the hybridizing fragments from both endogenous and antisense *bipA* transcripts used in Northern blot analysis (thick lines) and RT-PCR products expected in both cases (thin lines with an arrowhead at each end). The large black arrows represent promoters, and the open boxes with an arrow inside represent *bipA* sense and antisense genes. The empty open boxes represent terminators. The lollipop symbols indicate transcription start points. The primers used are represented by small arrows. (B) RT-PCR products from mRNA of the *lpr*66 and TGP-3 control strains (lanes 2 and 3) and antisense transformants (lanes 4 to 9). Lanes 1 and 10 contained size markers (λ PstI and 100-bp ladder). Oligonucleotides ORNABiPAS1, ORNABiPAS2, and Op*gdh* were used as the primers. The 1,570-bp band corresponds to the *bipA* sense transcript, and the 986-bp band corresponds to the *bipA* antisense mRNA.

The quantitative correlation between antisense *bipA* gene expression and thaumatin secretion (Fig. 8B) showed that an increase in *bipA* antisense levels caused a decrease in thaumatin secretion up to a certain level of antisense *bipA* gene expression, above which the secreted levels of thaumatin did not decrease further.

Effect of *bipA* gene downregulation on secretion of homologous proteins. No apparent differences between antisense transformants and control strains were found when the α-amylase activity was quantified (Fig. 9A) or the protein was observed directly in SDS-PAGE gels (Fig. 9B). The levels of glucoamylase, as analyzed by Western blotting, were not different in the transformants and the control strains (Fig. 9C).

In summary, a defined level of *bipA* gene expression is required for optimal thaumatin secretion, whereas reduction of *bipA* gene expression by antisense RNA decreased thaumatin

secretion. It is interesting that this clear effect on thaumatin production was not observed with the homologous proteins α-amylase and glucoamylase (see Discussion).

DISCUSSION

In filamentous fungi, as in other organism, the BiPA-encoding gene has a basal expression level under normal growth conditions, and it is overexpressed in cellular stress situations, such as glucose starvation, heat shock, and conditions that trigger the UPR (25, 35, 38).

We observed in this work the existence of a clear UPR in *A. awamori* (increased *bipA* mRNA levels) following expression of the thaumatin gene (Fig. 2A and 6A). Increases in *bipA* transcript levels due to overexpression of a recombinant protein have been observed in various yeast and *Aspergillus* strains (23, 28, 32, 36). The same UPR phenomenon was also observed when we measured another chaperone, PDI, in the same thaumatin-overproducing strains.

The main conclusion of this work is that a defined level of *bipA* gene expression is required for optimal thaumatin secretion. When this level is reached, the secreted levels of thaumatin increase up to 2.5-fold.

Conversely, downregulation of the *bipA* gene by antisense RNA in *A. awamori* causes a large decrease (up to 80%) in the secreted levels of this heterologous protein.

Disruption of the *bipA* gene seems to be lethal for most organisms (17, 31). Therefore, attenuation of *bipA* expression by antisense RNA was successful. Decreases in the protein
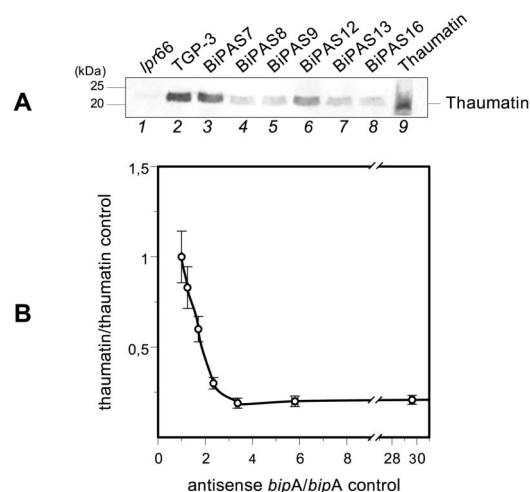

FIG. 8. (A) Immunoblots of secreted thaumatin in control strains and *A. awamori* antisense *bipA* transformants as revealed by anti-thaumatin antibodies (1:15,000). Lanes 1 and 2, *A. awamori* control strains *lpr*66 (untransformed) and TGP-3 (containing a *tha* expression cassette), respectively; lanes 3 to 8, *A. awamori* BiPAS transformants containing the *bipA* antisense cassette; lane 9, purified 22-kDa thaumatin from *T. daniellii* (50 ng). The molecular masses of markers are indicated on the left. (B) Correlation between thaumatin secretion and antisense *bipA* expression levels of the control strain and the different *A. awamori* transformants containing the antisense *bipA* cassette. The data are expressed in units relative to the control strain levels. From left to right the data points correspond to the following transformants: TGP3, BiPAS7, BiPAS12, BiPAS13, BiPAS8, BiPAS9, and BiPAS16.
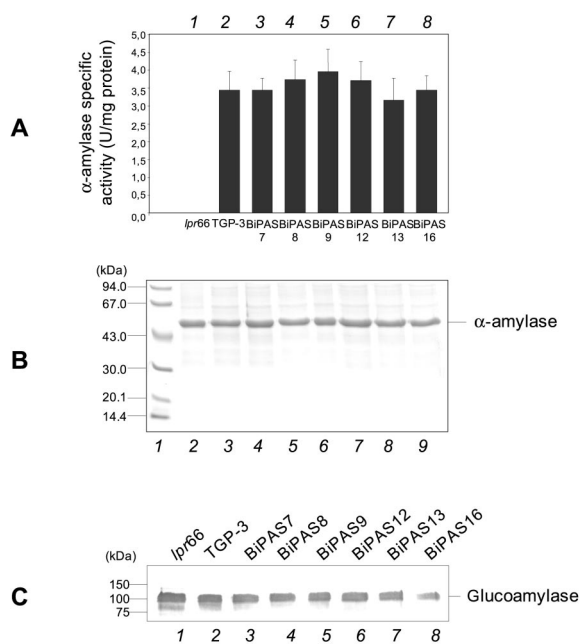
FIG. 9. Secreted homologous proteins α-amylase and glucoamylase in transformants containing the antisense cassette. (A) Extracellular α-amylase activity of *A. awamori* control strains *lpr*66 and TGP3 (bars 1 and 2, respectively) and transformants containing the *bipA* antisense cassette (bars 3 to 9). The error bars indicate standard deviations for three measurements. (B) SDS-PAGE of extracellular total proteins. Note that α-amylase is directly observed in Coomassie blue-stained gels as it is the most abundant protein. Lane 1, molecular mass markers; lanes 2 and 3, *A. awamori* control strains *lpr*66 and TGP-3, respectively; lanes 4 to 9, transformants containing *bipA* antisense cassette. (C) Immunoblots of secreted glucoamylase with anti-glucoamylase antibodies (1:30,000). Lanes 1 and 2, *A. awamori* control strains *lpr*66 and TGP-3, respectively; lanes 3 to 7, transformants containing the *bipA* antisense cassette. The molecular masses of markers are indicated on the left.

secretion level in different organisms were also observed by other authors when the *bipA* gene was downregulated (30).

It is interesting that the secreted levels of homologous proteins, such as α-amylase and glucoamylase, do not seem to be affected by these two strategies. These proteins, which are abundantly secreted by *A. awamori*, appear to have a lower chaperone requirement than the exogenous protein thaumatin. Presumably, secretion of the homologous α-amylase and glucoamylase proteins by *A. awamori* has adapted to a lower chaperone requirement, whereas plant thaumatin secretion is much more dependent on the BiPA (this study) and PDI (21) levels.

Increases in the secreted levels of other proteins due to overexpression of the *bipA* gene have been observed in the yeast *S. cerevisiae* (13, 16, 34). In other cases, no effect on secretion of specific proteins has been observed (6, 7, 15, 17, 30), and even decreases in the secreted levels of the proteins studied have been reported (6, 7, 37).

These different effects on protein secretion caused by overexpression or downregulation of the *bipA* gene could be due to (i) the multiple cellular functions in which the chaperone BiPA is involved; (ii) the importance of each function in a particular translocation process; and (iii) the different nature of the se-

creted proteins studied (5). It has also been reported that BiPA is not required for secretion of some specific proteins because they do not interact with BiPA upon transport through the endoplasmic reticulum (22).

Other chaperones, including the foldase PDI (21, 39), may also have a stimulatory effect on heterologous protein secretion (29); the effect of BiPA on thaumatin secretion may increase the effect reported for other chaperones. The increase in the amount of thaumatin produced in *bipA*-overexpressing transformants might be useful for development of strains of industrial interest.

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