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Expression of Nutritionally Well-balanced Protein, AmA1, in *Saccharomyces cerevisiae*

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Abstract Food yeast, *Saccharomyces cerevisiae*, is a safe organism with a long history of use for the production of biomass rich in high quality proteins and vitamins. AmA1, a seed storage albumin from *Amaranthus hypochondriacus*, has a well-balanced amino acid composition and high levels of essential amino acids and offers the possibility of further improving food and animal feed additives. In order to find an effective means of expressing *AmA1* in yeast, the gene was cloned into an episomal shuttle vector. Four different promoters were tested: the glyceraldehyde-3-phosphate dehydrogenase promoter, galactose dehydrogenase 10 promoter, alcohol dehydrogenase II promoter, and a hybrid ADH2-GPD promoter. The recombinant *AmA1* genes were then introduced into the yeast *Saccharomyces cerevisiae* 2805. Northern and Western blot analyses of the yeast under appropriate conditions revealed that *AmA1* was expressed by all four promoters at varying levels. An enzyme-linked immunosorbent assay demonstrated that the amount of AmA1 protein in the recombinant yeast was 1.3-4.3% of the total soluble proteins. The highest expression level was obtained from the hybrid ADH2-GPD promoter.

Keywords: AmA1, *Amaranthus hypochondriacus*, essential amino acid, yeast hybrid promoter

INTRODUCTION

Saccharomyces cerevisiae is a GRAS (generally recognized as safe) organism and has been used for many years as a food supplement and animal feed additive. Moreover, increasing the nutritional quality of the yeast that is added to food and feed is a recognized way of improving nutritional quality. One way of improving the nutritional quality of yeast is to use amino acid overproduction mutants, and yeast mutants, which accumulate up to 150 times as much methionine, 37 times as much threonine [1], and 3 to 17 times as much lysine, as the wild type have been reported [2]. Another approach involves introducing a heterologous gene encoding a protein with a high nutritional value. Genes encoding seed storage proteins, which are proteins consumed as nutrients during germination or the early stages of seedling growth, are good candidates. However, many storage proteins are deficient in at least one of the essential amino acids [3].

The grain amaranthus (*Amaranthus hypochondriacus*) is a pseudo-cereal with a high protein content, 17-19% of the seed dry weight, while most other grains have an average of about 10% protein [4]. The seed storage protein of amaranthus (molecular weight 35 kDa) is rich in essential amino acids, including lysine, tryptophan, and sulfur-containing amino acids [5]. Brazil nut 2S albumins, which also contain high levels of methionine and cysteine, have already been introduced into plants to improve their nutritional quality, however, these proteins are highly allergenic [6]. Animal feeding trials with a rat population eating the seed grain of *A. hypochondriacus* have shown that this grain is a suitable animal feed [7]. In addition, a hypersensitivity test in mice also showed that the AmA1 protein does not evoke any IgE response, thereby demonstrating that the protein is not allergenic [8]. Its full-length cDNA clone, *AmA1*, has already been cloned and characterized [9], and its deduced amino acid sequence shows a high proportion of essential amino acids, such as lysine, leucine, threonine, phenylalanine, valine, and sulfur-containing amino acids. In addition, its amino acid composition closely matches the values recommended by the World Health Organization [5]. Accordingly, the *AmA1* gene is a candidate that could be used to produce a protein with a high nutritional value.

In this study we used RT-PCR to clone the full-length cDNA of the 35-kDa seed storage protein from *A. hypochondriacus*. The gene was then introduced into *S. cerevisiae* 2805 using episomal shuttle vectors under the control of various promoters. The expression of *AmA1* was demonstrated by Northern and Western blot analyses, and the amount of protein expressed was measured by ELISA.

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MATERIALS AND METHODS

Total RNA Isolation and RT-PCR

The seeds of *A. hypochondriacus* were obtained from India. Early developing seeds of *A. hypochondriacus* were harvested and ground to a fine powder and the RNA from the homogenate was extracted according to the procedure described previously [10]. The first-strand cDNA was synthesized using a First-Strand cDNA synthesis kit according to the manufacturer's recommendations (Amersham Pharmacia Biotech, Piscataway, USA). RT-PCR was performed with primers p23 (5'-AGGAGCTCAAATGGCGGATT-3') and p24 (5'-TATAATTCTAACAATTATTTT-3') specific for the 5' and 3' ends of *AmA1* cDNA. The RT-PCR product was cloned into the *Sma*I site of pUC18 using a Sure-Clone ligation kit (Amersham Pharmacia Biotech, Piscataway, USA). The cloned *AmA1* was sequenced using a Sequenase version 2.0 DNA sequencing kit (USB, Cleveland, USA).

Expression of *AmA1* in *E. coli* and Antiserum Preparation

The *AmA1* gene was subcloned into a pRSET vector, which contains a six-histidine tag to facilitate the purification of the fusion protein. The resulting recombinant vector was transformed into *E. coli* BL21 and the transformed-*E. coli* was cultured to an optical density of 0.6 at 600 nm. The expression of the *AmA1* gene was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 2 mM. The induced culture was further incubated for 3 h and the AmA1 protein was purified using Ni-NTA resin. The samples were analyzed on a 10% SDS-PAGE. A polyclonal antibody against the purified AmA1 protein was raised in mouse ascites [11].

Construction of Recombinant Vectors and Yeast Transformation

All DNA manipulations were accomplished according to Sambrook *et al.* [12]. An episomal shuttle vector, yEP352 [13], was used to construct the yeast expression vectors. Four recombinant plasmids were made, placing the *AmA1* gene under the control of each of the following promoters: glyceraldehyde-3-phosphate dehydrogenase (GPD) [14], galactose dehydrogenase 10 (GAL10) [15], alcohol dehydrogenase II (ADH2) [16], and a hybrid ADH2-GPD promoter [17] (Fig. 2). Galactose-1-phosphate uridyl transferase (GAL7) terminator was used as the transcription termination signal. The resulting vectors were named, pGPDA, pGALA, pADHA, and pHYBA, respectively. The recombinant vectors were then introduced into *S. cerevisiae* 2805 (Mat α, pep4: His3, prb1-δ, Can1, Gal2, his3, ura3-52) using the lithium acetate based procedure [18]. The yeast was maintained in a YEPD medium (1% yeast extract, 2% peptone and 2% dextrose), and the transformants were screened on an uracil deficient selective medium (0.67% yeast nitrogen base without amino acids, 0.003% adenine and tryptophan, 0.5% casamino acid, 2% dextrose and 2% agar) at 30°C.

Northern Blot Analysis

The recombinant yeast cells were broken using glass beads, as described by Chomczynski and Sacchi [10]. The cells were then vortexed for 30 s and placed on ice for 30 s, three times, and the glass beads and cell debris were removed by centrifugation at 12,000 × g for 10 min at 4°C. The supernatant was then extracted twice with one volume of phenol:chloroform:isoamyl alcohol (25: 24:1). The aqueous phase was transferred to a fresh tube, mixed with an equal volume of isopropanol, and cooled to -20°C for at least 1 h to precipitate the RNA. After centrifugation in an Eppendorf centrifuge for 10 min at 4°C, the RNA pellet was dissolved in DEPC-treated water. The total RNAs (about 30 μg per lane) were separated on a 1% agarose gel (2.2 M formaldehyde) and blotted onto a Hybond membrane, as recommended by the manufacturer (Amersham Pharmacia Biotech, Piscataway, USA). Hybridization was performed in a church buffer (7% SDS, 1% BSA, 1 mM EDTA and 250 mM $NaPO_4$, pH 7.2) at 65°C [19]. The probe used was the *AmA1* gene labeled with α-[^{32}P]-dCTP using a random labeling kit (Amersham Pharmacia Biotech, Piscataway, USA) [20].

Western Blot Analysis and ELISA

The recombinant yeast cells were broken with glass beads in an ESB buffer (2% SDS, 80 mM Tris-Cl, pH 6.8, 10% glycerol, 1.5% DTT). The cells were then vortexed for 30 s and placed on ice for 30 s, three times. The glass beads and cell debris were removed by centrifugation at 12,000 × g for 10 min at 4°C, and the supernatant was transferred to a new 1.5 mL tube and its protein concentration measured using a Bradford protein assay kit (Promega, Madison, USA). Approximately 150 μg of the total proteins were separated on a 10% SDS polyacrylamide gel and then electroblotted onto a Hybond-C extra nitrocellulose membrane, as recommended by the manufacturer (Amersham Pharmacia Biotech, Piscataway, USA). The membrane was treated with antibody against AmA1 (1:200 dilution) and then with anti-mouse alkaline phosphatase-conjugated IgG (1: 7,000 dilution, Promega, Madison, USA). Alkaline phosphatase activity on the membrane was visualized with nitro blue tetrazolium chloride and 5-bromo-4-chloro-indolyl phosphate. ELISA was used to measure the amount of AmA1 protein expressed in the recombinant yeasts.

RESULTS AND DISCUSSION

Cloning and Sequencing of *AmA1*

Because the *AmA1* gene is known to be expressed in

Table 1. Percentage of essential amino acids of AmA1 in comparison to the World Health Organization recommended values.

Amino acid	% of total amino acids		
	Amaranthus (total protein)	AmA1[+]	WHO[#]
Trp	1.4	3.6	1.0
Met/Cys	4.4	3.9	3.5
Thr	2.9	5.1	4.0
Ile	3.0	6.1	4.0
Val	3.6	5.2	5.0
Lys	5.0	7.5	5.5
Phe/Tyr	6.4	13.7	6.0
Leu	4.7	9.2	7.0

[+] Calculated by considering the total residue number of each amino acid from the sequence and their respective molecular weights.
[#] From ref. Senft, 1980 [5].

early developing seeds of *A. hypochondriacus*, these seeds were used to isolate total RNA for RT-PCR. When the RT-PCR product was electrophoresed in a 1% agarose gel, a 1.2-kb fragment was observed, as expected (data not shown). A restriction enzyme map of the 1.2-kb fragment was constructed using *BamH*I, *Hinf*I, *Sph*I, and *Ssp*I, and this was found to be identical to that of the *AmA1* gene (data not shown). The nucleotide sequence of the 1.2-kb fragment was identical to that of the published *AmA1* gene (GenBank accession number Z11577), and its deduced amino acid composition showed high levels of essential amino acids, which compositionally closely matched the nutritional values recommended by the WHO (Table 1).

Protein Purification and Antibody Production

The *AmA1* gene was subcloned into a pRSET vector, which contained six histidine residues that aid in the purification of the fusion protein, and expressed in *E. coli*. The purified fusion protein so obtained was electrophoresed on a 10% SDS polyacrylamide gel and visualized by staining with Coomassie blue dye. As expected, the molecular weight of the fusion protein appeared to be 40 kDa. A mouse polyclonal antibody raised against the purified fusion protein showed immunoreactivity with the 40-kDa fusion protein (Fig. 1).

Expression of the *AmA1* Gene in Recombinant Yeasts

The *AmA1* gene was subcloned in yeast shuttle vectors with various promoters and a GAL7 terminator (Fig. 2) and the recombinant vectors were introduced into *S. cerevisiae* 2805. Five to 10 putative transformants for each vector were selected on an uracil-deficient medium, and the plasmid DNAs from these recombinant yeasts were isolated and reintroduced into *E. coli* to further confirm the yeast transformation. The transformants were cultured for five days and samples were

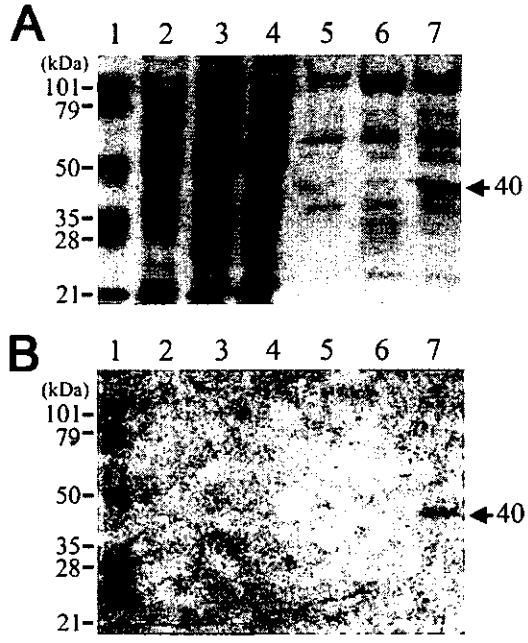

Fig. 1. SDS-PAGE and Western blot analysis of the recombinant AmA1 protein expressed in *E. coli*. (A) The SDS-PAGE shown displays crude extracts (lanes 2-4) and protein purified by Ni-NTA resin (lanes 5-7) from *E. coli* BL21, *E. coli* BL21 transformed with pRSET, and *E. coli* BL21 transformed with pRSET containing the AmA1 gene, respectively. Lane 1 contains low-range protein size markers (Bio-Rad). (B) This Western blot shows that the recombinant AmA1 protein is recognized by the polyclonal antibody against AmA1. The samples are as described in panel A. The numbers on the left and right refer to estimated sizes in kDa.

taken to obtain the growth curve for each of the recombinant yeasts. The observed growth patterns were similar to those of a previous study [21]. After three days of incubation, the growth entered an early stationary phase. No growth defects due to the expression of *AmA1* were observed (data not shown).

AmA1 expression was measured at the end of the 3-day incubation. The GAL10, ADH2, and hybrid ADH2-GPD promoters are all inducible, as such, they were induced with either 1% galactose (GAL10 promoter), or 1.5% ethanol (ADH2 and ADH2-GPD promoters) [17]. In the Northern blot analysis, all recombinant yeasts showed the expression of the *AmA1* gene except for the one transformed with pGALA (Fig. 3). However, expression of the GAL10 promoter was only detected under inducing conditions.

The expression of the *AmA1* gene was also observed when the yeasts harboring pADHA and pHYBA were cultured without ethanol induction. It has been previously reported that the ADH2 promoter is repressed by glucose and derepressed by nonfermentable carbon sources, such as glycerol or ethanol [22,23]. Generally, as yeasts grow, the glucose concentration of the medium drops to 1% of its initial value after 21 h of growth

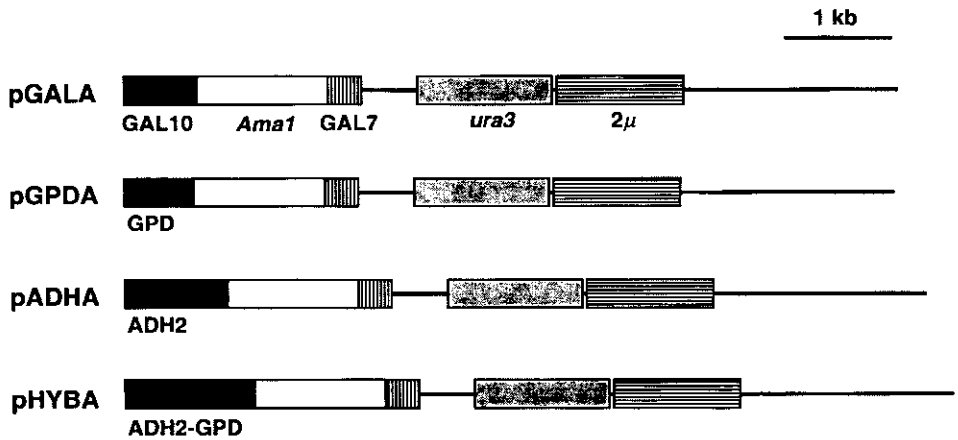

Fig. 2. Schematic diagram of recombinant yeast vectors used. The line shown represents plasmid DNA and the boxes represent the genes or their corresponding functional domains. The closed boxes are promoters, the open box is the *AmA1* coding region, and the shaded boxes are the *ura*3 selection cassette. Boxes with vertical and horizontal lines are the *GAL7* terminator and 2μ *ori* DNA, respectively.

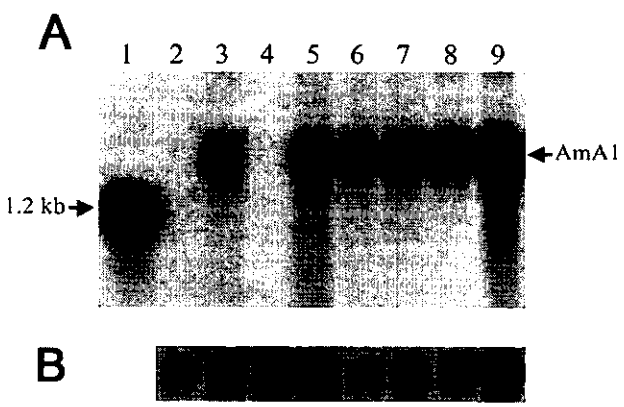

Fig. 3. Northern blot analysis of *AmA1* in recombinant yeasts. (A) The recombinant yeasts were probed with labeled *AmA1* to indicate expression of the gene at the mRNA level. Uninduced samples were grown in a YEPD medium, while induced samples were grown in a YEP medium supplemented with 1% galactose or 1.5% ethanol, as appropriate. The blot includes controls of *AmA1* cDNA (lane 1) and the total RNA of wild type yeast (lane 2). The level of mRNA was examined for recombinant yeast with pGPDA (lane 3), uninduced pGALA (lane 4), induced pGALA (lane 5), uninduced pADHA (lane 6), induced pADHA (lane 7), uninduced pHYBA (lane 8), and induced pHYBA (lane 9). (B) Northern blot analysis using glyceraldehydes-3-phosphate dehydrogenase as a probe indicates that a similar amount of RNA has been loaded for each sample.

[24]. This may also have been the case in the current experiment as the ADH2 promoter became derepressed by the depletion of glucose during growth.

Western blot analysis using a mouse polyclonal anti-AmA1 antibody was also in agreement with the Northern blot analysis. AmA1 proteins were detected in all the recombinant yeasts, but not in the wild type and uninduced recombinant yeast harboring pGALA. The AmA1 proteins from the recombinant yeasts migrated as a single band on 10% SDS-PAGE, of molecular weight 35 kDa, which is identical to that of the storage protein of *A. hypochondriacus* (Fig. 4).

As determined by ELISA, the amounts of AmA1 protein were approximately 1.3%, 2.7%, 1.5%, and 4.3% of the total soluble protein in a 3-day cultured recombinant yeast harboring *AmA1* under the control of pGPDA, pGALA, pADHA, and pHYBA, respectively. The hybrid ADH2-GPD promoter exhibited the highest expression level, and was 3 times higher than that of the GPD promoter. It has been previously reported that the GPD promoter is superior to the GAL10 promoter in a study on the expression of glucose oxidase [17], however, in the current the GAL10 promoter was found to be superior to the GPD promoter. It is possible that the productivity of a promoter depends on the target gene, rather than an intrinsic differences of the promoter strength.

AmA1 as a Nutritional Supplement

The results of amino acid composition analysis showed no increases in the levels of essential amino acids in the recombinant yeast, as compared with the wild type (data not shown). It is likely that the level of expression of the *AmA1* gene in the recombinant yeasts was too low to improve the overall nutritional quality of the yeasts. If the expression level of AmA1 is increased, it is possible that recombinant *AmA1*-expressing yeast can be used as a feed with a high nutritional quality. One strategy for augmenting protein expression involves synthesizing a protein as a translational fusion to another protein [25]. In yeast, heterologous gene expression can be increased up to several hundred-fold by expressing a foreign gene fused to the ubiquitin

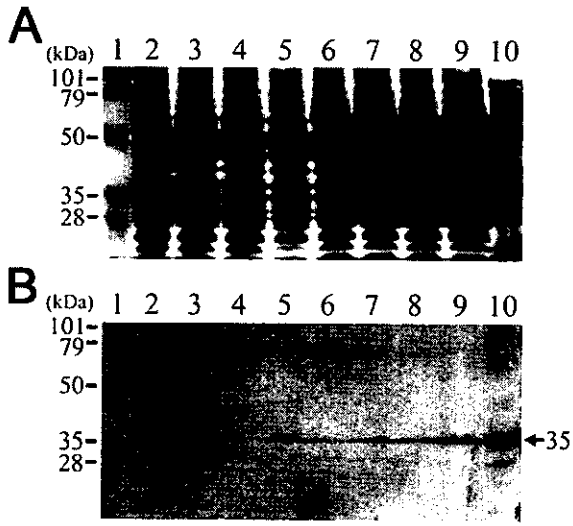

Fig. 4. SDS-PAGE and Western blot analysis of AmA1 proteins in recombinant yeasts. (A) SDS-PAGE analysis included low-range protein size markers (Bio-Rad; lane 1) and the crude extract of wild type yeast grown in YEPD (lane 2). Uninduced samples were grown in a YEPD medium, while the induced samples were grown in a YEP medium supplemented with 1% galactose or 1.5% ethanol, as appropriate. The profile of the crude protein extract is shown for recombinant yeast with pGPDA (lane 3), uninduced pGALA (lane 4), induced pGALA (lane 5), uninduced pADHA (lane 6), induced pADHA (lane 7), uninduced pHYBA (lane 8), and induced pHYBA (lane 9). The crude extract of seeds from *A. hypochondriacus* is also shown (lane 10). (B) The AmA1 protein in recombinant yeasts was identified by Western blot analysis with a polyclonal antibody against AmA1. The samples were as described in panel A. The numbers on the left and right refer to estimated sizes in kDa.

gene. In the resulting fusion protein, the ubiquitin is cleaved from the fusion protein by an endogenous yeast endoprotease (Ub-Xase), and thus the protein of interest accumulates [26]. The fusion of the *AmA1* gene with ubiquitin may increase the level of its expression in recombinant yeast, and this possibility will be explored in future research.

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