Enhanced Gene Expression by Fusion to Rice-ubiquitin in Yeast

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Chloramphenicol acetyl CoA transferase (CAT) and angiotensin- converting enzyme inhibitory peptide (ACEI) were fused to C-terminal region of rice ubiquitin to examine the level of transcripts or enzyme activities in yeast. When two chimeric genes under an inducible Gal1 promoter control were transformed into *Saccharomyces cerevisaie*, both CAT and ACE inhibitory activities were enhanced by three to four-fold as compared to those containing no ubiquitin gene. However, the levels of transcripts of ubiquitin fused and unfused genes were not significantly different each other. Therefore, it was suggested that the expression of foreign genes was post-transcriptionally enhanced by fusion of plant ubiquitin in heterologous organisms such as yeast.

Key words: *ubiquitin, ubiquitin-fusion, expression, rice, yeast.*

Ubiquitin is a highly conserved 76-amino acid residue protein found in all eukaryotic cells. The protein has been implicated in many vital cellular processes, such as protein turnover, chromatin structure, cell cycle control, DNA repair, and response to heat shock and other stresses.[1] It is found free or covalently joined, through its carboxyl terminal glycine residue, to the -amino group of a lysine residue in various cytoplasmic, nuclear, and integral membrane proteins. The conjugation of ubiquitin to proteins may alter their stability or serve as a recognition signal for proteolysis by an ATP-dependent non-lysosomal pathway. The protein conjugates with multi-ubiquitin chains are destined for proteolysis and disassambly. However mono-ubiquitinated conjugates do not serve as proteolysis intermediates and exist stably in the cell (for review, see 2-7).

The ubiquitin-dependent proteolytic pathway should become an useful tool for many aspects of plant biotechnology, such as enhancing the level of desired proteins by synthesizing them as ubiquitin fusion or reducing the level of undesired proteins by selective protein degradations.[8] Ecker *et al.* demonstrated that the yeast ubiquitin fusions give high levels of fusion proteins with simultaneous processing to yield authentic biologically active proteins.[9] Garbarino *et al.* suggested that the potato ubiquitin-translational fusion potentially mediates the increased translatability.[10] I report here that the rice ubiquitin-translational fusions of chloramphenicol acetyl-CoA transferase (CAT) and of angiotensin-converting enzyme inhibitor (ACEI) give higher levels of fused proteins as compared to respective unfused gene control.

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Abbrevations: ACEI, angiotensin-converting enzyme inhibitory peptide; CAT, Chloramphenicol acetyl CoA transferase; GAL, galactose-1-phosphate uridyltransferase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; TRP, tryptophan synthase; UB, ubiquitin.

Materials and Methods

Constuction of expression vector. The parent yeast *E. coli* shuttle vector used for expression of all gene constructions contains the galactose-inducible GAL1 promoter and GAP terminator, the TRP1 gene, which serves as a selectable marker in yeast, and the 2-μm circle origin of replication cloned into pBR322 is shown schematically in figure 1.

The rice ubiquitin gene was digested by SphI to generate monoubiquitin gene, and which was fused to the CAT and/or ACEI gene. Plasmid UBCAT and/or UBACEI contained a 228 bp of ubiquitin coding unit fused in frame to CAT and/or ACEI by site-directed mutagenesis.

The ACEI encodes angiotensin-converting enzyme inhibitor, 10 amino acid peptides (Gly-His-Lys-Ile-Ala-The-Glu-Arg) found in the acid-limited proteolysis of baker's yeast GAPDH, and has antihypertensive effects.[13] In the case of pACEI, we introduced the methionine codon into an amino-terminus of ACEI.

Gene expression. The genes under the GalI promoter were expressed by general methods.[14] Yeast cells were grown in synthetic complete medium to A_{600}=1.0. The culture was split into two tubes (10 ml each), and one of them was supplemented with 2% galactose to induce expression of the gene. After shaking overnight at 30°C, the cells were centrifuged, washed, and resuspended in lysis buffer (10 mM Tris pH 7.4, 100 mM LiCl, 10 mM EDTA, 0.2% SDS) for preparation of total RNA or in PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4) for enzyme assay of CAT or ACEI.

Northern hybridization. The yeast cells resuspended in 0.2 ml lysis buffer containing 200 mg glass beads were

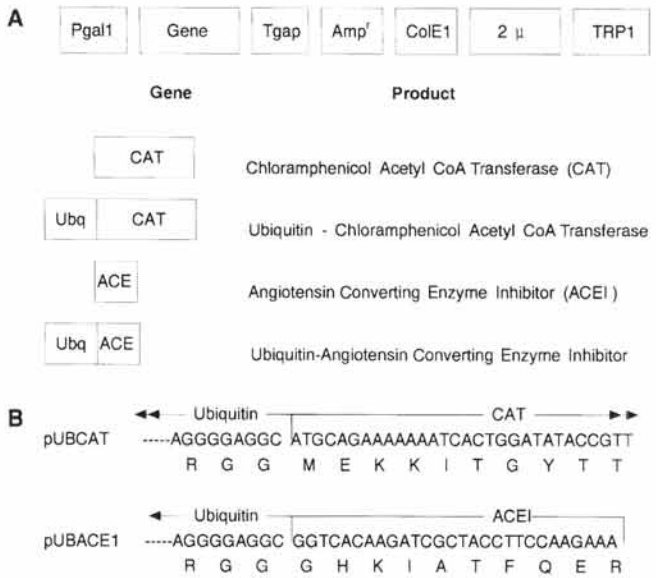

Fig. 1. Yeast expression vectors. A. Schematic representation of yeast expression vectors. The parent vector consisted of the galactose-inducible Gal1 promoter, transcriptional termination signals from GAP gene, the TRP-selectable marker for yeast, 2 μ yeast origin of replication, and amp^r gene and *ori* from pBR322. Genes of CAT, ubiquitin-fused CAT, ACEI, and ubiquitin-fused ACEI were cloned in between GAll promoter and GAP terminator of the parent vector. B. Nucleotide sequences of the junction of the UBCAT and the UBACEI translational fusion chimeric construct.

disrupted six times by vortexing on ice for 30 seconds. The lysate was centrifuged at 12,000 rpm for 5 min. The supernatant was collected, extracted with phenol: chloroform, and cooled on ice overnight after adding 0.5 volumn of 8 M LiCl. Total RNA pellet was precipitated by centrifugation at 12,000 rpm for 5 min, washed, and dissolved with DEPC-treated water. Total RNA 10 μg was seperated by 2% agarose gel electrophoresis containing 10% formaldehyde, transferred onto nylon membrane filter, and probed with the CAT gene or the region of the ACEI-GAP terminator.

CAT assay. The yeast cells containing CAT or UBCAT were grown in synthetic complete medium containing 2% glucose to A_{600}=1. Half of the culture was induced with 2% galactose at an indicated time (figure 3). The cells were harvested and washed in PBS buffer five times. They were then resuspended in TEN buffer (40 mM Tris-HCl pH7.5, 1 mM EDTA, 15 mM NaCl), homogenized by vortexing with glass beads on ice for 5 min, and centrifuged for 10 min at 4°C. Protein extracts (50 μg) were then assayed for CAT activity using CAT enzyme assay system (Promega).

Assay for ACE inhibitory activity. Protein extracts were prepared as in CAT assay. The ACE inhibitory activity was assayed by a modified method of Cushman and Cheng.[15] Protein extracts (50 μg) and 6.5 mM Hip-His-Leu were dissolved in a 100 mM sodium borate buffer (pH 8.3) containing 500 mM NaCl and incubated for 30 min with 3

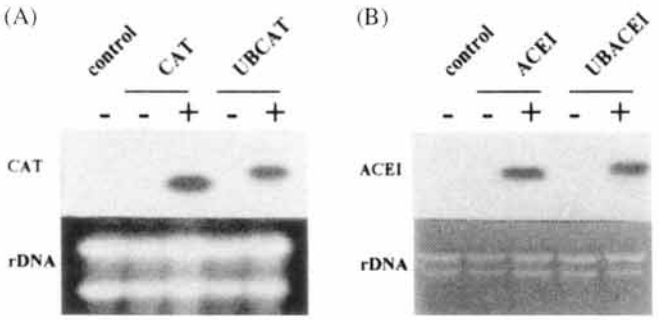

Fig. 2. Northern blot analysis of CAT and ACEI fused and/or unfused to ubiquitin. Expression of fused proteins was induced with 2% galactose (lane with at the top) or incubated without galactose (lane with at the top) for 15 h. Control yeast were not transformed. Ten microgram of total RNA isolated from yeast cells was separated on a formaldehyde-containing agarose gel and then transferred to a nylon membrane. The blot was probed with the CAT containing region (A) or the region of the ACEI and GAP terminator (B).

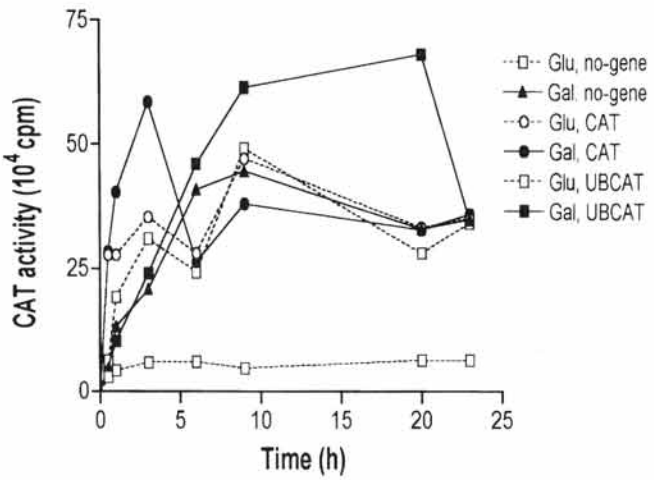

Fig. 3. CAT activity in yeast cells transformed with pCAT or pUBCAT construct. Cells were grown in synthetic complete medium supplemented with 2% glucose to log phase and then induced with 2% galactose. Protein extracts were prepared from yeast cells as described in "Materials and Methods". Enzymatic activity of CAT was measured in the presence of ^{14}C-chloramphenicol. Glu, glucose; Gal, galactose; -▲-, control, galactose; --○--, CAT, glucose; -●-, CAT, galactose; --❑--, UBCAT, glucose; -■-, UBCAT, galactose.

milliunits of ACE at 37°C.

Results and Discussion

Gene and expression vector. To test whether the rice-ubiquitin leader increase foreign protein and peptide, the rice-ubiquitin fusion protein/peptide was expressed in yeast. The genes (Fig. 1) were inserted both directly into the expression vector to express the authentic form of the protein/peptide and as fusions to the rice-ubiquitin gene to express them with an N-terminal ubiquitin leader. In the case of expression for only ACEI, we introduced the methionine codon into an amino-terminus of ACEI. The expression

vector pUBACEI contains the rice ubiquitin C-terminal glycine codon fused to the N-terminal glycine codon of the ACEI (Fig. 1B).

Ubiquitin fusion has no effect on the transcription or message RNA stabilization of fused genes. The effect of rice-ubiquitin fusion on the transcription or message stabilization of fused genes was analyzed in yeast containing the ubiquitin-fused and/or -unfused expression vectors (Fig. 1). The expression of these chimeric genes was induced with galactose. The transcripts of CAT and ACEI genes were observed only in the induced cells. The mRNA levels of CAT and ACEI were similar to those of UBCAT and UBACEI, respectively (Fig. 2). This suggests that the rice-ubiquitin fusion has no effect on the transcription or massage stabilization in yeast cells. However, it has been reported that the potato-ubiquitin fusion, though improved translability in potato was not associated with increased transcription rates or massage stability.[11] On the other hand, the yeast-ubiquitin fusion has no effect on transcription of a fused gene in yeast.

CAT activity is increased and delayed by ubiquitin fusion. High expression level of chimeric genes with the plant-ubiquitin fusion has been reported in potato,[10] maize[16] and tobacco.[17] The expression of chimeric gene fused to yeast-ubiquitin was also enhanced in yeast[9] and *E. coli*.[18] To test effects of rice-ubiquitin fusion on chimeric gene product in yeast, CATwas used as the reporter gene. To express CAT gene under GalI promoter of pCAT and pUBCAT, galactose was added to cells at 2%.

Figure 3 shows that CAT activities increased in ubiquitin-fused construct (pUBCAT). When CAT was expressed as an unfused state, CAT activity was detected from 0.5 h up to 3 or 4 h, and decreased at 6 h. However, in the case of pUBCAT, CAT activity increased up to 50×10^4 cpm by 20 h, even though the transcription levels of CAT were similar in pCAT and pUBCAT. This suggests that the ubiquitin fusion was responsible for the improved stability or translatability of the fusion protein. However, this approach has not resulted in a remarkable accumulation of the fusion protein, which may be explained by the fact that codon usage of rice-ubiquitin is unfavorable to yeast. Expression of β-glucuronidase (GUS) fused to potato-ubiquitin in potato has been reported.[11] In this case, GUS activity was five to tenfold higher than the unfused form. Moreover, UBGUS was processed to GUS after or during translation. In general, ubiquitin fusion protein is processed in the cytoplasm by endogenous hydrolase that recognized the C-terminus of ubiquitin.[6-8, 17]

ACE inhibitory activity enhanced by rice-ubiquitin fusion. To demonstrate that a fusion peptide can be produced in an enzymatic active form, we chose the ACE inhibitory peptide (ACEI). ACEI is a 10-amino acid peptide (Gly-His-Lys-Ile-Ala-Thr-Phe-Gln-Glu-Arg) corresponding to the residues 68-77 of yeast glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and has strong antihypertensive effects.[12, 13] The ACEI and UBACEI were expressed and assayed in yeast cells (see Materials and Methods). ACE inhibitory activity was assayed at 2, 6, and 15 h after adding galactose to the culture medium. Figure 4 shows that the ubiquitin fusion also resulted in higher level of ACE inhibitory activity. The rice-ubiquitin fusion increased the enzymatic activity of ACEI by threefold, strongly suggesting that ubiquitin fusion peptide is processed into rice-ubiquitin and authentic ACEI peptide.

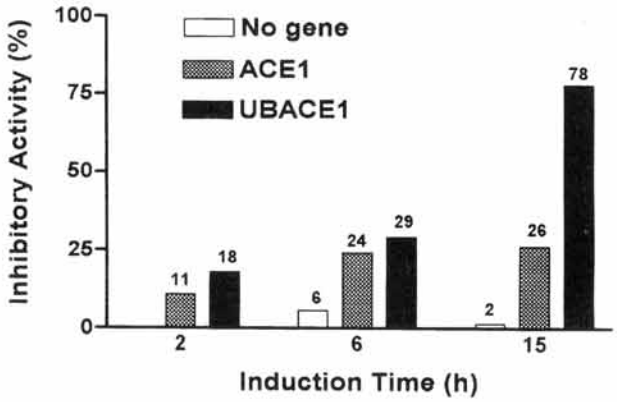

Fig. 4. ACE inhibitory activity in yeast cells transformed with constructs of pACEI or pUBACEI. The ACEI inhibitory activity was measured by a modified method of Cushman and Cheung.[15] Protein extracts were prepared from yeast cells induced with 2% galactose for indicated times. Hip-His-Leu (6.5 mM) and total yeast protein (10 μg) were dissolved in 100 mM sodium borate buffer (pH 8.3) containing 500 mM NaCl and incubated for 30 min with 3 milliunits of ACE at 37°C.

To express ACEI unfused to ubiquitin, we introduced the methionine residue to N-terminal glycine residue of ACEI peptide. By N-end rule, Met and Gly residues are classified into the same stabilizing residues. In fact, the *in vivo* half-lives of β-galactosidase derived from Ub-Met-β-gal and Ub-Gly-β-gal constructs in yeast were constant (>20h).[19] As in the cases of ACEI and UBACEI, although N-terminal residue of ACEI peptide was different, the metabolic stability of the peptide may be attributed to rice-ubiquitin fusion.

The mechanism by which protein or peptide expression is increased by ubiquitin as a leader sequence is still unknown. The ubiquitin may protect the amino terminus of the fusion peptide from proteolysis or may guide the fusion protein into a cellular compartment which is more protected from proteases. In summary, rice-ubiquitin was responsible for the improved translation and/or stability of foreign protein or peptide in yeast.

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