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Expression of *de novo* Designed High Nutritional Peptide (HEAAE) in Tobacco

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We have designed and constructed a gene encoding novel high essential amino acid encoding protein (HEAAE). The resultant DNA fragment was tested for *in vitro* and *in vivo* expression and then cloned into plant expression vector pBI121, under the control of the cauliflower mosaic virus 35S promoter. *Agrobacterium tumefaciens*, strain LBA4404, was subsequently transformed with this new construct and *Nicotiana tabacum var. Xanthi* transgenic plants were obtained. DNA analysis by Southern procedure confirmed the presence of the multi-copy number of genes in the transformed plants. Analysis of RNA and protein synthesized in these transgenic plants demonstrated the stable expression of this gene.

In many seeds, the storage proteins account for 50% or more of the total protein. Therefore, the protein quality of seeds is primarily determined by the storage proteins. With respect to human and animal nutrition, however, most seeds do not provide a balanced source of protein because of deficiencies in one or more of the essential amino acids (isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine).

There has been a great deal of effort to overcome these amino acid limitations by breeding and selecting for more nutritionally balanced varieties. Plants have been mutated in hopes of recovering individuals with more nutritious storage proteins. Neither of these approaches has been entirely successful, although some naturally occurring and artificially produced mutants of cereals were shown to contain a more nutritionally balanced amino acid composition. These mutations cause a significant reduction in the amount of storage protein synthesized and thereby result in a higher percentage of lysine in the seed; however, the softer kernels and low yield of such strains have limited their usefulness. The reduction in storage protein also causes the seeds to become more brittle; as a result, these seeds shatter more easily during storage. The lower levels of prolamin also result in flours with unfavorable functional properties which cause brittleness in the baked products (16). Thus, to date no satisfactory solution has been found for improving the amino acid composition of storage proteins.

Recently, many laboratories have tried to improve the nutritional quality of plant storage proteins by transferring heterologous storage protein genes from other plants (2) or by modifing the coding sequence of storage gene of target plants (7, 19). The development of recombinant DNA technology and the *Agrobacterium*-based vector system has made this approach possible. However, genes encoding storage proteins with a more favorable amino acid balance do not exist in the genomes of any major crop plant. Furthermore, the instability, low expression level, processing, and difficulty in modification of native or modified native storage proteins have limited the host plant range for their application.

One direct approach to this problem would be to modify the nucleotide sequence of genes encoding storage proteins so that they contain high levels of essential amino acids. Modified storage proteins have been created and expressed by changing their codon sequences. *In vitro* mutagenesis was used to supplement the sulfur amino acid codon content of a gene encoding β-phaseolin, a *Phaseolus vulgaris* storage protein (7), by the insertion of 15 amino acids, six of which were methionine. The inserted peptide was essentially a duplication of a naturally occurring sequence in the maize 15 kDa zein storage protein. However, this modified phaseolin achieved less than 1% of the expression level of normal phaseolin in transformed seeds because this insertion was made in part of a major structural element of the phaesolin trimer (12). In an another approach lysine and tryptophan encoding oligonucleotides were introduced at several positions into a 19 kDa α type zein complementary DNA by oligonucleotide-mediated

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mutagenesis (19) which suggested the possibility of creating high-lysine corn by genetic engineering.

There are alternative approaches that might be more practical. One of these is to transfer heterologous storage protein genes that encode storage proteins with higher levels of the desired amino acids. For this purpose, a chimeric gene encoding a Brazil nut methionine-rich protein which contains 18% methionine has been transferred to tobacco and expressed in the developing seeds (2). The accumulation of the methionine-rich protein in the seed of tobacco results in a significant increase (30%) by this transformation.

The synthetic genes have been used for the desired enhancement of nutritional quality of plants. The 292 bp synthetic gene (HEAAEI gene) which encodes high essential amino acid was expressed as CAT-HEAAEI fusion protein in potato (10, 18). However, structural instability limited the high level expression of this fusion protein in the potato system. Also, the content of essential amino acid was less than 40% of this fusion protein.

The most structurally studied storage proteins are the corn prolamines which are called zeins. Three types of zeins (α, β and γ) are synthesized on rough endoplasmic reticulum and aggregate within this membrane as protein bodies. The zein proteins readily self-associates to form protein bodies and are insoluble in water. The mechanisms responsible for protein body formation are thought to involve both hydrophobic and weak polar interactions between zeins (1, 19). Polar and hydrophobic residues, appropriately distributed along the helical surfaces, allow intra- and intermolecular hydrogen bonds and van der Waals interactions among neighboring helices. Therefore, based on the structural characteristics of zein, we designed and expressed the synthetic gene of HEAAE in *E. coli*. In this report, we report the expression of HEAAE in tobacco plant as a model system.

MATERIALS AND METHODS

Bacterial Strains and Vectors

A non-oncogenic *Agrobacterium* strain, *Agrobacterium tumefaciens* LBA4404 (Clontech, CA), was used as the host cell for pBI121 constructs of *heaae* (15).

Tobacco Transformation with HEAAE Gene

The disarmed *Agrobacterium* strain, LBA 4404 (Clontech, CA) was used for the transformation of tobacco. The procedure for the transformation of the intermediate vector pBI121 followed the method developed by Holster *et al* (8).

In vitro grown tobacco plants were used for the infection studies. Approximately 1 ml of tobacco cell suspension was dispensed on each culture medium in petri-dishes and covered with Whatman #1 filter paper. The explants were precultured for 1 to 2 days upside down on MS 104 medium (1.0 mg benzayladenine (BA), 0.1 mg napthaleneacidic acid (NAA) per ml of MSO) to allow initial growth. MSO medium contains 4.3 g MS salts (Gibco, BRL), 1 ml B5 vitamin stock (100 mg myo-inositol, 10 mg thiamine-HCl, 1 mg nicotinic acid and 1 mg pyridoxine-HCl per ml), 30 g sucrose and 0.8% agar per liter with pH 5.7. Explants were inoculated by immersing them in a resuspended culture of *A. tumefaciens* LBA4404.

The explants were incubated for two further days and then transfered to MS selection media (500 µg/ml carbenicillin or 300 µg/ml cefatoxim in MS 104). After three weeks, regenerated shoots, on the edges of leaf discs, were excised and put on the rooting medium (MSO with 0.6% agar, 300 µg/ml cefatoxim and 100 µg/ml kanamycine). Shoots which developed roots were assayed for β-glucuronidase activity (11). Root tips which turned blue in 5-bromo-4-chloro-3-indol-1-glucuronide (X-Gluc) solution after 15 h incubation were selected. The plantlets which developed roots after about two to three weeks and showed positive GUS activities were transfered to sterile Jiffy pots, and placed in magenta boxes. The plants were then transferred to bigger pots two weeks after and grown in the greenhouse.

About 0.1 g of leaf tissue was ground in an eppendorf tube containing 200 ml of GUS extraction buffer (50 mM $NaPO_4$, pH 7.0, 10 mM β-mercaptoethanol, 10 mM Na_2EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100). Ten ml of extract was added to 0.5 ml assay buffer (GUS extraction buffer with 1 mM methyl-umbelliferyl β-D-glucuronide (MUG)) and at regular intervals 100 ml aliquots of the mixture was added to 0.9 ml stop buffer (0.2 M Na_2CO_3). The MU concentration was determined using TKO 100 Spectroflurometer (Hoefer Scientific Inst., CA) exitation at 365 nm, emission at 455 nm.

Tobacco Genomic DNA Isolation and Southern Transfer

Tobacco leaf DNA was isolated by the procedure of Chen (3). Fresh tissue was ground with liquid nitrogen. The DNA was extracted with extraction buffer (168 g urea, 25 ml of 5 M NaCl, 20 ml of 1 M Tris-HCl pH 8.0, 16 ml of 0.5 M EDTA, 20 ml of 20% sarcosyl and 190 ml water). A phenol/chloroform was added and agitated vigorously. The mixture was left at room temperature for 15 minutes then centrifuged. For every 6 ml of the supernatant, 1 ml of 4.4 M ammonium acetate (pH 5.2) and 6 ml of isopropnol were added. The mixture was centrifuged and the DNA pellet was recovered by discarding the supernatant. The DNA pellet was washed with 80% cold ethanol, vacuum dried and dissolved in TE buffer.

The DNA was transferred on Duralon-UV membrane (Stratagene, CA) by capillary blotting. Probe for the hybridization was prepared by using a Random primed DNA labeling kit (Boehringer Mannheim, IN). The exact

heaae-4 (290 bp) fragment was used as the DNA template for probe production. The membrane was prehybridized (10% dextran sulfate, 50% deionized formamide, 1% SDS, 0.9 M NaCl and 200 μg/ml denatured sonicated salmon sperm DNA) for 2 h at 42°C and hybridize with heat denatured probe (10^7 cpm/ml of hybridization solution) at 42°C for 12 to 18 h with constant agitation in a water bath. The blot was washed with 1×SSC, 0.1% SDS solution for 10 minutes at room temperature. The second washing was done at 60°C with 1×SSC, 0.1% SDS solution. The blot was dried and exposed for autoradiographic detection.

mRNA Isolation and Detection

Plant tissues were ground with liquid nitrogen and then added to a 1 : 1 mixture of RNA extraction buffer (100 mM LiCl, 1% SDS, 100 mM Tris NaOH, pH 9.0, 10 mM EDTA) and phenol pre-heated to 90°C (1 ml buffer/g of tissue). The mixture was agitated, occasionally heated in a 90°C waterbath and put in a gyratory shaker for 5 minutes at room temperature. One ml chloroform was added for every gram fresh weight of plant material and shaken for 15-30 minutes at room temperature. The aqueous upper phase was taken by centrifugation. The RNA was precipitated with 1/3 volume of 8 M LiCl at 4°C. The pellet was washed with 2 M LiCl at 4°C and then washed with 80% ethanol, and finally vacuum dried. Poly A RNA was purified from total RNA using Hybond-mAP messenger affinity paper (Amersham) according to the manufacturer's manual. After running the gel, the RNA was transferred to a Duralon membrane and baked under vacuum at 80°C. The membrane was prehybridized (50% deionized formamide, 10% dextran sulfate, 1% SDS, 1 M sodium chloride and 100 mg/ml denatured sonicated salmon sperm DNA) for about 1 h at 42°C and hybridized at the same temperature with heat denatured probe (290 bp HEAAEII tetramer, 5×10^5 cpm/ml). The membrane was washed for 15 minutes with 2× SSC, 0.1% SDS and then, with 0.1×SSC, 0.1% SDS at 55°C for 5 minutes.

Protein Detection

The total protein was extracted from tobacco leaf tissue with extraction buffer (50 mM Tris-HCl pH 7.5, 5 mM DTT, 0.05% Triton X100, 50 mM EDTA and 0.19 mg/ml PMSF) and separated on denaturing polyacrylamide gels by gel electrophoresis. Samples (10 mg) were loaded on the 16.5% T (% of acrylamide+cross linker), 3% C (weight % of cross linker) Tricine-SDS polyacrylamide gel and separated. The proteins were subsequently transferred to Immobilon PVDF using a Hoefer trans-vac semi-dry transfer system. The membrane was then incubated in a blocking solution (5% (w/v) BSA, 0.9% (w/v) NaCl in 10 mM Tris-HCl pH 7.4) for 1 h at 37°C with gentle agitation. The membrane was thoroughly washed three times in 0.1% BSA, 0.9% NaCl, 10 mM Tris-HCl pH 7.4 for 10 minutes each. The blot was incubated with antiserum raised against HEAAE peptide diluted 100 fold in a volume of 20 ml buffer (1% BSA, 0.05% Tween 20 and 0.9% NaCl in 10 mM Tris-HCl pH 7.4) for 6 h at room temperature with gentle shaking. The blots were then washed as described above before incubation with the secondary antibody conjugates (alkaline phosphatase) in 1% BSA, 0.01% Tween 20. 0.9% NaCl in Tris-HCl pH 7.4 for 30 minutes at room temperature. The blots were washed again as described above. Detection of immunoreactive protein was carried out using alkaline phosphatase coupled to antirabbit IgG using 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) as substrate. Sixty six ml nitro blue tetrazolium (NBT, 50 mg/ml) and 33 ml BCIP substrates were mixed for every 10 ml alkaline phosphatase (AP) buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). The damp dried blots were incubated until the band was detected. The reaction was stopped by washing the blot with distilled water.

RESULTS AND DISCUSSION

Gene Construction and Transformation of HEAAE Gene into Tobacco

The 284 bp tetrameric gene (*heaae*-4) encoding this novel peptide was cloned into the plant expression vector pBI121 (pBI-*heaae*4). To obtain the pBI-tetramer, the 290 bp long *Xba*I-*Eco*RV fragment, containing the *heaae*-4, was cloned into the *Xba*I-*Sma*I site of pBI121. The 3' NOS terminator was inserted between *heaae*-4 and GUS (Fig. 1). This gene contains plant consensus sequences at the 5' end of the translation initiation site to optimize the expression of proteins *in vivo*. It was placed under the control of the 35 S cauliflower mosaic virus (CaMV) promoter in order to permit the stable expression of this gene in tobacco.

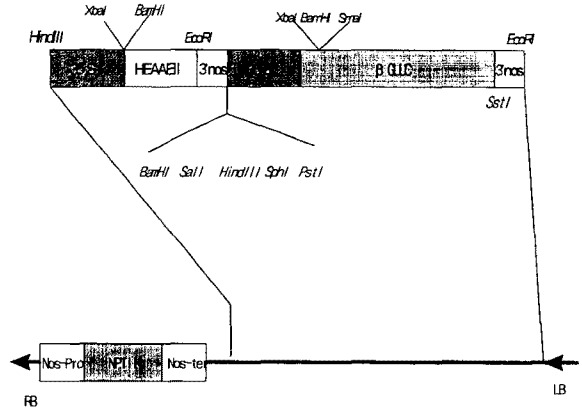

Fig. 1. Restriction map of binary vector pBI-*heaae*4 constructs for tobacco transformation.

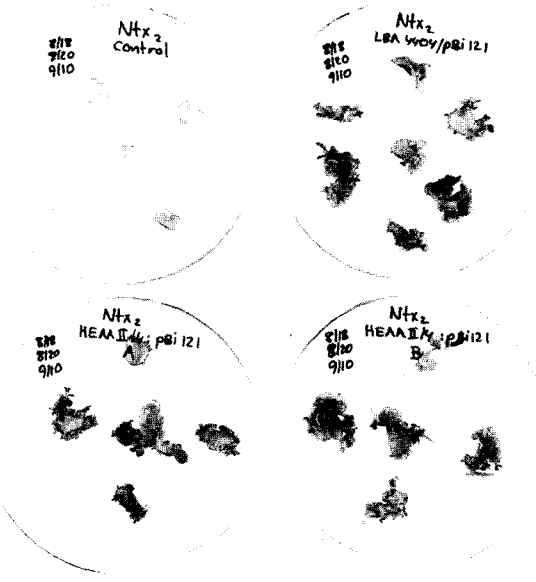

Fig. 2. Leaf disc tranformation.
Top left, untransformed tobacco leaf discs. Top right, transformed with *A. tumefaciens*/pBI121. Bottom right and left, transformed with pBI-tetramer. Three weeks after transfer, shoot appeared from all leaf discs except control plate.

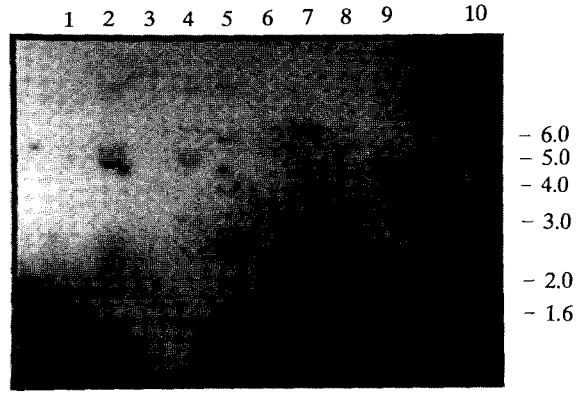

Fig. 3. Southern analysis of DNA isolated from transgenic tobacco plants.
The tobacco genomic DNAs were purified from leaves and digested with *Hin*dIII. The probe used was the 293 bp *heaae*-4 fragment. The plasmid DNA containing the pBI-tetramer was used as positive control which is corresponding to five copies (5×)(lane 10). The number on the top are: lane 1, plant #1; lane 2, #9; lane 3, #2; lane 4, #11; lane 5, #13; lane 6, #7; lane 7, #17; lane 8, #22; lane 9, #29. The numbers to the right of the gel are size markers in kb. Plant numbered #2 is DNA purified from a pBI121-transformed plants, while numbered #7 is from a nontransformed control.

Leaf discs, transformed with LBA4404 carrying the *heaae*-4 gene, gave about 5 to 7 shoots two to three weeks after infection. A total of 565 kanamycin-resistant shoots were regenerated from 120 leaf discs (Fig. 2). These shoots were excised from the leaf discs and transferred to new media to grow for two more weeks, and then transferred to rooting media. After three weeks in rooting medium, 126 rooted shoots were analyzed for β-glucuronidase. Root tips of 56 out of 126 plants showed various levels of GUS activity.

Not all the kanamycin-resistant shoots showed the GUS positive result. Although kanamycin resistance was due to the expression of neomycin phosphotransferase (NTP II gene), regeneration of nontransgenic shoots in the presence of kanamycin had been reported (9). Therefore, escapes from the screening based on kanamycin sensitivity might have occurred in the nontransformed plants, making them kanamycin resistant. It was often observed that the expression level of physically linked genes co-transferred to the host plant genome could vary independently, even if they were separated by as little as 600 bp (4, 5, 14). Therefore, it might be more reliable to screen the transformed plants based on the two different methods.

Expression of HEAAE in Tobacco

Thirty six plantlets which showed high levels of β-glucuronidase activity were transplanted into jiffy pots. After establishment of the plants, a more accurate fluorogenic assay for GUS activity was done to quantify the expression level of this gene. Some of these transformed tobacco plants showed higher levels of β-glucuronidase activity compared to other plants and these plants were selected for future analysis.

A distinct 1.4 kb *Hin*dIII band (include the CaMV 35S promoter and 3' NOS terminator) was detected using the *heaae*-4 fragment (290 bp) as a probe (Fig. 3). The copy number of the correct band varied among the plants, and ranged from 1 to 5 by densitometeric measurement compared to a positive control. Additional multiple positive bands were observed with the expected size band of 1.4 kb, from most of the transformed plants. All the extra bands which appeared were bigger than 1.4 kb and showed different patterns between the individual plants. These results suggested that the *heaae*-4, alone or with neighboring genes, might have inserted into several sites in the chromosomes with or without DNA rearrangement. One possible explanation for these multiple insertions of the HEAAE gene into the chromosome might be its repeated structure of DNA sequences. These repeated sequence regions might be active or not depening on the position of insertion in the chromosome.

Efficient expression of inserted *heaae*-4 in tobacco plants was tested by RNA and protein analyses. The polyA RNA was isolated and then analyzed using the *heaae*-4 DNA as probe. Bands were observed which correspond to the expected size (500 base long) from all the samples which were analyzed (Fig. 4). It was found that multiple inserted HEAAE genes did not actively produce

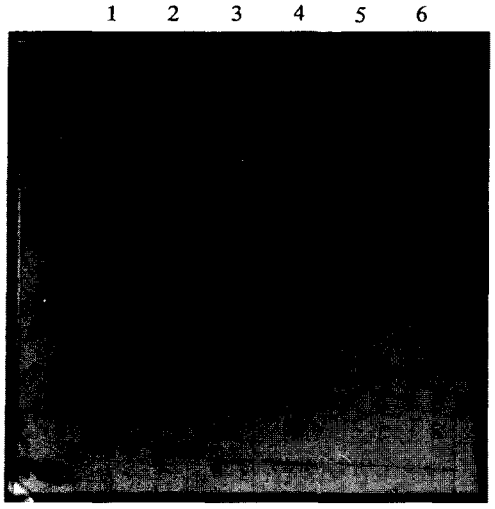

Fig. 4. Nothern blot of mRNA isolated from transgenic tobacco plants.
For each lane, 5 μg of mRNA were loaded. The probe used was the 293 bp *heaae*-4 fragment. The number on the top are: lane 1, plant #2; lane 2, #1; 3, #17; lane 4, #7; lane 5, #22; lane 6, #29. Plant numbered #2 is mRNA purified from a pBI121-transformed plants, while numbered #7 is from a nontransformed control. The number to the right are size markers in kb.

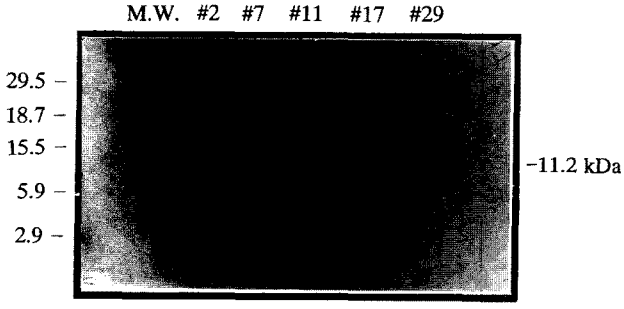

Fig. 5. Western immunoblot of transgenic plants.
For each lane 10 μg of leaf-extracted total protein were loaded. A 11.2 kDa HEAAE-4 proteins appeared with plant numbered #17. The numbers to the left indicate the size of molecular markers (1st lane) in kDa. Plant numbered #2 and #7 are pBI121 and nontransformed plants, respectively.

transcripts. However, the levels of transcription of the HEAAE genes was dramatically different among the transformed plants. Transformed plant #17 accumulated 5- to 50-fold more transcripts compared to the other transformed plants. Such differences in accumulation are explained by the effect of position or multi-copy number insertion. The expression levels of the *heaae*-4 and its neighboring GUS gene correlated with each other in some transformed plants but not all. These results suggests that the level of expression can be dramatically different between two closely linked genes. The HEAAE-4 (11.2 kDa) were detected from plant #17 by western immunoblot analysis (Fig. 5). No small degradation products were observed in tobacco unlike *E. coli*. The level of expression was not high enough for the protein body formation in plant tissue (data not shown). However, we might achieve stable high expression by optimizing the tissue specific expression (6, 13, 17) system. In this paper, we report the stable expression of the *de novo* designed HEAAE-4 in the tobacco system.

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