

EXPRESSION OF A SYNTHETIC GENE FOR IMPROVED PROTEIN QUALITY IN TRANSFORMED POTATO PLANTS

M.S. YANG^a, N.O. ESPINOZA^b, P.G. NAGPALA^a, J.H. DODDS^b, F.F. WHITE^c, K.L. SCHNORR^d and J.M. JAYNES^{a*}

^aDept. of Biochemistry, Louisiana State University & A.M. College and Louisiana State University Agricultural Center, Baton Rouge, LA 70803 (U.S.A.), ^bInternational Potato Center, P.O. Box 5969, Lima (Peru), ^cDept. of Plant Pathology, Kansas State University, Manhattan, KS 66506 (U.S.A.) and ^dDept. of Veterinary Microbiology and Parasitology, Louisiana State University School of Veterinary Medicine, Baton Rouge, LA 70803 (U.S.A.)

(Received October 6th, 1988)

(Revision received April 20th, 1989)

(Accepted May 18th, 1989)

The essential amino acid composition of plant foodstuffs is a very important nutritional property, especially in the developing countries where people often depend heavily on plant proteins from a single source. In these situations, it would be highly beneficial to 'engineer' the plant to produce proteins with a balanced essential amino acid content. Recombinant DNA technology and *Agrobacterium*-based vector systems offer a novel approach to modify plant proteins and raise the possibility of increasing their nutritive value. Methods are described for the inoculation of in vitro potato plants with *Agrobacterium rhizogenes* containing, within the Ri plasmid, a synthetic gene sequence coding for a protein high in essential amino acids. This synthetic gene fragment (HEAAE-DNA), 292 basepairs in length, codes for a protein composed of about 80% essential amino acids. Plants were regenerated from the *Agrobacterium*-induced hairy roots and we present data that these transformed plants contain the synthetic gene insert. Furthermore, our evidence shows that this novel gene was transcribed and translated within the transformed plant. Tubers from regenerated potato plants were subjected to analysis for introduction and expression of this gene. Integration of the gene into the plant genome and its expression into mRNAs and HEAAE-proteins have been demonstrated using Southern, northern and western analysis.

Key words: *Agrobacterium*; essential amino acids; potato; synthetic gene

Introduction

Over the last decade the techniques for gene transfer in plants using *Agrobacterium* vectors have been well developed using model systems such as tobacco and petunia [1,2]. Interest, however, is now moving towards economically important crop plants and the potato (*Solanum tuberosum*) is an outstanding candidate. The potato is an important calorie and protein source in many developed and developing countries with total production yielding about 95 million tons of tubers worth about 23,275 million dollars. The nutritional value of potato protein, although relatively high, is, like most plant proteins, deficient in certain essential amino acids, e.g. lysine and methionine [3]. The

expression of a synthetic gene, along with normal protein production within the tuber, could increase the overall nutritional value of the potato. Fortunately, for genetic engineering studies, the potato has many advantages over other major crop plants. The potato is easily manipulated in tissue culture [4], both in regeneration and methods of propagation [5], and it is susceptible to infection by *Agrobacterium tumefaciens* and *A. rhizogenes*, the gene vector system most commonly used.

We have constructed gene fragments that code for synthetic proteins rich in essential amino acids. A synthetic gene, 292 bp long, has been constructed, cloned, and expressed in bacteria [6]. This was done in bacteria first in order to facilitate the analysis of the gene fragment and its protein product. This novel gene fragment, HEAAE-DNA (High Essential Amino Acid

*To whom correspondence should be sent.

READING DIRECTION OF TOP STRAND (A)->->->

AATTCGGGGATCGTAAGAAATGGATGGATCGTCATCCATTTCTTCATCCATTTCTTAC
TTAAGCCCCTAGCATTCTTTACCTACCTAGCAGTAGGTAAAGAAGTAGGTAAAGAATG

GATCCATCCATTTCTTAAAGAAATGGATGAAGAAATGGATGACGATCCATCCATTTCTT
CTAGGTAGGTAAAGAATTCTTTACCTACTTCTTTACCTACTGCTAGGTAGGTAAAGAA

CATCCATTTCTTCATCCATTTCTTACGATCAAGAAATGGATGAAGAAATGGATGAAGA
GTAGGTAAAGAAGTAGGTAAAGAATGCTAGTTCTTTACCTACTTCTTTACCTACTTCT

AATGGATGAAGAAATGGATGCATCCATTTCTTAAAGAAATGGATGAAGAAATGGATGAA
TTACCTACTTCTTTACCTACGTAGGTAAAGAATTCTTTACCTACTTCTTTACCTACTT

GAAATGGATGACGATCGATCGTAAGAAATGGATGACGATCCATCCATTTCTTACGATC
CTTTACCTACTGCTAGCTAGCATTCTTTACCTACTGCTAGGTAGGTAAAGAATGCTAG

CCCGAATT
GGGCTTAA

<-<-<-READING DIRECTION OF BOTTOM STRAND(B)**SEQUENCE OF PROTEIN A**

GlyAspArgLysLysTrpMetAspArgHisProPheLeuHisProPheLeuThrIleHisProPheLeu->
LysLysTrpMetLysLysTrpMetThrIleHisProPheLeuHisProPheLeuHisProPheLeuThr->
IleLysLysTrpMetLysLysTrpMetLysLysTrpMetLysLysTrpMetHisProPheLeuLysLys->
TrpMetLysLysTrpMetLysLysTrpMetThrIleAspArgLysLysTrpMetThrIleHisProPhe->
LeuThrIlePro

SEQUENCE OF PROTEIN B

GlyAspArgLysLysTrpMetAspArgHisProPheLeuThrIleAspArgHisProPheLeuHisPro->
PheLeuHisProPheLeuLysLysTrpMetHisProPheLeuHisProPheLeuHisProPheLeuHis->
ProPheLeuAspArgLysLysTrpMetLysLysTrpMetLysLysTrpMetAspArgHisProPheLeu->
HisProPheLeuLysLysTrpMetAspArgLysLysTrpMetLysLysTrpMetThrIleHisProPhe->
LeuThrIlePro

Fig. 1. The nucleotide sequence of HEAAE-DNA and the derived protein sequences. The HEAAE-DNA yields two proteins designated A and B. One or the other will be produced depending upon the orientation of the gene when it is fused to the CAT protein under the control of the plant promoter. Protein A, the best so far analyzed, is composed of about 80% essential amino acids (those which are underlined).

Encoding DNA), codes for proteins with a high content of essential amino acids found to be most deficient in plant-derived protein [7]. The sequence of HEAAE-DNA and resultant protein sequences are shown in Fig. 1. Proteins A and B represent polypeptide sequences derived from both possible reading directions of the HEAAE-DNA. That is, HEAAE-DNA was constructed symmetrically so that a protein, containing a high content of essential amino acids, would be produced no matter which strand of the synthetic DNA was read by a host cell's protein synthesis machinery. The compositions of protein A and B, considering the most deficient essential amino acids of

plant-derived proteins, are three-fold higher than those found in milk or egg protein [7]. It can be seen that with the proper supplementation of plant-derived foods, with proteins encoded by HEAAE gene fragments, their overall essential amino acid balance would be markedly improved.

Materials and Methods*Plant material*

Two potato clones K-2 (AH-78.5262), and K-7 (AH-79.5503) were used in these studies as they possess many of the most desirable traits important to the small farmer of the Develop-

ing World. These clones form part of the in vitro germplasm collection of the International Potato Center, Lima, Peru.

Bacterial strains and culture conditions

The agropine-producing *A. rhizogenes* strain R1000 was used as the host strain for all Ri plasmids in this study [8]. It was grown on nutrient plates which was solidified by the addition of 1.5% agar (YM). Transconjugant strains were cultured on the same media and, in addition, contained the following antibiotics: carbenicillin 200 µg/ml, kanamycin 50 µg/ml and gentamycin 50 µg/ml. *E. coli* strain HB101 was used throughout the experiments for the construction and maintenance of the vectors.

Plasmid construction

The plasmid pGA414, which has the chloramphenicol acetyl transferase (CAT) gene under the transcriptional control of the nopaline synthetase promoter, and the 3' end of the nopaline synthetase gene for polyadenylation, was used for the construction of a CAT-HEAAE fusion protein [10]. To locate the target gene within the T-DNA of an pBR325 has been described previously [6]. This pBR325 derivative, containing HEAAE-DNA, was designated pSP547. The plasmid, pGA414, has only one EcoRI site located in the middle of the CAT gene, which was used to clone the HEAAE-DNA, in phase, producing a CAT-HEAAE fusion protein [10]. To locate the target gene within the T-DNA of an *Agrobacterium* Ri plasmid, the plasmid pFW105 was used, which possesses a fragment of T-DNA for recombination purposes and wide host range properties and can replicate in *Agrobacteria*. The desired recombinant plasmids were designated pFW105.10.2 (would yield protein sequence B) and pFW105.10.3 (would yield protein sequence A), respectively. The primary characteristics of the plasmids used in this study can be found in Fig. 2.

Transformation of A. rhizogenes and selection of double recombinants

Purified plasmids were used to transform

wild type *A. rhizogenes* strain R1000. This was accomplished by inoculating a single colony of R1000 into 50 ml of yeast-mannitol-LB (YML) media and incubating at 28°C overnight while shaking. This culture was then diluted and incubated at 28°C for 4 h until the culture had attained logarithmic phase growth. The culture was centrifuged and resuspended in 5 ml of fresh YM and 0.3 ml of the cells were mixed with ~2 µg of plasmid DNAs which were immediately frozen in an ethanol-dry ice bath for 5 min and then placed at 37°C for 25 min. One hundred microlitres were then spread on selection plates.

As a result of transformation of *A. rhizogenes* with pFW105.10.2 and pFW105.10.3, 12 colonies of pFW105.10.2 and 8 colonies of pFW105.10.3 grew on MM plates with carbenicillin (200 µg/ml) and kanamycin (50 µg/ml). After conjugation of pPH1JI (this plasmid confers resistance to gentamycin and is incompatible with pFW105 derivatives) into the *A. rhizogenes* transformants, 6 colonies of pFW105.10.2 and 3 colonies of pFW105.10.3 were obtained, all of which were resistant to kanamycin, ampicillin, gentamycin (50 µg/ml), and sensitive to tetracycline. Southern analysis confirmed that the required recombinational event had taken place in all nine of the *A. rhizogenes* transconjugants (data not shown).

Propagation of plantlets and culture of roots induced by infection with Agrobacterium

The in vitro plants used in this study were derived from in vitro storage and nodal cuttings. Normally, four nodal cuttings were inoculated into each test tube which contained 4 ml of propagation medium (see Table I). These cuttings were maintained at 22°C under 16 h/day cycle at 3000 lux illumination. Under these conditions, young plantlets develop rapidly and provide sufficient material to initiate shaken liquid cultures.

In vitro-derived plants, approximately 6 cm in length, were wounded with a sterile needle, and a small quantity of the various *A. rhizogenes* clones were inoculated into the wound site. Hairy roots formed within several

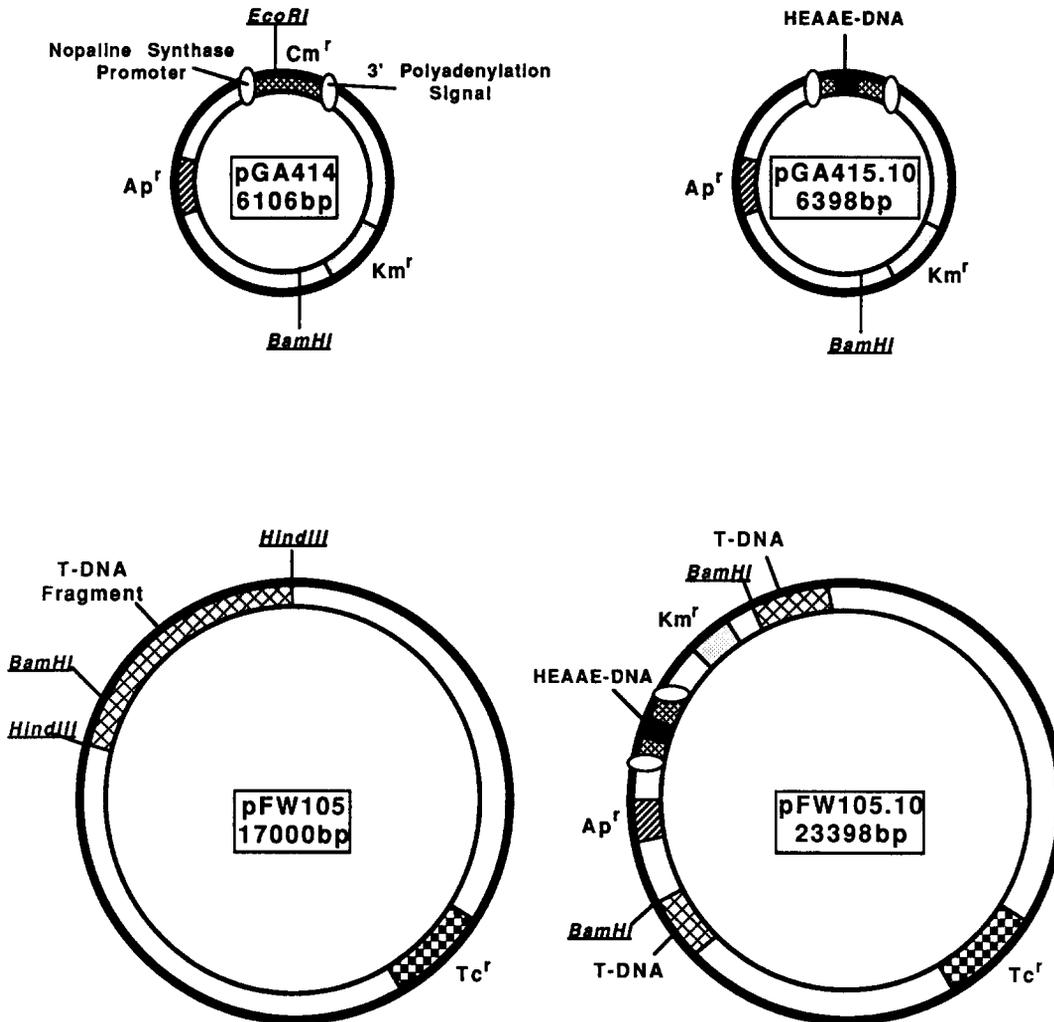


Fig. 2. Maps of the vectors used in these experiments as described in Materials and Methods.

Table I. Composition of culture media was based on Murashige and Skoog [27] with additions as indicated. Media were sterilized by autoclaving for 15 min. All numbers are in ppm unless otherwise indicated.

MS*	Propagation nodes	Propagation liquid	In vitro tuberisation	Plant regeneration from roots
Inositol	100.0	100.0	100.0	100.0
Ca-Pantothenic acid	2.0	2.0		
Benzylaminopurine		0.5	5.0	1.0
Gibberellic acid		0.4		10.0
Napthaline acetic acid		0.01		
Indole acetic acid				1.0
Chlorocholine chloride			500.0	
Thiamine	0.4	0.4	0.4	0.4
Sucrose (%)	3.0	2.0	8.0	0.5
Agar (%)	0.8			0.8
pH	5.5	5.5	5.5	5.5

weeks and were carefully cut into 4–6 cm long sections and deposited onto the surface of propagation medium in sterile 9 cm² petri dishes.

Propagation of in vitro tubers

Tubers were induced in vitro on plantlets regenerated from transformed material by the addition of chlorocholine chloride (CCC) to the medium together with a 3-fold increase in the concentration of sucrose and benzylaminopurine (BAP). This addition was achieved in two ways: firstly, by removing the existing propagation medium and replacing it with medium containing CCC, BAP and 8% sucrose, or, secondly, by adding stock CCC, BAP and sucrose solution to the flask containing the original propagation medium. The flasks, after addition of CCC stimulus, were transferred to total darkness without agitation at 22°C. In vitro tuber transformation was analysed 8–10 weeks later.

DNA extraction and Southern analysis

To confirm the incorporation of HEAAE-DNA into the plant genome, in vitro tubers were harvested and DNAs were extracted as follows: 10 g of fresh plant tissue were frozen with liquid nitrogen immediately and ground in cold mortar and pestle [11]. Twenty-five milliliters of homogenization buffer (20 mM MES, 20 mM KCl, 25 mM NaCl, 10 mM β -mercaptoethanol, 600 mM sucrose, 40% (v/v) glycerol, 400 μ g/ml EtBr) were added and mixed by inverting gently. The homogenate was filtered through two layers of cheese cloth and centrifuged at 1500 g for 30 min at 4°C. The supernatant was discarded and 7.5 ml of nuclei lysis buffer (100 mM Tris—HCl, pH 8.0, 20 mM NaCl, 10 mM EDTA, 1% (w/v) lauryl sarcosine) were added and the pellet was dispersed by a spatula. A quantity (7.5 g) of CsCl and 1 ml of ethidium bromide (4.4 mg/ml) were added and mixed gently. The solution was clarified by centrifugation at 10 000 rev./min at 4°C for 10 min and the supernatant fraction was collected and its density was readjusted to 1.55 with CsCl followed by ultracentrifugation in a Ti-75

rotor at 64 000 rev./min at 20°C for 16 h. The chromosomal DNA band was removed and extracted with TE-saturated isopropanol to remove ethidium bromide. The DNA solution was dialyzed and 5 μ g of purified DNA was digested to completion with BamHI, and the fragments were run on an 0.8% agarose gel. The gel was blotted to nitrocellulose filter paper [12] and was hybridized either with a CAT-HEAAE fusion gene probe (pSP547) or HEAAE-DNA specific oligonucleotides which were labeled by phosphorylation with either [α -³²P]dATP or [γ -³²P]ATP, respectively [13,14].

The labeled DNA (spec. act., 10⁸ cpm/mg) was mixed with 4 ml of a probe solution and poured into a seal-a-bag containing the nitrocellulose filter and placed in a 70°C water bath overnight. The filter was removed and washed as described [10].

The synthetic oligonucleotide (ATCCATCC-ATTTCTTA) used for the hybridization experiment was produced on an Applied Biosystems Model 380A DNA synthesizer and was purified according to the manufacturer's recommendation.

Northern analysis and RNA detection

Approximately 15 g of tuber tissue from control and transformed plants were mixed with 50 ml of RNA buffer and homogenized with a Polytron™ mixer set at 50% power. The homogenate was filtered through 8X cheese-cloth and spun at 7000 rev./min for 10 min at 4°C. To the supernatant, 20% SDS and 0.2 M EDTA, were added to a final concentration of 1% and 0.015 M, respectively. This mixture was twice-extracted with an equal volume of phenol/chloroform and once with an equal volume of chloroform. Total crude RNA was recovered by ethanol precipitation. This RNA preparation was dissolved in double-distilled H₂O and mixed with an equal volume of 4 M LiCl and allowed to incubate at 4°C overnight. Poly A⁺ RNA was purified using Hybond-mAP™ according to the manufacturer's instructions (Amersham). Northern analysis and RNA detection were conducted as described [15,16]. The RNA was fractionated on

1% agarose-formaldehyde gels. Purified radiolabeled HEAAE-DNA was used as the probe under conditions previously described.

Preparation of CAT antiserum

Two New Zealand white rabbits were immunized intramuscularly with 1 ml of a solution of 36 µg/ml chloramphenicol acetyl transferase (CAT) in Freund's Complete Adjuvant. Each rabbit received a second immunization (36 µg of CAT in Freund's Incomplete Adjuvant, intramuscularly) and a final immunization (36 µg of CAT in 0.15 M phosphate buffered saline, subcutaneously) 3 and 7 weeks following the first injection. The rabbits were then bled by cardiac puncture, while under fentanyl-inapsine restraint, 3 days after the final injection.

Western analysis and protein detection

Tuber tissue was ground up by mortar and pestle and subsequently sonicated (10 s at 50 W) in extraction buffer (50 mM Tris-HCl, pH 7.5, 5 mM dithiothreitol, 0.05% Triton X-100, 50 mM EDTA, 0.19 mg/ml phenylmethylsulphonyl fluoride). The solution was clarified by centrifugation at 10 000 × *g* for 10 min at 4°C, the supernatant fraction concentrated with acetone and the protein concentration was determined [17]. Ten microliters (50 µg) of each sample was mixed with 3 µl of sample buffer and heated at 95°C for 10 min. The samples were subjected to polyacrylamide gel electrophoresis at 120 V until the tracking dye had reached the bottom of the gel (12% acrylamide for the analyzing gel and 3.9% acrylamide for the stacking gel). After electrophoresis the samples were electroblotted onto nitrocellulose filter paper in transfer buffer (25 mM Tris, pH 7.0, 192 mM glycine, 20% (v/v) methanol) at 70 V for 3 h [18]. Protein, on the filter, was detected by using horseradish peroxidase and CAT specific antibodies [20]. Non-specific binding to the filter was reduced by soaking the filter with blocking buffer (50 ml/100 cm², 1% gelatin, 0.1% Tween 20 in PBS: 10 mM Na-phosphate, pH 7.4, 0.15 M NaCl, 3 mM KCl) for 1 h with gentle rocking. Antiserum, diluted (1:100) in blocking buffer, was added (25 ml/100 cm²) and

incubated for 2 h at room temperature with gentle rocking. The filter was removed and washed three times for 5 min each with 500 ml washing buffer (0.05% Tween 20 in PBS) and incubated in enzyme conjugate (goat anti-rabbit IgG, peroxidase conjugated, diluted 1:600 in washing buffer, 25 ml/100 cm²) for 2 h at room temperature and then washed as above. The filter was then incubated in substrate solution (30 mg of 4-chloro-1-naphthol in 5 ml of ethanol, 50 ml of 0.05 M Tris, pH 6.8 and 20 ml of 30% hydrogen peroxide). After color development, the filter was washed three times with washing buffer, one time with distilled water and air-dried. Densitometric analysis was used to quantify the amount of proteins detected in the western blot.

Results

Infection with A. rhizogenes and regeneration of potato plants

Two colonies from each of pFW105.10.2 and pFW105.10.3 were used to infect potato plants. Hairy roots began to emerge from the wound sites 2 weeks after test plants were wounded and inoculated with wild type or transformed *A. rhizogenes* (Fig. 3A). These roots were excised approximately 4 weeks later and used to regenerate transformed plants (Fig. 3B). In all cases tested, plantlets could be regenerated from the transformed roots. These plantlets were removed and propagated by single node cuttings (Fig. 4A). The material was also transferred for liquid media propagation and, at an appropriate stage, in vitro tubers were induced. These in vitro tubers (Fig. 4B) were small, normally 3–5 mm in diameter, but are known to be morphologically and biochemically identical to field produced tubers [19]. This method allows for efficient screening of the tubers from transformed regenerated plants for the presence of the HEAAE-DNA, mRNA and protein products.

Detection of HeAAE-DNA integration and segregation in transformed potato plants

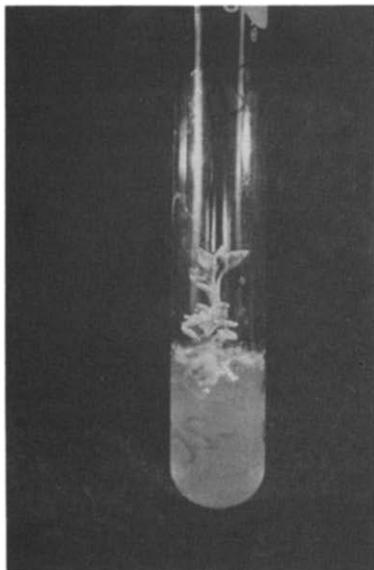
Results of Southern hybridization, with

A**B**

Fig. 3. Hairy roots from potato plants infected with *A. rhizogenes*. (A) Hairy roots start to develop from the wound site where the plants were infected with control and modified *A. rhizogenes* 4 weeks post-infection. (B) Hairy roots can be transferred to regeneration media and shoots begin to form in approximately 4 weeks (clockwise from top left).

pSP547 (CAT-HEAAE fusion gene probe), of DNAs isolated from minitubers produced by transformed control and treatment plants are shown in Fig. 5A. Hybridization, of isolated

A



B

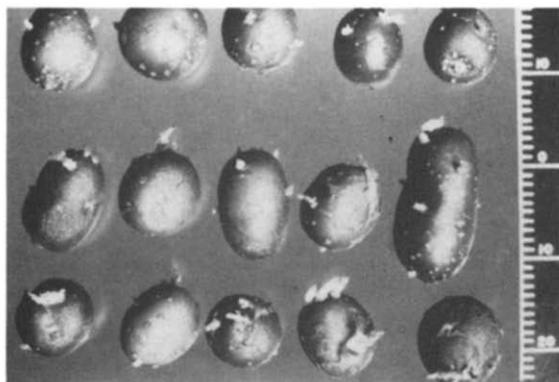


Fig. 4. Regenerated transformed potato plant and transformed in vitro potato tubers. (A) The transformed plants were propagated by placing single node cuttings in culture tubes as described in Materials and Methods. (B) Tubers from control and treatment transformed plants were induced in vitro as described in Materials and Methods. The 'minitubers' were small, normally 3–5 mm in diameter, but were morphologically and biochemically identical to the field produced tubers.

DNAs from tuber tissue derived from these plants (digested with BamHI), with the pSP547 probe, revealed a single band corresponding to 6.4 kb in size. This is as expected, because the BamHI digestion excises a 6.4-kb internal fragment corresponding to the pGA415.10 derivatives. However, one plant appears to have undergone an unexplained recombinational event (plant K2.1). Since the positive control represents 5 copies of HEAAE-DNA, densitometric analysis indicates that the transformed plants tested have about 4–6 copies of HEAAE-DNA per haploid genome. Similar hybridization results were obtained utilizing the oligonucleotide probe specific for HEAAE-DNA indicating that the gene fragment was still present within the CAT gene coding sequence (Fig. 5B).

Expression of HEAAE-DNA and its quantitation in transformed potato plants

The results of poly⁺ RNA hybridization with the pSP547 probe is shown in Fig. 6A. Distinct bands were detected which correspond to a mRNA size class of approximately 1100 bases. This agrees with the expected size class of about 1000 bases which would be derived from the CAT-HEAAE mRNA.

The CAT protein is known to be approximately 25 kDa in mass and the HEAAE-protein, based on computer analysis, is about 13 kDa in mass. Combined, the fusion protein should be about 38 kDa in mass which is in agreement with the observed band on the western blot (Fig. 6B). Minor bands are observed of lower molecular weight which, we presume, to be degradation products of the 38 kDa protein.

The amount of protein detected does not necessarily match with what would be expected from the level of mRNAs isolated. The K-2 transformed plants, in particular, have a low level of protein detected even though the amount of mRNAs are comparable in both K2 and K7 varieties. A possible explanation is a higher degree of degradation of the proteins in the K-2 plants. Densitometric analysis of western blot data (Table II) shows that the amount of protein detected comprises 0.02–0.35% of

total plant protein. Material isolated from K-2.1 minitubers (lane 3) contains approximately ~ 11 ng of detected proteins which corresponds to $\sim 0.02\%$ of total plant protein. On the other hand, material purified from K-7.2 minitubers

(lane 6) has ~ 177 ng of protein detected which corresponds to $\sim 0.35\%$ of total plant protein.

To make a significant impact on the essential amino acid content of crop plants, the synthetic genes must be expressed efficiently within the plant cell. The amount of HEAAE-protein which must be produced within the tuber, to significantly enhance the essential amino acid content of the potato, has yet to be ascertained. However, according to our calculations, if enough of the modified potato were fed to a 20kg child to satisfy 20% of their caloric needs (600 g), then about 25% of the protein in the potato tuber must be expressed as HEAAE-protein in order to provide the proper balance of essential amino acids. It is a significant challenge to achieve this level of expression of the HEAAE-protein.

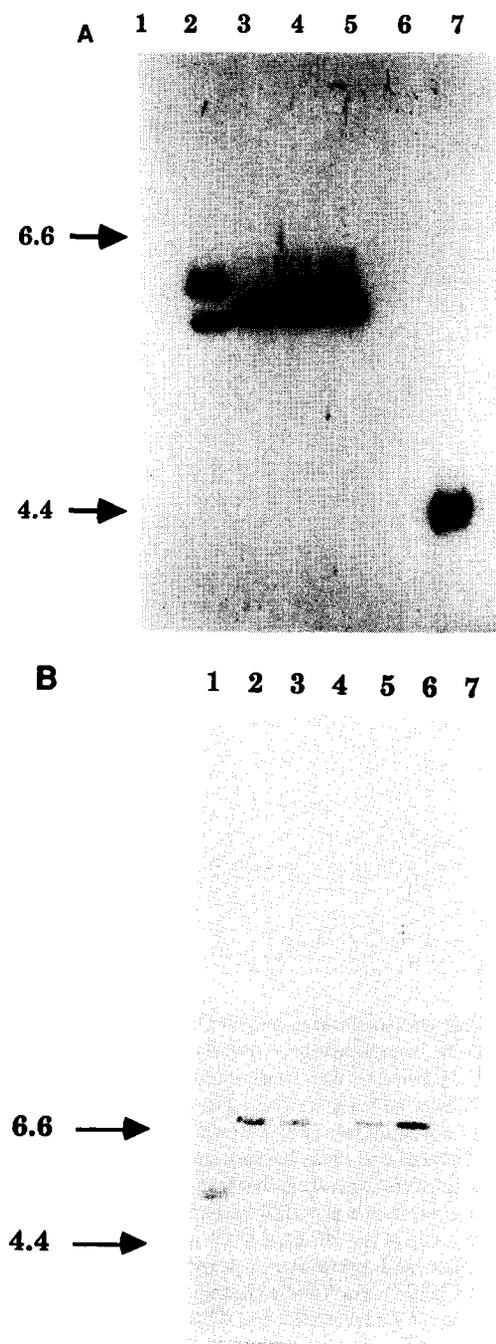


Fig. 5. Southern hybridization analyses of DNAs isolated from transformed potato plants utilizing both (A) pSP547 (CAT-HEAAE fusion) and (B) HEAAE-oligonucleotide probes. To demonstrate the integration of HEAAE-DNA into the plant genome, plant nuclear DNAs were extracted from 'minitubers' induced from several selected transformed plants and subjected to Southern hybridization analysis with the above-mentioned radiolabeled probes. Condition for the analyses are described in Materials and Methods. (A) Lane 1, the negative control, contains DNA extracted from minitubers obtained from a regenerated K2 plant (designated K2*) which had been transformed with *A. rhizogenes* containing pFW105. Lanes 2 and 3 contain DNAs purified from minitubers obtained from two separate plants (derived from potato clone K2 designated: K2.1 and K2.2) which had been transformed with *A. rhizogenes* containing pFW105.10.3. Lanes 4 and 5 contain DNAs isolated and analyzed in a similar fashion except they were obtained from minitubers derived from two separate transformed and regenerated plants from potato clone K7 (designated K7.1 and K7.2). Lane 6 contains DNA extracted from negative control K7 minitubers (designated K7*) obtained from a plant which had been transformed with *A. rhizogenes* containing pFW105. Lane 7 is the positive control pSP547 DNA treated with BamHI. (B) Lane 1 contains the positive control pSP547 DNA treated with BamHI. Lanes 2 and 3 contain DNAs purified from K2.1 and K2.2 minitubers, respectively. Lane 4 contains minituber-DNA from K2*. Lanes 5 and 6 contain DNAs isolated from K7.1 and K7.2 minitubers, respectively. Lane 7 contains minituber DNA from K7*. All of the DNAs isolated from minitubers were treated with BamHI prior to fractionation on agarose gels. The numbers to the left of the figure are molecular size markers in kilobase pairs.

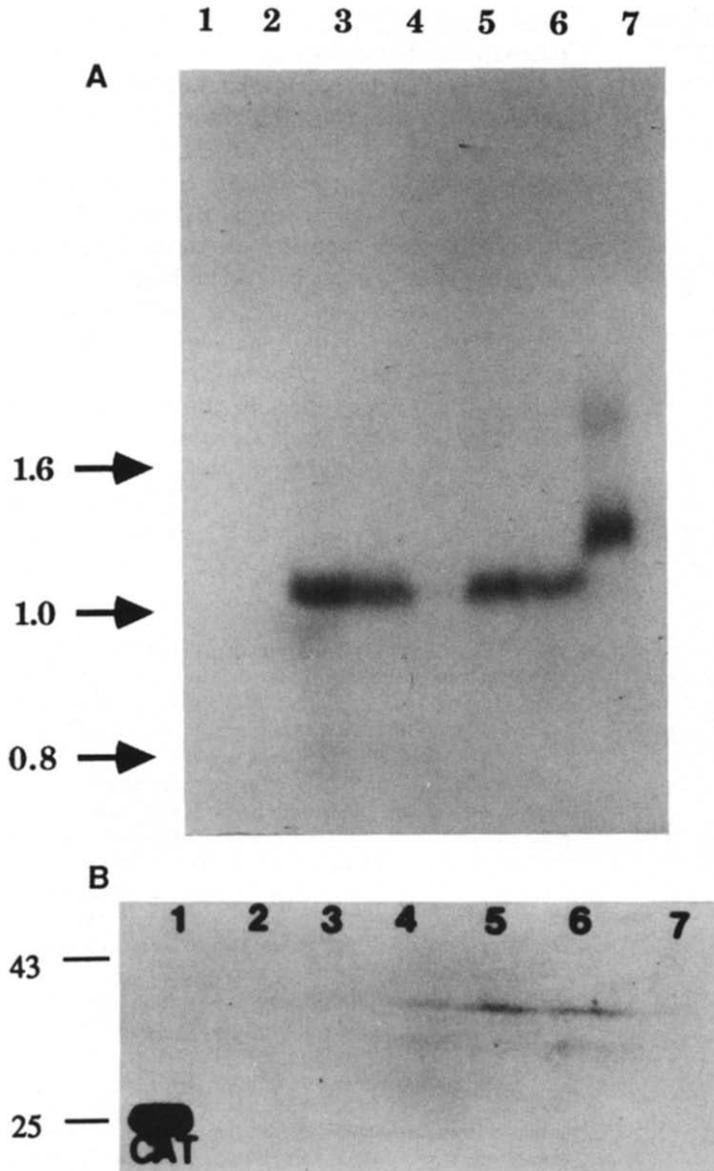


Fig. 6. Analysis for expression of HEAAE-DNA from minitubers obtained from transformed potato plants. (A) To demonstrate the expression of HEAAE-DNA as mRNA, poly A⁺ RNAs were isolated from minitubers derived from transformed potato plants and subjected to northern analysis utilizing a pSP547 probe as described in Materials and Methods. Lane 1 contains the negative control: poly A⁺ RNAs extracted from minitubers obtained from K2*. Lanes 2 and 3 contain poly A⁺ RNAs purified from minitubers obtained from K2.1 and K2.2, respectively. Lane 4 contains the negative control: poly A⁺ RNAs extracted from minitubers obtained from K7*. Lanes 5 and 6 contain RNAs isolated and analyzed in a similar fashion except they were obtained from minitubers derived from K7.1 and K7.2. Lane 7 is the positive control single-stranded Hha I fragment purified from pBR325 which contains the complete gene sequence of the CAT protein and is ~1350 bases in length. The numbers at the left of the figure are in kilobases. (B) To determine the level of expression of the HEAAE-DNA as protein, total proteins were isolated from minitubers from transformed plants. Lane 1 contains CAT protein as a positive control. Lane 2 contains proteins purified from minitubers obtained from the control plant K2* while lanes 3 and 4 contain total proteins purified from minitubers from K2.1 and K2.2. Lanes 5 and 6 contain total proteins extracted from K7.1 and K7.2 plants while lane 7 contains the proteins purified from the control K7* minitubers.

Table II. Quantitation of western blot data of transformed potato plants by densitometric analysis.

Sample		Total integration	Adjusted integration	Amount of proteins (ng)	% total proteins
Lane 1	CAT Protein	26860.0	26681.0	3000.0	
Lane 2	Plant K2*	179.0	0.0	0.0	
Lane 3	Plant K2.1	280.0	101.0	11.0	0.02
Lane 4	Plant K2.2	523.0	344.0	39.0	0.08
Lane 5	Plant K7.1	1127.0	848.0	107.0	0.21
Lane 6	Plant K7.2	1752.0	1573.0	177.0	0.35
Lane 7	Plant K7*	184.0	0.0	0.0	

Discussion

The experiments described in this paper demonstrate that the technologies exist to genetically engineer the potato using synthetic gene sequences. They also illustrate some of the advantages that can be obtained by the use of synthetic genes [20]. These experiments were carried out using wildtype *A. rhizogenes* which still contained the genes for 'hairy' root formation from which plants could be regenerated. However, these genes can cause some abnormalities in the phenotype of the regenerated plant (unpublished observations with transgenic sweet potatoes, although, we observed no demonstrable differences in potatoes). To overcome this potential problem, we have also utilized 'disarmed' vectors and have regenerated potato plants from cultured leaf-discs.

Tubers from transformed regenerated potato plants were analyzed for HEAAE-DNA integration into the plant genome and subsequent expression into mRNA and protein. Utilizing Southern analysis, the integration of the gene into the plant genome was demonstrated by hybridization of plant nuclear DNAs with the pSP547 probe (CAT-HEAAE fusion). Similar results were obtained when a synthetic oligonucleotide probe, specific to HEAAE-DNA, was used. Furthermore, mRNAs, with the expected length for CAT + HEAAE-DNA, have been detected by the hybridization of poly A⁺ RNAs with the pSP547 probe in northern blot analysis. Also, proteins have been analyzed by western blot techniques using antibodies

against CAT protein. In this case, CAT-HEAAE proteins, with the expected molecular weight (38 kDa), have been detected. Densitometric analysis of western blot data has shown that CAT-HEAAE proteins comprise about 0.02–0.35% of the total plant protein. By calculation, there would be up to 1.1 percentage increase of essential amino acids in these transgenic plants.

To effectively express a foreign gene within the plant, it needs proper modulators such as strong promoters and some sequences to enhance the translation. For example, it was reported that the cauliflower mosaic virus 35S promoter, under some conditions, is about one hundred times more active than the nopaline synthetase promoter [21]. It was also reported that duplication of the cauliflower mosaic virus 35S promoter sequences could create a strong enhancer in plants [22]. Other modulators, which have been known to enhance the translation of genes such as plant viral leader sequences [23], can also be used to enhance translation of foreign genes. Given the appropriate modulators, we believe it should be possible to achieve the level of expression required to satisfy the need mentioned above. Future success would make it possible for a child to satisfy a major portion of their essential amino acid requirement with just such a modified potato.

Another important consideration, when designing a synthetic protein to enhance the essential amino acid content of crop plants, is protein stability. Our data suggests that, at least in certain cultivars, the HEAAE-protein

was unstable. Due to recent advances in protein chemistry, we can speculate on the structure and stability of new proteins much better than before [24]. Indeed, we have designed a new protein of high essential amino acid content (designated HEAAE-II) which should be more stable as it folds and aggregates reminiscent of known plant storage proteins [25] (unpublished results in JMJ laboratory).

It would also be highly desirable to cause the expression of the HEAAE-proteins predominantly in the plant material which is consumed. The potato, like many important plants, contains its food deposits in a specialized organ, in this case a tuber. The ultimate goal of this research is to obtain high level expression of HEAAE-DNA only within the tuber. In the case of potatoes, patatin, the major potato tuber protein, is a family of 40 kDa glycoproteins that constitute forty percent of the soluble protein in tubers [26]. Therefore, the transcriptional control regions of the patatin gene would be a good candidate to obtain the tissue specific expression of the HEAAE-DNA.

When the techniques of synthetic protein design and gene synthesis are applied to the genetic engineering of this economically important basic food crop, it is expected that the work's culmination will yield a modified potato with superior nutritional quality.

Although significant improvement of essential amino acid content in potato plant protein has not been achieved due to the low level of expression, a system has been developed which should, in time, allow for the improvement of the quality of plant proteins by using synthetic genes.

The insertion of this and other genes may serve to improve the quality of potato production. Through its germplasm distribution network, the International Potato Center will make any improved varieties available to developing country national potato programs.

Acknowledgement

This research was sponsored by the Agency for International Development, Washington, D.C. Project number 936-5542.

References

- 1 A. Crossway, J. Oakes, J. Irvine, B. Ward, V. Knauf and C. Shewmaker, Integration of foreign DNA following microinjection of tobacco mesophyll protoplasts. *Mol. Gen. Genet.*, 202 (1986) 179–185.
- 2 R. Beachy, Z. Chen, R. Horsch, S. Rogers, N. Hoffmann and R. Fraley, Accumulation and assembly of soybean b-conglycinin in seeds of transformed petunia plants. *EMBO J.*, 4 (1985) 3047–3053.
- 3 N.O. Espinoza, R. Estrada, P. Tovar, J. Bryan and J.H. Dodds, Specialized Potato Technology, Vol. 1, International Potato Center, Lima, Peru, 1984, pp. 1–20.
- 4 J.H. Dodds, CIP Collaborative research on tissue culture and genetic engineering for potato improvement, in: CIP Circular, Vol. 15, International Potato Center, Lima, Peru, 1987, pp. 1–5.
- 5 N.O. Espinoza, R. Estrada, D. Silva-Rodriguez, P. Tovar, R. Lizarraga and J.H. Dodds, The potato: a model crop plant for tissue culture. *Outlook Agric.*, 15 (1986) 21–27.
- 6 J.M. Jaynes, P. Langridge, K. Anderson, C. Bond, D. Sands, C. Newman and R. Newman, Construction and expression of a synthetic DNA fragment coding for polypeptides with elevated levels of essential amino acids. *Appl. Micro. Biotechnol.*, 21 (1985) 200–220.
- 7 R. Hansen, Food intake index, in: *Nutritional Quality Index of Food*, AVI, 1979, pp. 169–543.
- 8 G.A. Huffman, F.F. White, M.P. Gordon and E.W. Nester, Hairy-root-induced plasmid: physical map and homology to tumor-inducing plasmids. *J. Bacteriol.*, 157 (1984) 269–276.
- 9 G. An, B.D. Watson, S. Stachel, M.P. Gordon and E.W. Nester, New cloning vehicles for the transformation of higher plants. *EMBO J.*, 4 (1985) 277–284.
- 10 T. Maniatis, E. Fritsch and J. Sambrook, in: *Molecular Cloning: A Laboratory Manual*, CSH, 1982, pp. 122–369.
- 11 P. Chee, R. Klassy and F. Slightom, Expression of a bean protein phaseolin minigene in foreign tissues. *Gene*, 41 (1986) 47–57.
- 12 P. Goldsbrough, S. Gelvin and B. Larkins, Expression of maize zein genes in transformed sunflower cells. *Mol. Gen. Genet.*, 202 (1986) 374–381.
- 13 R. Horsch, R. Fraley, S. Rogers, P. Sanders, A. Lloyd and N. Hoffmann, Inheritance of functional foreign genes in plants. *Science*, 223 (1984) 496–498.
- 14 R. Horsch, J. Fry, N. Hoffmann and D. Eichholtz, A simple and general method for transferring genes into plants. *Science*, 223 (1985) 1229–1231.
- 15 C. Sengupta-Gopalan, N. Reichert, R. Barker, T. Hall and J. Kemp, Developmentally regulated expression of the bean β -phaseolin gene in tobacco seed. *Proc. Natl. Acad. Sci. U.S.A.*, 82 (1985) 3320–3324.
- 16 D. Rabussay, L. Uher, G. Bates and W. Piastuch, Electroporation of mammalian and plant cells, *Focus*, 9 (1987) 1–5.
- 17 M. Bradford, A rapid and sensitive method for the

- quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, 72 (1976) 248–254.
- 18 H. Towbin, T. Staehelin and J. Gordon, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.*, 76 (1979) 4350–4354.
- 19 P. Tovar, R. Estrada, L. Schilde-Rentschler and J.H. Dodds, Induction and use of in vitro tubers, in: CIP Circular, Vol. 13, International Potato Center, Lima, Peru, 1985, pp. 1–6.
- 20 J.M. Jaynes and J.H. Dodds, Crop plant genetic engineering: science fiction or science fact? *Outlook Agric.*, 16 (1987) 1–5.
- 21 P. Sanders, J. Winter, A. Barnason, S. Rogers and R. Fraley, Comparison of cauliflower mosaic virus 35S and nopaline synthase promoters in transgenic plants. *Nucl. Acid Res.*, 15 (1987) 1543–1558.
- 22 R. Kay, A. Chan, M. Daly and J. McPherson, Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. *Science*, 236 (1987) 1299–1302.
- 23 S. Jobling and L. Gehrke, Enhanced translation of chimaeric messenger RNA containing plant viral untranslated leader sequence. *Nature*, 325 (1987) 622–625.
- 24 K.R. Shoemaker, P.S. Kim, E.J. York, J.M. Stewart and R.L. Baldwin, Tests of the helix dipole model for stabilization of α -helices. *Nature*, 326 (1987) 563–567.
- 25 M. Matzke, A. Susani, A. Binns, E. Lewis, I. Rubenstein and A. Matzke, Transcription of a zeine gene introduced into sunflower using a Ti plasmid vector. *EMBO J.*, 3 (1984) 1525–1531.
- 26 S. Rosahl, R. Schmidt, J. Schell and L. Willmitzer, Isolation and characterization of a gene from *Solanum tuberosum* encoding patatin, the major storage protein of potato tubers. *Mol. Gen. Genet.*, 203 (1986) 214–220.
- 27 T. Murashige and F. Skoog, A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.*, 15 (1962) 473–497.