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Improving peanut protein quality: Expression of a synthetic storage protein

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Peanut is an affordable legume used in most households. It represents one of the most important protein supplies worldwide. However, peanut proteins are deficient in several essential amino acids (EAA), like most plant proteins; whereas plants are the main source of dietary proteins consumed by humans and livestock. This could lead to protein malnutrition in areas where people diet relies on one or two staple foods. Based on its high nutritional value, peanut is a good candidate for genetic biofortification. This study is aimed at expressing an EAA-rich artificial storage protein (ASPx) into peanut seeds for increased nutritive value. The ASPx derived gene was introduced into peanut via *Agrobacterium*-mediated transformation. Molecular analysis of regenerated kanamycin resistant plants using polymerase chain reaction (PCR) and Southern hybridization indicate the stable integration of one copy ASPx gene in transgenic plants. The expression of the ASPx in transgenic peanuts seeds was detected by mass spectrometry (multiple reaction monitoring). Amino acids analysis showed an increase of 12 to 19% of most EAA (Val, Tyr, Phe, Iso, Leu, Met) in a transgenic line. The results show that the nutritional quality of peanut could be improved.

Key words: Genetic biofortification, essential amino acid, storage protein, peanut.

INTRODUCTION

With the growth of the world's population, global food demand is tremendously increasing. The increase of the production of animal-based protein would require more water and land, resulting in negative environmental effect (Henchion et al., 2017). Legumes are not only the second most important food source (Kouris-Blazos and Belski, 2016), but also good sources of dietary proteins (Singh, 2017), and cheap alternatives for populations, especially in developing countries where many cannot afford meat or dairy products (Maphosa and Jideani, 2017). With the increased incidence of metabolic diseases, plant proteins are appropriate source of protein without the concern of cholesterol (Maphosa and Jideani, 2017). However, with respect to human and animal nutrition, most seeds do not provide a balanced diet in protein because of deficiencies in one or several essential amino acids (EAA). This last decade estimates indicate that at least half of the world's population suffer from diseases caused by insufficient

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supplies of essential nutrients (Zhu et al., 2010; McGuire, 2015; Tien Lea et al., 2016). The lack of EAA in human diet can lead to protein deficiency, which negatively affects the growth of children, therefore hinders the development of affected countries. Possible solutions include dietary diversification and food supplementation but, these strategies are difficult to implement in developing countries, because poverty is widespread, and populations' dietary habits rely, most of the time, on couple of food crops. Thus, biofortification becomes a good alternative for sufficient and sustainable production of nutritionally improved foods (Saltzman et al., 2013).

Peanut (*Arachis hypogaea* L.) is a nutrient-dense legume providing over 30 essential nutrients and phytonutrients (Toomer, 2018). It is a major source of plant proteins in most tropical and subtropical regions of the world; and it is already used as base in some ready-to-use therapeutic food. However, the protein of peanut meal is of low quality as several EAAs (cysteine, methionine, threonine, lysine, isoleucine and valine) occur in limiting concentration (Andersen et al., 1998; Venkatachalam and Sathe, 2006). Because it is one of the cheapest staple foods and one of most consumed legumes in many countries of the world (Arya et al., 2016), peanut is a good candidate crop for protein biofortification. One of the strategies to enhance the level of aminoacids in food crops is to transfer genes encoding proteins with high content of essential amino acids (Zhang et al., 2003; Pérez-Massot et al., 2013). In this study, we report the successful genetic transformation of peanut hypocotyls using an amino acid rich artificial storage protein (ASPx) gene and the expression of the gene in the peanut seeds.

MATERIALS AND METHODS

Plant material

Seeds of peanut plant cultivar "Georgia Green" (Runner Market type) were obtained from Birdsong Peanut Co (Georgia, USA) and used in this study. The cultivar was selected, because it is the most commercially grown in the Southeast region of the USA.

Plasmids and bacterial strains

Vector construction

ASPx gene and protein: An ASPx and its corresponding gene were designed by Dr Jesse Jaynes (Tuskegee University, USA). The ASPx has been designed for stable expression *in vivo*. It is composed of 124 amino acids, arranged in 4 helical repeating monomers. It has about 75% of essential amino acid (EAA), including methionine (16%), lysine (13%), threonine (13%), isoleucine (10%), tryptophan (10%), valine (6.5%), phenylalanine (3%) and leucine (3%). In this study, the ASPx gene (420 bp) was synthesized by Operon Biotechnologies, Inc (Huntsville, AL USA) and carried into the pPCR-Script plasmid (Figure 1).

Plasmid pDK612 construction: The plasmid used for the genetic transformation of peanut in this study was constructed in two steps from the pPCR-Script. In the first step, the 420 bp ASPx gene was amplified using 2 primers, pf_ASPx and pr_ASPx, designed with *BamHI* and *BglII* restriction sites. The amplified polymerase chain reaction (PCR) fragment was cloned into TOPO TA vector (Invitrogen Inc, CA USA), resulting into the plasmid Topo-ASPx. In the second step, the 0.5kb *BglII*/*BamHI* fragment of ASPx gene from Topo-ASPx was subcloned into the plasmid pLAU2 (Idnurm et al., 2017) between the CVMV promoter and the E9 terminator. The resulting plasmid was pDK612. The gene arrangement from the right border to the left border of the T-DNA is p35S-nptII-NOS pCVMV-ASPx-E9. Plasmid amplification was done in TOP10 chemically competent *E. coli* cells from Invitrogen Inc.

Agrobacterium transformation: The plasmid pDK612 was mobilized into *Agrobacterium tumefaciens* strain EHA 105 by electroporation (Bio-Rad Gene Pulsar, Biorad, Hercules, CA). Bacteria were kindly donated by Dr. M. Egnin, (Tuskegee University, USA). Transformed *Agrobacterium* were used to infect peanut hypocotyls.

***Agrobacterium* preparation for peanut transformation:** For transformation, glycerol stocks of EHA105/pDK612 were prepared from a single colony of streaked plates grown at 28°C, divided into 300 µl aliquots and stored at -80°C. A day before transformation, the glycerol preps were used to inoculate liquid YEP medium supplemented with appropriate antibiotics (kanamycin or hygromycin 50 mg/l, along with rifampicin 15 mg/L). Cultures were incubated at 28°C for 16-20 h at 180-200 rpm (Gyromax 720 Orbital Shaker). For peanut transformation experiments, bacteria were pelleted for 10 min at 3500 *g* in a Beckman JA-20 rotor at 4°C. Pellets were resuspended in ½ MSi medium (half-strength MS -Murashige and Shoog- medium with 1 g/L myo-inositol and without sugar) (Egnin et al., 1998) and the OD_{600} was adjusted to 0.8-1.0 prior to the infection of peanut hypocotyl explants.

Genetic transformation, regeneration and selection of transgenic plants

Explants preparation

Mature peanut seeds were surface sterilized with 20% chlorox (2 times 30 min), followed by several rinses with sterile water. Embryo axes were excised from the seeds and germinated onto MS basal medium (Murashige and Shoog, 1962) supplemented with TDZ 10 µM (MSTDZ 10). Hypocotyl pieces (5-8 mm) from 5-6-day-old seedlings were used as explants for transformation. All cultures were incubated at 26±2°C under 16/8 h light/dark period.

Transformation and production of primary transformants

A modified method of Egnin et al. (1998), described by Dodo et al., (2008), was used in this experiment. Hypocotyl explants were rinsed three times for 20 min each in ½ MSi solution, and then immersed in an *Agrobacterium* suspension (OD = 0.9) for 10 min, blotted on sterile tissue papers, and co-cultivated inverted on MS0 medium (MS basal salts and vitamins, 100 mg/L myo-inositol, 30 g/L sucrose) for 5 days.

Selection: After the 5-day co-cultivation period, infected explants were transferred onto resting medium (MSTDZ and carbenicillin 400 mg/L) for 1-2 weeks, and then onto the selection medium (MSTDZ, carbenicillin 400 mg/L, kanamycin 200 mg/L) until appearance of shoot primordia. Elongated shoots were then transferred onto rooting medium (MS0, NAA 5 µM, Carbenicillin 100 mg/L, Kanamycin 50 mg/L). Explants were sub-cultured every 2-3 weeks. Well rooted putative transgenic shoots with well-developed roots were acclimatized for 1 week and transferred to the green house for

Figure 1. Aminoacids sequence of the ASPx.

Table 1. Sequences of primers used in the study.

Gene amplified	Primer name	Sequence	Amplicon length (Kbp)	References
ASPx gene	Pf_ASPx	5'-CTGGATCCGTTGATGATCGAGG-3'	0.5	This study
	Pr_ Aspx	5'-GGCTACTGACTCTAGAATTCGCG-3'	0.5	
CAMV35S	Primer 1 (35S-F)	5′-CAGAGGCAAGAGCAGCAGC-3′	1	Dodo et al. (2008)
	Primer 2 (35S-R)	5′-GCTGGGGTATCGATCACTGTCACAATGG-3′		
NPT2	NPT2 Fwd	5'-GCATACGCTTGATCCGGCTACC-3'	0.25	Matsumoto and Fukui (1996); Peyret et al. (2019)
	NPT2 Rev	5'-TGATATTCGGCAAGCAGGCAT-3'		
CAMV35S	Forward 35S	5'GAAGGTGAAGGTGACGACACTA3'	0.2	This study
	Reverse 35S	5'CTGTGGGTCAGCATTCTTTCTG3'		
	Probe 35S	5'FAM-TCACCACTGATAATGAGAAGGTTAGCC-TAMRA3'		
ASPx gene	ASPx-Fwd	5'GAAGGTGAAGGTGACGACACTA3',	0.2	This study
	ASPx- Rev	5'CTGTGGGTCAGCATTCTTTCTG3'		
ASPx mRNA	ASPx_FP	5'-TGGACGCATGATCGAGGAAAT-3'	0.1	
	ASP_RP	5'-TCGGCTTACACCCAGTAGGT-3'		

seeds production.

Production of subsequent generations of transgenic plants and seeds

Seeds from T0, T1 and T2 plants were germinated either through tissue culture or directly in soil to produce T1, T2 and T3 seeds.

Molecular and biochemical analysis

Genomic DNA was extracted from freeze-dried leaves using the DNeasy Plant kit (Qiagen Inc., Valencia, CA, USA). Young leaves of kanamycin resistant plants, as well as of wild type (WT) and non-transformed tissue culture control (TC) plants were used for the extraction. PCR and Southern blot analysis were performed following the standard protocols of Sambrook et al., (1989). The ASPx fragment was labelled using the AlkPhos direct labeling and detection system with CDP-Star and used as probe. For PCR amplification, different primers targeting the CAMV35S, NPT2 and ASPx gene were used (Table 1).

Taqman PCR amplification and analysis were performed using the Light Cycler 480 system (Roche Diagnostics, Corporation, Indianapolis, IN, USA), and following the manufacturer's procedure. The primers and probe were targeting the CaMV35S promoter. The sequences, presented in Table1, were designed using the software program Primer Express (Perkin-Elmer, Applied Biosystems, Foster City, CA).

Total RNA was extracted from T2 and T3 seeds using the Ambion RNAqueous-4PCR Kit (Life Technologies, Inc.). Reverse transcription was performed using the Superscript® II First-Strand Synthesis System for RT-PCR kit (Invitrogen, Inc.). About 400 ng of total RNA were used to synthesize the first strand of cDNA with the Oligo-dT primers. The first-strand cDNA obtained was amplified directly in a 25 µl reaction mixture containing primers and probe specific to the ASPx gene (FP 5'-TGGACGCATGATCGAGGAAAT-3'; RP 5'-TCGGCTTACACCCAGTAGGT-3'; probe FAM–TGAGACATGGATGAAAACCGTGATGGA-BHQ-1. Samples included a non-reverse transcriptase (nrt) control to ensure that amplification is not obtained from contaminated genomic DNA.

Crude protein was extracted from transgenic, as well as wild type seeds following a modified method described by Koppelman et al., (2001). Protein content of supernatants was determined using the Bradford assay kit (Bio-Rad) and equal amounts of proteins were loaded on each lane for gel electrophoresis (Bradford, 1976). Tris-glycine SDS-PAGE were run according to Laemmli, (1970) using a Bio-Rad Criterion cell system (Bio-Rad, Hercules, CA USA). Peptides bands around 15 kDa were excised from the 1-D gels to perform a multiple reaction monitoring experiment on the triple 5600 Qtrap mass spectrometer (Tandem MS/MS) (Applied Biosystems). Samples were digested *in vitro* using trypsin which cleaves on the carboxyl side of arginine (R) and Lysine (K). The digested peptides were then filtered by the quadrupole mass filter to select peptides. Based on the predicted cleavages of the ASPx protein with trypsin (Figure 2), the masses of resulting peptides (parent masses) and the fragmentation patterns (daughter ions) were predicted to

MIEEIMKKFETWMKTVMELWTKIMTYWVGPGRMIEEIMKKFETW

MKTVMELWTKIMTYWVGPGRMIEEIMKKFETWMKTVMELWTKI

MTYWVGPGRMIEEIMKKFETWMKTVMELWTKIMTYWV

Figure 2. ASPx protein tryptic cleavage pattern.

construct a diagnostic scan of the protein of interest. A positive control (expressed ASPx protein) was used to make method development scan. The first of the 3 quadrupoles selected for the parent mass (parent ion) of the desired analyte. The second quadrupole dissociated the parent ions by collision with an inert gas (N2) into daughter ions (b and y ions). The third quadrupole selected for one of the daughter ions. The parent–daughter ion combination allowed for a highly specific and sensitive diagnostic tool for detection and/or quantification for specific protein(s) in complex solutions. T3 seeds protein extracts were used for the experiment.

Nutritional analysis

Total protein and amino acids composition of transgenic peanut seeds from lines 2 and 6 were compared to those of the wild type (WT) peanut seeds. Total protein was analyzed by Kjeldahl according to AOAC 981.10. The amino acid composition of transgenic and wild type seeds was determined by using a C18 HPLC column equipped with an Alpha Plus Amino Acid Analyser (Biochrom, Cambridge, UK). The seeds were hydrolyzed with 6M HCl for 24 h at 110°C in an oil bath. Samples were then dried in an evaporator at 50°C, and dissolved in 5ml Na-Citrate buffer (0.2 M, pH 2.2) by vortexing for 30min. Samples were then ready to be loaded on the column for amino acid composition analysis (Lebet et al., 1994). Increment (%I) or decrement (%D) of aminoacids was calculated as followed:

(I%) = [(Transgenic seeds amino acid content − control seeds amino acid content) / control seeds
amino acid content] *100

Statistical analysis

Nutritional analysis was performed in triplicates. Means were calculated and compared by ANOVA using the SAS version 9.2 software. Tukey test was used to separate means.

RESULTS AND DISCUSSION

Production of transgenic plants and seeds expressing the ASPx gene

Primary transformants

Twenty-six independent kanamycin-resistant plants were obtained following the transformation of 479 hypocotyls explants. Eleven plants tested positive for the Southern blot of the PCR products. Ten T0 plants successfully rooted. Primary transgenic plants produced few pods (0 to 3). It has happened that primary transformants produced few seeds, but the main reason in our study was that plants were infected by the spotted-wilt disease.

Subsequent generations

From the T1 seeds, T2 seeds were produced with a germination success rate of 27.77%. Seeds from 2 transgenic lines (line 2 and line 6) successfully germinated along with a tissue culture and wild type control. T3 seeds from the same lines were produced from T2 seeds with about 50% germination success rate. The low germination rate of T1 seeds was primarily due to the small size of most seeds.

Molecular analysis of transgenic peanut plants

Putative transgenic T0 plants

During the selection for putative transgenic T0 plants, kanamycin-resistant plants were screened by PCR for the presence of the transgene. Primers specific to the CaMV35S promoter were used to amplify the putative transgenic plants along with the plasmids pDK612 and pDK30 as controls. A clear, defined and unique band was not obtained from the putative transgenic despite optimization of the PCR conditions. A smear along with unspecific bands showed on the PCR gels. So, to confirm the presence and the authenticity of the bands, PCR products were blotted onto nylon membrane for Southern hybridization (Figure 3). A 1 kb PCR amplified fragment of the CaMV35S promoter was used as probe. Out of 26 kanamycin resistant (KanR) plants, 11 plants were Southern blot positive, producing about 58% of escapes. The wild type and tissue culture control were Southern blot negative. Nine plants resulted from individual explants and 2 from the same explants, thereby 10 different transformation events occurred during the experiment, producing 10 transgenic lines.

The high number of escapes (58% of the kanamycin resistant) could be explained by the selection regime. Infected hypocotyl explants were cultured on a resting medium (MSTDZ) without kanamycin for 2 weeks before their transfer on the selection medium (MSTDZ + kanamycin). Dodo et al., (2008) also observed a high rate of escapes (over 50% due to the delay of the application

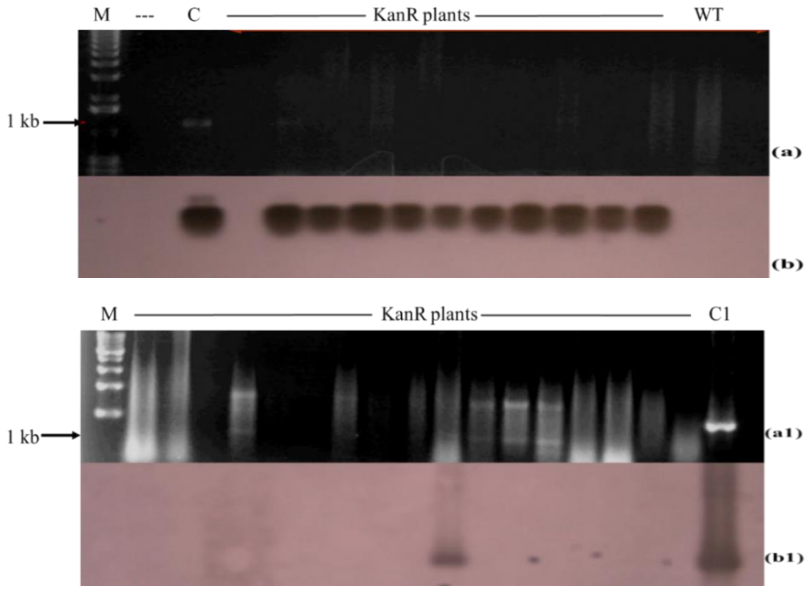

Figure 3. Molecular screening of transgenic T0 peanut plants. (a) and (a1) agarose gel of PCR targeting the CaMV35S promoter. (b) and (b1) PCR products probed with non-radioactive; Alkphos-labelled CaMV35S promoter. WT: wild type; M: 1kb step DNA ladder promega; C: plasmid pDK26; C1: plasmid pDK30.

of a selection pressure).

T1 plants

The inheritance of the ASPx transgene in the transgenic T1 plants was assessed by PCR and qPCR, targeting respectively a 250 bp fragment of the nptII gene and a 200 bp fragment of the transgene. Figure 4 shows the presence of the npt 250 bp and ASPx 200 bp fragments in the gels and Figure 5a shows the amplification curve obtained from the real-time PCR. Lines 2 and 6 plants tested positive.

Copy number analysis

Quantitative PCR: Transgene copy number was estimated by an absolute quantification of the ASPx transgene using a standard curve obtained by the amplification of the ASPx transgene in a 10-fold serial dilutions series of pDK612. Linearized plasmid has been used for estimation accuracy. The threshold value was plotted against the log DNA concentration. The standard curve was a linear regression line between Ct and log10 of transgene copies/reaction (calculated from standard plasmid DNA concentration). The amplification factor of the reaction was 1.95 and the resulting efficiency was 95%. The correlation R^2 between the Cp values and the log DNA concentration was 0.99. The amplification curves of the plasmid standard and transgenic plants are shown by Figure 5. Table 2 shows the results of the copy number obtained from the Taqman PCR. The precise number of integrated transgenic sequences was obtained from the ratio between the absolute quantity of the transgene and the genome copy number of each transgenic peanut line in T0 and T1 plants (Gadaleta et al., 2011). Transgene copy numbers ranged from 1 to 3 in the T0 transgenic lines analyzed. Lines 2 and 6 have 1 transgene copy. Results also showed that T0 plants were hemizygous and T1 plants homozygous.

Southern blot: Copy number of the ASPx transgene was confirmed by Southern blot. Ten (10) µg genomic DNA from T1 line 2 plants were digested with *BglII* which makes a single cut in the T-DNA region. A 400 bp ASPx fragment was labelled with Alkaphos direct DNA labeling kit (Amersham, Inc. USA) and used as probe to hybridize the blots. Results show that the analyzed plants carry a single copy of the transgene (Figure 6).

Expression of ASPx gene in the seeds

At the mRNA level

Expression of the ASPx at the RNA level was determined by RT-PCR using primers targeting a 100 bp fragment of the ASPx gene and total RNA extracted from T2 and T3 seeds in 2 lines. Figure 7 showed presence of the

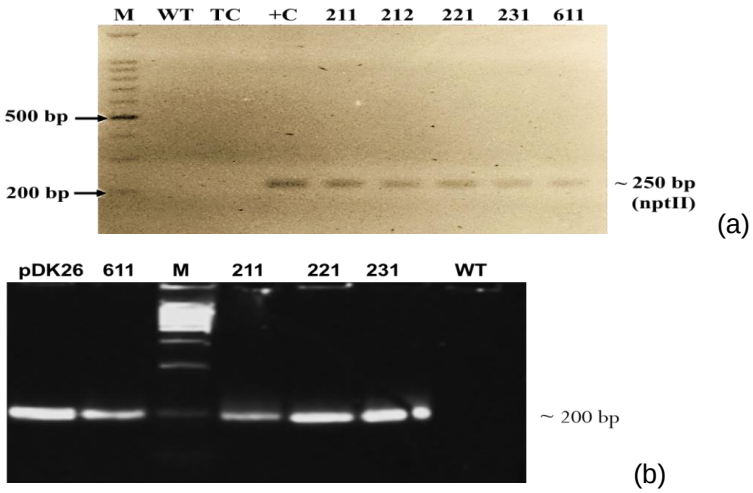

Figure 4. (a) PCR products T1 transgenic plants (targeting npt2 gene); (b) PCR products of T1 transgenic plants (targeting ASPx gene); M: 100 bp DNA ladder (Promega); WT: negative control; TC: Tissue culture control; +C & pDK26: Positive control; 211, 212, 221, 231 & 611: lines 2 and 6 - T1 plants.

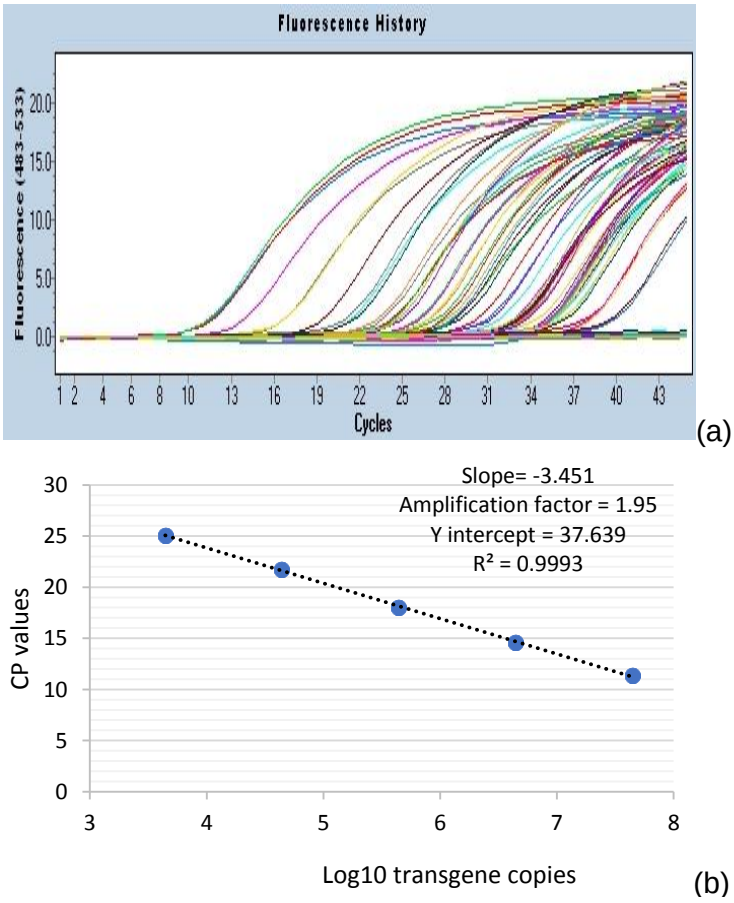

Figure 5. Copy number analysis of transgenic plants by qPCR. (a) Amplification curves of the serial dilutions of plasmid PDK26 (standard) and transgenic T0 and T1 plants. (b) Standard curve obtained from the serial dilutions.

Table 2. Determination of transgene copy number.

Lines or plants	Initial [(a)] absolute transgene copies / rxn	Initial [(b)] number of genomes /rxn	Estimated CN per number of genomes	Estimated CN per haploid genome	Zygosity
T0 line 1	12172	5273	1.2	0.6	
T0 line 2	**1478**	**710**	**1.0**	**0.5**	
T0 line 3	46228	10914	2.1	1	
T0 line 4	14869	4570	1.8	0.9	Hemizygous
T0 line 6	**10831**	**5516**	**1.0**	**0.5**	
T0 line 8	141813	23152	3.1	1.5	
T0 line 9	22413	9947	1.1	0.6	
T0 line 10	7090	1859	1.9	1	
T1 line2 (221)	751874	371520	2,0	1	
T1 line 2 (231)	15685	8551	1,8	0.9	Hemizygous
T1 line (661)	5941	2650	2,2	1.1	

a: Initial absolute transgene copies obtained from the standard curve; b: Initial genome copies calculated from the amount of transgenic plants DNA used in the PCR.

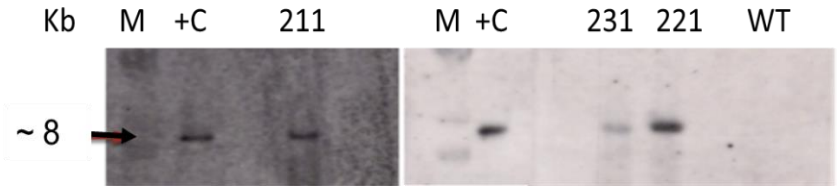

Figure 6. Molecular analysis of T1 progeny plants. Southern hybridization of *BglII*-digested DNA using ASPx probe. M-lambda *HindIII;* DNA marker (Promega); 5-negative control; C-positive control; 211, 231, 221: T1 transgenic plants.

fragment, indicating that the ASPx gene is actively transcribed to mRNA in transgenic seeds.

Protein profile of the transgenic seeds by SDS-PAGE

The expression of the APSx protein (presence of the 15 kDa ASPx protein) was first investigated by SDS-PAGE of proteins extracted from transgenic seeds along with WT and TC controls. The presence of the ASPx protein was not clearly distinguishable between negative controls (WT, TC) and transgenic seeds at the expected size, due to the presence of existing proteins at the same size in non-transgenic peanut seeds, especially the Arah2 peanut protein (Figure 8). However, differences are observed in the complete profile. Most transgenic seeds, if not all, showed an altered profile compared to the WT and TC. Some seeds showed reduction of proteins bands at the size of major allergens in peanut, especially Arah1 and Arah3 (between 75-35kDa). Most seeds showing reduction around the Arah1 protein area, showed accumulation of protein between 10 and 20 kDa. Even though the mechanism of storage protein accumulation in the proteins bodies of legumes is not fully understood, it seems to exist a compensatory effect between the different storage proteins in a crop. As storage proteins are source of nitrogen for the new developing seed, reduction of the expression of some key proteins might increase the expression of others and vice versa, in order to maintain a minimum pool of storage proteins necessary for the germination process..

Multiple reaction monitoring (MRM)

Since SDS-PAGE results did not clearly show the presence of the ASPx protein, mass spectrometry was chosen as a tool to detect the expected ASPx protein in the transgenic seeds. Crude proteins extracted from transgenic and wild type seeds were resolved on SDS-PAGE and proteins bands around 15 kDa were cut for

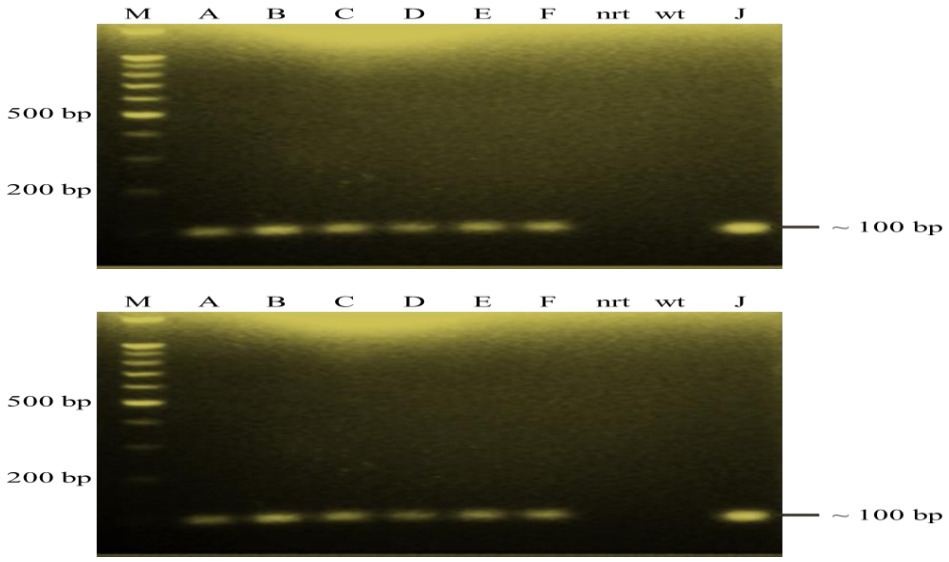

Figure 7. RT-PCR (Reverse-transcriptase polymerase chain reaction) of T2 and T3 seeds. 2% Agarose gel of products. M: 100 bp DNA ladder (Promega); A-F: transgenic T2 seeds; 1-6: transgenic T3 seeds; wt: wild type; J: ASPx positive control. nrt: non-reverse transcriptase negative control.

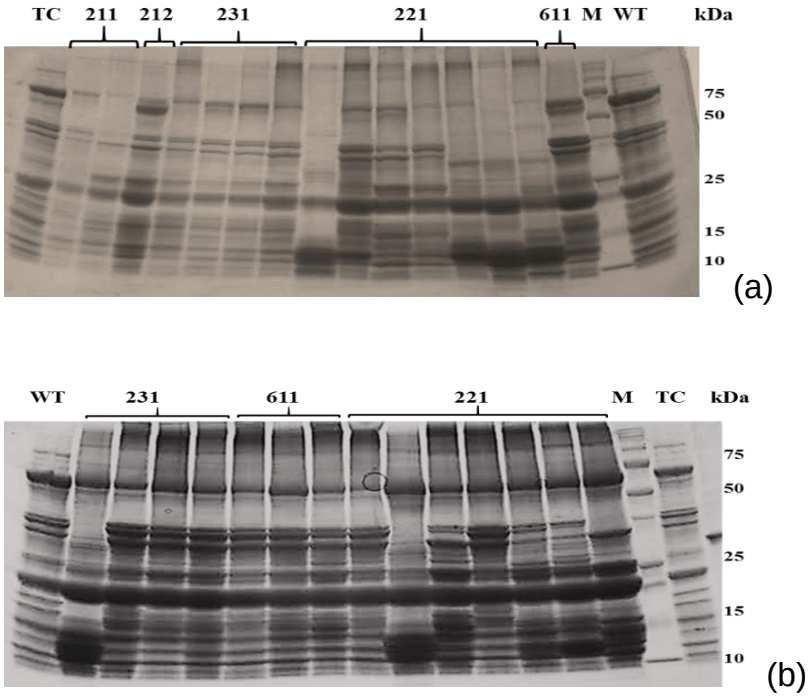

Figure 8. Protein profile of T2 (a) and T3 (b) seeds. WT: wild type; M: Protein standards; TC: Tissue culture 211, 212, 221, 611: Transgenic seeds.

targeted analysis of the ASPx protein using the MRM method on the triple 5600 Qtrap mass spectrometer (Tandem MS/MS) (Applied Biosystems). Spectrometer profile obtained showed the presence of the ASPx protein in the protein extracts analyzed. Figure 9 represents an example of chromatograms obtained during the analysis.

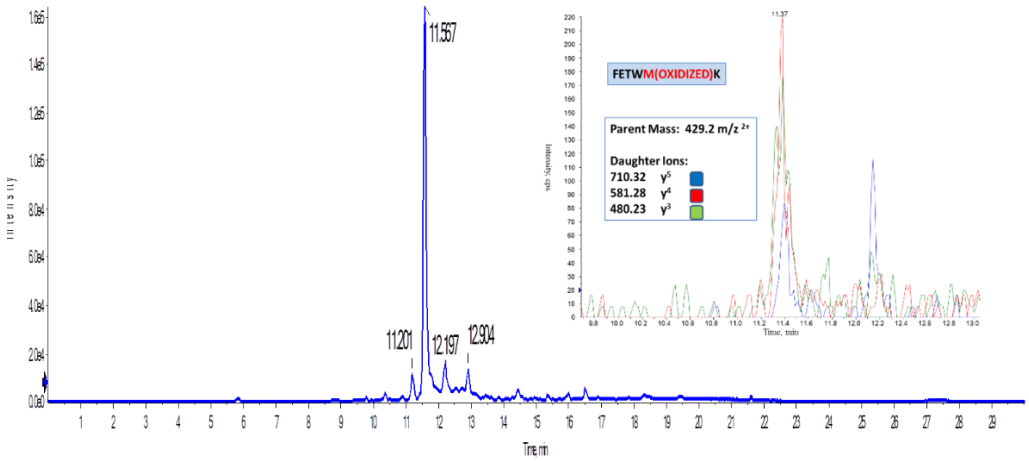

Figure 9. MRM scan profile ofthe ASPx-derived peptide sequences (positive control and T3 sample).

Table 3. Amino acid composition of peanut from transgenic and non-transgenic seeds (%).

Amino acids	WT	Line2	Line6
Aspartic Acid	2.42^{b}	2.88^{a}	2.55^{b}
Threonine	0.63^{a}	0.71^{a}	0.66^{a}
Serine	0.92^{b}	1.06^{b}	0.93^{b}
Glutamic Acid	3.42^{b}	4.24^{a}	3.95a^{b}
Proline	0.91^{a}	1.07^{a}	0.94^{a}
Glycine	1.11^{a}	1.22^{a}	1.15^{a}
Alanine	0.73^{a}	0.84^{a}	0.77^{a}
Valine	0.88^{b}	1.05^{a}	0.91^{ab}
Isoleucine	0.75^{b}	0.88^{a}	0.78^{b}
Leucine	1.47^{b}	1.68^{a}	1.51^{b}
Tyrosine	0.98^{a}	1.11^{a}	0.96^{a}
Phenylalanine	1.23^{b}	1.42^{a}	1.21^{b}
Lysine	0.77^{b}	0.88^{a}	0.86^{a}
Histidine	0.52^{a}	0.61^{a}	0.54^{b}
Arginine	2.58^{a}	3.02^{a}	2.66^{a}
Methionine	0.39^{b}	0.44^{a}	0.42^{a}
Cysteine	0.06^{a}	0.05^{a}	0.05^{a}
Total Amino Acids	19.77^{b}	23.16^{a}	20.85^{b}
Crude protein			

WT: wild type; Line2: Transgenis seeds from line 2; Line 6: Transgenic seeds from line 6; Values are means and numbers with different superscript are statistically different ($p<0.05$) according to Tukey's studentized test.

Nutritional analysis of transgenic seeds

Total protein content and the amino acids composition of transgenic seeds from lines 2 and 6 and from WT negative control seeds were analyzed (Table 3). Protein content of WT seeds (23.94%) is not statistically different from line 2 (24.52%), and line 6 (25.92%) ($P\geq0.05$) seeds. Unlike the total protein content, line 2 has a significantly higher total amino acids content (23.16%) than transgenic line 6 (20.85%) and WT (19.77%) at level $P<0.05$. Line 6 and WT are not statistically different. ($P\geq0.05$). Overall, line 2 showed a significant increase in individual amino acids, especially in EAA compared to the line 6 and WT. EAA presented between 12 and 19% increase for line 2,

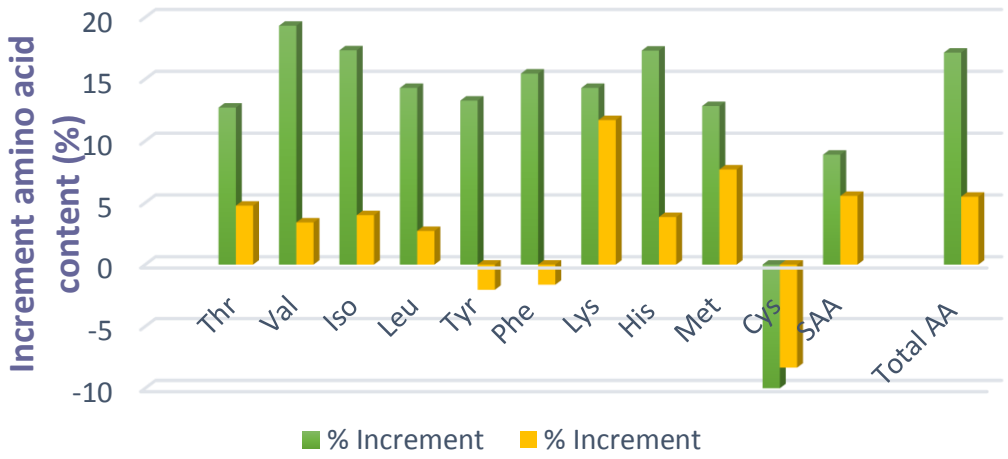

Figure 10. Percentage of the essential amino acids above the wild type control. Line 2: In Green; Line 6: in yellow; Increment is calculated by (transgenic seeds amino acid content − control seeds amino acid content) ÷ control seeds amino acid content.

especially in five of the limiting amino acids in peanut, Val, Iso, Leu, Met and Threonine. Line 6 had lower increase compared to line 2.

The difference in the expression levels of the ASPx protein in the transgenic plants is a phenomenon that has been reported in several transgenic crops and might be due various factors including position effects, transgene rearrangements and developmental stage of the tissue (Zhang et al, 2003; Yang et al., 2016). However, several authors obtained increase level of aminoacids in tubers and roots (Zhang et al., 2003; Wong et al., 2015). Concerning total protein content, some authors have also noticed either no significant difference in transgenic seeds, leaves or tubers, or very slight increase, even there was significant difference in the amino acids (Zhang et al., 2003; Yang et al, 2016). Results suggest that the overall protein content must be regulated in peanut seeds, or the expression of ASPx might have caused the suppression or the down-regulation of the expression of some other proteins.

We have noted a decrease (1-2.5%) in the level of Phe and Tyr, the aromatic amino acids in line 6. (Zhang et al, 2003) made the same observation in some biofortified transgenic cassava roots. A decrease in cysteine level was also noted in both transgenic (-10% in line 2 and -8% in line 6) compared to the WT. Molvig et al., (1997), Wong et al. (2015) and Yang et al. (2016) reported a decrease of some amino acids following the expression of a sunflower seed albumin gene in lupins seeds or the increase of lysine in transgenic rice grains. The increase of one sulphur-containing amino acids can make up the decrease of the other, because both cysteine and methionine can be synthesized from one another (Bin et al., 2017; Cohen et al., 2017), as they are synthesized via the aspartate (Asp) family pathway. Increments and decrements are better shown by Figure 10.

Conclusion

In this study, the ASPx gene encoding a protein with 75% EAA was successfully integrated into the peanut genome and expressed into seeds via *Agrobacterium*-mediated transformation and direct organogenesis. For stable expression levels in transgenic plants, the ASPx gene was linked to the KOZAK translational enhancer. An increase of at least 4 limiting amino acid in peanut was obtained for transgenic lines, along with few decreases in the level of some amino acids. This result shows that the protein quality of peanut seeds could be improved. For further study, better increase level of amino acids should be investigated by coding the ASPx gene under the control of dicots seed-specific promoters such as the bean β-phaseolin, the β-conglycinin, the napin, and the soybean lectin promoters. Also, the allergenic status of the transgenic seeds should be evaluated, as well as the bioavailability of the new protein.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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