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Assessment of Seed Protein Quality of A Transgenic Chickpea Event Expressing Cry2Aa Protein

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ABSTRACT

Background: Evaluation of the nutritional composition of genetically modified (GM) crops is mandatory for their deregulation. Chickpea is known for its high-quality protein and demonstrating that the seed protein quality of transgenic chickpea remains unaltered is important for its acceptance. Amino acid content, seed storage protein profile and the digestibility of chickpea protein are important determinants of seed protein quality. Thus, in the present study, we assessed the effect of *Bt* (*Cry2Aa*) gene expression on the Bt chickpea seed protein quality.

Methods: We assessed the amino acid profile, *in vitro* protein digestibility and factors affecting protein digestibility like trypsin inhibitor, tannins and phytic acid contents of the transgenic Bt chickpea expressing a codon modified *Cry2Aa* gene and its non-transgenic counterpart. Furthermore, the seed storage proteins were also fractionated and separated on SDS-PAGE followed by mass spectroscopy of the major peptides.

Result: Amino acid profile and factors affecting protein digestibility revealed no significant variations between transgenic and non-transgenic chickpeas. Seed storage protein profile confirmed the presence of legumin, vicilin and albumin. No potential change in the digestibility pattern of seed proteins was revealed. Our findings suggest no potential unintended changes in chickpea seed protein quality due to the expression of *Cry2Aa* gene.

Key words: Bt chickpea, Genetically modified, Protein digestibility, Seed protein, Unintended.

INTRODUCTION

Genetically modified (GM) crops have benefitted agricultural sector across the globe. GM crops are stringently evaluated prior to release in the field. The commercial release of genetically modified (GM) crops requires a detailed nutritional assessment to establish the GM crops as safe as conventionally bred crops. These assessments assure that the changes made in a crop genome by introducing gene(s) to improve trait(s) are safe for humans and the environment by establishing a substantial equivalence between the GM crops and their non-GM counterparts (Kuiper *et al*., 2001).

Chickpea (*Cicer arietinum* L.), one of the highly nutritious legumes, is the third most important pulse crop worldwide and the most important grain legume in India. In India, the production of chickpea suffers significant (40-90%) yield losses, annually due to pod borers (*Helicoverpa armigera)*, (Sharma, 2001). The development of transgenic (Bt) chickpea expressing a high level of Cry2Aa protein provided a new strategy for insect-resistance (Acharjee *et al*., 2010). However, like other GM crops, such as rice (Gayen *et al*., 2013; Gayen *et al*., 2016), soybean (Chiozza *et al*., 2020), pigeon pea (Mishra *et al*., 2017), corn (Rayan *et al*., 2015), wheat (Akhtar *et al*., 2020), nutritional equivalence assessment of Bt chickpea was essential to ascertain no difference in the the nutritional quality compared to the non-transgenic parent. In the nutritional context, chickpea is known as a source of high-quality protein and thus the acceptability of transgenic chickpea is highly dependent on it. Total protein content, amino acids (AAs) content and their bioavailability upon ingestion and anti-nutrient content are the important determinants of seed protein quality (Singh *et al*., 1993). The seed storage proteins are also one of the factors that determine seed protein quality (Shewry *et al*., 2008). Thus, in this study we assessed the seed protein quality of Bt chickpea lines.

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MATERIALS AND METHODS

Homozygous transgenic Bt chickpea lines expressing a Cry2Aa gene (Acharjee *et al*., 2010) in its advanced generation along with its non-transgenic counterpart were selected for the present study. Seeds harvested from transgenic and non-transgenic events were used for analyses. The study was carried out at Assam Agricultural University, Jorhat, India in the year 2017-18. The experiments were repeated twice and the analyses for amino acids and anti-nutrients were carried out in three biological replicates.

Estimation of amino acid content

Amino acids content of chickpea seeds were estimated from finely ground chickpea seed samples commercially by the service provider, Sandor Life Sciences Pvt. Ltd., Hyderabad, India. Chickpea seeds (about 2 g) were finely homogenized with metabolite extraction buffer. The extract obtained was subjected to organic solvent precipitation by treating with five volumes of SDS buffer with protease inhibitors and 0.1% Tris-buffered phenol. Pellet obtained was air-dried and dissolved in 50 mM ammonium bicarbonate buffer.

About 50 µl of the obtained sample was digested with 2 ml of 6N HCl for 15 min. After digestion, 7 µl of the sample was loaded on to an HPLC (Shimadzu, Model CBM 20 A) system and quantified using standards (Sigma, Ltd., USA). Crude proteins was analyzed by the Kjeldahl method (The Association of official Analytical Chemists, 2000).

Antinutrients analysis

The phytic acid content was measured colorimetrically at 510 nm using 2,2 bipyridine and sodium phytate used as standard following the protocol by Ahmad *et al*. (2013). Tannin content was estimated by the Folin Denis method described in the AOAC (1995). The trypsin inhibitor activity was determined following a modification of American Oil Chemist's Society Official Method, 2009 (Coscueta *et al*., 2017). Chickpea samples were extracted with 0.01N NaOH, mixed with trypsin and benzoyl-DL-arginine-nitroanilide hydrochloride (BAPNA) and absorbance was measured using a spectrophotometer at 410 nm. The trypsin inhibitor activity (TIA) were expressed as trypsin inhibitor units (TIU) per milligram of the extracted sample using the following expression:

$$TIU = \frac{100\ Y(\text{Absorbnce control-Absorbnce sample})}{X} DV,$$

Where
100 = Factor to convert 0.01 unit Abs in TIU units.
D = Dilution factor of supernatant.
V = Extraction volume.
X = Aliquot used in the assay.
Y = Final reaction volume in the cuvette.

Statistical analysis

The data were analyzed in triplicate and statistical significance between the samples was obtained by t-test at $p \leq 0.05$ using the SPSS software.

***In vitro* protein digestibility**

In vitro digestibility of seed proteins was evaluated by transient pepsin hydrolysis (mimics simulated gastric fluid) followed by trypsin (mimics simulated intestinal fluid) following the method described previously (Chavan *et al*., 2001; Wang *et al*., 2010). Pepsin digestion was carried out in a ratio of 100:1 (w/w) of seed proteins and pepsin, respectively, in an acidic environment using 0.1 M HCl for 120 min. The pepsin digested proteins were then neutralized with 1.0 M phosphate buffer (pH 8.0), followed by the addition of trypsin (substrate/enzyme ratio of 100:1, w/w). Aliquots were removed from each tube after 0, 10, 60 and 120 min of incubation, mixed with sample buffer (4X SDS-PAGE loading) and loaded on to sodium-do-decyl sulfate-polyacrylamide gel electrophoresis(SDS-PAGE) along with pre-stained molecular weight markers (from 10 to 130 kDa). Quantitative analysis of protein digestibility was carried out using the multienzyme method (Hsu *et al*., 1977).

Seed storage protein fractionation

Seed storage protein fractionation was carried out according to the protein fractionation protocol essentially developed by Rubio *et al*. (2014). Defatted chickpea flour was extracted using (1:10 w/v) 0.2 M borate buffer (0.2 M boric acid, 0.2 M borax), pH 8 containing 0.5 mol L^{-1} NaCl and centrifuged at15000 rpm for 45 min at 4°C. The supernatant obtained was adjusted to pH 4.5 with glacial acetic acid and centrifuged (15000 rpm, 30 min, 4°C). The sediment (1) obtained was re-dissolved in borate buffer and dialyzed against distilled water to extract legumins 11S fraction. Following extensive dialysis the supernatant obtained was centrifuged. The sediment (2) obtained was stored at -80°C as the vicilin fraction(7S). And the supernatant thus obtained was subjected to 82% $(NH_4)_2SO_4$ precipitation and centrifuged (12000 rpm, 30 min, 4°C). The sediment (3) was dialyzed for 72 hrs against distilled water to obtain albumin fraction and is stored at -80°C. A 4-12% linear gradient Mini-Protean TGX Precast gel was used to separate about 40 µg of each protein fraction at a constant voltage of 150 V. A pre-stained molecular protein marker was also loaded onto the SDS-gel. Coomassie Brilliant Blue solution was used for staining the protein bands.

Protein identification by mass peptide fingerprinting

Identification of protein was carried out following the protocol used in previous studies (Padaria *et al*., 2014). The major bands were excised out of the gel from each fraction, based on similar reports on chickpea (Chang *et al*., 2012). The gel pieces were destained and dehydrated using acetonitrile; incubated with iodoacetamide, followed by an ammonium bicarbonate solution. The samples obtained were digested with trypsin solution at 37°C and vacuum dried. The dried samples were re-suspended in TA (Tris-acetate) buffer. The peptides obtained were mixed with Alpha-cyano-4-hydroxycinnamic acid (HCCA) in 1:1 ratio and 2 µl of the mix was spotted onto the matrix-assisted laser desorption ionization (MALDI) plate. It was then analyzed on the MALDI TOF/TOF ULTRAFLEX III instrument and the the peptide mass fingerprint was obtained using FLEX ANALYSIS SOFTWARE. The peptide mass fingerprinting data obtained were submitted to Mascot search of the NCBI database to identify the protein. The parameters for protein identification were; a) peptide mass tolerance: ±380 ppm, b) taxonomy: Viridiplantae, c) fixed modification: carbamidomethylation of cysteine and d) variable modification: methionine oxidation.

RESULTS AND DISCUSSION

GM crops are commercialized after a comprehensive food

safety assessment. Studies assessing the nutrient composition and quality in various transgenic crops have increasingly revealed the importance of such criteria in establishing biosafety. Amongst the nutritional assessment, seed protein quality of transgenic chickpea is important, if consumed. A comparative analysis is mostly adopted to evaluate that the transgenic and their non-transgenic counterparts are nutritionally equivalent. Several studies indicated that GM crops are considered safe if their nutritional composition is similar or data are within the range reported for their conventional counterparts (Mishra *et al*., 2017; Cho *et al*., 2016; Gayen *et al*., 2013; Junhua *et al*., 2005; Oberdoerfer *et al*., 2005; Wang *et al*., 2012). Therefore, we assessed the key composition determining seed protein quality of chickpea and compared the data with the non-transgenic parent (Jukanti *et al*., 2012), USDA, (2018).

Total protein and amino acid content in Bt chickpea

Total protein and amino acid contents are the critical determinants of the nutritional quality of chickpea seed protein. Total protein content revealed no significant difference between the transgenic and non-transgenic chickpea seeds (Table 1). The amino acid contents exhibited nearly identical amino acid profiles between the transgenic (Bt) and non-transgenic chickpea seeds except for the glutamic acid content (Fig 1). However, the overall amino acid profile was similar to the values previously reported (USDA,2018). A similar observation on amino acid composition was reported on transgenic rice expressing a Cry1Ac gene (Park *et al*., 2012) and transgenic pigeon pea expressing Cry1AcF and Cry2Aa gene (Mishra *et al*., 2017) which were considered safe in regard to nutritional equivalence to other commercial varieties.

Anti-nutrients content in Bt chickpea seeds

High levels of anti-nutrients in chickpea seeds can reduce their nutritional value, as well as the digestibility of chickpea protein (Alajaji *et al*., 2006; Esmat *et al*., 2010). The levels of these anti-nutrients like phytic acid, tannins and trypsin inhibitor in the Bt chickpea event were comparable to their non-transgenic chickpea seeds (Table 1) and were also within the range reported for chickpea varieties (Jukanti *et al*., 2012). Similar studies were also reported in transgenic pigeon pea (Mishra *et al*., 2017), rice (Cho *et al*., 2016) confirming them to be safe.

***In vitro* protein digestibility and seed storage protein analysis in Bt chickpea**

The bioavailability of a protein is mostly dependent on its digestibility by gastric, pancreatic and intestinal peptidases and were partly determined using *in vitro* digestibility assays. Pepsin-trypsin digestion of chickpea protein isolates indicated that seed proteins from all the samples were hydrolyzed within the first ten minutes of pepsin digestion

Table 1: Protein content and anti-nutrient content of Bt chickpea event and its non-transgenic counterparts.

	Non-transgenic chickpea	Bt chickpea	Reference range
Crude protein (g/100 g)	25.48±0.173	27.60±0.311	3.80-10.20
Tannin (mg/100 mg)	1.178±0.003	1.181±0.004	Trace amount
Phytic acid (mg/100 mg)	0.314 ±0.094	0.312±0.068	Trace amount
Trypsin inhibitor (unit/mg)	15.30±0.021	17.20±0.128	6.90-19.00
In vitro protein digestibility %	87.60±0.424	86.87±0.597	48.00-89.010

Values are mean±S.E; n = 3: (*) = p value < 0.05.

Reference Sources: (Jukanti *et al*., 2012; USDA, 2018).

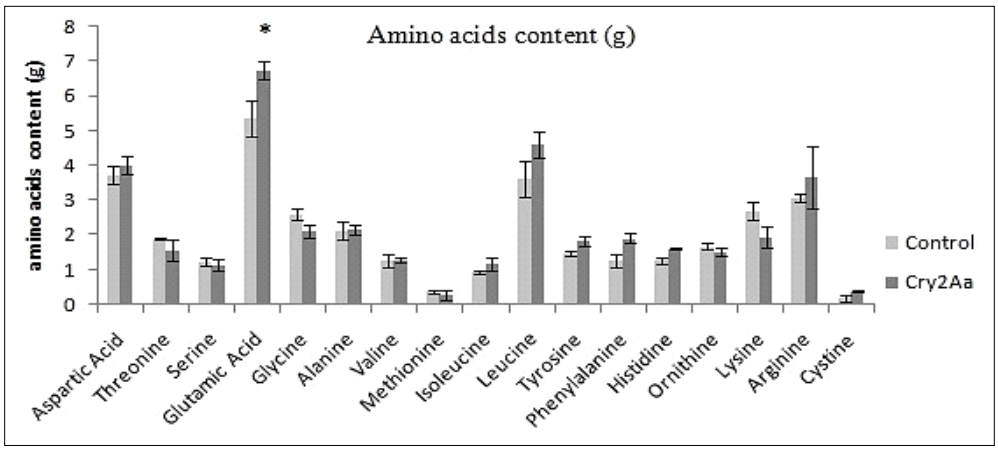


Fig 1: Bar diagram representing amino acids content of seeds of Bt chickpea expressing Cry2Aa protein and non-transgenic chickpea. Control is the non-transgenic chickpea sample and Cry2Aa is the Bt chickpea sample expressing Cry2Aa protein. Amino acid content is expressed as g of amino acid per 100 gm of chickpea seed sample. (*p-value < 0.05, **p-value < 0.01, ***p-value < 0.001, p-value >0.05 non-significant) bars represent mean value ± standard error.

resulting in the formation of polypeptides of low molecular weight (<25 kDa) (Fig 2a). Pepsin treated samples on digestion with trypsin resulted in peptides of molecular weight < 20-15 kDa (Fig 2b and Fig 2c). The polypeptides observed even after digestion had a similar banding pattern in both the transgenic and its non-transgenic counterpart, which is similar to a report on chickpea seed protein digestion (Wang *et al*., 2010). The quantitative evaluation of *in vitro* digestibility using a multienzyme system (Table 1) was also in agreement with earlier reports (Jukanti *et al*., 2012;

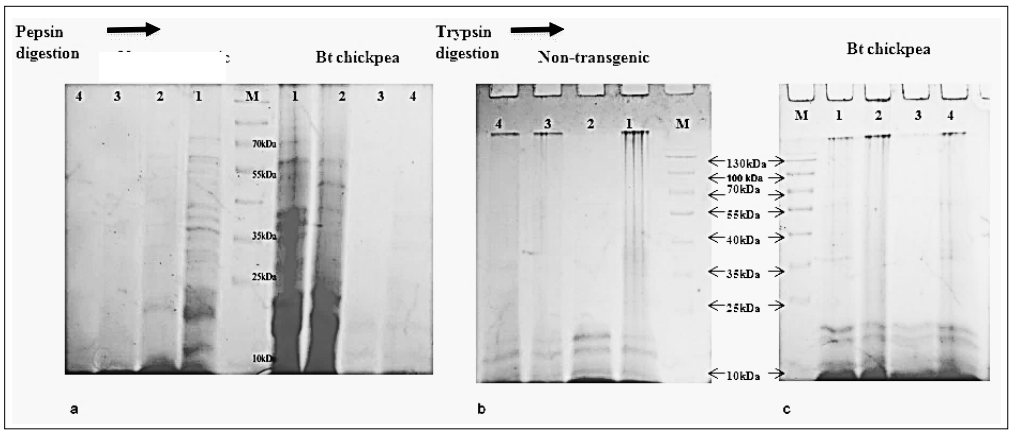


Fig 2: *In vitro* digestibility of seed protein of Bt chickpea and non-transgenic chickpea samples at various time points. Protein extract from chickpea seeds were digested with pepsin and trypsin sequentially and loaded on to the gel; Fig 2a: Digestion of protein isolates with pepsin at different time intervals (M- Protein ladder; 1, 2, 3, 4 represents the digested protein samples at 0,10, 60,120 min); Fig 2b: Pepsin digested protein isolates of non-transgenic chickpea digested with trypsin at different time intervals, (M- Protein ladder: 1, 2, 3, 4 represents the digested protein samples at 0,10,60,120 min); Fig 2c: Pepsin digested protein isolates of Bt chickpea digested with trypsin at different time interval, (M- Protein ladder: 1, 2, 3, 4 represents the digested protein samples at 0, 10, 60, 120 min).

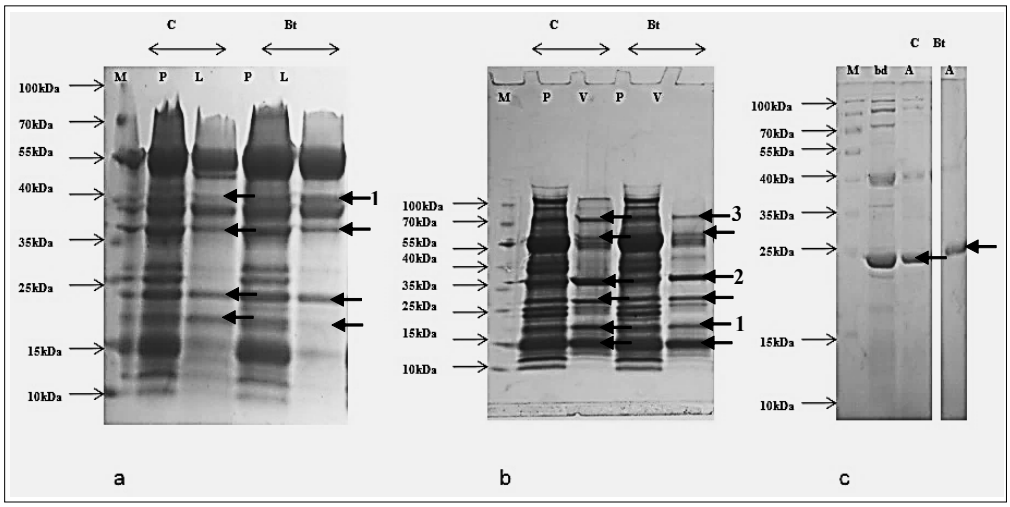


Fig 3: Seed storage protein fractions of the Bt Chickpea event and its non-transgenic counterpart. Seed storage protein fractionated on the basis of their pI and solubilities from total seed protein extracted using borate buffer. C represents the non-transgenic chickpea (control) sample and Bt is the Bt chickpea sample expressing Cry2Aa protein; Fig a: Molecular weight (M),Total protein(P), Legumin (L), fraction. Bands in the lane indicate the peptides of probable molecular weight (marked with an arrow) (legumin α-subunits ~ 40.6, ~39.5 kDa and legumin β-subunits with MW ~23.5, ~22.5 kDa) for legumin fractions as reported by Chang *et al*., 2012; The numbering 1 indicates the peptide sent for identification using for mass peptide fingerprinting and confirming the protein fraction; Fig b: Molecular weight (M), Total protein (P), Vicilin (V), Fraction. Bands in the lane indicate the peptides of probable molecular weight (V- ~70.2, ~50.7, ~35.0, ~33.6, ~18.9 and ~15.5 kDa for vicilin fractions as reported by Chang *et al*., 2012. The numbering 1,2,3 are the major peptides sent for peptide fingerprinting for identification and confirming the protein fraction; Fig c: Molecular weight (M), Albumin fraction from non-transgenic chickpea sample before dialysis (bd), Albumin fraction after dialysis (A), Fraction; The electrophoretic band in the lane indicates the probable molecular weight, 25 kDa, for albumin fractions as reported by Chang *et al*., 2012.

Table 2: Putative identification by mass peptide fingerprinting of electrophorectic bands of different protein fractions extracted from seed of transgenic chickpea using Mascot Search Engine.

Band	Homologous protein	Score	Coverage	Uniprot /NCBI Prot Accession no.
Albumin (Cry2Aa)	Albumin-2- like [*Cicer arietinum*]	116	72	XP_004516876
Legumin 1 (Cry2Aa)	Legumin-J like [*Cicer arietinum*]	125	32	XP_004495100
Vicilin 1 (Cry2Aa)	Vicilin like [*Cicer arietinum*]	262	46	XP_004496704
Vicilin 2 (Cry2Aa)	Vicilin like [*Cicer arietinum*]	174	55	XP_012569154
Vicilin 3 (Cry2Aa)	Vicilin like [*Cicer arietinum*]	92	37	NP_001296635

Esmat *et al*., 2010). Thus, the results suggest that the accumulation of Bt protein does not induce any unintended effects on seed protein digestion of Bt chickpea.

In chickpea, the major seed storage proteins are albumin, globulin, prolamin and glutelin. Among these, we analyzed albumin and globulin (Legumin and Vicilin) as they are the major determinants of seed protein quality (Singh and Jambunathan, 1982; Chavan *et al*., 2001; Chang *et al*., 2012). Both the transgenic (Bt) and non-transgenic chickpea samples revealed an almost similar protein profile (Fig 3) and the major proteins bands eluted from the gel were confirmed through identification by MS/MS (Table 2).

Electrophoretic profiling demonstrated that legumin (11S) fractions showed major electrophoretic bands of legumin α-subunits corresponding to MW ~ 40.6 and ~39.5 kDa and legumin β-subunits with MW ~23.5 and ~22.5 kDa, Vicillin (7S) fraction with MW ~70.2, ~50.7, ~35.0, ~33.6, ~18.9 and ~15.5 kDa and albumin with ~25kDa in both transgenic and non-transgenic chickpea lines similar to study reported by Chang *et al*. (2012). The mass peptide finger printing data of the major bands of each fraction (in both transgenic and non-transgenic chickpea) confirmed each fraction to be of legumin (band 1- Fig 3a), vicilin (band 1; band 2; band 3- Fig 3b) and albumin (~25 kDa- Fig 3c). The present findings reveal no alteration in the seed storage protein fractions of the transgenic Bt chickpea event compared to its non-transgenic counterpart. Similar results were also reported on genetically modified corn (Rayan *et al*., 2015), which was considered to be nutritionally equivalent to its traditional counterparts.

CONCLUSION

The results obtained confirm that the seed protein quality of transgenic Bt chickpea is comparable to its non-transgenic counterpart, suggesting no unintended effects of Bt protein (Cry2Aa) accumulation on the seed protein, thereby indicating its safety. This study thus provides the initial step for the food safety assessment of this Bt chickpea line.

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