# Comparative Proteomic and Nutritional Composition Analysis of Independent Transgenic Pigeon Pea Seeds Harboring *cry1AcF* and *cry2Aa* Genes and Their Nontransgenic Counterparts

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Ⓢ *Supporting Information*

**ABSTRACT:** Safety assessment of genetically modified plants is an important aspect prior to deregulation. Demonstration of substantial equivalence of the transgenics compared to their nontransgenic counterparts can be performed using different techniques at various molecular levels. The present study is a first-ever comprehensive evaluation of pigeon pea transgenics harboring two independent *cry* genes, *cry2Aa* and *cry1AcF*. The absence of unintended effects in the transgenic seed components was demonstrated by proteome and nutritional composition profiling. Analysis revealed that no significant differences were found in the various nutritional compositional analyses performed. Additionally, 2-DGE-based proteome analysis of the transgenic and nontransgenic seed protein revealed that there were no major changes in the protein profile, although a minor fold change in the expression of a few proteins was observed. Furthermore, the study also demonstrated that neither the integration of T-DNA nor the expression of the *cry* genes resulted in the production of unintended effects in the form of new toxins or allergens.

**KEYWORDS:** *nutritional analysis, pigeon pea, proteome, transgenics*

## INTRODUCTION

Pigeon pea (*Cajanus cajan* L.) is a perennial legume belonging to the family Fabaceae grown in tropical and semitropical regions of Asia and Africa. Pigeon pea has a unique place in Indian farming, and the subcontinent accounts for about 90% of the global production. Pigeon pea is regarded as an "orphan crop" despite being a rich source of essential nutrients. The crop encounters various biotic and abiotic stresses that result in tremendous yield losses. In recent years, molecular breeding and transgenic technology-based crop improvement programs have delivered immense hope toward combating these stresses. Among biotic factors, sterility mosaic disease (SMD), pod fly, fusarium wilt, and pod borer (*Helicoverpa armigera*) are important, which substantially damage the crop and result in significant yield losses.[1] The success of insecticidal "cry" gene-based insect resistance has opened avenues for its use in the development of insect-resistant varieties in numerous crops including pigeon pea.[2,3] However, the major concern in the development of transgenics involves introduction of foreign genes, which when integrated into the host genome at random positions may affect the function of endogenous genes and result in alteration of various metabolic pathways, leading to unintended effects. Biosafety assessment of genetically modified organisms (GMOs) for the absence of undesirable phenotypic and physiological effects assumes tremendous significance, especially in food crops that are transgenically modified.[4,5] Nontargeted technologies involving comparative transcriptomics, proteomics, and metabolomics have gained popularity in the biosafety assessment of GMOs.[6−8] The advantage in using these techniques is that the end point of any metabolic activity can be measured and compared. In this direction, the focus of this paper has been the comprehensive evaluation of independent transgenics of pigeon pea for two novel *cry* genes (*cry1AcF* and *cry2Aa*) on the basis of their proteomic and nutritional composition. To the best of our knowledge, this is the first ever report evaluation of transgenic pigeon pea for substantial equivalence.

## MATERIALS AND METHODS

**Plant Material.** Selected transgenic events of pigeon pea homozygous for *cry1AcF* and *cry2Aa* (11-7-3b with *cry2Aa* in cv. Pusa 992 and Ev-3 with *cry1AcF* in cv. TTB7) were raised in contained net houses along with their nontransformed counterparts. The plants were grown to maturity and harvested, and seeds of uniform shape and similar weight were selected to carry out nutritional and proteomic analyses. The selected events were earlier evaluated for transgene integration and bioefficacy against the target insect, *H. armigera*, using standardized protocols.[3] All of the experiments were carried out in three technical replicates.

**Nutritional Analysis.** The major components for analysis in the present study were based on their importance and abundance in pigeon pea. The following are the components that were assessed in the present study.

*Estimation of Amino Acid Content.* Finely ground powder of transgenic and nontransgenic pigeon pea seeds (100 mg) was used for

quantification of essential amino acids. A Waters AccQ-Tag Chemistry package kit (Waters India Pvt. Ltd.) was used for sample preparation, derivatization, and analysis per the manufacturer's instructions. The derivatized samples including standards were subjected to HPLC analysis by Eurofins Analytical Services India Ltd., detected using a fluorescence detector (LC-FLD), and expressed as g/100 g.

*Estimation of Reducing Sugars, Raffinose, and Starch.* Transgenic and nontransgenic seeds (100 mg) were finely powdered, extracted twice with 80% ethanol at 95 °C, pooled, and dried at 80 °C for 2 h. The residue was dissolved in 10 mL of distilled water, and reducing sugars were estimated.[9,10] Raffinose was estimated according to the manufacturer's protocol (Megazyme International, Ireland) and expressed as mg/100 g dry weight. For total starch estimation, soluble sugars were initially removed as explained above and estimation of starch was carried out.[11]

*Quantification of Mineral Ions.* About 100 mg of pigeon pea seeds was used to estimate total $Fe^{2+}$, $Zn^{2+}$, and $Ca^{2+}$ concentrations as per the published protocol.[12] The powdered seed samples, which were digested in a diacid solution of $HNO_3$ and $HClO_4$, were subjected to an atomic absorption spectrometry (ECIL, AAS 4141) system that was calibrated for $Fe^{2+}$, $Zn^{2+}$, and $Ca^{2+}$.

*Estimation of Antioxidant Potential.* Antioxidant potential in the transgenic and nontransgenic seeds was estimated following two methodologies: (a) Sample extract was prepared by centrifuging overnight-soaked seed powder (100 mg) (with acetone) at 1509g for 10 min, and the resultant supernatant (100 μL) was used for estimation of antioxidant activity by the cupric reducing-antioxidant capacity (CUPRAC) method.[13] The antioxidant capacity was expressed as Trolox equivalent (μmol TE/g) using the formula

$$\mu\text{mol TE/g} = \left[\frac{A_f}{\varepsilon_{TR}}\right]\left[\frac{V_f}{V_S}\right] \times r\left[\frac{V_{initial}}{m}\right]$$

where $V_{initial}$ = initial volume, $m$ = weight of sample, $r$ = dilution factor, $V_f$ = final volume, $V_s$ = volume of aliquot, $A_f$ = absorbance, and $\varepsilon_{TR}$ = $1.67 \times 104$ L/mol·cm. (b) Free radical scavenging activity was measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay.[14,15] Sample extract was prepared as described for CUPRAC assay. The DPPH radical scavenging activity (S%) was calculated using the equation

$$\text{scavenging activity (S\%)} = \left[\frac{A_{control} - A_{sample}}{A_{control}}\right] \times 100$$

where $A_{control}$ is the absorbance of the blank (containing all reagents except the extract solution) and $A_{sample}$ is the absorbance of the test sample.

*Estimation of Total Phenolics and Tannins.* Total phenolic content (TPC) per gram of seed powder was determined following the published protocol.[16] Calibration plot was expressed as gallic acid (2−10 μg/mL) equivalents/g DW. Tannins were estimated by using the vanillin−HCl method.[17] Catechin solution (0−100 ppm) was used for the standard curve, and the results were expressed as mg of catechin equiv/g DW.

**Estimation of Antinutrient.** The antinutrient factor, α-amylase inhibitor, was estimated in the transgenic and nontransgenic seeds following the published methology.[18] The percentage of α-amylase inhibition was calculated by using the formula

$$\% \text{ of } \alpha\text{-amylase inhibition} = 100 \times (\Delta A_{control} - \Delta_{sample}/\Delta A_{comtrol})$$

$$\Delta A_{control} = A_{test} - A_{blank}$$

$$\Delta A_{test} = A_{test} - A_{blank}$$

**Proteomic Analysis and MS/MS Identification.** All the procedures for protein extraction and two-dimensional gel electrophoresis (2-DGE) were carried out following the standardized methodology[19] with some modifications. In short, for protein isolation, 1 g of seed was homogenized in liquid nitrogen in a prechilled pestle and mortar, suspended in phosphate buffer (pH 7.4), and centrifuged at 5000g for 10 min at 4 °C. The supernatant was further resuspended in cold 10% (w/v) TCA and acetone containing 0.1% (v/v)

β-mercaptoethanol and allowed to precipitate at −20 °C for 2 h. The suspension was centrifuged at 24700g for 15 min at 4 °C in a refrigerated high-speed centrifuge. The pellet was resuspended in chilled acetone containing 0.1% β-mercaptoethanol (v/v) and centrifuged at 24700g for 15 min at 4 °C; the process was repeated three or four times. Final washing of the pellet was carried out with chilled 100% acetone. The pellet was air-dried at room temperature and stored at −80 °C. After 24 h, pellets were resuspended in rehydration buffer (7 M urea, 2 M thiourea, 2% (w/v) CHAPS) and centrifuged at 24700g for 5 min at room temperature. The total proteins in the supernatant were quantified[20] using BSA as a standard and further used for proteomic analysis.

**2-DGE.** Isoelectric focusing (IEF) was carried out using Ettan IPGphor III (GE Healthcare) 13 cm IPG strips (pH 3−10, GE Healthcare). For this, 250 μg of protein in 250 μL of rehydration buffer with (1% (v/v) IPG buffer (pH 3−10) and 1% (w/v) dithiothreitol) was loaded into the IEF tray, and active rehydration was carried out at 20 °C for 16 h. The second dimension was carried out in SDS-PAGE after equilibration of the IPG strips in equilibration buffer (6 M urea, 2% (w/v) SDS, 30% (v/v) glycerol, 1% (w/v) DTT, and 50 mM Tris-HCl (pH 8.8) with 0.002% bromophenol blue and DTT (10 mg/mL)) for 15 min and later replaced with iodoacetamide (25 mg/mL). The equilibrated strips were used for the 2D electrophoresis and image acquired following the published protocol.[19] After electrophoresis, the gels were stained with colloidal CBB R-350 (GE Healthcare) and scanned with an Image Scanner (GE Healthcare) using silver Fast (Epson IT8) 8ba (vers. 6.5.5r3) software at a resolution of 300 dpi; brightness and contrast were set to default. The analysis was carried out in three technical replicates.

**Gel Image Acquisition and Data Analysis.** Gel images were analyzed using Image Master 2D Platinum (7.0) (GE Healthcare). To confirm the spots detected by software, all the spots were inspected manually and edited as necessary before the statistical analysis of variance criteria ($p \leq 0.05$) was applied.

**In-Gel Digestion and MS/MS Identification of Peptides.** This process was performed according to the method of Mishra et al.[19] with some modifications. In brief, the differentially expressed protein spots were excised manually and washed with ultrapure water and kept for destaining with 100 μL of 100 mM $NH_4HCO_3$/acetonitrile (ACN) (50:50, v/v). Destained gel spots were dehydrated with 50 μL of ACN. For trypsin digestion, 10 μg/mL of sequencing grade trypsin (Promega) was added to each dried gel fragment and incubated for 45 min at 4 °C. Furthermore, 10 μL of 50 mM $NH_4HCO_3$ was added and kept for incubation at 37 °C for 16 h. After digestion, peptides were purified, concentrated, and used for spotting on a MALDI (MPT 384 target) plate. Peptide mass fingerprint (PMF) was measured using Ultraflex MALDI-TOF/TOF MS after calibration with external peptide calibration standard-II. The PMFs were analyzed with the protein search engine Mascot (Matrix Science, UK) against the NCBI nonredundant database. Search parameters were set as follows: peptide mass tolerance, 100 ppm; fragment mass tolerance, ±0.75 Da; taxonomy, Viridiplantae; fixed modification, carbamidomethylation of cysteine; and variable modification, methionine oxidation. Peptide masses of tryptic fragments and MS/MS fragment ion masses for the most intense peptides were sent as combined data for search against databases using Mascot.

**Statistical Analysis.** Each experiment was carried out with three technical replicates. The data were subjected to *t*-test analysis using ANOVA software, and difference between means was compared by the Duncan's multiple-range test at ($p \leq 0.05$). Statistical analysis of nutritional and antinutritional components was performed using Microsoft Office Excel 2007.

## ■ RESULTS AND DISCUSSION

"Substantial equivalence" is an important facet of biosafety assessment of transgenic events. Deregulation of transgenics is possible when it is demonstrated that the nutritional/phenotypic/molecular quality of the specific transgenic event is comparable with that of the nontransgenic counterpart. In this study, proteomics and nutritional analyses of transgenic pigeon pea and its
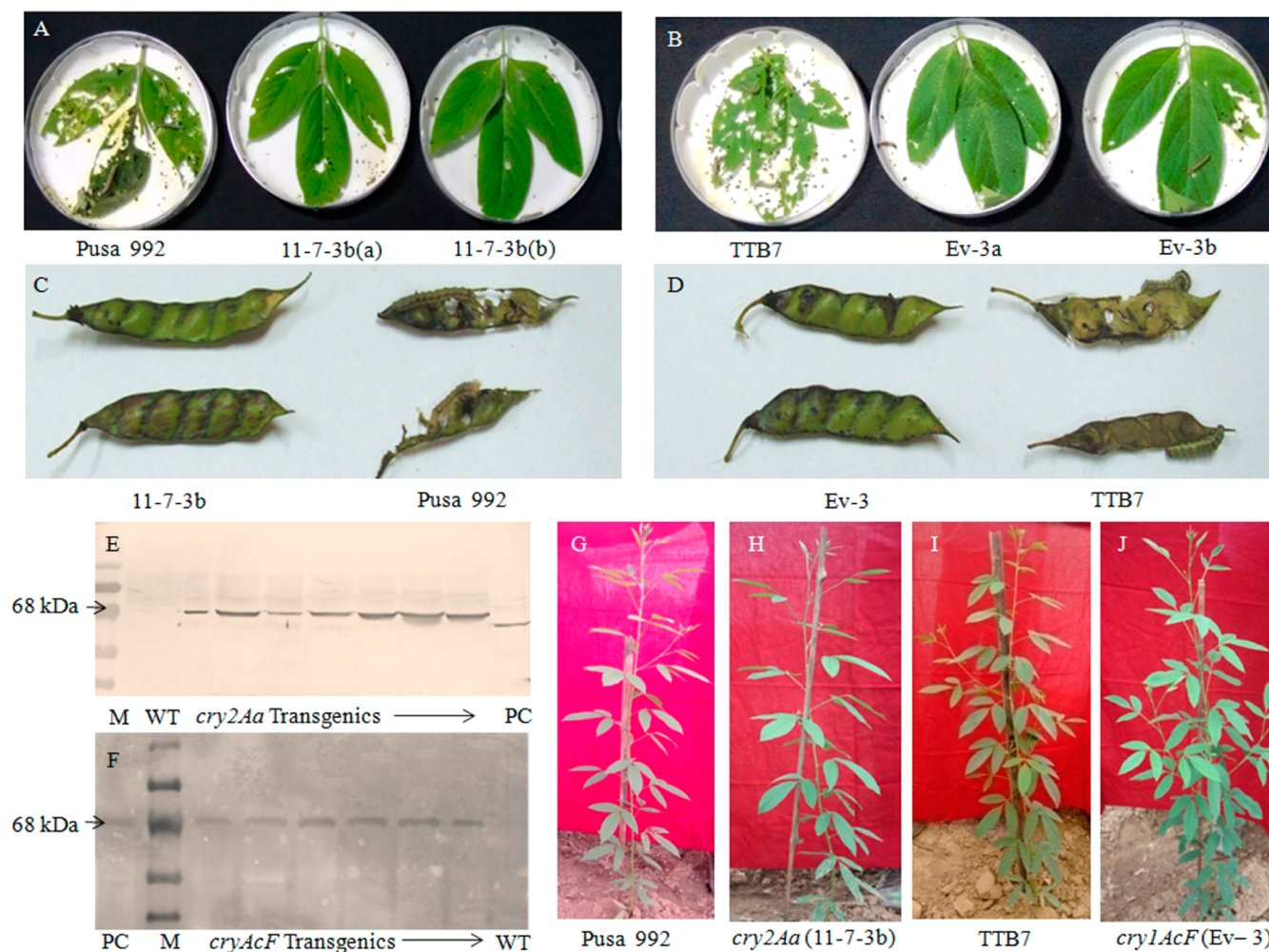
**Figure 1.** Efficacy analysis of the selected transgenic events against pod borer *H. armigera* using detached leaf and pod bioassay. Leaves of Pusa 992 and its transgenic event 11-7-3b (*cry2Aa*) (A) and TTB7 and its transgenic event Ev-3 (*cry1AcF*) (B) were subjected to in vitro challenging assay for 96 h with second-instar larvae of *H. armigera*. Pods of Pusa 992 and its transgenic event 11-7-3b (*cry2Aa*) (C) and TTB7 and its transgenic event Ev-3 (*cry1AcF*) (D) were subjected to in vitro challenging assay for 96 h with fourth-instar larvae of *H. armigera*. (a) and (b) depict replicates in each of the assays. (E) Western blot analysis to confirm the expression of cry2Aa in transgenic event 11-7-3b. (F) Western blot analysis to confirm the expression of cry1AcF in transgenic event Ev-3 and absence in the respective nontransgenics [M, protein marker, WT, wildtype or nontransgenic, PC, purified cry protein (50 ng)]. Plant phenotype of pigeon pea cv. Pusa 992 (G) and its transgenic with *cry2Aa* (11-7-3b) (H). Pigeon pea cv. TTB7 (I) and its transgenic with *cry1AcF* (Ev-3) (J).

nontransgenic counterpart were carried out to assess the risk associated with the transgene integration. Transgenics to combat pod borer (*H. armigera*) were developed in pigeon pea harboring two highly effective *cry* genes, *cry1AcF* and *cry2Aa* (unpublished data). To the best of our knowledge, this paper is the first-ever comprehensive evaluation of a food crop, pigeon pea, demonstrating lack of unintended effects in stable transformed events.

Promising pigeon pea transgenics were selected for the present study following stringent molecular and bioefficacy analysis against *H. armigera* with focus on unaltered phenotype and yield (Figure 1). Seeds of the two best events (11-7-3b with *cry2Aa* and Ev-3 with *cry1AcF*) with normal plant and pod phenotype and comparable yield were selected for the comprehensive biosafety analysis.

**Nutritional Analysis of Transgenic and Nontransgenic Pigeon Pea.** Pigeon pea is best recommended for a balanced vegetarian diet because of its protein, carbohydrate, and mineral contents. Nutritional analysis of transgenic events (11-7-3b and Ev-3) and their nontransgenic counterparts is shown in Table 1

along with the reference range of the respective components wherever available. It was observed that there was no difference in the quantity of carbohydrates such as glucose, raffinose oligosaccharides, and reducing sugar in both transgenics and nontransgenics (Table 1). Likewise, amino acid and total protein contents in pigeon pea seeds are the most important components that designate pigeon pea as a "food protein source", and acceptability of pigeon pea transgenics is largely dependent on these constituents. It was observed in the present study that the concentrations of essential amino acids were similar between the transgenics and their respective nontransgenics.

**Antioxidative Activities in Pigeon Pea Seeds.** Other important components pertaining to antioxidant capacity of pigeon pea and reduced occurrence of antinutritional factors along with the important minerals were well within the reference range, demonstrating the substantial equivalence of the transgenic events with respect to nutritional composition (Table 1).[21]

**Comparative Proteomic Analysis of Transgenic and Nontransgenic Pigeon Pea.** Because proteins are end points

**Table 1. Nutritional Analysis of Nontransgenic and Transgenic Pigeon Pea Seeds**

| analyte | PUSA 992 | cry2Aa11-7-3b | TTB7 | cry1AcF Ev-3 | reference range |
|---|---|---|---|---|---|
| **starch test** | | | | | |
| glucose (mg/100 mg) | 12.6 ± 0.17 | 14.43 ± 0.15 | 27.9 ± 0.06 | 30.5 ± 0.09 | not available |
| **sugar test**[a] | | | | | |
| reducing sugar (mg/100 mg) | 1.35 ± 0.01 | 1.3 ± 0.12 | 1.07 ± 0.02 | 1.63 ± 0.09 | not available |
| raffinose oligosaccharides (mg/100 g) | 4.70 ± 0.21 | 4.98 ± 0.08 | 3.82 ± 0.09 | 3.96 ± 0.05 | 0.24−1.05 g/100 g |
| **DPPH radical scavenging activity**[a] | | | | | |
| activity (%) | 24.26 ± 0.01 | 30.04 ± 0.02 | 10.63 ± 0.14 | 16.53 ± 0.14 | not available |
| antioxidant activity ($\mu$mol TE/mg) | 0.77 ± 0.0 | 0.86 ± 0.0 | 1.08 ± 0.0 | 1.46 ± 0.0 | not available |
| **minerals (mg/100 g)**[a] | | | | | |
| calcium | 119.53 ± 3.89 | 121.68 ± 3.38 | 122.17 ± 1.92 | 123.23 ± 2.28 | 94.6−120.8 |
| iron | 4.17 ± 0.32 | 4.2 ± 0.32 | 4.1 ± 0.16 | 4.37 ± 0.23 | 3.9−4.6 |
| zinc[b] | 2.73 ± 0.15 | 2.86 ± 0.23 | 2.93 ± 0.23 | 2.9 ± 0.33 | 2.3−2.5 |
| **secondary metabolite**[a] | | | | | |
| total phenol (mg/g) | 4.56 ± 0.21 | 4.42 ± 0.07 | 4.51 ± 0.08 | 4.23 ± 0.12 | 3.0−18.3 |
| tannin[b] (mg/g) | 0.31 ± 0.01 | 0.29 ± 0.02 | 0.39 ± 0.01 | 0.38 ± 0.01 | 0.0−0.2 |
| **$\alpha$-amylase inhibitor (units/g)**[b] | | | | | |
| before heat treatment | 19.1 ± 0.55 | 14.11 ± 0.57 | 14.9 ± 0.38 | 10.27 ± 0.40 | |
| after heat treatment | 23.3 ± 0.35 | 25.87 ± 0.19 | 13.93 ± 0.29 | 17.26 ± 0.39 | 22.5−34.2 |
| **amino acid (g/100 g)**[c] | | | | | |
| alanine | 0.74 ± 0.03 | 0.74 ± 0.03 | 0.83 ± 0.08 | 0.87 ± 0.02 | 0.972 |
| arginine | 1.33 ± 0.05 | 1.33 ± 0.16 | 1.45 ± 0.08 | 1.43 ± 0.11 | 1.299 |
| aspartic acid | 1.6 ± 0.07 | 1.35 ± 0.22 | 1.6 ± 0.34 | 2.01 ± 0.04 | 2.146 |
| cysteine + cystine | 0.24 ± 0.02 | 0.16 ± 0.01 | 0.22 ± 0.03 | 0.31 ± 0.02 | 0.25 |
| glutamic acid | 3.4 ± 0.09 | 3.33 ± 0.1 | 3.49 ± 0.08 | 4.07 ± 0.36 | 5.031 |
| glycine | 1.35 ± 0.06 | 1.42 ± 0.04 | 1.58 ± 0.13 | 1.49 ± 0.11 | 0.802 |
| histidine | 0.71 ± 0.02 | 0.69 ± 0.02 | 0.69 ± 0.02 | 0.79 ± 0.04 | 0.774 |
| isoleucine | 0.74 ± 0.03 | 0.76 ± 0.02 | 1.14 ± 0.08 | 1.01 ± 0.04 | 0.785 |
| leucine | 1.32 ± 0.03 | 1.38 ± 0.02 | 1.53 ± 0.11 | 1.47 ± 0.11 | 1.549 |
| lysine | 1.32 ± 0.01 | 1.26 ± 0.02 | 1.63 ± 0.10 | 1.51 ± 0.05 | 1.521 |
| methionine | 0.23 ± 0.02 | 0.22 ± 0.01 | 0.13 ± 0.07 | 0.17 ± 0.02 | 0.243 |
| phenylalanine | 1.73 ± 0.04 | 1.73 ± 0.03 | 1.67 ± 0.11 | 2.1 ± 0.09 | 1.858 |
| proline | 0.94 ± 0.04 | 1.11 ± 0.05 | 1.3 ± 0.067 | 1.23 ± 0.02 | 0.955 |
| serine | 0.82 ± 0.03 | 0.81 ± 0.01 | 1.19 ± 0.04 | 1.2 ± 0.16 | 1.028 |
| threonine | 0.74 ± 0.03 | 0.73 ± 0.01 | 0.79 ± 0.03 | 0.89 ± 0.03 | 0.767 |
| tyrosine | 0.41 ± 0.02 | 0.41 ± 0.03 | 0.46 ± 0.02 | 0.46 ± 0.06 | 0.538 |
| valine | 0.96 ± 0.01 | 0.91 ± 0.02 | 1.18 ± 0.02 | 1.21 ± 0.13 | 0.937 |

[a]Singh (1988).[28] [b]Genotypic variation. [c]USDA National Nutrient Database for Standard Reference.[27]

of metabolic pathways, any change in the total proteome would lead to unintended effects in the physiology and thereafter phenotype of the plant. In this direction, proteomic tools offer a high-throughput platform to assess the changes on a comparative basis. There are several studies on the use of proteomic profiling in transgenic crops[22−25] that provide support to biological safety. 2-DGE was performed to visualize protein profiles of transgenic events 11-7-3b (cry2Aa) and Ev-3 (cry1AcF) and their non-transgenic counterparts (Figure 2). Comparative proteomic profiling by Image Master platinum 2D (IMP) software version 7.0 (GE Healthcare, Piscataway, NJ, USA) as well as manual analysis demonstrated minimal or no variations between transgenics and the respective nontransgenic counterparts. The 2DE analysis of the gels showed approximately 470 protein spots present in each of the gels of transgenics and nontransgenics (Figure2). Protein spots showing a difference in relative intensity of ≥2.5-fold were considered for analysis and, accordingly, 11 and 10 spots were selected in cry2Aa and cry1AcF events, respectively, and were used for MS/MS analysis (Supporting Information). Five spots could be successfully identified, of which 3 belonged to the cry2Aa event and 2 belonged to cry1AcF as summarized in Table 2. Coincidentally, both

transgenics demonstrated a slight up-regulation in the expression of $\beta$-1,3-glucanase, a protein that plays an important role in growth and development as well as in pathogen response.[26] Furthermore, carbonic anhydrase, an important player in photosynthesis, was seen to be up-regulated in the event with cry2Aa, and a putative transmembrane protein was up-regulated in the event with cry1AcF. However, these variations did not induce any unintended effect in the phenotype as evident from the nutritional compositional analysis described earlier. Alternatively, these proteins could lead to better performance of the transgenics under field situations.

The present study prudently demonstrates substantial equivalence of the selected transgenic pigeon pea seeds with their nontransgenic counterpart based on nutritional composition analyses and proteome profiling. The subtle differences observed in the nutritional compositional analysis cannot be considered biologically relevant because all of the significantly different mean values were within reference ranges (wherever available) prescribed for pigeon pea.[27,28] Furthermore, protein spots with >2.5-fold variation were monitored, and the differentially expressed proteins identified were mostly involved in metabolic activities and were not allergenic or toxic. These results thus
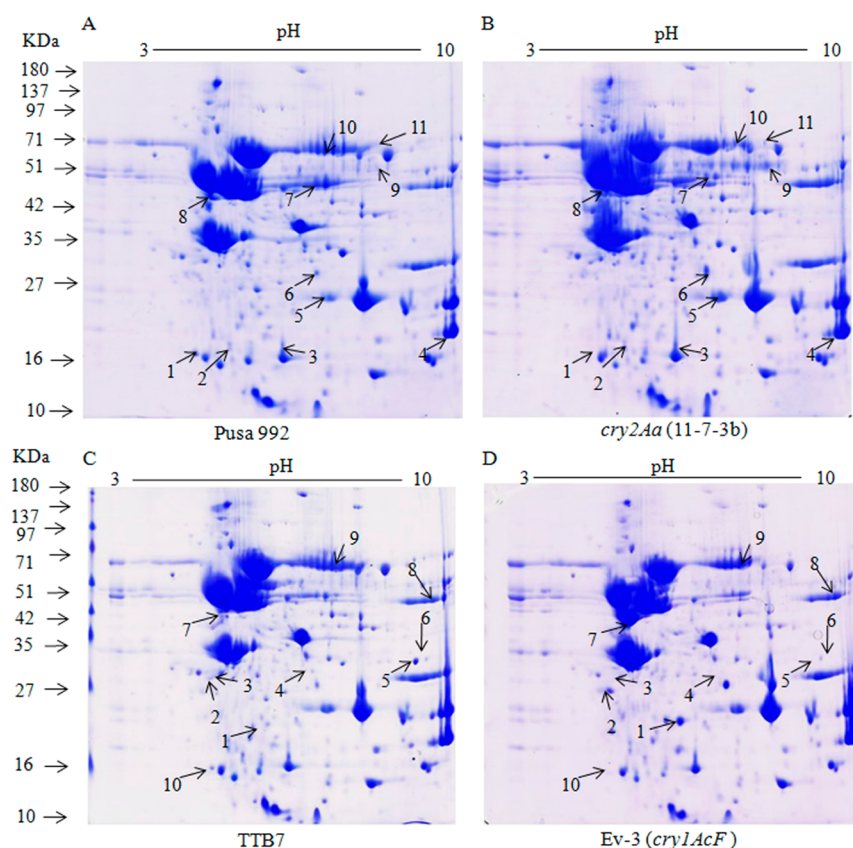
**Figure 2.** Two-dimensional electrophoresis gels (2DGE) of pigeon pea seed proteins showing the position of proteins (indicated by numbers) selected for MS/MS analysis. Total seed protein was separated on 13 cm IPG pH 3−10, stained with colloidal coomassie R-250. (A) and (B) depict 2DGE profiles of pigeon pea cv. Pusa 992 and transgenic *cry2Aa* (11-7-3b), respectively; (C) and (D) are 2DGE profiles of pigeon pea cv. TTB7 and transgenic *cry1Acf* (Ev-3), respectively.

**Table 2. MS/MS Identification of Differentially Expressed Proteins in Transgenic and Nontransgenic Pigeon Pea Seeds**

| spot ID | protein accession no. | protein name | function | fold change expression | MS/ MS score | protein seq coverage (%) | mol wt calcd/exptl | pI calcd/exptl |
|---|---|---|---|---|---|---|---|---|
| | | | **Pusa 992 versus *cry2Aa* 11-7-3b** | | | | | |
| 4 | gil41584408 | endo-β-1,3-glucanase, partial | hydrolase activity, acting on glycosyl bonds | ↑2.61 | 45 | 5 | 23/25 | 7.9/9.8 |
| 10 | gil1009122791 | penta tricopeptide repeat-containing protein | m-RNA processing | ↓2.17 | 16 | 2 | 95/69 | 6.8/8.6 |
| 5 | gil734390961 | carbonic anhydrase, chloroplastic | reversible hydration of carbon dioxide | ↑2.60 | 180 | 17 | 36/28 | 5.8/7.5 |
| | | | **TTB7 versus *cry1AcF* Ev-3** | | | | | |
| 1 | gil545627505 | β-1,3-glucanase | carbohydrate metabolism, hydrolase activity, hydrolyzing *O*-glycosyl compounds | ↑3.66 | 38 | 4 | 37/23 | 5.7/6.5 |
| 7 | gil357437697 | transmembrane protein, putative | RNA synthesis | ↑15.5 | 10 | 28 | 90/41 | 8.8/5.5 |

confirm that the nutritional quality of transgenic pigeon pea seed was comparable to that of the respective nontransgenic counterparts with no unintended effects due to the integration of both transgenes.

## ASSOCIATED CONTENT

**Ⓢ Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b05301.

> Differentially expressed proteins in transgenic and nontransgenic pigeon pea seeds (PDF)

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## Notes

The authors declare no competing financial interest.

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