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**Comparative proteomic and nutritional composition analysis of independent transgenic
pigeon pea seeds harboring *cry1AcF* and *cry2Aa* genes and their non-transgenic
counterparts**

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Abstract

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

Key words: nutritional analysis, pigeon pea, proteome, transgenics

39 Introduction

40 Pigeon pea (*Cajanus cajan* L.) is a perennial legume belonging to the family Fabaceae grown
41 in tropical and semitropical regions of Asia and Africa. Pigeon pea has a unique place in
42 Indian farming and the sub-continent accounts for about 90% of the global production.
43 Pigeon pea is regarded as an 'orphan crop' despite being a rich source of essential nutrients.
44 The crop encounters various biotic and abiotic stresses that results in tremendous yield losses.
45 In the recent years, molecular breeding and transgenic technology-based crop improvement
46 programmes have delivered immense hope towards combating these stresses. Among biotic
47 factors, sterility mosaic disease (SMD), pod fly, fusarium wilt and pod borer (*Helicoverpa*
48 *armigera*) are important which substantially damage the crop resulting in significant yield
49 losses¹. The success of insecticidal 'cry' genes-based insect resistance has opened avenues for
50 its use in development of insect resistant varieties in numerous crops including pigeon pea^{2,3}.
51 However, the major concern in the development of transgenics involves introduction of
52 foreign genes, which when integrated into the host genome at random positions may affect
53 the function of endogenous genes resulting in alteration of various metabolic pathways
54 leading to unintended effects. Biosafety assessment of GMOs for the absence of undesirable
55 phenotypic and physiological effects assumes tremendous significance especially in the food
56 crops that are transgenically modified^{4,5}. Non-targeted technologies involving comparative
57 transcriptomics, proteomics and metabolomics have gained popularity in the biosafety
58 assessment of GMOs^{6, 7, 8}. The advantage in using these techniques is that the end point of
59 any metabolic activity can be measured and compared. In this direction, the focus of this
60 article has been comprehensive evaluation of independent transgenics of pigeon pea for two
61 novel cry genes (*cry1AcF* and *cry2Aa*) on the basis of their proteomic and nutritional
62 composition. This is the first ever report of comprehensive evaluation of transgenic pigeon
63 pea for substantial equivalence.

64

65 **Material and Methods**

66 **Plant material.** Selected transgenic events of pigeon pea homozygous for *cry1AcF* and
67 *cry2Aa* (11-7-3-b with *cry2Aa* in cv. (cultivar) Pusa 992 and Ev-3 with *cry1AcFin* cv. TTB7)
68 were raised in contained net houses along with their non-transformed counter parts. The
69 plants were grown to maturity, harvested and seeds of uniform shape and similar weight were
70 selected to carry out nutritional and proteomic analysis. The selected events were earlier
71 evaluated for transgene integration and bioefficacy against the target insect, *H. armigera*
72 using standardized protocols³. All the experiments were carried out in three technical
73 replicates.

74

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

78 **Estimation of Amino acid content.** Finely ground powder of transgenic and non-transgenic
79 pigeon pea seeds (100 mg) was used for quantification of essential amino acids. Waters
80 AccQ-Tag Chemistry package kit (Waters India Pvt. Ltd.) was used for sample preparation,
81 derivatization and analysis as per manufacturer's instructions. The derivatized samples
82 including standards were subjected to HPLC analysis by Eurofins Analytical Services India
83 Ltd., detected using a fluorescence detector (LC-FLD) and expressed as g/100g.

84

85 **Estimation of Reducing Sugars, Raffinose, and Starch.** Transgenic and non-transgenic
86 seeds (100 mg) were finely powdered, extracted twice with 80% ethanol at 95 °C, pooled and
87 dried at 80 °C for 2h. The residue was dissolved in 10 ml of distilled water and reducing
88 sugars were estimated^{9, 10}. Raffinose was estimated according to manufacturer's protocol

(Megazyme International, Ireland) and expressed as mg/100g dry weight. For total starch estimation, soluble sugars were initially removed as explained above and estimation of starch was carried out¹¹.

Quantification of mineral ions. About 100 mg pigeon pea seeds were used to estimate total Fe²⁺, Zn²⁺ and Ca²⁺ concentration as per the published protocol¹². The powdered seed samples, which were digested in a di acid solution of HNO₃ and HClO₄, were subjected to Atomic Absorption Spectrometry (ECIL, AAS 4141) system that was calibrated for Fe²⁺, Zn²⁺ and Ca²⁺.

Estimation of antioxidant potential. Antioxidant potential in the transgenic and non-transgenic seeds was estimated following two methodologies:

(a) Sample extract was prepared by centrifuging overnight-soaked seed powder (100 mg) (with acetone) at 1509 g for 10 min and the resultant supernatant (100 µl) was used for estimation of antioxidant activity by Cupric reducing-antioxidant capacity (CUPRAC) method¹³. The antioxidant capacity was expressed as Trolox equivalent (µmol/ TE/g) using the formula,

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

Where, V_{initial} = initial volume; m = weight of sample; r = dilution factor; V_f = final volume; V_s = volume of aliquot; A_f = absorbance; $\epsilon_{TR} = 1.67 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$.

(b) Free radical scavenging activity was measured by DPPH (2,2-diphenyl-1-picrylhydrazyl) assay^{14, 15}. Sample extract was prepared as described for CUPRAC assay. The DPPH radical scavenging activity (S%) was calculated using the following equation.

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

Where, A_{control} is the absorbance of the blank control (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 protocol¹⁶. Calibration plot was expressed as gallic acid (2-10 µg/ml) equivalents/g DW. Tannins were estimated by Vanillin-HCl method¹⁷. Catechin solution (0-100 ppm) was used for standard curve and the results were expressed as mg of catechin equivalent/g DW.

Anti-nutrient alpha amylase inhibitor. The anti-nutrient factor, alpha amylase inhibitor was estimated in the transgenic and non-transgenic seeds following the published methodology¹⁸. The percentage of alpha amylase inhibition was calculated by the following formula.

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

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

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

Proteomic analysis and MS/MS identification. All the procedures for protein extraction and 2-DGE (2-dimensional gel electrophoresis) were carried out following the standardized methodology¹⁹ with some modifications. In short, for protein isolation, 1 g seed was homogenized in liquid nitrogen in a pre-chilled pestle and mortar, suspended in phosphate buffer (pH 7.4) and centrifuged at 5000 g for 10 min at 4 °C. The supernatant was further re-suspended in cold 10% (w/v) TCA and acetone containing 0.1% (v/v) β-mercaptoethanol and allowed to precipitate at -20 °C for 2h. The suspension was centrifuged at 24,700 g for 15 min at 4 °C in a refrigerated high-speed centrifuge. Pellet was re-suspended in chilled acetone containing 0.1% β-mercaptoethanol (v/v) and centrifuged at 24,700 g for 15 min at 4 °C; the

process was repeated 3–4 times. Final washing of pellet was carried out with chilled 100% acetone. The pellet was air dried at room temperature and stored at -80 °C. After 24h, pellets were re-suspended in rehydration buffer (7M urea, 2M thiourea, 2% (w/v) CHAPS) and centrifuged at 24,700 g for 5 min at room temperature. The total proteins in the supernatant were quantified²⁰ using BSA as a standard and further used for proteomic analysis.

2D-Gel Electrophoresis. Isoelectric focusing (IEF) was carried out using an Ettan IPGphor III (GE Healthcare) 13cm 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 equilibrating 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 protocol¹⁹. 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 analysed 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 applying the statistical analysis of variance criteria ($p \leq 0.05$).

In gel digestion and MS/MS identification of peptides. This process was performed according to 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 μ l of 100 mM NH_4HCO_3 /acetonitrile (ACN) (50:50, v/v). Destained gel spots were dehydrated with 50 μ l of ACN. For trypsin digestion, 10 μ g ml^{-1} of sequencing grade trypsin (Promega) was added to each dried gel fragment and incubated for 45 min at 4 $^\circ\text{C}$. Further, 10 μ l of 50 mM $\text{NH}_4\text{-HCO}_3$ was added and kept for incubation at 37 $^\circ\text{C}$ for 16 h. After digestion, peptides were purified, concentrated and used for spotting on 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 non-redundant 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 most intense peptides were sent as a combined data for search against databases using Mascot.

Statistical analysis. Each experiment was carried out with three technical replicates. The data was subjected to t-test analysis using ANOVA software and difference between the means was compared by the Duncan's Multiple Range Test at ($p \leq 0.05$). The Statistical analysis of nutritional and anti-nutritional components were performed using Microsoft office excel 2007.

Results and Discussion

“Substantial equivalence” is an important facet of biosafety assessment of transgenic events. De-regulation of transgenics is possible when it is demonstrated that the nutritional/phenotypic/molecular quality of the specific transgenic event is comparable with the non-transgenic counterpart. In this study, proteomics and nutritional analysis of

transgenic pigeon pea and its non-transgenic counterpart was carried out to assess risk associated with the transgene integration. Transgenics to combat pod borer (*H. armigera*) were developed in pigeon pea harbouring two highly effective *cry* genes, *cry1AcF* and *cry2Aa* (unpublished data). To the best of our knowledge, this report 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 A-J). Seeds of two best events (11-7-3-b 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 non-transgenic pigeon pea. Pigeon pea is best recommended for a balanced vegetarian diet because of its protein, carbohydrate and mineral content. Nutritional analysis of transgenic events (11-7-3-b and Ev-3) and its non-transgenic counterpart is shown in Table 1 along with the reference range of the respective components whichever available. It was observed that there was no difference in the quantity of carbohydrates like glucose, raffinose oligosaccharides and reducing sugar in both transgenics and non-transgenics (Table 1). Likewise, amino acid and total protein content in pigeon pea seeds are the most important components that designate pigeon pea as “food protein source” and acceptability of pigeon pea transgenics is largely dependent on these constituents. It was observed in the present study that the concentration of essential amino acids were similar between the transgenics and their respective non-transgenics.

Antioxidative activities in pigeon pea seeds. Other important components pertaining to antioxidant capacity of pigeon pea and reduced occurrence of anti-nutritional 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 non-transgenic pigeon pea. Since proteins are end points 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 reports on the use of proteomic profiling in transgenic crops^{22, 23, 24, 25} that provide support to biological safety. 2D gel electrophoresis was performed to visualise protein profiles of transgenic event-11-7-3b (*cry2Aa*) and Ev-3 (*cry1AcF*) and its non-transgenic counterparts (Figure 2). Comparative proteomic profiling by Image Master platinum 2D (IMP) software version 7.0 (GE healthcare, USA) as well as manual analysis demonstrated minimum or no variations between transgenics and the respective non-transgenic counterparts. The 2DE analysis of the gels showed approximately 470 protein spots present in each of the gels of transgenics and non-transgenics (Figure 2A-D). 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 (Supplementary table 1). Five spots could be successively identified of which 3 belonged to the *cry2Aa* event and 2 belonged to *cry1AcF* as summarized in Table 2. Coincidentally, both the transgenics demonstrated a slight upregulation in the expression of β -1, 3-glucanase, a protein that plays an important role in growth and development as well as in pathogen response²⁶. Further, 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

235 variations did not induce any unintended effect in the phenotype as evident from the
236 nutritional compositional analysis described earlier. Alternatively, these proteins could lead
237 to better performance of the transgenics under field situations.

238 The present study prudently demonstrates substantial equivalence of the selected
239 transgenic pigeon pea seeds with their non-transgenic counterpart based on nutritional
240 composition analyses and proteome profiling. The subtle differences observed in the
241 nutritional compositional analysis cannot be considered biologically relevant since all the
242 significantly different mean values were within reference ranges (whichever available)
243 prescribed for pigeon pea^{27, 28}. Further, protein spots with more than 2.5 fold variation were
244 monitored and the differentially expressed proteins identified were mostly involved in
245 metabolic activities and were not allergenic or toxic. These results thus confirm that the
246 nutritional quality of transgenic pigeon pea seed was comparable to that of the respective
247 non-transgenic counterparts with no unintended effects due to the integration of both the
248 transgenes.

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252

253 **Supportive information**

254 Supplementary Table 1: List of differentially expressed proteins in transgenic and non-
255 transgenic pigeon pea seeds

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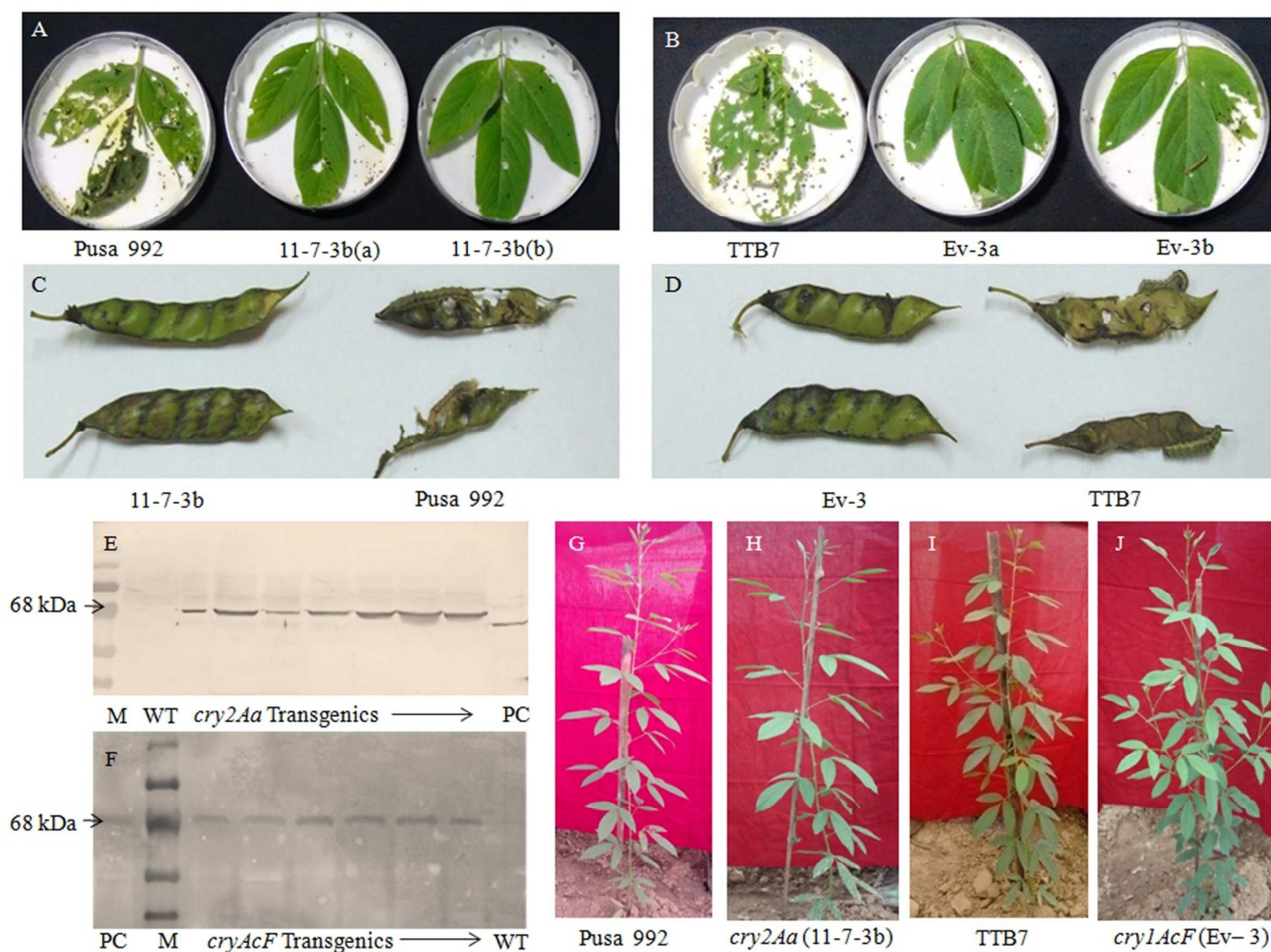
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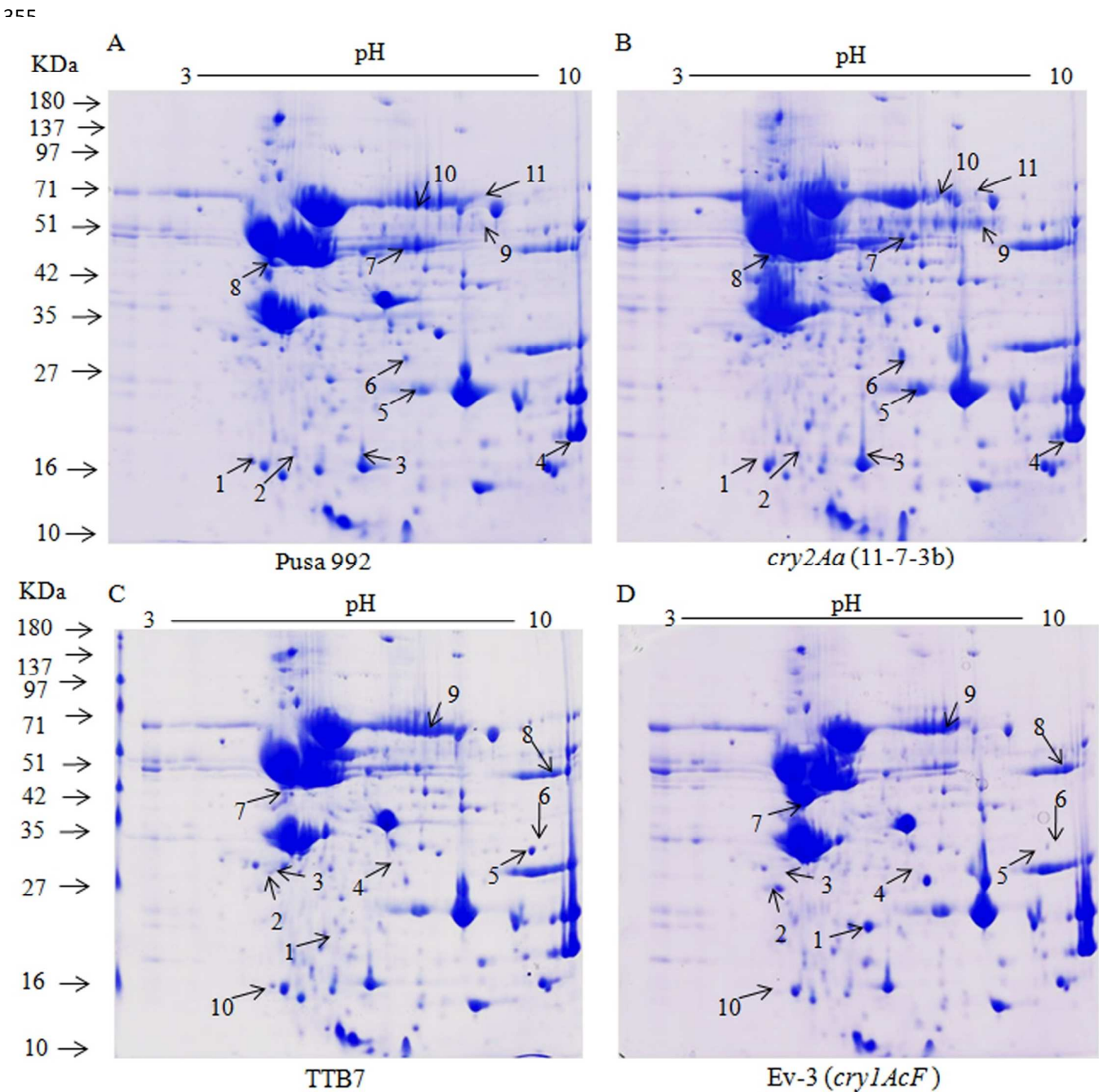
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Figure Legends

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); TTB7 and its transgenic event Ev-3 (*cry1AcF*) (B) were subjected to *in vitro* challenging assay for 96 h with 2nd instar larvae of *H. armigera*. Pods of Pusa 992 and its transgenic event 11-7-3b (*cry2Aa*) (C); TTB7 and its transgenic event Ev-3 (*cry1AcF*) (D) were subjected to *in vitro* challenging assay for 96 h with 4th instar larvae of *H. armigera*. a & b depict replicates in each of the assays. (E) Western blot analysis to confirm the expression of *cry2Aa* in the transgenic event 11-7-3b; (F) Western blot analysis to confirm the expression of *cry1AcF* in the transgenic event Ev-3 and absence in the respective non-transgenics [M-protein marker, WT-wildtype or non-transgenic, 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).



349 2. Two-dimensional electrophoresis gels (2DGE) of pigeon pea seed proteins showing the
350 position of proteins (indicated by numbers) selected for MS/MS analysis. Total seed
351 protein was separated on 13 cm IPG pH 3-10, stained with colloidal coomassie R-250.(A)
352 and (B) depict 2DGE profiles of pigeon pea cv. Pusa 992 and transgenic *cry2Aa*(11-7-3b)
353 respectively; (C) and (D) are 2DGE profiles of pigeon pea cv. TTB7 and transgenic
354 *cry1Ac*(Ev-3).



362 Table1. Nutritional analysis of non-transgenic and transgenic pigeon pea seeds

Analytes	PUSA 992	<i>cry2Aa11-7-3b</i>	TTB7	<i>cry1AcFEv-3</i>	Reference Range
Starch test					
Glucose (mg/100mg)	12.6 ± 0.17	14.43 ± 0.15	27.9 ± 0.06	30.5 ± 0.09	Not available
Sugar test*					
Reducing sugar (mg/100mg)	1.35 ± 0.01	1.3 ± 0.12	1.07 ± 0.02	1.63 ± 0.09	Not available
Raffinose oligosaccharides (mg/100g)	4.70 ± 0.21	4.98 ± 0.08	3.82 ± 0.09	3.96 ± 0.05	0.24-1.05(g/100g)
DPPH radicle scavenging activity*					
Activity %	24.26 ± 0.01	30.04 ± 0.02	10.63 ± 0.14	16.53 ± 0.14	Not available
Antioxidant activity (μmol TE/mg)	0.77 ± 0.0	0.86 ± 0.0	1.08 ± 0.0	1.46 ± 0.0	Not available
Minerals (mg 100g⁻¹) *					
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***	2.73 ± 0.15	2.86 ± 0.23	2.93 ± 0.23	2.9 ± 0.33	2.3-2.5
Secondary metabolite*					
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 (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⁻¹)***					
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/100g**					
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 ± .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

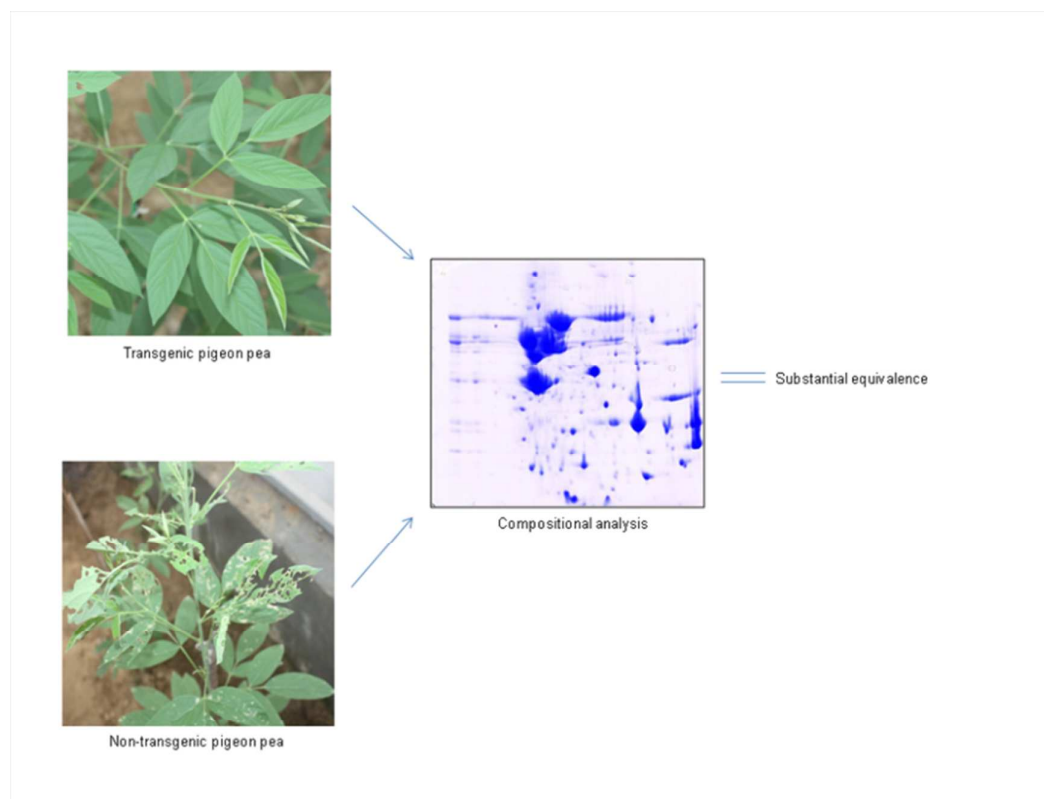
363 Source: *Singh U (1988)²⁸364 **USDA National Nutrient Database For standard Reference²⁷

365 ***Genotypic variation

366 Table 2. MS/MS identification of differentially expressed proteins in transgenic and non-transgenic pigeon pea seeds
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Spot ID	Protein Ac. No.	Protein Name	Function	Fold change expression	MS/MS Score	Protein seq coverage	Mol wt cal/exp	pI cal/exp
Pusa 992 vs cry2Aa 11-7-3b								
4	gi 41584408	Endo-beta-1,3-glucanase, partial	Hydrolase activity, acting on glycosyl bonds	↑2.61	45	5%	23/25	7.9/9.8
10	gi 1009122791	Penta tricopeptide repeat-containing protein	m-RNA processing	↓2.17	16	2%	95/69	6.8/8.6
5	gi 734390961	Carbonic anhydrase, chloroplastic	Reversible hydration of carbon dioxide	↑2.60	180	17%	36/28	5.8/7.5
TTB7 vs cry1AcF Ev-3								
1	gi 545627505	Beta-1,3-glucanase	Carbohydrate metabolism Hydrolase activity, hydrolyzing O-glycosyl compounds	↑3.66	38	4%	37/23	5.7/6.5
7	gi 357437697	Transmembrane protein, putative	RNA synthesis	↑15.5	10	28%	90/41	8.8/5.5

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