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Analytical Methods

Immunodiagnostic analysis of transgenic vegetative insecticidal prot[illegible] in genetically modified crops/produce

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ABSTRACT

In the process of development of insect resistant genetica[illegible] modified (GM) crops and also to evaluate the consistency in the expression of toxi[illegible] field condition[illegible] mmunological assays are commonly being used. We have developed immun[illegible] say to support labelling of vegetative insecticidal protein (Vip3A) based GM produce. The develope[illegible] LISA for measu[illegible]ement of Vip3A is a triple antibody sandwich procedure utilising a polyclonal captur[illegible] ntibody (anti-V[illegible]3A raised in mice) and a polyclonal detection antibody (anti-Vip3A raised in rabbits) [illegible]llowed by use[illegible] a third HRP-conjugated antispecies antibody (goat anti-rabbit IgG). The detection limi[illegible] r the dev[illegible]ped ELISA assay was 16 ppb of Vip3A protein. The quantification range [illegible]1–500 ppb [illegible] protein. The assay is sensitive, quick and reproducible can be utilise in regul[illegible]ng G[illegible]oduce.

1. Introduction

As market restrictions for various t[illegible]nsgenic cr[illegible]s continue, there is increasing interest among [illegible]ers in deter[illegible]ining the presence of genetically modified (G[illegible]M) co[illegible]nts in crops. [illegible]rowers producing non-GM grains for s[illegible]ecialty mark[illegible] need to verify that there is no GM contaminatio[illegible] or that contam[illegible]tion levels meet tolerance levels establish[illegible] by different regulatory authorities. The maximum allowab[illegible]evel is in[illegible]e range of 0.9–5% of GM content as set by different [illegible]ula[illegible]y authorities (Marmiroli et al., 2008).

Bacillus thuri[illegible]giensis (Bt) pro[illegible]es t[illegible]o known classes of vegetative insecti[illegible]al prote[illegible]s (Vip). T[illegible]e include the binary toxins Vip1 and V[illegible]2 with c[illegible]an specificity and Vip3 with lepidopteran specific[illegible] (P[illegible]g, Gil, Neisner, Van Rie, & Frutos, 2005). Vip3A represents a ma[illegible] discovery among insect toxins and shares no amino acid sequen[illegible]similarity with the widely used Bt endotoxins (Estruch et al., 1996). [illegible]ip3A is now part of different plants, e.g. – GM cotton and maize (Syngenta Seeds Inc., USA), GM cotton, tobacco and brinjal (ICGEB, New Delhi, India). The Vip3Aa (88.5 kDa) is highly insecticidal to several major lepidopteran pests, including the black cutworm (*Agrotis ipsilon*), fall armyworm (*Spodoptera frugiperda*), beet armyworm (*Spodoptera exigua*), tobacco budworm (*Heliothis virescens*), and corn earworm (*Helicoverpa zea*) (Estruch et al., 1996). It was observed that Vip3Aa kills insects by lysing insect midgut cells via cell membrane pore formation and mode of action is different from Bt endotoxins (Lee, Walters, Hart, Palekar, & Chen, 2003).

The detection of GM crops is useful in crop production to find out the expression of protein of interest during the growing season as well as for regulatory compliance on GM labelling. Quality control and genetic purity of legal transgenic seed is an important concern. The antibody-based immunoassay has been widely used to detect transgenic proteins in a variety of applications including testing in the breeding process, testing for unapproved events, and determining GM content ensuring compliance with non-GM labelling requirements. There are several immunoassays commercially available for detection of Cry1Ab and Cry1Ac in both plate format and dipstick format (Agdia Inc.; DesiGen; EnviroLogix Inc.; Strategic Diagnostics Inc.), but not for Vip3A. Recently, PCR assay (Singh, Ojha, Bhatanagar, & Kachru, 2008) and dipstick test (Kumar et al., 2010) for detection of Vip3A protein were developed. Syngenta has submitted validated methods for extraction and direct ELISA analysis of Vip3A in cotton seed to the US Environmental Protection Agency; however this is not available commercially or in public domain so far.

In this article, we described the development of an enzyme-linked immunosorbent assay (ELISA) for detection and quantification of Vip-S protein (a type of Vip3A), which has been expressed in GM cotton, tobacco, brinjal and maize. The developed assay is

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rapid, selective, and sensitive for monitoring protein levels in plant and related products. In addition, this could be used for product quality control, environmental risk assessment and other relevant studies. The current study apart from assisting farmers will help regulators and quarantine personnel to detect and track down the spread of approved, unapproved and unintentionally released GM organisms (GMOs) in the environment.

2. Materials and methods

2.1. Chemicals and reagents

Freund's complete and incomplete adjuvants, Protease inhibitor cocktail, isopropyl-b-D-thiogalacto-pyranoside (IPTG), ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), bovine serum albumin (BSA), polyvinylpyrrolidone (PVP-40), *p*-nitro blue tetrazolium chloride (NBT) and 5-bromo 4-chloro 3-indolyl phosphate toluidine (BCIP) were obtained from Sigma, USA. Luria Broth media, kanamycin and ampicillin were purchased from Himedia, India. HiTrap Protein-A column was obtained from GE Healthcare, USA. Bradford reagent was purchased from BioRad, USA. HRP-conjugated anti-rabbit IgG, Alkaline phosphatase conjugated goat anti-rabbit IgG and IDA-CL agarose column were purchased from Bangalore Genei, India.

2.2. Expression and isolation of Vip3A protein

For high expression of Vip3A protein, recombinant *Escherichia coli* clone pQE30 carrying *vip-S* gene (a type of *vip3A*) was used from International Centre for Genetic Engineering and Biotechnology, New Delhi, India). This clone was designed to express Vip3A using lac promoter by IPTG induction. In addition, the *vip-S* gene sequence is linked with six Histidine amino acid coding nucleotide sequences which facilitate the purification process by IDA-CL agarose affinity column.

Single isolated colony of *E. coli* was scrapped from culture plate and inoculated in 10 ml of 2% Luria broth (LB) media containing 25 μg/ml kanamycin and 50 μg/ml ampicillin antibiotics. The primary culture (seed culture) was incubated at 37 °C for 12–15 h with shaking. After 15 h, 1% of seed culture was inoculated to 250 ml of 2% LB media to produce secondary culture. The culture was incubated at 37 °C and 200 rpm. When the O.D. (A_{600}) of culture was reached 1.0, it was induced with 1 mM IPTG and incubated for 4 h more for high expression of Vip3A. Further, the bacterial cells were harvested at 10,000*g* for 10 min. The pellets obtained then were resuspended in 50 ml lysis buffer (50 mM Tris–HCl pH 8.0, 0.5 M NaCl, 2 mg/ml lysozyme) and incubated for 2 h at 37 °C and 200 rpm. The lysozyme treated bacterial solution was then sonicated with the help of sonicator at 50 Hz for 20 s pulse with interval of 3 min for 6 repetitions. Sonicated solution was then centrifuged at 10,000*g* for 10 min and supernatant was stored at 4 °C for the purification of the Vip3A protein.

2.3. Purification of Vip3A by affinity chromatography

IDA-Cl agarose column was recharged with Nickel–ions according to manufacturer's instructions and equilibrated with wash buffer (20 mM sodium phosphate, 0.3 M NaCl, pH 7.4). The supernatant containing Vip3A protein was passed through Ni-bound column. The bound protein was eluted with 25 mM increments of imidazole (25–500 mM). The fractions were analysed on 10% SDS–PAGE. Fractions were pooled and concentrated using Centricon-30 K (Millipore, USA). The protein was quantified by Bradford (1976) method and stored at 4 °C.

2.4. Raising of polyclonal antibodies in rabbits and Balb/c mice

Highly purified Vip3A protein was used as antigen for immunization of rabbit (New Zealand white male, 1.5 kg) and Balb/c female mice (~25 g). Animals were procured from animal facility of Indian Institute of Toxicology Research. Approximately, 100 μg (for mouse) and 200 μg (for rabbit) of the Vip3A protein was mixed with Freund's complete adjuvant (1:1 v/v ratio) and administered intramuscularly for rabbit and intraperitoneally for mouse. The first booster dose was prepared, similarly as stated above, but with Freund's incomplete adjuvant and given 30 days later. The three subsequent booster doses were given 15 days of interval. The fifth booster dose was given 4 weeks later the fourth booster and the animals bled 10 days later. The blood samples were drew from ear vein (rabbit) or by puncturing orbital plexus (mouse). The sera samples were collected and the antibodies titre was checked by double immunodiffusion assay and Western blot. The high titre antibodies were pooled and affinity purified using HiTrap Protein-A column (bed volume: 1 ml). The Vip3A antiserum samples were loaded on column and eluted with 100 mM glycine buffer, pH 3.0. The purified anti-Vip3A IgG was resolved on a 10% SDS–PAGE gel and stained with Coomassie brilliant blue-R 250 dye to check the purity of Vip3A IgG. The eluted immunoglobulin fractions were dialysed against PBS (Phosphate buffered saline, pH 7.4) and its protein content was measured at 280 nm using NanoDrop spectrophotometer (NanoDrop Technologies, USA).

2.5. Double immunodiffusion assay

The double immunodiffusion assay was performed to confirm the raised antibody against Vip3A protein. A 1.5% agarose solution was poured onto a round glass petriplate and after solidification, one central and 4 surrounding, (5–10 mm diameter) wells were prepared by piercing the gel. The recombinant bacterial purified Vip3A protein (1 μg, 50 μl) was placed in the central well. The pre-immune sera (50 μl) and raised anti-Vip3A sera (50 μl) samples were placed in the surrounding 04 wells. The agarose plate was maintained in a humid environment. After a night long diffusion step, results were observed.

2.6. Extraction of total protein from GM and non-GM leaf samples

About 200 mg of leaf samples were crushed in 2 ml of GM protein extraction buffer (50 mM sodium phosphate buffer pH 7.4, 2 mM DTT, 5 mM EDTA, 0.1% Triton X-100, 2% PVP-40, Protease inhibitor cocktail). This crude preparation of protein was left standing for 30 min at room temperature with gentle shaking and centrifuged at 12,000*g* at 4 °C for 10 min. The supernatant of crude preparation was used for the detection of Vip3A protein by Western blot and ELISA.

2.7. Western blot analysis

Western blot analysis was carried out using purified Vip3A protein to validate and determine the titer value of the raised antibody. The recombinant bacterial Vip3A protein (8 μg) was separated by a 10% SDS–PAGE and transferred to nitrocellulose membrane (0.45μ). Further, membrane was blocked with blocking buffer (5% fat free milk in PBS). After that, membrane was incubated for 2 h with different dilution of rabbit anti-Vip3A serum (1:5000–1:100,000 dilutions). Further, membranes were washed and incubated with secondary antibody (Alkaline phosphatase conjugated goat anti-rabbit IgG) for 1 h. Membranes were washed and the protein–antibody complex was visualised by addition of blot developer solution (0.1 M Tris–HCl buffer pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$, 150 μg/ml NBT, and 75 μg/ml BCIP). A similar Wes-

tern blot was also performed against proteins isolated from cotton leaves to characterise the Vip3A bearing GM crops.

2.8. Development of sandwich ELISA for Vip3A

An aliquot of 100 μl/well of capture antibody (100 ng mouse anti-Vip3A IgG) was coated on a 96 well ELISA plate. After overnight incubation at 4 °C, any excess antibody was washed with 10 mM PBS. Approximately 300 μl of blocking buffer (5% fat free milk in PBS, pH 7.4) was used to block any remaining space of ELISA well plate. The plate was incubated for 2 h at 37 °C with slow shaking. After washing with PBS, 100 μl of twofold dilution of purified Vip3A protein (3.9, 7.81, 15.6, 31.2, 62.5, 125, 200, 250, 500, 1000, 2000, 4000, and 8000 ng/ml) was added to the wells. The plate was incubated at 37 °C for another 1 h with continuous mild shaking to facilitate the interaction of recombinant Vip3A antigen to complex with anti-Vip3A-IgG antibody. Again, after four wash with PBST (PBS containing 0.05% Tween-20), about 100 μl of rabbit anti-Vip3A IgG (100 ng) was added and incubated for 1 h. Further, wells were 4 times washed with PBST and 100 μl of HRP-conjugated anti-rabbit IgG was added into each well and incubate at 37 °C for another 1 h with continuous mild shaking. Again, wells were washed with PBST and 100 μl of TMB substrate was dispensed into each well and reaction allowed to proceed for 30 min at room temperature (~30 °C). During the time, a gradually increasing blue colour was developed. The reaction was stopped by adding 100 μl of 2 N sulphuric acid. Finally, the resultant yellow colour was read at 450 nm using a Micro-plate reader (BioRad, USA). The amount of substrate hydrolysed is proportional to the amount of antigen in the test solution. A standard graph was p[lo]ted for the calculation of transgenic Vip3A proteins.

3. Results

3.1. Expression and purification of Vip3A protein

Vip3A protein was expressed by 1 mM IPTG in[duc]tion. A SDS–PAGE run of the expressed protein v[eri]fied expected molecular weight of 88.5 kDa for Vip3A prot[ein] a[nd] fairly high expression (Fig. 1a). The Vip3A protein thus produc[ed] was purified using IDA-CL agarose column. This r[eco]mbinant Vip[3A] protein has a tail of six Histidine amino acid w[hi]ch was incorporate[d] during creation of recombinant clone by [the] 8 oligon[u]cleotides, responsible for six Histidine amino acids. T[he] His-ta[g] makes use of the fact that Nickel gets covalently bound thro[ugh] histidine r[esi]dues of Vip3A, holding the entire Vip3A [pro]tein, allow[in]g othe[r] proteins to get eluted out of column and l[at]er on, [Vip3A] gets elute[d] exclusively, on addition of Imidazole. A ba[tch] elution [w]ith imidazole gradient yielded pure Vip3A protein frac[tion]s as s[ho]wn in Fig. 1b and c. The protein elution started at 100 m[M] imidazole, but the highly purified Vip3A protein fractions eluted at [2]5 mM imidazole concentration and continued up to 425 mM.

3.2. Antibody development and purification

The purified Vip3A protein was used to raise the antibody. The raised antibodies against Vip3A were confirmed by double immunodiffusion assay. After a night long diffusion step, clear white precipitin lines (semi-circular) were observed in both mouse and rabbit samples, confirming the presence of Vip3A recognising antibodies in the sera samples (Fig. 2a). The pre-immune sera gave no signal, confirming the possible efficacy of the raised antibody. Subsequent to confirmation of antigen–antibody interaction, sera sample was purified and the immunoglobulins resolved on a 10% SDS–PAGE gel. Two bands were observed, one was that of 53 kDa

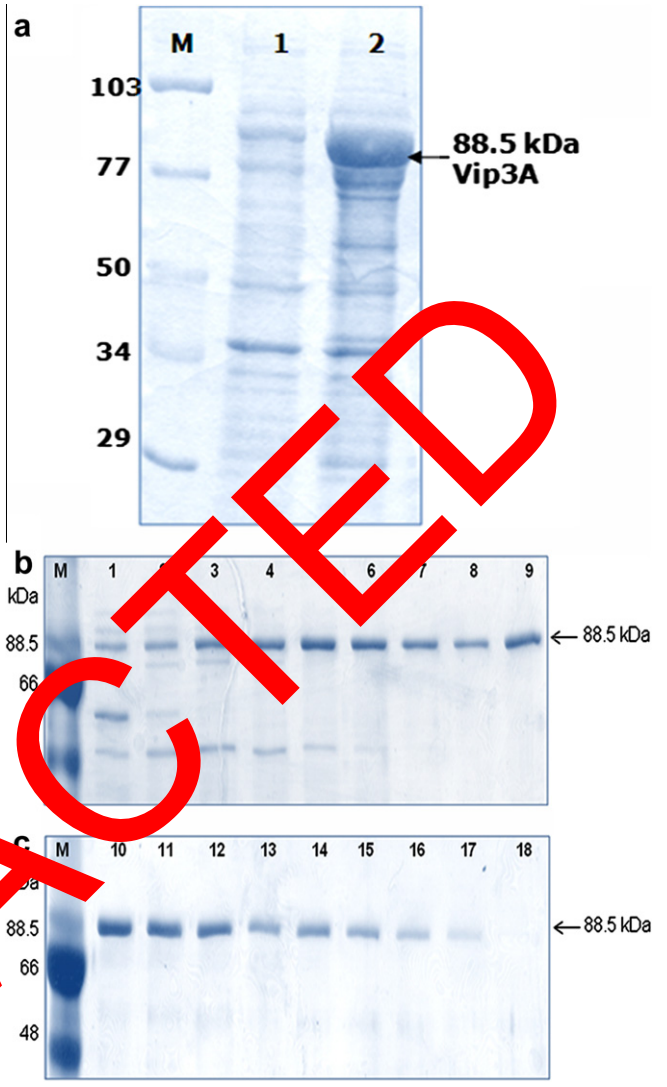

Fig. 1. (a) IPTG induction for Vip3A expression. Lane M, protein marker; lane 1, uninduced protein; lane 2, induced protein. (b) and (c) SDS–PAGE of Vip3A elutes: Vip3A protein purification using affinity column (IDA-Cl agarose). Lane M, marker; lanes 1–18, protein elution profile after batch elution with imidazole at 25 mM increments (25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425 and 450 mM, respectively).

of heavy chain and the other being 23 kDa light chain (Fig. 2b). The purified protein concentration of rabbit IgG–antibody was 2.84 mg/ml while that from mouse was 1.623 mg/ml.

3.3. Western blot analysis

Western blot was carried out to determine the titer value of purified Vip3A IgG. After Western blot analysis with different dilution of rabbit anti-Vip3A sera (1:5–100 k), a prominent band of 88.5 kDa was seen and the intensity was almost the same up to 100 k dilutions (Fig. 3a), indicating high titer response of the antibody. Similarly, another Western blot was also performed with leaf-extract of GM cotton samples to validate the raised antibody against GM samples. An 88.5 kDa band was clearly visible; however, there was no corresponding band in case of non-GM cotton leaf sample (Fig. 3b). The data from Western blot analyses of these fractions established that stable epitopes remain after the leaf has been subjected even to processing conditions typically employed by the food industry, thereby enabling development of an ELISA method.

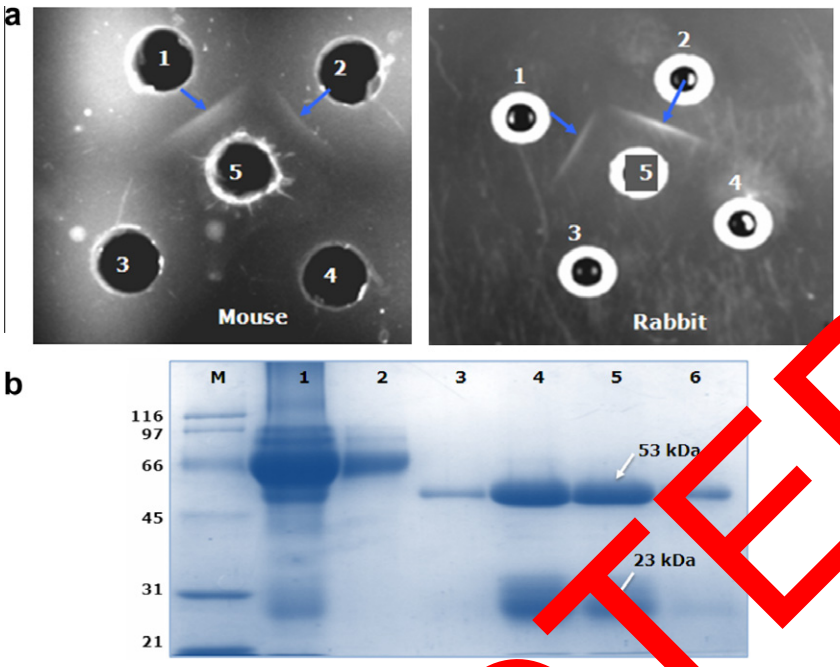

Fig. 2. (a) A double immunodiffusion assay for Vip3A antibody raised in mouse and rabbit. Wells 1 and 2, sera after immunization; wells 3 and 4, pre-immune sera; well 5, Vip3A antigen. (b) Purification of Vip3A-antisera. Lane M, marker; lane 1, serum; lane 2, flow through; lanes 3–6, eluted fractions (IgG).

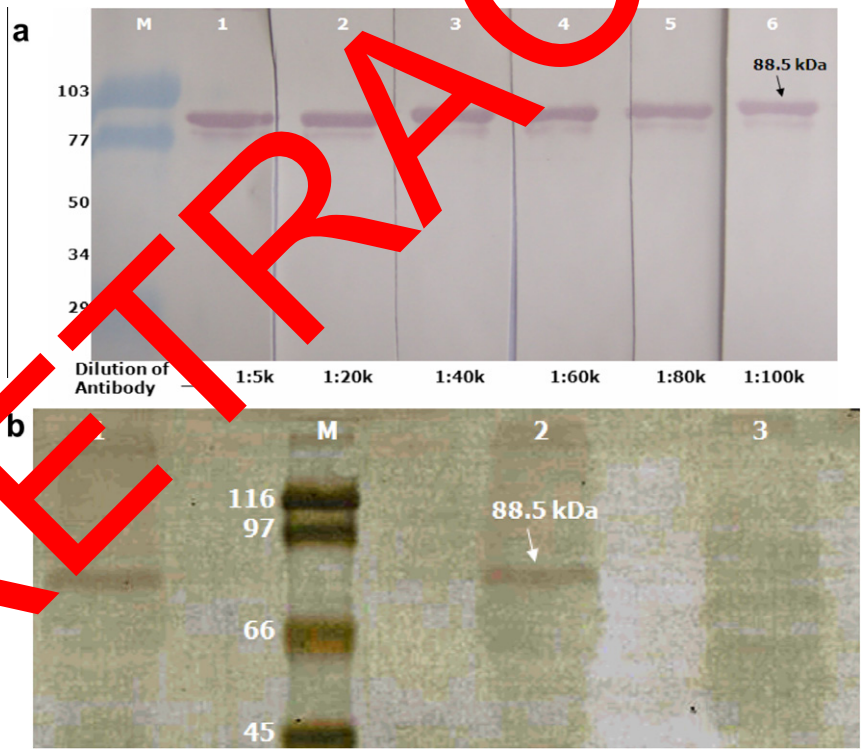

Fig. 3. (a) Determination of titer value of antibody by Western blotting. Lane M, marker; lanes 1–6, blots with sequentially increasing dilutions of antibody. (b) Detection and characterisation of transgenic Vip3A protein by Western blot analysis. Lane M, marker; lanes 1 and 2, Vip3A bearing GM cotton leaf; lane 3, non-GM cotton leaf.

3.4. Development of indirect sandwich ELISA for Vip3A protein

The optimum conditions and constituents for ELISA were determined through checker board analysis. Different ELISA formats were tried and finally indirect sandwich ELISA was chosen for further study. The developed ELISA for measurement of Vip3A was a triple antibody sandwich procedure utilising a polyclonal capture antibody (anti-Vip3A raised in mouse) and a polyclonal detection antibody (anti-Vip3A raised in rabbit) followed by use of a third HRP-conjugated antispecies antibody (goat anti-rabbit IgG). We judged the efficiency of the assay with different concentration of pure recombinant Vip3A protein. The typical curve for ELISA was obtained by plotting absorbance value (450 nm) against the concentration of Vip3A protein at log scale (Fig. 4a). To simplify the

calculations, a linear graph was generated using different concentration of pure Vip3A protein. The linearity of quantification was found in the range of 31–500 ng/ml of Vip3A protein concentration (Fig. 4b). A derived equation ($y = 0.001x + 0.041$) was obtained which could be used to calculate amount of an unknown Vip3A protein. The R^2 value was equal to 0.998 which was acceptable. The assay is not truly linear but in best fit form. In the present assay, a good test result could be obtained by considering of mean value of calculated through standard curve equation and point-to-point parameter of the graph. To force an inherently non-linear method to fit the best straight line may lead to inaccuracies in reporting value of the test samples. Those inaccuracies are most significant at the extremes of the standard curve, most often in the low end, but sometimes in the high e[illegible]ll.

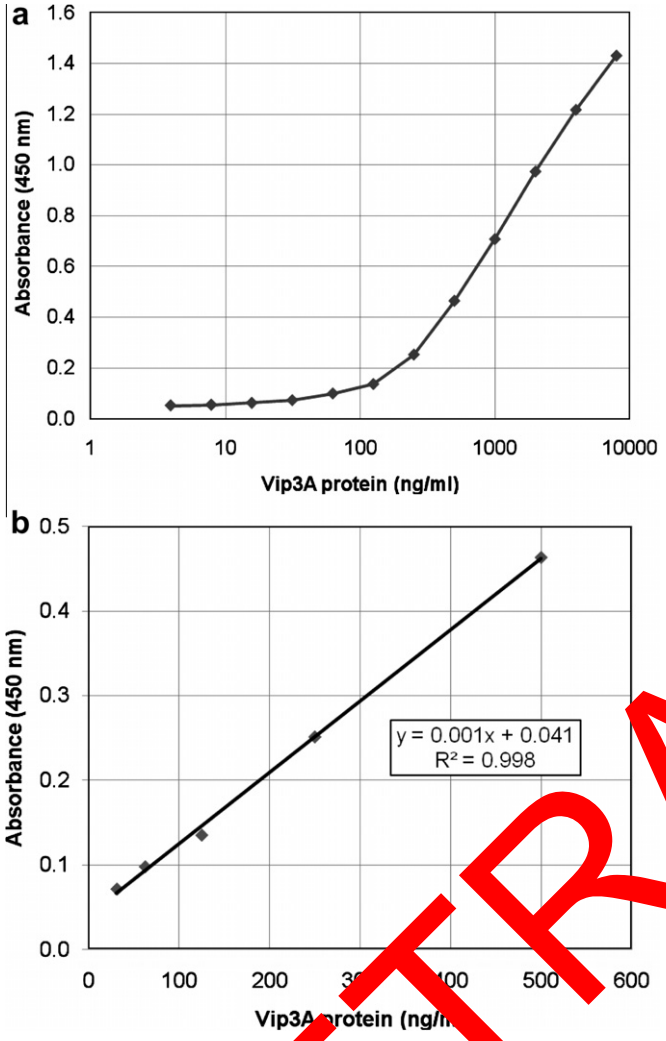

Fig. 4. (a) Indirect sandwich ELISA for Vip3A protein quantification (log scale). (b) Standard curve for Vip3A protein with indirect sandwich ELISA showing a linear relation between the optical density and Vip3A concentration.

3.5. Limit of detection (LOD) and limit of quantification (LOQ) of ELISA

The LOD was determined for the developed ELISA assay, i.e. the lowest quantity of a Vip3A antigen that can be distinguished from the absence of Vip3A (a blank value). To identify positive values from the blank value, standard deviation (SD) of blank value was multiplied by a factor of 3 and added to the blank value. The positive values are signals greater than blank value + 3 × SD of blank value. The results showed that minimum 16 ng/ml (16 ppb) concentration of the test sample could be detected as was evident from the colour developed in the ELISA plate. The LOQ of the developed assay was validated at 31 ng/ml (31 ppb) of Vip3A protein. The LOQ was determined by fortifying a population of negative GM brinjal samples at 31 ppb Vip3A. The mean recovery was 85% with a coefficient of variation of 6.4% [%CV = (standard deviation/mean) × 100].

3.6. Validation of the developed ELISA with GM leaf samples

The ELISA test was done with leaf extract of GM cotton, tobacco (gift from ICGEB, New Delhi, India) and brinjal (gift from Nirmal Seeds Pvt. Ltd., India) samples bearing Vip3A protein. Three different controls; environmental control (GM protein extraction buffer alone), negative control (respective non-GM sample) and positive control (Vip3A pure protein) were also used for accuracy of the test. A very clear colour development was visualised in all the test samples indicating marked expression of Vip3A protein in GM leaf samples. However, as expected varied Vip3A protein expression was evident from the colour developed in the wells (Fig. 5). The environmental and negative controls showed no colour development. To interpret the result quantitatively, the volume in ml of

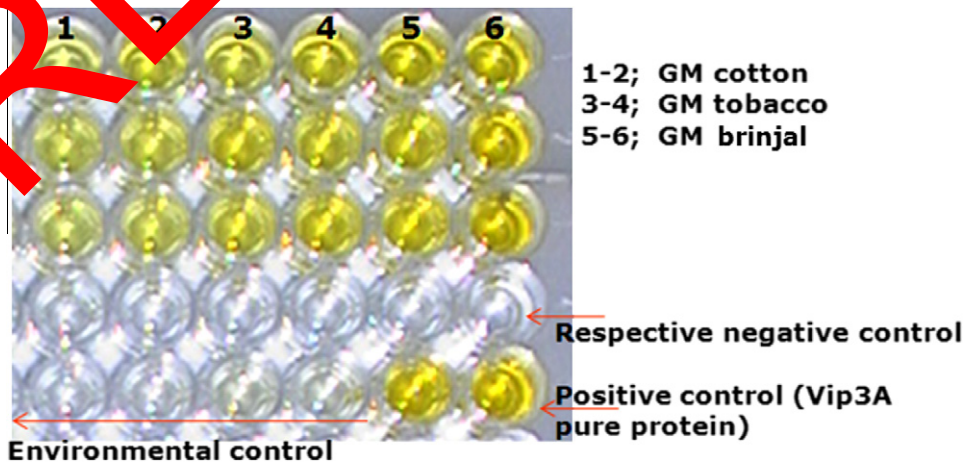

Fig. 5. Quantitative assay with GM cotton, tobacco and brinjal leaf samples. A typical plate showing colour after indirect sandwich ELISA. Lighter colour indicates lower concentration and the darker colour indicates higher concentration of Vip3A protein. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

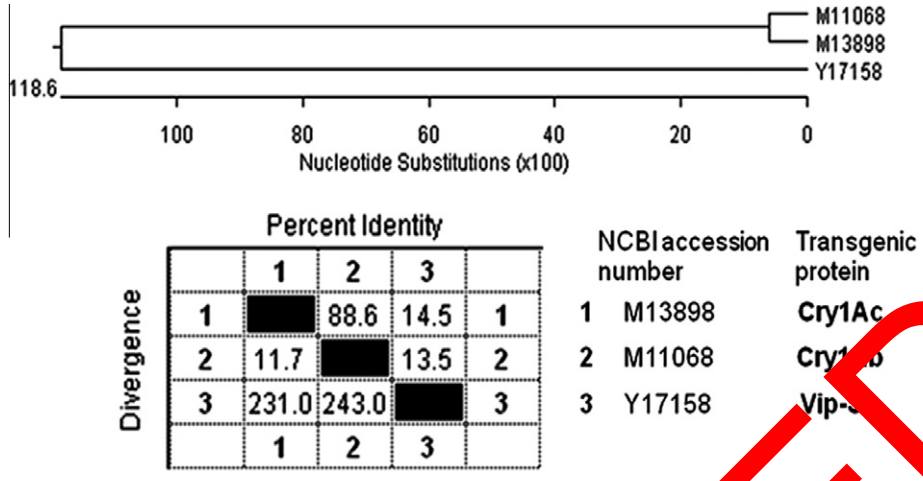

Fig. 6. Cross-reactivity of the developed assay. Identity and divergence analysis of Vip-S amino acids with Cry1Ac and Cry1Ab insecticidal proteins.

extraction buffer was divided by the weight of the leaf in gram to determine the dilution factor. The results from standard curve were multiplied by that dilution factor. The Vip3A protein was quantified after plate reading at 450 nm and found that expressed Vip3A protein in different GM samples was in the range of 20–50 μg/g of leaf tissue.

Further validation of the developed ELISA assay was done with second batch of GM brinjal samples. The amount of expressed Vip3A protein in GM brinjal samples was calculated using the standard curve. It was found that expressed Vip3A protein was in GM brinjal samples was in the range of 40–50 μg/g of leaf tissue.

3.7. Cross-reactivity of the developed assay

The cross-reactivity of the developed ELISA was checked with three available GM proteins (CP4-EPSPS, Cry1Ab and Cry1Ac). There was no colour development in any of the samples, implying no cross-reactivity of the developed ELISA assay (data not shown). In addition to this, amino acid sequence analysis was carried out with help of DNA-Star software to find out the possible cross-reaction of Vip3A with Cry1Ac and Cry1Ab insecticidal protein. It was found that the per cent identity of Vip3A amino acid with Cry1Ac and Cry1Ab was very feeble and divergence was very high (Fig. 6). Hence, this protein can not cross-reactive with Cry1Ac and Cry1Ab insecticidal protein. Regarding CP4-EPSPS, it is totally different non-insecticidal protein.

4. Discussion

As an essential part the process of development of insect resistant transgenic plants and also to evaluate the consistency in the expression of toxin under field conditions, immunological assays are commonly used. A rapid, selective, and sensitive method for monitoring protein levels in plant and related products is of significance for product quality control, environmental risk assessment, and other relevant studies. The ELISA assay being described in this report is for vegetative insecticidal protein (Vip3A) based GM plant products. Insecticidal gene *vip3A* is now being the part of GM crops (cotton and maize) developed by Syngenta (CERA, 2010) and GM crops (cotton, tobacco and brinjal) developed by ICGEB, New Delhi.

The antibody-based immunoassay has been widely used to detect transgenic proteins in a variety of applications including testing in the breeding process, testing for unapproved events, and determining GM content ensuring compliance with non-GM labelling requirements. There are several immunoassays commercially available for detecting different GM proteins like Cry1Ab, Cry1Ac, Cry2Ab, Cry1Ia5, CP4-EPSPS in ELISA plate format. However, till date, no commercialised immunoassay for Vip3A protein detection in GM crops/produce is reported. Hence, the need for an easy and reliable detection for Vip3A based GM plants was very urgent and crucial. This study involved the development of immunological based detection methods to manage labelling of Vip3A based GM products.

Before the final assemblage of ELISA system, raised antibodies were purified and then tested with Vip3A pure protein as well as with GM protein extracted from leaves. In the present study, a combination of two polyclonal antibodies, raised in mouse and rabbit, was selected for the betterment of indirect sandwich ELISA so that it can recognise different epitope. The raised polyclonal rabbit anti-Vip3A antibody was of very high titer, as it works even at 1:100,000 dilutions. Three major factors were considered for the development of ELISA system; these are the optimal dilutions of antigen, antibody and conjugate. The optimal concentrations of these constituents were determined through titration analysis.

The usefulness of an ELISA system is its sensitivity and specificity of detection, which allows this system to be applied to complex biological material without the need for purification. The sensitivity and specificity of sandwich ELISA is dependent on the number of molecules of the first antibody that is bound to the solid phase, avidity of the first antibody for the antigen, avidity of the second antibody for the antigen, specific activity of the second antibody (Millipore, USA). The amount of the capture antibody that is bound to the solid phase can be adjusted easily by dilution or concentration of the antibody solution. The avidity of the antibodies for the antigen can only be altered by substitution with other antibodies. The specific activity of the second antibody is determined by the number and type of labelled moieties that it contains.

However, it should be noted that the antibody–antigen reaction that triggers the detection signal may also occur with partially degraded Vip3A protein if the epitopic structure to which the antibodies respond is still preserved. In the indirect sandwich ELISA, the immunological activity of Vip3A was reduced by the extraction method required to solubilise Vip3A protein from GM products. For GMO detection by ELISA it is very difficult to properly solubilise a desired protein without any inhibitors (Terry, Harris, & Parkes, 2002). So, a common solubilisation buffer for Vip3A from cotton, tobacco and brinjal was developed which improved the sensitivity of the assay. The extraction buffer should not solubilise the phenolics or any other compound which may act as inhibitors for antigen–antibody interaction.

After the development of the indirect sandwich ELISA, its cross-reactivity was checked with different GM and non-GM samples and no cross-reactions were seen. Now, the developed assay was sufficient enough in terms of sensitivity and specificity. The indirect sandwich ELISA test for detection of Vip3A protein displayed a sensitivity of 16 ng/ml and almost linear response up to 500 ng/ml. The working range and linearity of the curve was determined through best fit strategies. An easy way to determine the optimal curve fit routine is by "backfitting" the signals of standards as unknowns. If the standards when backfit as unknowns do not give back their nominal values it may have artifacts introduced by inappropriate assumptions or restrictions in the curve fit algorithm. Lower level of the standard curve is LOD and if the test value is above the upper range, there will be need for sample dilutions.

The need for detection of the Vip3A in brinjal, cotton, maize and tobacco can be met by use of the developed ELISA tests. However, sample extraction steps may be necessary to minimise matrix effects. The sandwich immunoassay is absolutely a simple format, less time-consuming and requires very limited 1 day training for running the assay. The high degree of reproducibility shown by low coefficient of variation (CV = 6.4%) value confirms that this format is a reproducible, robust and accurate test format for detection for GM samples containing Vip3A protein (Zel, Gruden, Cankar, Stebih, & Blejec, 2007).

The results generated in the developed assay are in ng/ml of Vip3A protein/g of sample which can be easily converted into μg/g of leaf sample. The guidelines for acceptable GMO contamination in a sample have been set in terms of %GMO in a sample by weight. The two sets of measurement units are quite different. Attempts to equate ng or ppb with %GMO contamination are complicated the fact that the expression of same gene in different GM plants could vary greatly. However, for each GM plants (cotton, tobacco brinjal and maize) bearing Vip3A protein, an attempts could be tried in case of availability of certified reference material (CRM). Right now, no CRM is available for Vip3A protein.

Our test kits are comparable with the existing commercial kits available for different GM proteins. The sensitivity of the developed assay is almost equal to reported ELISA kits for transgenic protein (Fantozzi et al., 2007; Wang et al., 2007) or commercially available ELISA test kits (Agdia; Biogenetic Services; DesiGen; EnviroLogix; Neogen; Romer Labs; Strategic Diagnostics Inc.). The ELISA based tests developed during this study for Vip3A bearing GM plants and product thereof would surely be of immense use to all regional food laboratories, research centers, crop breeding centers, farmers and exporters enabling GM labelling and governmental regulatory compliance. The assay is sensitive, quick and reproducible can be utilise in regulation of GMOs.

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