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A Quantitative Immunopolymerase Chain Reaction Method for Detection of Vegetative Insecticidal Protein in Genetically Modified Crops

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ABSTRACT: Vegetative insecticidal protein (Vip) is being employed for transgenic expression in selected crops such as cotton, brinjal, and corn. For regulatory compliance, there is a need for a sensitive and reliable detection method, which can distinguish between approved and nonapproved genetically modified (GM) events and quantify GM contents as well. A quantitative immunopolymerase chain reaction (IPCR) method has been developed for the detection and quantification of Vip protein in GM crops. The developed assay displayed a detection limit of 1 ng/mL (1 ppb) and linear quantification range between 10 and 1000 ng/mL of Vip-S protein. The sensitivity of the assay was found to be 10 times higher than an analogous enzyme-linked immunosorbent assay for Vip-S protein. The results suggest that IPCR has the potential to become a standard method to quantify GM proteins.

KEYWORDS: Vegetative insecticidal protein, immuno-PCR, genetically modified crops, polyclonal antibody

INTRODUCTION

Because insect pests cause tremendous loss in the yield of several crops,[1] the crops are continuously being genetically modified (GM) by incorporating insect resistant traits. *Bacillus thuringiensis* (*Bt*) is an important source of insecticidal proteins, which has been used commercially as a biopesticide to control insect pests for the past several decades.[2] Several *Bt* insecticidal genes have been extensively used for the development of insect-resistant GM crops. Vegetative insecticidal proteins (Vip) are a novel group of exotoxins produced by *Bt* during the vegetative growth phase from the cell into growth medium.[3] These proteins are grouped into three families including Vip1, Vip2, and Vip3 (subgroup: Vip3A and Vip3B),[4] where Vip3A has been found to have insecticidal activity against several lepidopteran pests[5] and employed for transgenic expression in crops. The mechanism of action of Vip3A is quite different from other known *Bt* toxins, and the insecticidal activity has been found to be ∼260-fold higher than δ endotoxins (Cry1A) against *Agrotis ipsilon*.[3] These characteristic features of Vip3A protein signify its importance and suitability for being a part of GM crops conferring insect resistance.

Syngenta Seeds Inc. has developed GM cotton events (COT102, COT202, and VipCot) and GM corn events (MIR162, BT11 × MIR162, BT11 × MIR162 × MIR604) having synthetic *vip3A* transgene. Recently, some of these varieties have been commercialized for the cultivation and usage in several countries.[6] Some of these transgenic varieties having *vip-S* (*vip3Aa9*) transgene are also developed by private companies in India, which are under field trials and awaiting an approval to launch in the market. The regulatory need to monitor and verify the presence of GM materials in the products has increased with the expansion of the cultivation of GM crops. The labeling legislation and trade requirements differ from one country to another, leading to the necessity for the development of reliable and sensitive methods for the detection of GM varieties.

Several protein-based detection methods have been employed for screening GM materials, for example, enzyme-linked immunosorbent assay (ELISA), dipstick, immunopolymerase chain reaction (IPCR), immunoblotting, and insect bioassay. The IPCR method combines the advantages of specificity of immunoassay and sensitivity of PCR. However, IPCR can detect only products of gene expression, and it relies on the affinity of reporter DNA-labeled antibody to bind to specific antigens. Furthermore, the bound reporter DNA can be readily detected by end point or real-time PCR. Taking advantage of its high sensitivity, the IPCR assay is extensively being used in healthcare, medicine, and immunological diagnostics. Previously, Allen et al.[7] have developed an IPCR method for the detection of *Bt* Cry1Ac protein; however, the IPCR assay for the detection of Vip protein has not been reported so far.

Keeping in view the expanding cultivation of Vip bearing GM crops in the future, there is a need to develop a highly sensitive detection method to detect Vip protein for regulatory monitoring. The goal of the present work is to develop a quantitative IPCR assay for measuring expression of the levels of Vip proteins in different GM samples. This assay can also be applied to detect any unapproved event in GM foods, thereby preventing its entry into the food chain.

MATERIALS AND METHODS

Chemicals and Reagents. Goat antirabbit IgG, goat antimouse IgG, DAB (3-3′-diaminobenzidine tetrahydrochloride), tetramethylbenzidine (TMB), and Ni-nitrilotriacetic acid (NTA) CL column were purchased from Bangalore Genei (India). Sulfo-succinimidyl-6-biotinamido-hexanoate (sulfo-BNHS), complete and incomplete Freund's adjuvant, ethylenediaminetetraacetate (EDTA), dithiothreitol (DTT), bovine serum albumin (BSA), polyvinylpyrrolidone (PVP-40), protease inhibitor cocktail, isopropyl-β-D-thiogalacto-pyranoside (IPTG), and streptavidin were obtained from Sigma (United States). The Bradford

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reagent was purchased from Bio-Rad (United States). QIAquick PCR purification kit and QuantiTect SYBR Green PCR kit were purchased from Qiagen (Hilden, Germany). All other chemicals and organic solvents were of analytical grade.

Vip-S Protein Expression and Purification. The recombinant Vip-S protein (a type of Vip3A) was expressed in *Escherichia coli* M15 using recombinant Vip-S expression vector pQE30. The expressed proteins (789 amino acids) carried a 6xHis tag at the N terminus, facilitating their purification by Ni-NTA affinity chromatography. The protein was purified using the protocol mentioned in Kumar et al.[8] The protein concentration was determined by Bio-Rad protein assay dye. After purification, protein was stored at 4 °C. The purity of the protein was verified on 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and staining the gel in Coomassie Brilliant Blue R-250.

Raising Polyclonal Antibodies. Two male New Zealand white rabbits (∼1.5 kg) and three Balb/c female mice (∼25 g), procured from the animal facility of Indian Institute of Toxicology Research, were immunized with purified Vip-S protein. The protein was emulsified in complete Freund's adjuvant before injecting into the animals. One immunization dose for rabbit contained about 100 μg of the protein, while one immunization dose for mouse contained 50 μg of the protein. The protein was injected intramuscularly in rabbit, while it was injected intraperitoneally in mouse. After 28 days, the first booster dose was given having the same amount of protein emulsified in Freund's incomplete adjuvant. The subsequent booster doses were given after a 15 day interval, and the last fifth booster dose was given after 1 month. The animals were bled, drawing blood from ear vein (rabbit), or by puncturing orbital plexus (mouse), 10 days later, and the sera were collected. The titer of the sera was determined by dot blot, and high titer sera were selected for further purification. The antiserum (IgG) was purified by HiTrap Protein-A column (GE Healthcare, United States). The concentration of purified antibody was measured at 280 nm by NanoDrop spectrophotometer (NanoDrop Technologies, United States) and stored in aliquots at −20 °C for further use. The specificity of antibody was determined using Western blot.

Western Blot Analysis. Western blot analysis was carried out using purified Vip-S protein to validate and determine the specificity of the raised antibodies. Ten microliters of each elute of recombinant Vip-S protein (100, 150, 200, 250, and 300 mM) was separated on 10% SDS-PAGE and transferred onto nitrocellulose membrane (0.45 μm pore size). Three SDS-PAGE gels were run, and the protein was transferred onto nitrocellulose membranes. Furthermore, the membranes were incubated in blocking buffer (5% fat free milk) to block open binding sites on membrane. After they were washed three times with PBST (10 mM phosphate-buffered saline, pH 7.4, containing 0.05% Tween-20), membranes were incubated for 2 h each with horseradish peroxidase (HRP)-conjugated anti-His antibody (1:1000 dilution), rabbit anti-Vip-S sera (1:10,000 dilution), and mouse anti-Vip-S sera (1:10000 dilution), respectively. Furthermore, the last two membranes were washed and incubated with secondary antibodies (HRP-conjugated goat antirabbit IgG and goat antimouse IgG, respectively) for 1 h. Membranes were washed, and the protein−antibody complex was visualized by the addition of blot developer DAB solution.

Preparation of Biotinylated Antibody. The antibody was conjugated with biotin using sulfo-BNHS. Freshly prepared sulfo-BNHS (50 μg) was mixed with 500 μg of mouse anti-Vip-S antibody in 1 mL of 100 mM sodium phosphate buffer (80.3 mM Na_2HPO_4, 19.7 mM NaH_2PO_4, pH 7.4). The tube was wrapped in aluminum foil to prevent the reaction from light, incubated, and rotated at 50 rpm at room temperature for 2 h. The unconjugated biotin was removed by using Centrisep spin column (Princeton Separations). The concentration of purified biotinylated antibody was measured by NanoDrop spectrophotometer and stored in dark at 4 °C.

Preparation of Biotinylated Reporter DNA. The reporter DNA (296 bp sequences), used in this assay, was previously reported by Zhang et al.[9] The biotinylated reporter DNA was prepared by PCR using plasmid pUC19 (New England Biolabs, United Kingdom) as a template DNA, forward biotin linked primer (5′ biotin-CCC GGA TCC CAG CAA TAA ACC AGC CAG CC-3′) and unmodified reverse primer (5′- GCC AAC TTA CTT CTG ACA AC-3′). Each 25 μL of PCR reaction mixture contained 1× *Taq* Buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.4 μM forward primer, 0.4 μM reverse primer, 0.7 U *Taq* polymerase (Fermentas, United States), and about 25 ng of template plasmid DNA. PCR amplification was performed in an epigradient Mastercycler (Eppendorf, Germany). The program followed initial denaturation at 94 °C for 60 s, denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 70 °C for 45 s, for 35 cycles. The final extension was carried out at 70 °C for 5 min. The PCR product was analyzed by 1.5% agarose gel electrophoresis following ethidium bromide staining. The 296 bp amplified product, thus obtained, was purified using QIAquick PCR purification kit and quantified by NanoDrop spectrophotometer.

Preparation of Leaf Extract of GM and Non-GM Samples. About 100 mg of non-GM and GM brinjal leaf samples was crushed in 500 μL of protein extraction buffer (100 mM sodium phosphate buffer, pH 7.0, 2 mM DTT, 5 mM EDTA, 0.1% Triton X-100, 2% PVP-40, and protease inhibitor cocktail) separately. This crude preparation of protein was incubated for 30 min at room temperature with gentle shaking. Next, the samples were centrifuged at 10000 rpm at 4 °C for 10 min. The supernatant of crude preparation was used for quantification of Vip-S protein in the IPCR assay. The leaf extract of non-GM brinjal was used for preparation of spiked samples.

Assay Design for IPCR Protocol. The IPCR assay was done in eight-well Nunc TopYield strips (Nunc, United States) in direct sandwich format. The steps of IPCR assay was followed as described by Niemeyer et al.[10] The entire IPCR assay was divided into four parts: microplates coating with antibody, protein immobilization, assembly of signal-generating immunocomplex, and real-time PCR signal detection. All samples were run in triplicates.

Surface Coating of Antibody. Rabbit anti-Vip-S antibody was diluted with coating buffer (50 mM sodium−carbonate buffer, 15 mM Na_2CO_3, and 35 mM $NaHCO_3$, pH 9.6) to 3 μg/mL, and 30 μL of the diluted antibody was added to each well of strips. The strips were sealed and incubated overnight at 4 °C to adsorb onto the walls. The wells were washed three times for 1 min with 240 μL of 1× TBS buffer (Tris-buffered saline: 20 mM Tris-Cl, 150 mM NaCl, pH 7.5) with shaking. The surface was blocked for remaining sites with 240 μL of blocking buffer (1× TBS, 1% BSA, and 0.05% Tween-20) for 12 h at 4 °C. The blocked wells were washed three times for 1 min with 240 μL of TETBS per well (1× TBS, 5 mM EDTA, and 0.05% Tween-20) at room temperature with shaking.

Protein Immobilization. Ten-fold serial dilutions of the Vip-S pure protein (1 μg/mL to 10 pg/mL) were prepared in 1× TBS buffer, and 30 μL of each dilution was added to each well of strips. For sample analysis, 30 μL of GM and non-GM brinjal extracts was added in its respective wells. The Vip-S spiked samples (10 and 1000 ng/mL) were also included in the assay to observe the matrix interference. In no antigen (NA) control, 30 μL of TBS buffer was added. The strips were then incubated for 45 min at room temperature with constant shaking and washed twice for 30 s and twice for 2 min with 240 μL of TETBS per well.

Assembly of Signal-Generating Immunocomplex. The biotinylated detection antibody (mouse anti-Vip-S) was diluted to a concentration of 1 μg/mL in TETBS. Thirty microliters of the dilution was added to each well of the strips. The strips were incubated for 45 min at room temperature with constant shaking and washed twice for 30 s first and twice for 2 min again with 240 μL of TETBS per well. A stock solution of

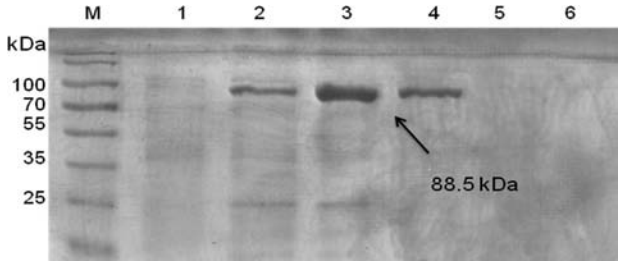

Figure 1. SDS-PAGE of Vip-S elutes: Vip-S protein purification using affinity column (NTA). Lane M, marker; lanes 1−6, protein elution profile after batch elution with imidazole at 50 mM increments (100, 150, 200, 250, 300, and 350 mM, respectively).

streptavidin was diluted to a concentration of 25 nM in TETBS. Thirty microliters of streptavidin was added to each well and incubated for 45 min at room temperature with constant shaking at 100 rpm, and the strips were washed with TETBS as described earlier. The biotinylated reporter DNA was diluted to a concentration of 50 pM in TETBS, and 30 μL of the dilution was added to each well of the strips. The strips were then incubated for 45 min at room temperature with constant shaking and washed seven times with 240 μL of TETBS per well (four times for 30 s and threetimes for 4 min each). Strips were finally washed two times with 240 μL of Milli-Q water. Furthermore, real-time PCR was performed in the same TopYield strips.

Signal Detection by Real-Time PCR. The real-time PCR was performed using a QuantiTect SYBR Green PCR kit on a thermocycler (iCycler iQ system, Bio-Rad). The assay was carried out with varying dilutions of Vip-S protein along with spiked samples. Three controls were also included in the assay; non-GM control and NA control both included all of the steps except addition of protein, and no template control (NTC) contained the real-time PCR master mix only. The PCR master mix (20 μL/well) contained 1× SYBR Green master mixture, 0.4 μM forward primer (5′-CAG CAA TAA ACC AGC CAG CC-3′), 0.4 μM reverse primer (5′-GCC AAC TTA CTT CTG ACA AC-3′), and sterile distilled water. The strips were sealed with optical sealing cover foil (Applied Biosystems). The reporter DNA was amplified by using initial denaturation at 95 °C for 15 min followed by 30 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s, and primer extension at 72 °C for 30 s. The emitted fluorescence by SYBR Green was recorded during amplification (287 bp). After automatic baseline correction, the threshold cycle (Ct) values were determined by setting a threshold line in the exponential phase of the amplification curves, reading out the fractional cycle number at which the amplification curve crosses the threshold line. The average Ct value of each sample was calculated. The amplified reporter DNA and primer−dimer product were distinguished by their respective Melting Curves.

Sandwich ELISA for Vip-S. Sandwich ELISA was performed using same antibodies as used in the real-time IPCR assay. The ELISA was performed in Nunc TopYield IPCR strips. It comprised a direct sandwich assay, and the detection antibody was linked via streptavidin to enzyme HRP. A 10-fold dilution series was prepared ranging from 0.001 to 10000 ng/mL of the Vip-S protein. The steps of antibody coating, antigen immobilization, and addition of biotinylated antibody were the same as for IPCR assay. Thereafter, 30 μL of diluted streptavidin−HRP conjugate was added to each well and incubated at room temperature for 1 h with shaking. Subsequently, 100 μL of TMB was added and incubated for 30 min. The blue color was developed gradually, and the reaction was stopped by adding 100 μL of 2 M sulfuric acid to each well, and the acid stops reagent caused the color change to yellow. The absorbance was measured at 450 nm using a microtiter plate reader (BD Biosciences, United Kingdom).

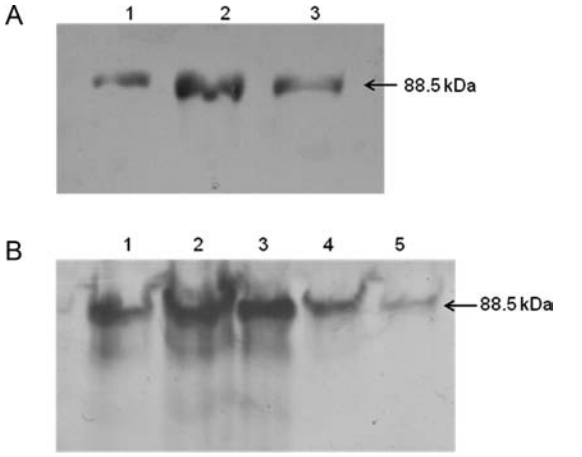

Figure 2. (A) Western blot analysis using anti-His-tag antibody: lanes 1−3 showing Vip-S protein fraction eluted with 150, 200, and 250 mM, respectively. (B) Western blot analysis using mouse anti-Vip-S antibody: lanes 1−6 showing Vip-S protein fraction eluted with 100, 150, 200, 250, and 300 mM, respectively.

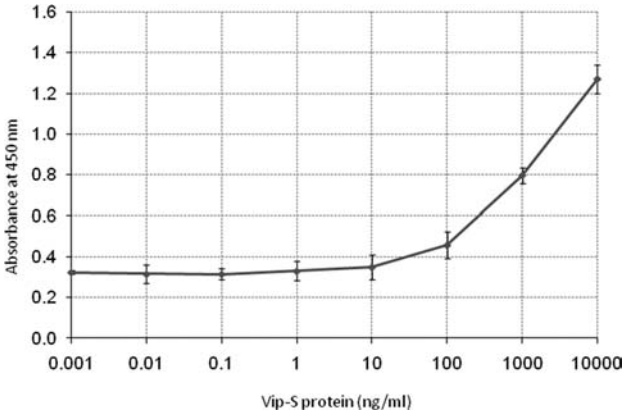

Figure 3. Direct sandwich ELISA for Vip-S protein quantification (dilutions of Vip-S protein made ranging from 0.001 to 10000 ng/mL). The detection limit was determined as 10 ng/mL. The average blank value was 0.27 ± 0.02.

RESULTS AND DISCUSSION

Identification of Recombinant Protein and Polyclonal Antibody Development. Following IPTG induction in the culture propagation, a significant amount of Vip-S protein was expressed. Because the expressed Vip-S protein was His-tagged, it was purified by Ni-NTA affinity column using a range of 150−250 mM imidazole as eluent (Figure 1). These elutes were resolved on SDS-PAGE, and a marked 88.5 kDa band was visualized on the gel, which confirmed the presence of Vip-S protein in elutes. The presence of 6xHis sequences tagged to the protein of interest in elutes was further confirmed by Western blot using anti-His IgG (Figure 2A). Furthermore, the titer value of raised polyclonal antibody was tested by dot blot, and it was observed that the antibody can detect at 1:100000 sera dilution. Finally, the specificity of raised antibody was confirmed by Western blot using mouse anti-Vip-S IgG (Figure 2B) and rabbit anti-Vip-S IgG (data not shown).

Sandwich ELISA for Vip-S. A series of 10-fold dilution of Vip-S protein, ranging from 0.001 to 10000 ng/mL, was used to generate

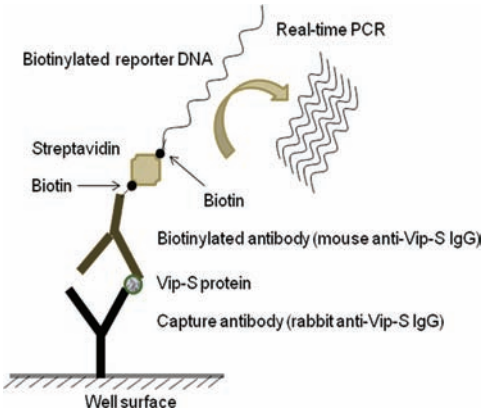

Figure 4. Schematic of sandwich IPCR: captured antibody (rabbit anti-Vip-S) and detection antibody (mouse anti-Vip-S) with sandwiched Vip-S protein. The reporter DNA was attached with mouse anti-Vip-S antibody via streptavidin. The bound reporter DNA was amplified by real-time PCR using SYBR Green dye.

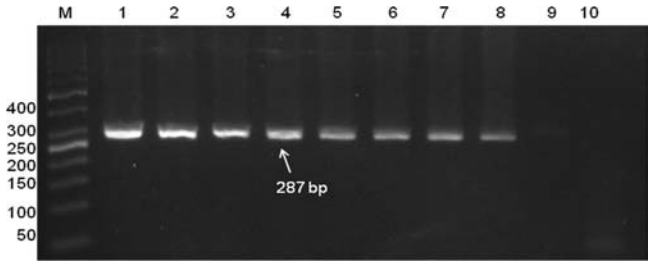

Figure 5. Conventional IPCR for Vip-S. Lane M, marker; lanes 1−7, showing 10-fold serially diluted Vip-S protein ranging from 1000 to 0.001 ng/mL; lanes 8, no antigen control (NA); and lanes 9 and 10, no template control (NTC).

a standard ELISA curve. Furthermore, a standard curve was obtained by plotting the absorbance at 450 nm against the protein concentration (Figure 3). The limit of detection of the assay was calculated as the value greater than an average of blank values ± three standard deviations of the blank control values (0.27 ± 3 × 0.02). The limit of detection for ELISA was established at 10 ng/mL or 300 pg of Vip-S protein. The linear quantification range was approximately in between 100 and 10000 ng/mL.

Quantitative Analysis of Vip-S by IPCR. A schematic of sandwich IPCR assay is shown in Figure 4. The IPCR assay was similar to sandwich ELISA except that the detection antibody (mouse anti-Vip-S IgG) that was linked to a reporter DNA (296 bp) instead of a conventional enzyme. The assay included a combination of two polyclonal antibodies recognizing different epitopes of Vip protein. The first antibody, raised in mouse, was linked to a reporter DNA molecule via streptavidin linker. The second, raised in rabbit, was used as a coating antibody. Initially, IPCR assay was optimized by conventional PCR to ascertain optimal amounts of reporter DNA, antibody, and streptavidin used in the assay. A gradual decrease in band intensity of PCR amplicon (287 bp) was observed on agarose gel, which represented the serial dilution of Vip-S protein (1000 to 0.001 ng/mL) in the assay (Figure 5). However, in negative control, a very faint band was also observed. It could possibly be due to the nonspecific binding of reagents. To minimize the effect of nonspecific binding in the assay, real-time PCR was opted. The quantitative IPCR

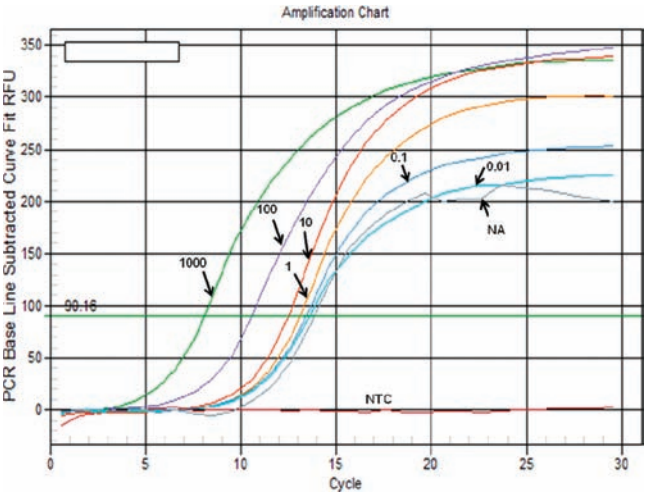

Figure 6. Amplification curves of the dilution series of Vip-S protein ranging from 0.01 to 1000 ng/mL. Two negative controls (NA and NTC) were also run in parallel.

assay was repeated five times, and the results were found to be the same.

Because PCR signal amplification is extremely sensitive, the presence of even a very low amounts of nonspecifically bound reporter DNA molecules can lead to a strong background signal. The real-time PCR method permits the quantification of specific to nonspecific signal ratio, thereby discarding other background signals. The detection of background signal in negative control could be because of nonspecific adsorption of assay reagents (e.g., detection antibody, streptavidin, and biotinylated DNA-label) to the vessel surface. The background detection also limits the sensitivity of IPCR.[11]

Real-time PCR was performed directly onto the washed TopYield strips using PCR master mix. Although, the reporter DNA has a *Bam*HI restriction site, which can detach the DNA from the protein−antibody complex. The detached DNA thus can be transferred to new tubes to minimize the background signals. The bound reporter DNA was successfully amplified without any interference.

After real-time PCR, the amplification curves were obtained (Figure 6), and the mean Ct value of each sample was calculated. To identify and discriminate positive signals from background signals originating in NA control, the following steps were taken. Typically, a potentially positive signal is defined as the concentration giving a difference of 3 × standard deviation (SD) for Ct values of NA lower than that given for NA (NA ± 3 × SD). Furthermore, the detection limit of IPCR assay was defined as the concentration giving a difference of 0.53 Ct (3 × SD) lower than that given for the NA control (mean Ct value 13.56); the cut off thus was 13.03 cycles. The quantitative IPCR displayed the minimum detection of 1 ng/mL (30 pg or 1 ppb) of Vip-S protein. This result showed that IPCR assay was 10-fold more sensitive than an analogous ELISA. The developed IPCR assay was also 100 times more sensitive than dipstick test developed recently by Kumar et al.[8] For the quantification of protein in unknown samples, the mean Ct values were plotted against the concentrations of the Vip-S pure protein, which showed a linear quantification of protein to a certain range (Figure 7). The linear range of quantification was 10−1000 ng/mL, which displayed a regression coefficient of 0.99.

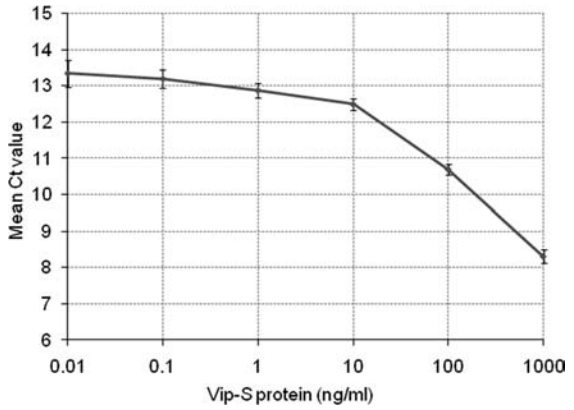

Figure 7. Semilogarithmic plot of different dilutions of Vip-S protein and mean Ct values. The linear quantification range of Vip-S protein was 10−1000 ng/mL. The mean Ct value of NA was 13.56 ± 0.18. The detection limit was determined as 1 ng/mL of Vip-S protein.

The developed IPCR assay has detection limit in ng/mL range, whereas the sensitivity of IPCR assay was also reported even up to fg/mL range.[12] The developed assay will have a novel application in the testing of Vip based GM produce. Furthermore, the detection limit of developed IPCR assay can be increased by using reporter DNA linked to the antibody through chemical conjugation,[11] which in turn decreases the nonspecific binding; thus, the sensitivity of detection is increased. Furthermore, a monoclonal antibody against Vip-S was also tested, but the observed sensitivity of the detection was very low. An IPCR method has been developed for *Bt*-Cry1Ac protein with detection limit 21.6 ng or 216 ng/mL.[7] However, the developed assay in this paper has the potential to detect up to pg levels of Vip-S transgenic protein, indicating high sensitivity in comparison to previously developed IPCR for Cry1Ac. Also, in this study, a combination of two polyclonal antibodies, raised in rabbit and mouse, were used, which possibly recognized different epitopes in the Vip-S protein, resulting in more specific and sensitive detection limit. Furthermore, the sensitivity of the test could be improved with the use of affinity purified polyclonal and monoclonal antibodies on Vip-S protein linked column with a higher specific activity.

Cross-Reactivity of IPCR Assay. The cross-reactivity and specificity of the IPCR test developed for Vip-S was tested with available nontarget *Bt*-insecticidal *δ*-endotoxins (Cry1Ac and Cry1Ab). The assay showed negligible cross-reactivity with Cry1Ac/Cry1Ab proteins (0.1−0.01%) (Table 1). Vip insecticidal proteins has no structural homology with *δ*-endotoxins,[3] indicating no possibility of cross-reactivity in the assay. On the basis of the data, it is anticipated that the developed IPCR assay could be highly specific for Vip type proteins.

Validation of IPCR Assay with GM Samples and Spiked Samples. To assess the validity of the proposed method, the test was performed with GM brinjal samples bearing Vip-S protein (provided by Nirmal Seeds Pvt. Ltd.) along with two Vip-S spiked samples. The mean Ct value for each sample was calculated (Table 1). The spiked Vip-S protein in non-GM brinjal leaf extract was considered an unknown sample for calculating assay recovery. The assay recoveries were observed approximately 80 and 110%. The values were within ±25% accepted range of theoretical value[13] and thus considered it acceptable. The amounts of expressed Vip protein in the three

Table 1. Data Showing the Quantitative IPCR of GM Brinjal Samples Bearing Vip Protein, Vip-S Spiked Samples, and Cry Proteins

sample	mean Ct value ± SD	result
Vip-S spiked sample (1000 ng/mL)	10.74 ± 0.07	80% recovery
Vip-S spiked sample (10 ng/mL)	14.33 ± 0.43	110% recovery
GM brinjal 1	14.17 ± 0.09	75 ppb protein
GM brinjal 2	14.16 ± 0.15	75 ppb protein
GM brinjal 3	14.13 ± 0.09	75 ppb protein
non-GM brinjal	15.55 ± 0.60	
Cry1Ab (100 ng/mL)	13.25 ± 0.45	∼ 0.1% cross-reactivity
Cry1Ac (100 ng/mL)	13.35 ± 0.05	∼ 0.01% cross-reactivity

batches of GM brinjal samples were checked with the developed assay and found ∼75 ng/g of leaf in all samples. The results indicate that the developed assay has the potential to detect the transgenic protein in the GM samples successfully.

Till very recently, several varieties of GM cotton and GM corn bearing Vip3A protein have been developed by Syngenta Seeds Inc., but there are no detection kits (PCR/protein based) available in the market so far. However, Singh et al.[14] have developed a PCR-based detection methodology for *vip3A*-type gene in the GM crops/produce. Syngenta has also submitted validated methods for extraction and direct ELISA analysis of Vip3A in cotton seed to the U.S. Environmental Protection Agency (EPA) and found to be acceptable by the U.S. EPA.[15] Previously, we have successfully developed and reported a protein-based immunochromatographic strip assay for the detection of Vip3A type protein in GM crops, which detects up to 100 ng/mL of protein.[8] However, no IPCR assay is available for the detection of Vip protein as yet. This assay can also provide the detection of other commercially released varieties of GM cotton and GM corn bearing Vip3A protein (Table 2) due to the structural similarity among protein sequences of Vip that is being used in the development of GM crops (Table 3). In the present study, the developed IPCR assay was not tested on various commercially released GM crops due to unavailability of the samples in India. Furthermore, the evaluation of the assay using a greater range of samples from different GM products is needed.

This quantitative IPCR assay for the detection of transgenic protein has a better performance than other protein-based detection methods such as ELISA and dipstick. This assay can successfully differentiate the GM and non-GM samples where the expression levels of transgenic protein will be very low. It is also suitable to identify the authorized and nonauthorized GM materials, which have been released into the market. The developed IPCR assay for Vip has several advantages such as the requirement of less amount of protein and a higher sensitivity as compared to that of other protein-based detection methods, although it has a few disadvantages such as it is time-consuming and requires expensive instruments as well as a suitable laboratory setup. Additionally, the assay relies on the specific interaction between antigen and antibody; therefore, the availability of the specific antibody against transgenic protein is required before developing an IPCR assay for the detection of any other transgenic protein.

Table 2. List of Commercially Available GM Crops Bearing Vip Protein[a]

S.N.	Vip protein	transgenic events	year	country	NCBI accession no.
1.	Vip3Aa20	MIR 162 corn	2008−2010	United States, Australia, Taiwan, Brazil, Mexico, Philippines	ABG20429
		BT11x MIR162 corn	2009	United States	
		BT11 x MIR162 x MIR604 corn	2009	United States	
2.	Vip3Aa19	COT102 cotton	2005	United States, Australia, Mexico	ABG20428
3.	Vip-S[b] (Vip3Aa9)	Brinjal (under trail)		India	CAA76665

[a] Source: CERA.[6]. [b] Developed by ICGEB, India.

Table 3. Structural Similarity Among Three Different Variants of Vip Proteins (Vip3A19, Vip3A20, and Vip-S) Used in GM Crops (Analyzed by DNA-STAR Software)[a]

Percent Identity

Divergence	1	2	3	
1		99.9	99.6	1
2	0.1		99.7	2
3	0.4	0.3		3
	1	2	3	

MIR Vip3Aa20
Vip3Aa19
Vip-S

[a] The percent identity among proteins was >99%; however, divergence was <0.4%.

In conclusion, a highly sensitive and robust IPCR assay was developed for the detection of transgenic Vip-S protein in GM crops. As results suggest, Vip-S protein could be detected at a minimum of 1 ng/mL in the samples. The better performance of IPCR assay in comparison to other protein-based tests such as ELISA and the dipstick test was also observed. The developed IPCR assay can be successfully used for the detection of even trace amounts of Vip-S protein expressed in GM crops. The assay would also facilitate the monitoring of this protein to differentiate GM and non-GM crops. The improved detection limit, requirement of small sample volume, and compatibility with the biological matrices are the major advantages of the developed IPCR assay.

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