Cloning of BBTV (Banana Bunchy Top Virus) components and screening of BBTV using functionalized gold nanoparticles

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Abstract Banana bunchy top virus (BBTV) affects all varieties of banana plants and causes heavy economic loss in most of the banana cultivating areas. The BBTV genome comprises of six DNA components; in this study, we have cloned the six BBTV-DNA components from one of the BBTV-infected plants (Tri-8) and were submitted to GenBank. Analysis of the BBTV DNA-R component showed that it belonged to south Pacific group. Resistance against BBTV has not been observed so far in banana plants and removal and killing of the infected plants has been routinely practiced. Hence, early detection of BBTV infection would be desirable and various detection methods routinely employed include enzyme linked immunosorbent assay (antigen–antibody based) and molecular-based methods such as polymerase chain reaction (PCR), qPCR, or LAMP PCR. Most of these methods require enzymes or antibodies for detection and hence are expensive. Here, we report a visual detection method (AuNP probe assay) using gold nanoparticles (AuNPs) functionalized with an ssDNA-thiolated probe (CR1). This method is based on the hybridization of the functionalized AuNPs with the target DNA (BBTV). In the AuNP probe assay, the functionalized AuNPs retains red colour when BBTV DNA is present, and in the absence of BBTV DNA, the colour of the functionalized AuNPs changes to purple when salt is added. The AuNP probe assay was compared with PCR for the detection of banana plants and it was found that AuNP probe assay was better than PCR in detecting BBTV infection (86.5%

for AuNP probe assay and 65% for PCR). The AuNP probe assay was found to be highly specific to BBTV and was found to detect up to 1 pg/μl of the plasmid (pTZBBTri 4, BBTV DNA) mixed with healthy banana DNA.

Keywords Banana bunchy top virus · Gold nanoparticles · AuNP probe assay · Viral detection · Polymerase chain reaction

Introduction

Banana is one of the most important food crops such as rice, wheat, and maize and it is cultivated in about 10.3 million ha in the tropical and subtropical regions (Heslop-Harrison and Schwarzacher 2007). At present, banana is grown in 130 countries. Total production of banana is estimated at 139 million tons from 10.3 million ha in 2012 (Kumar et al. 2015). Banana plants are affected by various viruses. Among them, BBTV is one of the most important viral diseases that were reported in many countries and leads to loss in the production of bananas in Australia, Africa, and Asian countries (Dale 1987; Oben et al. 2009; Kumar et al. 2011). Banana and plantain (*Musa* spp.) are mostly grown as perennial crop cultivated in many developing countries with India being one of the major producer. Banana bunchy top disease (BBTD) had its impact in Tamil Nadu, India, where the production area was significantly reduced from 18,000 to 2000 ha (Kesavamoorthy 1980) (dessert banana cultivar Virupakshi Pome group, AAB). Recently, in India, BBTD outbreaks during 2007–2010 in several districts in Andhra Pradesh as well as in Maharashtra leads to losses worth US\$50 million (Kumar et al. 2015).

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Banana bunchy top virus (BBTV, family Nanoviridae, genus Babuvirus) is transmitted by the aphid *Pentalonia nigronervosa* (Magee 1927). BBTV is the worst among all the viruses affecting banana plants leading to 100% loss in fruit yield and has been recorded in all countries cultivating banana across the globe (Stainton et al. 2015). The BBTV is an ssDNA virus and comprises of six genome components each (~1.1 Kb long) and are termed DNA-R, DNA-U3, DNA-S, DNA-M, DNA-C, and DNA-N (King et al. 2011). Based on the DNA-R sequences, the BBTV isolates were grouped into two: Asian and south Pacific groups (Karan et al. 1994). The Asian group comprises of the isolates from Philippines, Taiwan, China, Japan, Indonesia, and Vietnam and the south Pacific group includes India, Pakistan, Egypt, Australia, Burundi, Tonga, Myanmar, Fiji, and USA-Hawaii and isolates from Africa (Stainton et al. 2015). Since BBTV is a destructive disease, and so far, no resistant banana varieties have been identified, it is imperative to detect this disease early and quarantine the infected plants. Hence, various methods were developed for the detection of BBTV based on antibodies (protein-based methods). Several research groups have produced monoclonal and/or polyclonal antisera to BBTV coat protein and enzyme linked immunosorbent assay (ELISA) method was developed and evaluated (Wu and Su 1990a; Thomas and Dietzgen 1991; Hu et al. 1993; Wanitchakorn et al. 1997; Geering and Thomas 1996; Thomas 1991; Su et al. 2003). It was found that double antibody sandwich ELISA (DAS-ELISA) and triple antibody sandwich ELISA were found to be effective for BBTV detection (Wu and Su 1990b; Hu et al. 1993; Geering and Thomas 1996).

Polymerase chain reaction (PCR)-based detection methods were developed by several authors for BBTV detection (Hu et al. 1996; Hafner et al. 1997; Dietzgen et al. 1999; Furuya et al. 2004; Mansoor et al. 2005). In our lab, a PCR-based detection method for BBTV was developed using unique primers that could detect many components of the BBTV DNA simultaneously (Anandhi et al. 2007). Recently, quantification of BBTV through real-time PCR using SYBR green was reported (Fu et al. 2009; Watanabe and Bressan 2013). Real-time TaqMan PCR was also developed for the detection of BBTV and could detect as low as 2.76 copies of BBTV DNA (Chen and Hu 2013). Another method, loop-mediated isothermal amplification (LAMP) assay, was developed for the detection of BBTV, which takes about 90 min and detects up to 1 pg/μl cloned BBTV DNA when mixed with extracted DNA from healthy banana plant (Peng et al. 2012). Even though most of the PCR-based methods and antibody-based methods are routinely used, they are cumbersome, need sophisticated equipments to record and interpret data, and are expensive. One major drawback in PCR-based methods is the inhibition of Taq polymerase by contaminants such as

polyphenolics and polysaccharides present in the extracted banana DNA. Hence, there is a need for simpler, sensitive, and cost-effective methods for the detection of BBTV.

Gold nanoparticles (AuNPs) have shown tremendous potential as diagnostic tool in the detection of many human pathogens. The AuNPs have the following advantages over other nanoparticles as these could be easily prepared, inert, easily functionalized with varied molecules and possess surface plasmon resonance (SPR). The SPR property of AuNPs has been utilized to develop visual, colorimetric detection method by Mirkin et al. (1996). Both unmodified AuNPs and functionalized AuNPs have been utilized to develop visual detection methods. Examples of utilization of unmodified AuNPs for detection of human pathogens such as HCV and plant pathogens such as maize chlorotic mottle virus (Shawky et al. 2010; Liu et al. 2015). Many methods have been developed that utilize modified or functionalized AuNPs for detection of pathogens as functionalization with a probe (nucleotide or antibody) results in better specificity of the assay. These methods have also been shown to have better sensitivity and shorter turnover time than conventional molecular methods such as PCR and ELISA. Many human pathogens such as *Mycobacterium tuberculosis*, *Leishmania* spp., and *E. coli* and plant pathogens such as tomato leaf curl virus have been developed with functionalized AuNPs (Baptista et al. 2006; Soo et al. 2009; Padmavathy et al. 2012; Andreadou et al. 2014; Larginho et al. 2015; Dharanivasan et al. 2016). The paper-based gene sensor was developed for the detection of BBTV using a probe-conjugated AuNP (Wei et al. 2014). Though this method could detect BTB DNA up to (0.13 aM), prior amplification of BBTV by PCR is needed. Hence, a simple, visual detection method using functionalized AuNP devoid of amplification steps was attempted.

Materials and methods

Banana plant material

Banana leaf samples ($n = 104$) were collected from various regions of Tamil Nadu, Kerala, and Assam, India and few control banana samples (healthy, BBTV infected, and BSV infected) were gifted by Jain Irrigation systems, India. The plant materials *Azadirachta indica* and *Talinum triangulare* were obtained from Chennai, India to determine the specificity of the assay.

Isolation of total banana nucleic acid from banana plants

The total DNA was isolated from BBTV infected or healthy banana plants by the method described in our earlier work with few modifications (Anandhi et al. 2007).

Briefly, 2 g of leaf tissue was ground to a fine powder using liquid nitrogen followed by the addition of 5 ml of extraction buffer [100 mM Tris (pH 8.0), 50 mM EDTA, 500 mM NaCl, and 10 mM β -mercaptoethanol]. The mixture was vortexed and 660 μ l of 10% SDS was added, mixed thoroughly, and incubated at 65 °C for 10 min. Then, 1.6 ml of 5 M potassium acetate was added and centrifuged at 8000 rpm for 15 min at 4 °C. The supernatant was transferred into a fresh tube and equal volume of isopropanol (100%) was added and incubated at –20 °C for 30 min. The total DNA was precipitated by centrifugation at 8000 rpm for 15 min at 4 °C. The pellet was air-dried and resuspended in 700 μ l of sterile distilled water. DNA was further treated with RNase A (10 mg/ml) for 2 h followed by proteinase K (40 mg/ml) treatment at 37 °C for 90 min. The mixture was extracted with phenol–chloroform and chloroform. The total DNA was precipitated with ethanol (100%) and washed with 70% ethanol and the pellet was air-dried and resuspended in 100 μ l of Milli Q water.

Cloning and sequencing of BBTV DNA components

The BBTV DNA components were amplified by PCR using Pfu polymerase (MBI Fermentas) and primer pair either (i) BBTVSL1 (5'YGCYCRGGACGGGACAT3') and BBTVSL2 (5'CKGGGGGTAATAATARTC3') designed in this study or (ii) specific full-length primers and the reaction conditions are given in Table 1. Typically, the PCR reaction mixture consisted of total genomic DNA (~50 ng), Pfu polymerase buffer (1 $\times$), Pfu polymerase (5 U), 0.2 mM of each dNTP, 1 μ M of each primer, and MilliQ water in a total volume of 30 μ l and carried out in PCR machine (G-storm). The PCR products were analyzed in agarose gel followed by A-tailing and further cloned into pTZ57R/T vector using InsTAclone PCR cloning kit (MBI Fermentas) as per manufacturer's instructions. The transformed colonies were then screened for the presence of the six BBTV components by PCR using component specific primers (Table 1). The clones carrying the BBTV DNA components were then purified using Nucleospin Plasmid isolation kit (Macherey–Nagel) as per manufacturer's instructions. The plasmids were then sequenced using M13F and M13R primers at Scigenom, Kerala, analyzed by blastn tool, and submitted in GenBank (Table 2).

Detection of BBTV in banana plants by PCR

The typical PCR reaction mixture consisted of either *Taq* DNA Polymerase—1 U (Merck), reaction buffer (Merck) (10 mM Tris–HCl 9.0, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% Gelatin), 0.2 mM of each dNTP or Amplicon master mix and 1 μ M of each primer (BBT2.2F and BBT2.2R),

and total DNA from banana plants (50 ng) in a reaction volume of 20 μ l. The PCR condition is provided in Table 2 and the PCR products were analyzed on 1% agarose gel and documented.

Designing of probe for BBTV detection

A consensus sequence corresponding to the common region major (CR-M) of BBTV DNA components was identified by clustalW analysis and a probe (5'(thiol-C6)CACGCTATGAAATACAAGACGC3') was designed and synthesized from Sigma (Sigma Aldrich) with 5' thiol modification. The probe was suspended in MilliQ water at 100 μ M concentration, further diluted to 10 nM concentration using MilliQ water. The thiolated probe (10 nM) was activated by treating with dithiothreitol (DTT) (100 mM). One ml of 10 nM thiolated probe was treated with 100 μ l of DTT and incubated for 30 min at RT and purified using NAP25 column as per manufacturer's instruction (GE Lifesciences) and used for conjugation of gold nanoparticles (AuNPs).

Synthesis of AuNPs and conjugation of gold nanoparticle with the probe

Gold nanoparticles (AuNPs) were synthesized using sodium citrate reduction method (Grabar et al. 1995). One gram of gold chloride [$HAuCl_4 \cdot xH_2O$ (SRL, India, Cat. No 074745)] was suspended in 1 ml of MilliQ water and used for the synthesis of gold nanoparticles. Briefly, 50 ml of MilliQ water and 5 μ l of gold chloride were mixed in a spoutless beaker and a round bottom flask filled with water was kept on top to prevent evaporation and the mixture was brought to boil. Then, 750 μ l of 1% trisodium citrate dihydrate (Merck) was added and boiled for 10 min. The mixture turns blue and then to cherry red by the end of 10 min, and the mixture was allowed to cool to room temperature (RT). The final volume of synthesized AuNPs was 50 ml and was stored in 50 ml falcon tube at 4 °C until further use. The freshly synthesized AuNPs were conjugated with activated thiolated probe by salt-ageing method as described by Hill and Mirkin (2006) with slight modifications. Briefly, to 15 ml of AuNPs, 300 μ l of activated thiolated probe was added and incubated in an orbital shaker at RT overnight for 110 rpm. Then, 297 μ l of 100 mM phosphate buffer (pH 7) was added and followed by addition of 165 μ l of 10% SDS. The mixture was further incubated for 30 min in orbital shaker at RT. Finally, six doses of 81 μ l of 2 M NaCl in 100 mM PBS (pH 7) were added to the mixture over a period of 2 days in an orbital shaker at RT. Then, the functionalized AuNPs were purified by centrifugation at 12,000 rpm for 10 min at RT in a 1.5 ml microfuge tube and washed with 500 μ l of

Table 1 List of primers and PCR conditions for BBTV components

Primer pair code	Nucleotide sequences	Target gene	PCR conditions	Amplicon size
BBT3.2F	5'GCTAGGTATCCGAAGAAATCC3'	BBTV DNA-S ORF (complete ORF) (unpublished data)	95 °C—5 min;	~ 500 bp
BBT3.2R	5'ATAAAGCTTTCAAACATGATATGT3'		95 °C—30 s; 45 °C—45 s; 72 °C—1 min; 34 cycles 72 °C—8 min	
BBT2.2 F	5'CGGGCAGGGACATGGGCTTT 3'	DNA-2 (Anandhi et al. 2007)	94 °C—4 min;	~ 750 bp
BBT2.2R	5'CGCCCTTGTAATTCATAGCGTGTGTATT3'		94 °C—30 s; 60 °C—45 s; 72 °C—1 min; 40 cycles 72 °C—10 min	
BBTVCoF	5'GGAATTCATGGAGTTCTGGGAATCG3'	BBTV DNA-C ORF (complete ORF) (un published data)	94 °C—5 min;	~ 500 bp
BBTVCoR	5'CCCAAGCTTTTAGAGTAATGTTACATC3'		94 °C—30 s; 60 °C—45 s; 72 °C—1 min; 40 cycles 72 °C—10 min	
BBTVSL1	5' YGCYCRGGACGGGACAT 3'	Any component (in this study)	94 °C—5 min;	~ 1.1 Kb
BBTVSL2	5' CKGGGGGTAATAATARTC 3'		94 °C—30 s; 58.5 °C—45 s; 72 °C— 1.2 min; 36 cycles 72 °C—10 min	
BBTVSF	5'ATCAAGAAGAGGCGGGTTGG3'	BBTV DNA-S full length (Islam et al. 2010)	94 °C—5 min;	~ 1.1 Kb
BBTVSR	5'GGATTTCTTCGGATACCTA3'		94 °C—45 s; 63 °C—60 s; 72 °C— 1.3 min; 34 cycles 72 °C—10 min	
BBTVM F	5'GTATATTAAGCAGCTCGTGAGG -3'	BBTV DNA-M full length (Islam et al. 2010)	95 °C—5 min;	~ 1.1 Kb
BBTVMR	5'TTCGGTACCTCAAAGAGCAAAACC3'		94 °C—30 s; 55 °C—45 s; 72 °C— 1.1 min; 27 cycles 72 °C—10 min,	
BBTVCF	5'TGCCTGACGATGTCAAGAGAGAG3'	BBTV DNA-C full length (Islam et al. 2010)	94 °C—5 min;	~ 1.1 Kb
BBTVCR	5'TAGCAGACCATTCCCAGAACTCC3'		94 °C—45 s; 63 °C—60 s; 72 °C— 1.3 min; 34 cycles 72 °C—10 min	

Table 1 continued

Primer pair code	Nucleotide sequences	Target gene	PCR conditions	Amplicon size
BBTVNF	5'TGGAAGAAAGTCGCCTCGCAAGG3'	BBTV DNA-N full length (Islam et al. 2010)	95 °C—5 min;	~ 1.1 Kb
BBTVNR	5'GCTCCAGAATCGACGCATGGTAC3'		94 °C—30 s; 65 °C—45 s; 72 °C- 1.1 min; 27 cycles 72 °C—10 min	

Table 2 Annotation of the features of the Viral DNA (BBTV) from this study (blast.ncbi.nlm.nih.gov)

Component	Accession no	Size (nt)	Predicted ORF size (location)	Predicted protein (pI/aa/kDa)	TATA box location (sequence)	Poly A location (AATAAA)	CR-M size (location)	CR-SL size (location)
DNA R (Partial)	KJ513015	934	861 (73–933)	Rep (8.30/286/33.53)	22–30 (CTATAAATA)	502–508	–	–
DNA U3 (Partial)	KJ513016	1035	234 (117–350)	Unknown protein (11.15/77/9.061)	84–92 (CAATAATTA) 230–238 (CTATAAATA)	462–467 517–522 881–886	90 (702–791)	–
DNA S	KJ513017	1075	528 (213–740)	Coat protein (9.80/175/20.03)	189–197 (CTATAAATA)	979–984	90 (786–875)	1051–1044
DNA M	KJ513018	1046	354 (282–635)	Movement protein (9.96/117/13.85)	261–269 (CTATAAATA)	171–176 950–955	91 (756–846)	1022–1044
DNA C	KJ513019	1018	(486) 240–725	4.75/161/18.94	(CTATTTAAA) 188–196	110–115 477–482 745–750	91 (821–911)	994–944
DNA N	KJ513020	1086	(465) (276–740)	5.39/154/17.41	(CTATTAATA) 221–229	155–160 736–741 763–768 984–989	91 (800–890)	1071–1044

resuspension buffer [10 mM phosphate buffered saline (PBS) pH 7.4; 0.1% SDS; 150 mM NaCl]. Finally, the functionalized AuNPs were then suspended in 50 µl of resuspension buffer, covered in aluminium foil, and stored at RT until further use.

AuNP probe assay for BBTv detection

The total DNA from banana plants (100 ± 10 ng/µl) were diluted (1:10) with MilliQ water and utilized for AuNP probe assay. The assay was performed in PCR machine and has following steps—diluted total DNA (5 µl) was denatured at 95 °C for 5 min, and then, 5 µl of functionalized AuNP was added and annealing was done at 65 °C for 10 min. Finally, 5 µl of 10 mM PBS containing 2 M NaCl prepared in MilliQ water was added and the results were recorded within 20 min. The clone (pTZBBTri4) that carried the BBTv DNA-U3 component

containing CR-M region was used as positive control, whereas MilliQ water and healthy banana plant DNA served as negative controls.

Specificity and sensitivity of AuNP probe assay

Various DNA (total DNA from plants and clones) were tested to confirm the specificity and sensitivity of AuNP probe assay. To confirm the specificity of the assay, total DNA (10 ± 2 ng/µl) from healthy banana (negative control), BBTv-infected banana (positive control), and BSV-infected banana (negative control) (gift from Jain irrigation systems, India) and other plants (*Azadirachta indica* and *Talinum triangulare*) belonging to other genomes were tested against the functionalized AuNPs. The clones (1 ng/µl) (pETBBSO.1—carrying the ORF of BBTv DNA-S component and pETBBCo.1—carrying the ORF of BBTv DNA-C component) (unpublished work) were tested against functionalized AuNPs to determine the

specificity. The plasmid (pTZBBTri4—BBTV DNA-U3 component carrying the CR-M region) mixed with healthy banana DNA was used to test the sensitivity of the AuNP probe assay. The various concentrations tested with the functionalized AuNPs were 1 ng/μl, 1 pg/μl, and 1 fg/μl mixed with total DNA from healthy banana plant (10 ± 2 ng/μl). To further validate the sensitivity of AuNP probe assay, five different concentrations of plasmid pTZBBTri4 (100, 10, 1 ng/μl, 1 pg/μl and 1 fg/μl) mixed with healthy banana DNA (10 ± 2 ng/μl) were analyzed by PCR using BBT2.2F and BBT2.2R primers.

Results

Cloning of viral DNA components from India and its analysis

The BBTV DNA (six components) were amplified by PCR using gene specific primers or primer pair BBTVSL1 and BBTVSL2. The PCR resulted in an amplicon of ~1.1 Kb in size and were cloned into pTZ57RT vector using InsTA cloning kit. The clones were screened for the presence of individual BBTV DNA components (DNA-R, U3, S, M, C, N) and then sequenced at Scigenom, Kerala, India. The sequences were analyzed using blastn tool and the sequences were then submitted to GenBank with the following accession numbers—KJ513015, KJ513016, KJ513017, KJ513018, KJ513019, and KJ513020. The phylogenetic analysis of the DNA-R component of BBTV isolated from a banana sample (Trivandrum 8) (red arrow) and other available BBTV DNA—R sequences showed that it belonged to south Pacific group (Fig. 1). Various features of the BBTV DNA components obtained in the current study are presented in Table 2. Each of the BBTV components obtained in this study had CR-M region, common region stem-loop (CR-SL) region, and a unique ORF except for DNA-R and DNA-U3 (Table 2). Further analysis of the CR-M regions by clustalW showed that the CR-M region had a consensus sequence of around 20 nucleotide (5'CACGCTATGAAATACAAGACGC3') that was designated as CR1 and a probe (ssDNA) with 5' thiol modification [5'(thiol-C6)CACGCTATGAAATACAAGACGC3'] was synthesized from Sigma and utilized for AuNP probe assay. The ORF of BBTV DNA-S and DNA-C components was isolated using specific primers, cloned and utilized for AuNP probe assay.

Screening of banana plants by PCR

Total DNA was isolated from banana plants ($n = 104$), diluted and utilized for PCR screening and AuNP probe

assay. The plants included both symptomatic and asymptomatic plants (at the time of sample collection) and PCR analysis was done using BBT2.2F and BBT2.2R primers. This primer was designed and showed good potential for the detection of BBTV by PCR in our earlier work; hence, this primer was utilized in this study also (Anandhi et al. 2007). The PCR would result in an amplicon of ~750 bp corresponding to DNA-U3 component of BBTV. Of the total 104 samples, 65% ($n = 68$) banana samples were positive for BBTV viral DNA (Fig. 2).

Synthesis and conjugation of AuNPs with CR1 probe

AuNPs synthesized by citrate reduction method were red in colour and the size of the nanoparticles was found to be $\sim 17 \pm 3$ nm by scanning electron microscopy (SEM) (Fig. 3). The AuNPs were then conjugated using the activated thiolated probe (CR1) by salt-ageing method. The functionalized AuNPs were red in colour due to surface plasmon resonance (SPR) phenomenon, which was utilized to develop the AuNP probe assay. The AuNP probe assay involves mixing of denatured total DNA and functionalized AuNPs followed by the addition of salt. Subsequently, the colour of the reaction mixture was documented. The functionalized AuNPs would retain red colour when they are bound to target sequences (CR-M region of BBTV), but in the absence of CR-M region of BBTV, the electron cloud of AuNPs gets neutralized resulting in purple colour (Fig. 4).

Specificity of the AuNP probe assay

The specificity of the AuNP probe assay was demonstrated by three different experiments. In the first experiment, total DNA from banana plants (healthy, BBTV infected, and BSV infected) were analyzed by the AuNP probe assay and it was found that only BBTV-infected banana was positive (red colour) (Fig. 5a). PCR analysis of these samples with BBT2.2F and BBT2.2R primers showed that only BBTV-infected banana had a faint band (~750 bp) correlating with the positive sample (pTZBBTri4) (Fig. 5b). In the second experiment, total DNA from two plants (*Azadirachta indica* and *Talinum triangulare*) belonging to other genomes were tested and were found to be negative (purple colour) (Fig. 6a). To further confirm the specificity of the probe, two plasmids (pETBBSO.1—carrying the ORF of BBTV DNA-S component and pETBBCo.1—carrying the ORF of BBTV DNA-C component) were assayed and both the samples were found to be negative (purple colour) (Fig. 6b).

Fig. 1 Phylogenetic relationship based on nucleotide sequences of DNA R of Trivandrum (this study) with previously reported south Pacific and Asian isolates of BBTV rooted with ABTV. The evolutionary history was inferred using the neighbour-joining method (Kimura 1980)

Sensitivity of the AuNP probe assay

To test the sensitivity of the AuNP probe assay, plasmid (pTZBBTri4) carrying BBTV DNA-U3 component containing CR-M region was mixed healthy banana sample and three concentrations (1 ng/μl, 1 pg/μl, and 1 fg/μl) were tested and compared with PCR assay. It was found that the AuNP probe assay was able to detect BBTV DNA up to 1 pg/μl, whereas PCR was able to detect only up to 10 ng/μl (Fig. 7a).

Screening of banana plants with AuNP probe assay

The AuNP probe assay was performed for all the 104 banana samples along with healthy banana samples and assay was carried out in duplicates to avoid false positives or negatives. It was found that 90 samples were positive with the AuNP probe assay, whereas only 68 samples were positive with PCR. A representative picture of the AuNP probe assay of the banana samples from Assam (ASM) was provided (Fig. 7b).

Fig. 4 Schematic representation of AuNP probe assay for detection of BBTv

Fig. 5 **a** AuNP probe assay was performed with MilliQ water as negative control. *H* healthy, *BSV* banana streak virus, *BBTV* banana bunchy top virus. **b** PCR analysis of healthy banana, BSV, BBTv, and pTZBBTri4 (positive control) using BBT2.2 F and BBT2.2 R

Fig. 6 **a** Specificity of AuNP probe assay was tested using total DNA from two plants *A. indica* and *T. triangulare* and were found to be negative. **b** AuNP probe assay was tested with plasmids—pETBBso.1

and pETBBCo.1 that carried ORF of BBTv-DNA-S and DNA-C, respectively, and were found to be negative

Fig. 7 **a** Comparison of the sensitivity of AuNP probe assay with PCR (using BBT2.2F and BBT2.2R) using different concentrations of plasmid (pTZBBTri4) mixed with healthy banana DNA. AuNP probe assay was able to detect BBTV up to 1 pg/ μ l, whereas PCR detected

10 ng/ μ l. **b** Detection of BBTV in banana samples using the AuNP probe assay. Plant DNA (ASM 1 to 24). *P* positive, *H1* *H2*, *H3* healthy banana samples, *N* negative

that our BBTV belonged to south Pacific group. This correlated with earlier reports that BBTV isolates from India belong to south Pacific regions though few BBTV isolates belonging to Asian group have been reported (Karan et al. 1994; Stainton et al. 2012).

Each of the six BBTV DNA components obtained in this study carried one unique ORF and CR-M and CR-SL regions except for DNA-R and DNA-U3 components (Table 2). The components (DNA-R and DNA-U3) were obtained from the BBTV library that was generated with primers BBTVSL1 and BBTVSL2. When the common regions (CR-M and CR-SL) of the BBTV DNA components obtained were analyzed for conserved regions, only the CR-M region showed the presence of a conserved region of 20 nt (5' CACGCTATGAAATACAAGACGC3') and was designated as CR1 and a 5' thiol-modified probe [5' (thiol-C6) CACGCTATGAAATACAAGACGC3'] was synthesized to facilitate attachment of the probe to AuNPs. The probe was designed in the common region of BBTV genome as it would facilitate better detection of BBTV in banana plants, as CR-M region is present in all the six DNA components of BBTV.

The various methods employed routinely for BBTV detection include molecular methods (PCR, qPCR, and LAMP PCR) (Hu et al. 1996; Hafner et al. 1997; Dietzgen et al. 1999; Furuya et al. 2004; Mansoor et al. 2005; Fu et al. 2009; Peng et al. 2012; Watanabe and Bressan 2013) and protein-based methods such as ELISA, Immuno-capture PCR (Wu and Su 1990b; Hu et al. 1993; Geering and Thomas 1996). Even though these methods are specific and sensitive, they employ costly chemicals or enzymes; time consuming (few hours) and laborious. Hence, we have developed a simple, visual detection method utilizing functionalized AuNPs.

Recently, many detection methods have been developed using gold nanoparticles (AuNPs) for the detection of many pathogens or disease in humans and few plant pathogen (Baptista et al. 2006; Soo et al. 2009; Padmavathy et al. 2012; Andreadou et al. 2014; Larguinho et al. 2015; Dharanivasan et al. 2016). The ease of preparation, characterization, and functionalization of AuNPs has paved way for development of simple, visual detection or imaging methods for varied applications. One of the key properties that have resulted in the development of visual detection

methods using AuNPs is surface plasmon resonance (SPR). The AuNPs are naturally red in colour and in the presence of salt or acid gets neutralized, thereby becoming purple in colour due to loss of electrons cloud (Mirkin et al. 1996). The AuNPs synthesized by citrate reduction method were found to be $\sim 17 \pm 3$ nm in size (Fig. 3) and were conjugated to activated thiolated probe (CR1, ssDNA) and utilized for AuNP probe assay. The functionalized AuNPs (CR1 conjugated) would retain red colour in the presence of BBTv (target DNA) but would turn purple in the absence of BBTv when assayed with salt (Fig. 4).

The specificity of the AuNP probe assay was tested using plant DNA [banana plants—healthy, BBTv infected, and BSV infected: *A. indica* and *T. triangulare*] and plasmid DNA (ORF of DNA-S and DNA-C and pTZBBTri4). The assay was able to detect BBTv DNA in the BBTv-infected banana and in the positive control (pTZBBTri4) as evidenced by the red colour (Figs. 5a, 6a). The plasmid pTZBBTri4 served as positive control, since it consisted of the CR-M region of BBTv DNA-U3 component. The colour of the assay turned purple for other samples (*A. indica* and *T. triangulare* DNA and plasmids carrying ORF regions of BBTv) (Fig. 6a, b) due to the absence of BBTv DNA (CR-M region). The negative results in other plants showed that the probe does not have any cross-reactivity to other plant genomes and could be safely utilized for viral detection.

The amount of BBTv DNA present in asymptomatic banana plants is highly variable and difficult to quantify, so the sensitivity of the AuNP probe assay was tested with different concentrations of plasmid DNA carrying BBTv genome mixed with healthy banana DNA. It was found that the assay was able to detect up to 1 pg/ μ l (Fig. 7a), whereas PCR was able to detect only up to 10 ng/ μ l (Fig. 7a) and this confirmed that AuNP probe assay was sensitive than PCR. To further confirm the applicability of the developed assay, 104 banana plant samples were analyzed by PCR and AuNP probe assay. The plants were collected from different regions of Tamil Nadu, Kerala, and Assam. The banana plants collected in this study include both asymptomatic and symptomatic plants for BBTv infection at the time of sample collection. It was found that PCR was able to detect only 68 samples, whereas AuNP probe assay detected 90 samples. The number of samples detected by PCR was less and this could be due to the presence of low levels of BBTv DNA or contaminants such as phenolics and polysaccharides. The samples that were positive in PCR were also found to be positive in AuNP probe assay. This showed that the developed assay was not only sensitive as it detected more samples than PCR and lower concentrations of BBTv DNA (1 pg/ μ l) but also specific and reliable. Another advantage of the AuNP probe assay is requirement of low

amounts of total DNA (~ 10 ng/ μ l or less) for detection and fast turnover time (20–30 min). The routine detection methods such as ELISA, PCR, and LAMP PCR (Peng et al. 2012) require 60 min to few hours for completion and further separation on gels and/or instruments for documentation; this new method is a simple visual detection method. In addition, the molecular- and protein-based methods involve costly chemicals or reagents or antibodies to perform, and the AuNP probe assay needs low concentrations of chemicals and, hence, is cost effective. Finally, the AuNP probe assay involves only three steps (denaturation of target, hybridization of probe, and addition of salt) and, hence, avoids carryover contamination due to processing of the products and results in direct detection. One major limitation of the AuNP probe assay is that coloured DNA samples (brown or black—due to phenolics) could not be detected directly. This method of detecting BBTv utilizing functionalized AuNPs has been filed for patent (Indian Patent filed: Patent No: 2117/CHE/2013—A method for Detecting BBTv).

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Compliance with ethical standards

Conflict of interest Authors declare no conflict of interest.

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