Highly efficient immunodiagnosis of Large cardamom chirke virus using the polyclonal antiserum against *Escherichia coli* expressed recombinant coat protein

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Abstract Large cardamom chirke virus (LCCV), genus *Macluravirus*, family *Potyviridae* is an important constrain in large cardamom production in India. Purification of LCCV from large cardamom tissues is difficult and therefore immunodiagnostic reagents are not available. In the present study, we have successfully expressed coat protein (CP) gene of LCCV in *Escherichia coli*. The purification of expressed protein by Ni-NTA affinity chromatography was inefficient due to precipitation of protein during renaturation. We have optimized a simple, inexpensive and efficient method for purification of the expressed CP through gel extraction with 5 % SDS followed by renaturation in Milli-Q water, which resulted in high yield (4.7 mg/ml) and good quality of the protein. A higher titer (1:256,000) polyclonal antibody (PAb) to the recombinant CP was produced, which strongly recognized LCCV in crude leaf extract and showed minimal background reaction with the healthy leaf extract in enzyme linked immunosorbent assay (ELISA) and dot immunobinding assay (DIBA). The sensitivities of the ELISA and DIBA were 5 and 0.1 ng of expressed protein, respectively. Both the ELISA and DIBA were validated with 100 % accuracy in detecting LCCV in field samples. The PAb differentiated *Cardamom mosaic virus*, another close relative of LCCV. Our study is first to report highly efficient immunodiagnosis with PAb to

E. coli expressed recombinant CP of a virus under the genus *Macluravirus*. The antigen expression construct and PAb developed in the present study will be useful in production of virus free planting materials of large cardamom.

Keywords Large cardamom chirke virus · LCCV · Coat protein gene expression · ELISA · DIBA

Introduction

Large cardamom (*Amomum subulatum*) is cultivated as a cash crop under the shady and moist environment of eastern Sub-Himalayan Mountains. Sikkim state and Darjeeling district of West Bengal are the major large cardamom producing areas in India, where the crop is vital to the rural livelihood security. Large cardamom production is affected by two chronic viral diseases known as chirke and foorkey [14, 17]. Chirke disease characterized by streak mosaic symptoms on leaves was known to occur since 1958 [14]. Chirke disease causes gradual decline in plant growth and results in significant yield loss in the third year of infection [14–16]. Large cardamom chirke virus (LCCV), a new virus species under the genus *Macluravirus*, family *Potyviridae* was identified as the virus associated with the chirke disease [10]. LCCV, a (+)ssRNA virus is vectored by aphids, *Rhopalosiphum maidis*, *Brachycaudus helichrysi* and *Myzus persicae* in a non-persistent manner [10, 15].

Large cardamom is predominantly grown through vegetative propagation of suckers. Although, several aphid vectors are known, LCCV primarily spreads through planting material. For propagation of virus free planting material of large cardamom, sensitive diagnostic reagents and methodology are necessary. Purification of LCCV

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particle from large cardamom leaf tissues is difficult and that limits production of adequate quantity of high quality antiserum for the immunodiagnosis of LCCV. Polyclonal antiserum to the purified preparation of LCCV from large cardamom leaf tissues was raised but the quality was not adequate for efficient detection of the virus by enzyme linked immunosorbent assay (ELISA) [10]. Prokaryotic (*E. coli*) expression of plant viral antigen provides several advantages in antigen preparation over the traditional methods and the recombinant antigen has utilized to develop immunodiagnosis of several plant viruses [2, 4, 6, 12, 13, 18]. However there is no report of expression of recombinant CP in bacteria and its application in immunodiagnosis of LCCV. In this study, we report the expression of highly immunogenic recombinant CP of LCCV in *E. coli*, production of high titer polyclonal antiserum to the recombinant CP and its application in efficient detection of LCCV.

Materials and methods

RT-PCR and cloning of LCCV coat protein gene

Total RNA was isolated from 100 mg of infected large cardamom leaf tissues according to Chomczynski and Sacchi [3]. A pair of primers BM336F (gagctcgctccattagcaccagttacg) and BM337R (ctcgagttagtatatgtgtgcgcgag) with *SacI* and *XhoI* restriction sites, respectively, were designed to amplify the coat protein gene based on the 3' genome sequence generated in our previous study on LCCV [10]. The first strand cDNA was synthesized using Revertaid First Strand cDNA Synthesis Kit (Fermentas, Maryland, USA) in 20 µl of mixture containing 4 µl of 5× reaction buffer, 2 µl of 10 mM dNTP mix, 1.0 µl of RiboLock™ RNase inhibitor (20 U/µl), 2.0 µl of 10 µM reverse primer, 1.0 µl of RevertAid™ M-MuLV Reverse Transcriptase (200 U/µl) and 10 µl (~1.0 µg) of RNA template. The reverse transcription was conducted at 42 °C for 60 min followed by inactivation at 70 °C for 5 min in a thermal cycler (Biometra T Personal, Goettingen, Germany). PCR was performed in a 50 µl reaction mixture containing 10 µl of cDNA, 5 µl of 10× reaction buffer, 5 µl of 10 mM dNTP, 2 µl each of forward and reverse primer (10 µM primer), 0.25 µl (5 U/µl) of Ex Taq DNA polymerase (Takara, Japan) and nuclease-free water to make up the volume. The mixture was subsequently subjected to 35 cycles of PCR, each consisting of denaturing at 94 °C for 30 s, primer annealing at 55 °C for 30 s, and extension at 72 °C for 1 min with the final extension at 72 °C for 10 min. The amplified DNA fragment was cloned into pT&A vector (RBC, New Taipei city, Taiwan) and sequenced.

The LCCV CP gene was re-cloned into the prokaryotic expression vector, pET28a(+) (Novagen, Darmstadt, Germany). The expression construct was used to transform *E. coli* BL21 (DE3) strain. The clones were identified by restriction digestion with site-specific enzymes and confirmed by sequencing.

Expression of LCCV coat protein gene

The *E. coli* BL21(DE3) strain containing the pET28a-CP was grown overnight in 5 ml of Luria broth at 37 °C and induced for expression by adding 1.0 mM IPTG at 0.6 OD for 3 h and 6 h with vigorous shaking at 200 rpm. The bacterial cells were collected by centrifuging at 10,000×g at 4 °C for 10 min, dissolved in 0.01 M phosphate buffer (pH 7.4) and the samples were sonicated for 5 min on ice bath, for ten passes of 30 s. The soluble and insoluble fractions were separated by centrifuging the total bacterial lysates at 10,000×g at 4 °C for 10 min and resolved in 15 % SDS-PAGE. The gels were stained for proteins with Coomassie Brilliant blue (R250) in 50 % (v/v) methanol and 10 % (v/v) acetic acid.

Purification of protein by Ni-NTA column chromatography

The insoluble sonicated fraction containing the expressed protein obtained from one liter of 3 h induced bacterial culture was dissolved in 6 M urea. The protein was purified using Ni-NTA column chromatography (Novagen, Darmstadt, Germany) as per manufacturer's protocol. The purified protein was dialyzed using dialysis membrane (MWCO 12,000–14,000 Da) to remove the salt impurities with continuous stirring for 24 h in 20 mM Tris-HCl with 0.5 M NaCl (pH 7.9).

Purification of the protein by gel extraction

The insoluble sonicated fraction containing the expressed protein obtained from one liter of 3 h induced bacterial culture was dissolved in 1× sodium dodecyl sulphate (SDS) sample buffer and electrophoresed in 15 % SDS-PAGE and the gel was stained with Coomassie brilliant blue (R250) solution. The desired protein band was excised and destained in 10 ml of 1 % ammonium bicarbonate and 10 ml of methanol by keeping in a shaker for 15 min at room temperature. This step was repeated three times until the gel became colourless. The protein band was crushed in a pestle and mortar using different percentage of SDS (0.1, 0.5, 1, 3, 5 %) at a ratio of 1:2 (w/v) and centrifuged at 10,000×g at 4 °C for 10 min. The supernatant was passed through Whatman filter paper (pre-rinsed with sterile water) to remove the polyacrylamide gels. The filtrate was

kept in ice for 10 min to precipitate SDS and centrifuged at $10,000\times g$ at 4 °C for 5 min. The supernatant containing the protein was collected and the step was repeated 2–3 times to remove the SDS. Finally the protein was dialyzed against Milli-Q water for 12 h at 4 °C.

Production of polyclonal antiserum and Western blot analysis

The gel purified recombinant CP of LCCV (300 µg) was emulsified with an equal volume of Freund's complete adjuvant and injected intramuscularly in New Zealand white rabbits. After 7 days, three injections were administered intramuscularly each with 200 µg of purified protein emulsified with incomplete Freund's adjuvant at weekly intervals. The antiserum was isolated from rabbit blood after 41 days of immunization and stored at –80 °C with 50 % glycerol.

The expressed LCCV protein was resolved in SDS-PAGE and electro blotted on to nitrocellulose membrane (Sartorius, Germany) using semi dry blotting apparatus (Atto, Taito-ku, Tokyo) at 95 volts for 45 min. The membrane was blocked with 5 % skimmed milk powder overnight at 4 °C and then incubated with antiserum (1:5,000 dilutions) for 1 h at 37 °C. After washing, the membrane was incubated with goat anti rabbit IgG alkaline phosphate conjugate (Sigma Aldrich, St. Louis, USA) (1:20,000 dilutions) at 37 °C for 1 h. The protein band was visualized by adding BCIP/NBT (Genei, Bangalore, India).

Direct antigen coating ELISA (DAC-ELISA)

The plates were coated with 100 µl of leaf extracts prepared in carbonate buffer containing 2 % PVP and incubated overnight at 4 °C. After three washings with PBS-T, the wells were blocked with 0.5 % bovine serum albumin in PBS-T at 37 °C for 1 h. After three gentle washing with PBS-T, antiserum diluted with the PBS-TPO (PBST with 0.2 % ovalbumin and 2 % PVP) was added to the plates and incubated at 37 °C for 1 h. Plates were washed thrice and goat anti-rabbit IgG-AP conjugate (Sigma Aldrich, St. Louis, USA) diluted in PBS-TPO (1:20,000) was added for 1 h at 37 °C. Finally, the plates were washed thrice and substrate, *p*-nitro phenyl phosphate substrate was added. The absorbance was measured by the ELX 800 Universal Microplate Reader (BIO-TEK Instruments Inc., Winooski, VT, USA) after 1 h at 405 nm. DAC-ELISA was carried out in three replicates with different concentrations of protein and antiserum dilutions to determine sensitivity and antibody titer.

Dot immunobinding assay (DIBA)

Samples were prepared by grinding 100 mg of large cardamom leaf tissues in 0.5 ml of carbonate buffer and 5 µl

of the supernatant was spotted on nitrocellulose membrane (NCM) presoaked in PBS. The free protein binding sites on NCM were blocked using 5 % skimmed milk powder dissolved in PBS at pH 7.5 for 1.0 h. The membrane was treated for half an hour with the LCCV PABs diluted (1:5,000) in PBS at 37 °C, followed by incubation with goat anti-rabbit IgG-AP conjugate (1:20,000) for half an hour at 37 °C. The bound antigens were visualized using BCIP/NBT substrate. The blot was washed with PBS. Washing was carried out after each step either manually five times for 5 min at room temperature or with a 0.5 mm air brush (Killer Ink Ltd, UK) at 60 psi for 2 min at each step.

Results

Construction and expression of LCCV CP

The LCCV CP gene was successfully amplified from symptomatic large cardamom leaf tissues as about 800 bp DNA fragment and the sequence analysis revealed that the clone contained 810 nucleotides, the full length CP gene of LCCV. The sequence analysis of the prokaryotic expression construct pET28a-CP showed that the LCCV CP gene was in frame with His-tag at both the ends and PCR induced mutation was not observed.

After induction with 1.0 mM IPTG at 37 °C for 3 and 6 h, *E. coli* BL21(DE3) cells harboring the expression construct pET28a-CP exhibited expression of ~37 kDa protein. Whereas, no expression of the LCCV CP was found in the uninduced *E. coli* BL21(DE3) cells harboring the expression construct pET28a-CP. The major expressed protein was observed in insoluble fractions in SDS-PAGE (Fig. 1a).

Purification of the LCCV CP

Purification of urea denatured LCCV recombinant CP through Ni-NTA resin column showed precipitation of the protein during dialysis and resulted in low yield of 0.1–0.15 mg/ml from one liter of induced bacterial culture and also contained residual *E. coli* proteins.

Purification through gel extraction method showed no precipitation of protein during dialysis and no background of *E. coli* protein as judged by SDS-PAGE. The yield of the gel extracted protein was 0.03, 0.6, 1.2, 3.3 and 4.7 mg/ml, when protein was extracted with 0.1, 0.5, 1, 3 and 5 % of SDS, respectively. From a liter of induced bacterial culture, about 3 ml of protein was obtained after dialysis, use of 5 % SDS in purification resulted in isolation of about 8.1–12.6 mg LCCV CP.

Fig. 1 Expression of coat protein (CP) of Large cardamom chirke virus (LCCV) in *E. coli*. **a** SDS-PAGE **b** Western blot with the antiserum developed against the gel extracted recombinant CP. Lane 1: Marker, Lane 2: Uninduced BL21(DE3) *E. coli* cells harboring the

pET28a-CP, Lane 3, 4: Sonicated insoluble fractions at 3 and 6 h post induction, Lane 5: Ni-NTA purified protein, Lane 6: Gel extracted protein

Evaluation of polyclonal antibody and detection by ELISA

In the initial test, antiserum at 1:10,000 dilutions showed strong OD value of 0.63–1.1 with the diseased samples and low OD value of 0.14–0.17 with the healthy samples. In Western blot, the antiserum could recognize the expressed protein in crude bacterial lysate and also in purified preparation, whereas no reactivity was observed in the uninduced bacterial lysate (Fig. 1b). Western blot with the chirke affected large cardamom cv. Varlangey and a close relative of large cardamom Churumpa (*Ammomum dealbatum*) showed positive reactions but healthy large cardamom showed negative reactions indicating the antiserum to the recombinant CP was able to recognise LCCV in naturally infected plants (Fig. 2).

In ELISA with the 50 ng of expressed protein, the LCCV polyclonal antiserum showed high OD value of 2.47 at 1:8,000 dilutions. The OD value declined with the

increase of dilution of antiserum and at 1:256,000 dilutions, the OD value of 0.37 was obtained, which was higher than the average cut-off value for scoring positive reaction. When the protein level was reduced to 25, 10 and 5 ng, the titer of the antiserum also declined to 1:128,000, 1:64,000 and 1:32,000 dilutions, respectively (Fig. 3a).

The antiserum at 1:10,000 dilution could efficiently detect LCCV in 1:5 dilution of crude plant sap and partially purified virus (0.62–2.36 OD), whereas it showed very low ELISA readings (0.16–0.17 OD) with *Cardamom mosaic virus* (CdMV) in small cardamom leaf sap (Fig. 3b). A total of 63 symptomatic large cardamom leaf samples collected from three different cultivars and one wild type grown in eight different places in Darjeeling district of West Bengal and Sikkim were tested to validate the accuracy of ELISA based detection (Table 1). LCCV was detected in all the symptomatic samples of large cardamom cultivars Varlangey, Ramsey and Sawney where the OD value ranged from 0.49 to 1.0. A relative of large cardamom, Churumpa showed exceptionally high OD value of 2.7–3.27 (Table 1) revealing the presence of LCCV in high titer in this plant. ELISA was repeated with additional 48 randomly collected field samples from Kalimpong. Only 14 samples that exhibited chirke symptoms were positive in ELISA, whereas the remaining 34 asymptomatic samples were negative (Data not shown).

Fig. 2 Western blot analysis of LCCV. Chirke affected field samples and the antiserum developed against the recombinant coat protein of LCCV were used. M: Pre stained marker, Lane 1: Gel extracted LCCV CP, Lane 2: Chirke affected Churumpa (*Ammomum dealbatum*), Lanes 3 and 4: Chirke affected large cardamom cv. Varlangey, Lane 5: Healthy large cardamom

Detection by DIBA

Initially, DIBA experiments were carried out with 1.0 h of incubation at each step of blocking and probing with primary and secondary antibody conjugate, and washing five times manually, which showed efficient detection of the virus in the diseased samples. The procedure, however, took about 5 h to complete and showed some background. Reduction in the incubation period at each step to half an

Fig. 3 Evaluation of polyclonal antiserum to the recombinant coat protein of LCCV. **a** DAC-ELISA showing titer of the antiserum with different concentration of expressed protein. Dotted line indicates cut-off value for scoring positive reaction, which was based on twice the average healthy readings (OD 0.26). **b** Detection of LCCV and *Cardamom mosaic virus* (CdMV) from large cardamom and small cardamom, respectively, by DAC-ELISA using the LCCV antiserum at 1:10,000 dilutions. Crude extracts were prepared in 1:5 dilutions from leaf samples. Partially purified virus was used as positive control and healthy readings were used as negative control. OD at 405 nm was recorded 1 h after adding *p*-nitro phenyl phosphate (1.0 mg/ml)

Table 1 Detection of Large cardamom chirke virus in large cardamom leaf samples showing streak mosaic symptoms by DAC-ELISA and DIBA using the antiserum developed against *E.coli* expressed recombinant coat protein

Sample number ^a	Place	Genotype	No positive/no tested ^b	ELISA OD at 405 nm ^c
1–9	Kashyem, Darjeeling	Ramsey	9/9	0.57–0.92
10–16	Kashyem, Darjeeling	Varlangey	7/7	0.54–0.95
17–22	Halpoo, Darjeeling	Varlangey	6/6	0.73–0.97
23–27	Kolbung, Darjeeling	Varlangey	5/5	0.74–0.97
28–32	Payong, Darjeeling	Varlangey	5/5	0.51–0.84
33–46	Pangthang, East Sikkim	Sawney	14/14	0.51–0.89
47–48	Ramding, South Sikkim	Ramsey	2/2	0.49–0.66
49–51	Assam-Lingzey, East Sikkim	Varlangey	3/3	0.86–0.98
52	Kalimpong, Darjeeling	Churumpa	1/1	2.72–3.278
53–63	Kalimpong, Darjeeling	Varlangey	11/11	0.65–1.0

^a Leaf samples were collected during May 2013. Each sample represents a clump

^b Each sample was tested by DAC-ELISA and DIBA. Sample 52: the samples were collected from four different tillers of the same clump and tested by DAC-ELISA

^c The reaction was considered positive if sample OD was more than twice of healthy control value (0.16–0.19) OD at 405 nm

hour and application of mechanized blot washing system using an air brush reduced background as well as the total processing time to about 2.5 h.

The LCCV antiserum at 1:5,000 dilutions showed strong positive signal with 1.0–0.1 ng of expressed protein. With the reduction of protein quantity the detecting signal

Discussion

Large cardamom plants are very slow growing, leaf tissues are highly fibrous and turn brown on storage or after extraction. Due to lack of suitable propagative host for LCCV, there is no choice other than cardamom tissues to use as a source of virus purification. While purification of LCCV, filamentous particles get aggregated resulting in loss of yield [10]. Several such issues impose constraints in preparing adequate quantity of high quality antigen of LCCV. The present study overcomes these constraints in antigen preparation by expressing highly immunogenic CP of LCCV in *E. coli*. Purification of *E. coli* expressed protein is generally conducted by immobilized metal ion affinity chromatography [1]. In the present study, His-tag based purification using Ni-NTA column was attempted, but the purified protein showed precipitation resulting in the loss of protein during renaturation and in addition to the target protein additional minor protein bands were observed in SDS-PAGE. The CP gene of CdMV infecting small cardamom was shown to express in *E. coli* BL21 cells and reported aggregation of expressed protein during purification resulting in low yield [5]. Similar problem was found with Potyviruses, *Potato virus A* and *Pepper vein banding virus* [7, 11]. A Ni-NTA method for purification

reduced, however the protein as low as 1.0–0.1 ng could be detected by DIBA. Similarly, efficient detection was obtained in the crude sap of infected large cardamom leaf at a dilution of 1:5–1:10. The intensity of the developed spot gradually decreased as the dilution of sap increased. However, the virus was detectable in the sap dilution up to 1:100 (Fig. 4). The LCCV antiserum showed very weak detection signal with CdMV leaf extract in small cardamom at only 1:5 dilution. Healthy cardamom leaf extract however did not show any positive reaction with the antiserum at any dilution. Sixty three symptomatic large

are symptomatic and samples 64–70 are healthy large cardamom. **a** Blot was washed in PBS manually 5 times for 5 min or **b** spraying PBS with the help of a 0.5 mm air brush at 60 psi for 2 min

was not efficient, in the present study, we have optimized a simple and inexpensive method of purification of the LCCV expressed CP in *E. coli*. The method involves resolving of sonicated bacterial lysate on SDS-PAGE, extraction of specific protein band by crushing gel fragments with 5 % SDS followed by renaturation in Milli-Q water. The method resulted in purification of about 8.1–12.6 mg of LCCV CP from a liter of induced bacterial culture. The purified protein showed no precipitation and no contamination of *E. coli* protein. Purification of protein band from SDS-PAGE was previously demonstrated by electroelution and sonicated extraction using low level (0.1 %) SDS [8]. In the present study, target protein was isolated with higher percentage (5 %) of SDS and renatured protein in Milli-Q water retained immunogenicity of the protein. Immunization of rabbit with the purified protein, resulted in production of high titer antiserum (1:256,000 with 50 ng of protein), which strongly recognized LCCV in crude leaf extract and didn't show high background with the healthy cardamom leaf extract.

Two immunodiagnostic techniques ELISA and DIBA for LCCV were optimized and validated with the field samples. ELISA and DIBA were highly sensitive in detecting about 5 ng and 0.1 ng of expressed protein, respectively. Accuracy of the diagnostic test was 100 % as all the symptomatic field samples collected from different cultivars and places showed positive results in both ELISA and DIBA. LCCV was detected in several popular cultivars of large cardamom, where ELISA showed moderate to high OD values. A close relative of large cardamom, Churumpa, which is taller plant type having longer and broader leaves growing luxuriously showing brighter streak mosaic symptoms compared to the cultivars of large cardamom showed very strong ELISA readings. The results were confirmed by repeated testing. Although, only a single clump was examined in the present study, Churumpa may serve as a high titer source and a better propagative host for LCCV, which needs further studies. The antiserum specifically detected LCCV differentiating CdMV, a close relative infecting small cardamom. In our previous studies antiserum produced against purified virus particles from large cardamom tissues showed a very low titer and high background and low level cross reactivity with CdMV [10]. In the present study, the antiserum to the *E. coli* expressed proteins of LCCV couldn't detect CdMV, however very low reactivity was observed in DIBA. Immunodiagnosis of LCCV by DIBA was approximately 50 times more sensitive than ELISA. We have successfully improvised immunoblot washing method by using air brush which reduced processing time and also improved the background. Our DIBA methodology provides opportunity to cut down 50 % time over traditional process of blot development.

Large cardamom is a plantation crop, which is maintained for a long time through vegetative propagation of sucker. Horizontal spread of LCCV in the plantation through aphid vectors seems to be low and the vector species *R. maidis*, *B. helichrysi* and *M. persicae* colonize seldom on large cardamom and do not transmit the virus at high rate [10]. Planting materials derived from the infected clumps are the major source of spread of LCCV. Virus free planting material is a highly desirable preventive measure against circulation of LCCV. The efficient immunodiagnosics developed in the present study will be useful in producing virus free planting materials, monitoring disease spread and incidence and estimating level of resistance in large cardamom genotypes. *E. coli* expression of CP of macluravirus has been shown for CdMV [5] and *Alpinia mosaic virus* [9]. Our study for the first time demonstrates a successful application of recombinant CP in highly efficient immunodiagnosis of a member under the genus *Macluravirus*.

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