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ORIGINAL ARTICLE

Molecular characterization and infectivity analysis of tomato leaf curl New Delhi virus isolates infecting potato

Arjunan Jeevalatha[1,2] · G. Vanishree[1] · Sundaresha Siddappa[1] · Ravinder Kumar[1] · Priyanka Kaundal[1] · Ashwani Kumar[1] · Swarup Kumar Chakrabarti[1]

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Abstract

Nucleotide sequence of complete genome of a new isolate (KAN-6) of tomato leaf curl New Delhi virus (ToLCNDV) from Kanpur, Uttar Pradesh, India was determined. Sequence analysis indicated that it shared maximum identity to ToLCNDV isolates from pumpkin and ashgourd. Infectious clones of isolate KAN-6 along with two other ToLCNDV isolates (MOD-21 & FAI-19) obtained from potato fields of Modipuram and Faizabad, India were produced and used in symptom expression studies in *N. benthamiana* and potato plants through agro-inoculation. These isolates produced different symptoms both in *N. benthamiana* and potato. Severe symptoms of yellow mottling, downward curling and stunted growth were observed in *N. benthamiana* plants inoculated with KAN-6. MOD-21-inoculated plants also showed downward curling, stunted growth, but yellow mottling was observed only in older leaves whereas FAI-19-inoculated plants produced only downward curling symptoms. In case of potato, typical symptoms of apical leaf curl disease were observed in cultivar Kufri Pukhraj inoculated with MOD-21 and KAN-6 that are similar to those produced by virus-infected plants in the field. However, MOD-21 produced more prominent yellow mosaic symptoms as compared to KAN-6. FAI-19 produced only restricted yellow spots in Kufri Pukhraj. Only mild symptoms appeared in KAN-6 and no symptoms were observed in MOD-21- and FAI-19-inoculated Kufri Bahar plants which is known to show lowest seed degeneration under field conditions. Analysis of genomic components indicated that these isolates had 94.8–94.9% and 87.9–97.3% identity among them in DNA A and DNA B, respectively. The results of the study indicate the association of ToLCNDV isolates of different symptomatology with apical leaf curl disease of potato. This is also a first experimental demonstration of Koch's postulate for a begomovirus associated with apical leaf curl disease of potato.

Keywords Begomovirus · Infectious clones · Symptoms · ToLCNDV · Potato

Introduction

The potato is susceptible to many diseases, some of which are widespread and others are localized. There are more than forty tuber transmitted viruses reported on potato of

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✉ Arjunan Jeevalatha
A.Jeevalatha@icar.gov.in

G. Vanishree
Vanishree.G@icar.gov.in

Sundaresha Siddappa
Sundaresha.S@icar.gov.in

Ravinder Kumar
Ravinder.Kumar2@icar.gov.in

Priyanka Kaundal
priyanka_kaundal@yahoo.co.in

Ashwani Kumar
aryan.verma07@gmail.com

Swarup Kumar Chakrabarti
skc_cpri@yahoo.co.in

[1] ICAR-Central Potato Research Institute, Shimla, Himachal Pradesh 171 001, India

[2] Present Address: ICAR-Indian Institute of Spices Research, Kozhikode, Kerala 673 012, India

which eight are reported to cause economic losses in India. Apical leaf curl disease (ALCD) caused by tomato leaf curl New Delhi virus (ToLCNDV) is one among them and it is one of the most important viral diseases of potato in India (Jeevalatha et al. 2017a; Kreuze et al. 2020; Lakra 2002). The virus spreads to potato due to a change in planting date of potato which coincided with an increased whitefly population. Symptoms appear as curling/crinkling of apical leaves with distinct mosaic symptoms due to primary infection (Garg et al. 2001; Usharani et al. 2004). In case of secondary infection, the entire plant show severe leaf curling and stunting symptoms (Sohrab et al. 2013). Initially, sporadic incidence of the disease was reported in 1996 at Hisar in Haryana (Lakra 2003), later severe infection was observed in western UP and other parts of northern India (Chandel et al. 2010; Saha et al. 2014). About 40–75% infection in the Indo-Gangetic Plains and up to 100% infections from Hissar (Haryana) were observed with heavy yield losses in susceptible varieties (Lakra 2002; Venkatasalam et al. 2005). This virus is also reported from other parts of the country (Jeevalatha et al. 2013, 2014, 2017a). Its incidence is on the rise, menacing the cultivation of potato across the country.

Usharani et al. (2004) confirmed that the virus causing apical leaf curl disease in potato is a strain of ToLCNDV belonging to the genus *Begomovirus* within the family *Geminiviridae*. After a decade, diversity of ToLCNDV infecting potato from different parts of the country was studied based on coat protein gene and complete genome (Jeevalatha et al. 2017a). ToLCNDV, a bipartite begomovirus, is the most predominant virus in northern India, infecting elite cultivars of tomato (Kushwaha et al. 2015; Sahu et al. 2010). It also infects other crops such as okra, cucurbitaceous crops, eggplant and papaya (Chigurupati et al. 2012; Ito et al. 2008; Pratap et al. 2011; Raj et al. 2008). ToLCNDV is reported to be transmitted to potato from cucurbit crops grown nearby potato fields (Sohrab et al. 2013) and may be easily transmitted between tomato, cucurbits and potato. The component of bipartite begomoviruses known as DNA-A encodes for all factors required for virus replication, overcoming host defences, insect transmission, and control of gene expression; while DNA-B component encodes factors required for inter- and intra-cellular movement in host plants (Rojas et al. 2005; Moriones et al. 2017). Recombination and pseudo-recombination are very frequent phenomena occurring between as well as within species and genera of geminiviruses and are significant contributors to begomovirus evolution (Polston and Anderson 1997; Kumar and Chakraborty 2018) led to gain the ability to infect new hosts (Lefeuvre and Moriones 2015).

Infectious clones are used for characterizing the function of viral components in virus infections (Babu et al. 2018; Basu et al. 2018) and vector transmission (Pan et al. 2020). They also provide highly reproducible and simple inoculation method which facilitates host–virus interaction studies and crop breeding. This is especially useful for viruses that cannot be transmitted to host plants by mechanical inoculation (Pasin et al. 2019). Infectious clones are widely used in begomovirus studies including tomato leaf curl viruses (Jailani et al. 2016; Jyothsna et al. 2013; Malik et al. 2011; Qadir et al. 2019; Shafiq et al. 2019; Vinoth Kumar et al. 2015). In the present study, we cloned and sequenced a new isolate (KAN-6) of ToLCNDV from potato field of Kanpur, Uttar Pradesh, India. Infectious clones of KAN-6 and two other isolates (MOD-21 and FAI-19) of ToLCNDV were developed and infectivity studies were carried out in *Nicotiana benthamiana* and potato. In this study, association of tomato leaf curl New Delhi virus, a bipartite begomovirus, with apical leaf curl disease of potato was experimentally demonstrated. Thus, proving Koch's postulate for the first time for a begomovirus associated with apical leaf curl disease of potato.

Materials and methods

Cloning of viral components

The leaf samples showing typical symptoms of potato apical leaf curl disease such as leaf curling, mosaic and stunting were collected from potato field in Kanpur and stored at – 80 °C until further use. Total DNA was extracted from the leaves using GenElute™ Plant Genomic DNA Miniprep kit (Sigma-Aldrich, Missouri, USA) following the manufacturer's instructions. The concentration and quality of DNA were checked using a Nanodrop 2000 spectrophotometer (Thermoscientific, Leon-Rot, Germany). The viral DNA was amplified using the TempliPhi™ amplification kit (GE Healthcare) as follows: 80 ng of total DNA (2 µl) was added to 5 µl of the sample buffer, heated to 95 °C for 3 min to denature the DNA, chilled on ice, and combined with 5 µl of reaction buffer plus 0.2 µl of enzyme mix. The reaction mixtures were incubated for 18 h at 30 °C, followed by inactivation of the enzyme at 65 °C for 10 min. The resulting concatamers were subjected to digestion with *Xba* I enzyme to release the unit-length viral genome. Restricted products were checked in 1% agarose gel stained with Ethidium bromide and visualized under UV light. An approximately 2.7 kb band was observed after digestion with *Xba*I enzyme. The digested RCA product fragments of ≈ 2.7 Kb were gel purified using the MinElute gel extraction kit (Qiagen, Hilden, Germany). The eluted fragment was cloned in pUC18 vector at *Xba* I site following standard protocols (Sambrook et al. 1989). Colony PCR analysis was performed with CP gene-specific primers (Forward primer-5′-AAAGTCATGTGTGTTAGTGATGTTACC-3′ and Reverse primer-5′-TAGAAATAGATCCGGATTTTC

AAAGTA-3′) and positive colonies showing an expected size of 491 bp amplicon were selected for DNA A analysis. Remaining negative colonies were used for DNA B analysis. Restriction digestion of the plasmids isolated from the colonies released an expected size of approx. 2.7 kb fragment.

Sequencing and analysis

The recombinant clones were sequenced using M13 forward and reverse primers and four internal primers (designed at approximate 500 bp interval) in the automated DNA sequencer (ABI PRISM ™ 3500 Genetic Analyzer from Applied Biosystems). The nucleotide sequences were assembled using DNAbaser to form contigs, the peaks were checked and manually edited. The complete nucleotide sequences are available in GenBank under the accession numbers, KX951455-KX951456. Multiple sequence alignments were made and per cent identity was calculated using Bioedit software. Phylogenetic dendrograms were generated using Neighbor joining algorithm of MEGA software version 4.0.2 and only bootstrap values > 80% are shown. Isolates, MOD-21 (KC874508 and KC874500) and FAI-19 (KC874505 and KC874497) reported earlier by Jeevalatha et al. (2017a) were used along with KAN-6. Genome organization of the tomato leaf curl New Delhi virus isolates of the present study is depicted in Supplementary Fig. 1.

Construction of infectious clones

To perform symptom expression studies, infectious clones were produced and artificially inoculated to *N. benthamiana* and potato plants through agro-infection. To develop partial dimers of DNA A components of MOD-21 and FAI-19, initially 2253 bp fragment was released from pUC18/DNA A recombinant plasmid by *Xba*I and *EcoR*I and ligated with plant transformation vector pRI101 digested with the same enzymes to develop 0.8 mer. Later, the complete DNA A (2740 bp) was released from pUC18/DNA A recombinant plasmid by digestion with *Xba*I enzyme and inserted at *Xba*I site pRI101-0.8 mer to get 1.8 mer tandem repeat of DNA A (pRI101-MA1.8 and pRI101-FA1.8). To develop partial dimers of DNA A of KAN-6 isolate, initially 1342 bp fragment was released from pUC18/DNA A recombinant plasmid by *Sac*I and *EcoR*I and ligated with plant transformation vector pRI101 digested with the same enzymes to develop 0.4 mer. Later, the complete DNA A (2739 bp) was released from pUC18/DNA A recombinant plasmid by digestion with *Sac*I enzyme and inserted at *Sac*I site pRI101-0.4 mer to get 1.4 mer tandem repeat of DNA A (pRI101-KA1.4). The partial dimers of DNA B were developed by cloning ≈ 1800 bp fragment of DNA B released from pUC18-DNA B recombinant plasmid using *Xba*I and *EcoR*I enzymes and cloning in pRI101 to generate 0.6-mer of DNA B. Then, the full-length DNA B was released from pUC18-DNA B using *Xba*I enzyme and cloned at *Xba*I site of pRI101-0.6 mer to develop 1.6-mer tandem repeat of DNA B (pRI101-MB1.6, pRI101-FB1.6 and pRI101-KB1.6).

Agro-inoculation studies in *N. benthamiana*

The recombinant plasmids (MA, MB, FA, FB, KA and KB) were mobilized into *Agrobacterium tumefaciens* strain EHA105 through freeze–thaw method. Young seedlings (3–4 leaf stage) of *Nicotiana benthamiana* were inoculated with infectious clones by agro-infiltration method. Empty binary vector, pRI101 in *A. tumefaciens* was used for mock inoculation on control plants. The *A. tumefaciens* cultures were incubated at 28 °C and 200 rpm for 48 h (OD600 = 1) in Yeast Extract Mannitol (YEM) medium (pH 6.8) containing kanamycin (50 µg/ml) and rifampicin (20 µg/ml). *Agrobacterium* cells were harvested, resuspended in induction buffer [10 mM MES, pH 5.5, along with 200 µl of acetosyringone (ACS)] and incubated at 50 rpm for 3 h at room temperature. Then, the *Agrobacterium* cells were again harvested, resuspended in infiltration buffer (5 mM MES, pH 5.5) and used for agro-infiltration on *N. benthamiana* plants (Senthil Kumar and Mysore 2014). Three-to-four leaf stage plants were used and five plants were inoculated for each culture. The inoculated plants were maintained under 16/8 h light/dark periods, 18,000 lx, 28–30 °C, and 85% relative humidity for 3–4 weeks in the controlled growth chamber until they were scored for symptoms and analyzed for viral DNA (Kanakala et al. 2013). The experiment was repeated thrice for confirmation.

Agro-inoculation studies in potato

The potato cultivars, Kufri Pukhraj (susceptible) and Kufri Bahar (resistant) were agro-inoculated by tuber sprout method followed by stem injection. Germinating seed tubers were inoculated with *Agrobacterium* cells (mixture of DNA A and DNA B; 1:1 ratio) in a rotary shaker for 4 h after pricking around the sprouts and planted in sterile pot mixture. Then again 15 days after planting, the grown plants were inoculated with *Agrobacterium* cells through stem injection (Supplementary Fig. 2). Four replicates were maintained for each treatment and the experiment was repeated thrice. The inoculated plants were maintained under controlled condition along with control plants and observed periodically for symptom expression.

Viral DNA quantification

Viral DNA was quantified in agro-inoculated plants using qPCR assay with coat protein gene-specific primers. Total

DNA was extracted from systemically infected *N. benthamiana* plant leaves collected at 10, 15, 20, and 25 days after inoculation (DAI) and potato plants at 30 days after planting (DAP)/30 days after first inoculation using GenElute™ Plant Genomic DNA Miniprep kit (Sigma-Aldrich, Missouri, USA) following the manufacturer's instructions and the concentration and quality of DNA was checked using a Nanodrop 2000 spectrophotometer (Thermoscientific, Leon-Rot, Germany).The qPCR assays were carried out in an ABI PRISM HT7900 (Applied Biosystems) with the following thermal cycles: 50 °C for 2 min, 95 °C for 10 min; 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Each 15 µl reaction mixture contained 10 µl of Power SYBR Green PCR Master mix, 1.0 µl each of 5 µM forward and reverse primers, 1 µl of cDNA templates and 2 µl of nuclease free water. The primers used are PALCV-CPF: 5′-ACCGTCGTCCTACAGGAT CTC-3′ and PALCV-CPR: 5′-GCTCGGTTCATTGTCAAA CATGT-3′. The qPCR reactions were carried out in triplicates and average values were considered for quantification.

Results and discussion

Cloning and sequence analysis

The DNA A of isolate KAN-6 was 2739 bp long with a maximum of 96.0–96.8% identities to ToLCNDV isolate from pumpkin (JN129254) and ash gourd (JN208136). It shared 94.8% identity to MOD21 and FAI-19 and 94.9–95.2% identities to other ToLCNDV isolates from potato (Supplementary Table 1). The DNA B of KAN-6 isolate was 2693 bp long and had a maximum of 93.4% identity to DNA B of ToLCNDV isolates from pumpkin (AM286435) and ash gourd (JN208137). It shared 87.5–88.0% identity to DNA B of ToLCNDV isolates from potato (Supplementary Table 2). The per cent identities of these isolates at DNA A level to ToLCNDV is 93.3–96.8% which are above the threshold cut-off value 91% for species demarcation for begomoviruses based on DNA A (Brown et al. 2015). These results confirm that the begomovirus that infect potato is an isolate of ToLCNDV. It supports the report of Usharani et al. (2004) who initially found that the begomovirus infecting potato in India as ToLCNDV. These isolates (MOD-21, FAI-19 and KAN-6) showed about 5% variation in DNA A components. MOD-21 had more identity to the already reported ToLCNDV isolates from potato. While FAI-19 and KAN-6 shared more identity to ToLCNDV isolates from chilli and pumpkin/ash gourd, respectively.

In a phylogenetic analysis based on DNA A components, MOD-21 was grouped with already reported potato isolates of ToLCNDV from Meerut, Palampur and Happur. FAI-19 was grouped with an isolate of ToLCNDV from chilli in Tumkur. While the isolate, KAN-6 was grouped with ToLCNDV isolates from pumpkin and ashgourd. However, all the three isolates were found in the same cluster in which the ToLCNDV isolates from different crops were observed (Fig. 1a). Phylogenetic analysis using DNA B components showed that both MOD-21 and FAI-19 isolates were grouped with the ToLCNDV isolates reported earlier from potato, whereas the isolate KAN-6 was found to form another cluster with ashgourd and pumpkin isolates with 100% bootstrap support (Fig. 1b).

Symptoms expression in *N. benthamiana*

Among the three infectious clones, symptom expression started earlier in KAKB (KAN-6)-inoculated *N. benthamiana* plants (at 10 DAI). Symptoms appeared at 15 DAI in MAMB (MOD-21) and FAFB (FAI-19)-inoculated plants (Supplementary Table 3). The symptoms progressed faster in KAKB (KAN-6)-inoculated plants with prominent yellow mottling, small young leaves and severe downward curling of leaves. MAMB (MOD-21)-inoculated plants showed typical downward curling, stunted growth and yellow mottling in older leaves. FAFB (FAI-19) produced mild symptoms, i.e. merely downward curling of leaves was observed (Fig. 2a–c). In general, agro-inoculation of DNA A alone resulted in downward curling of leaves in *N. benthamiana*. This indicates DNA A can maintain itself and cause infection. However, symptoms were milder when compared to those plants co-inoculated with the DNA B component. In bipartite begomoviruses, it is generally the DNA B genes (movement protein) that lead to development of symptoms (Hou et al. 2000; Hussain et al. 2005; Kleinow et al., 2020) and hence, plants co-inoculated with DNA B produced severe symptoms.

Symptoms expression in potato

Symptoms appeared at 20 DAP or first inoculation in Kufri Pukhraj which is highly susceptible to the virus. Kufri Pukhraj plants showed typical symptoms of apical leaf curl disease, i.e. curling of leaves and mosaic symptoms with stunted growth that are similar to those occurred in virus-infected plants in the field (Fig. 3) upon inoculation with MOD-21 and KAN-6, whereas FAI-19 produced only restricted yellow spots. However, MOD-21 produced more prominent yellow mosaic symptoms which had bright yellow patches as compared to KAN-6. No symptoms were observed in Kufri Bahar after inoculation with MOD-21 and FAI-19 which is known to show lowest seed degeneration due to ToLCNDV (Lakra 2003) and no/mild symptoms of apical leaf curl disease in both field and under artificial inoculation (graft inoculation) in glass house (Jeevalatha et al. 2012, 2017b; Lakra 2003). Only mild yellow restricted spots appeared in KAN-6-inoculated Kufri Bahar plants at

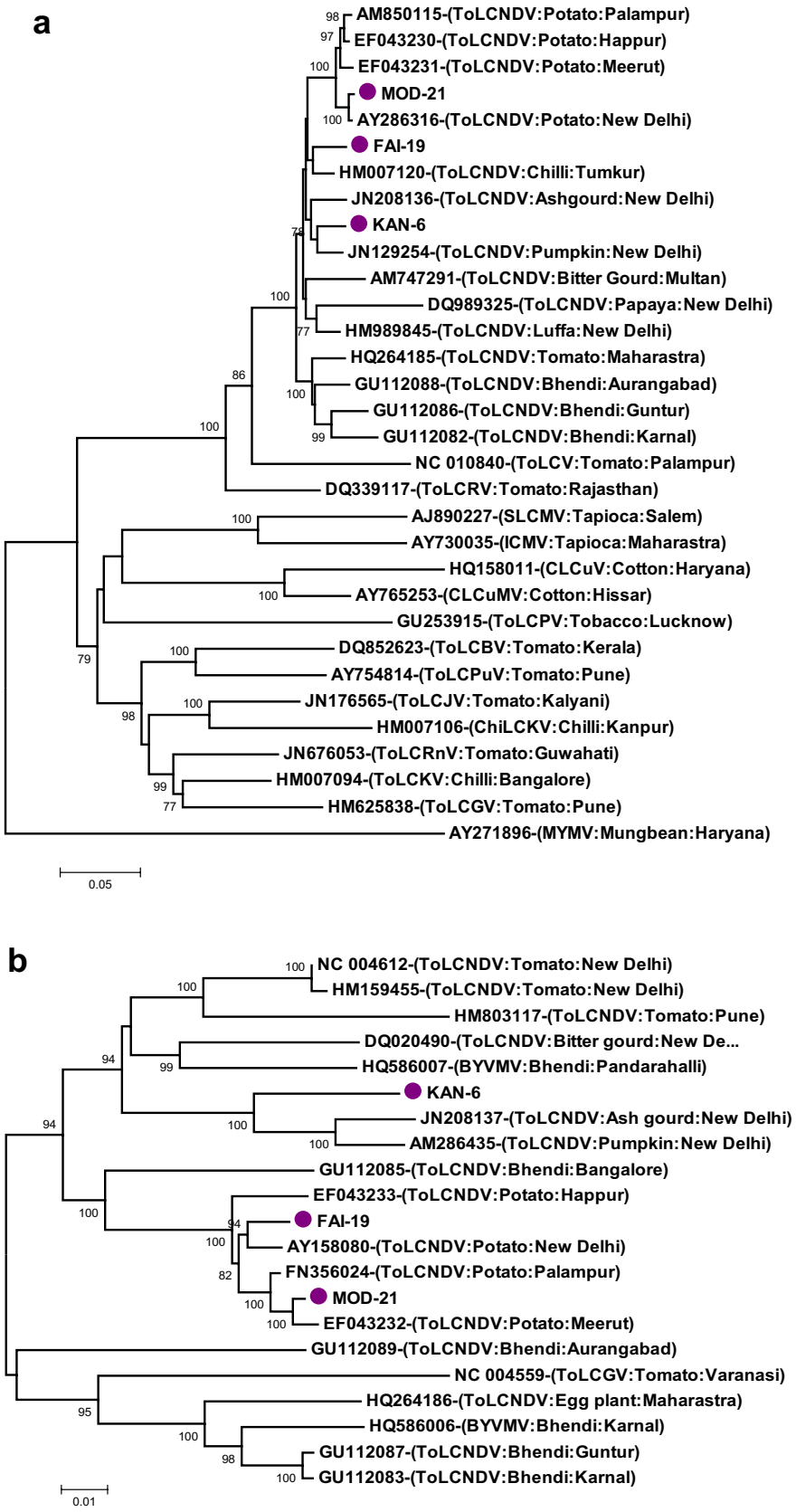


Fig. 1 Phylogenetic dendrogram generated based on DNA A (**a**) and DNA B (**b**) components of ToLCNDV isolates from potato and other begomoviruses (isolates of this study are marked with violet color dots)

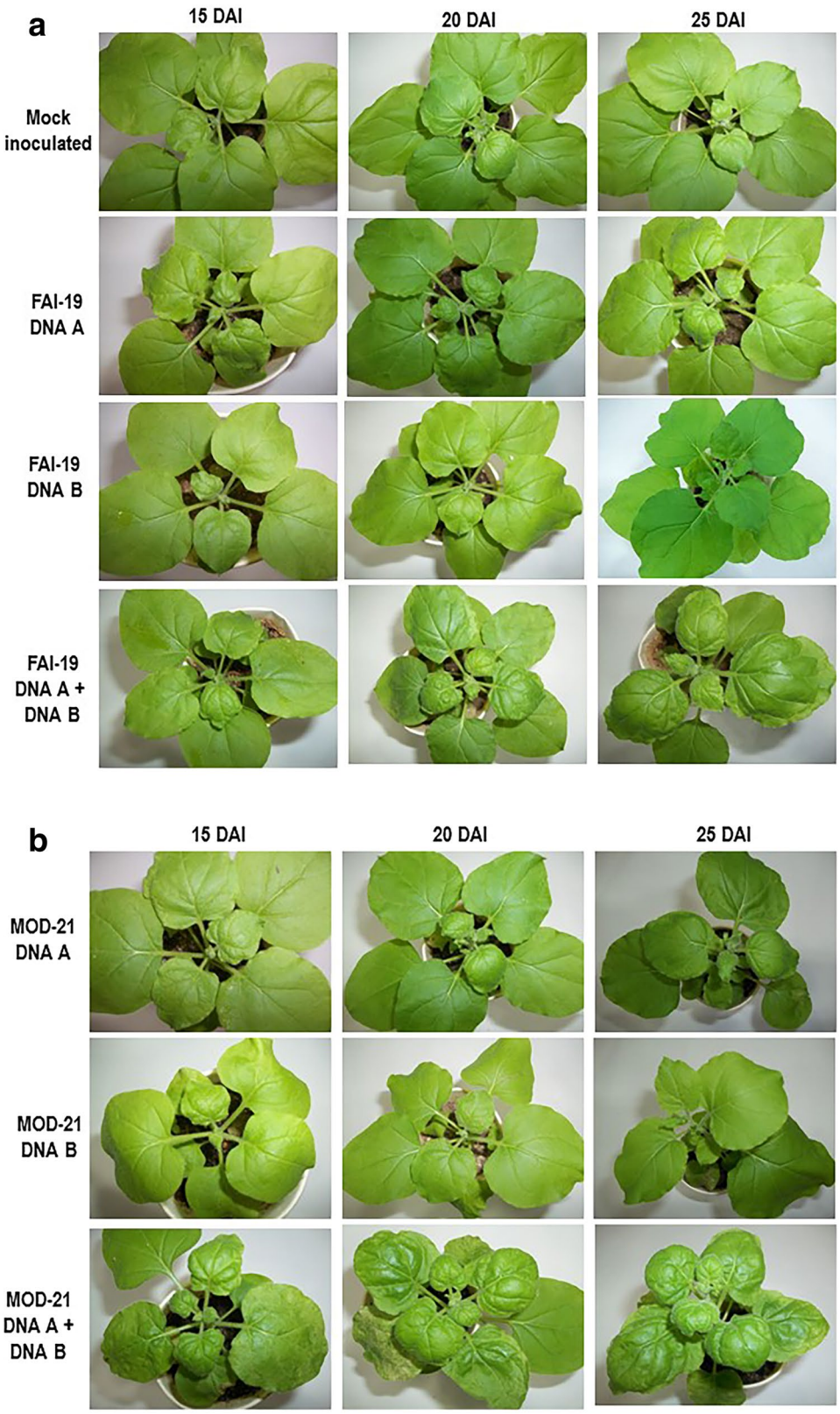
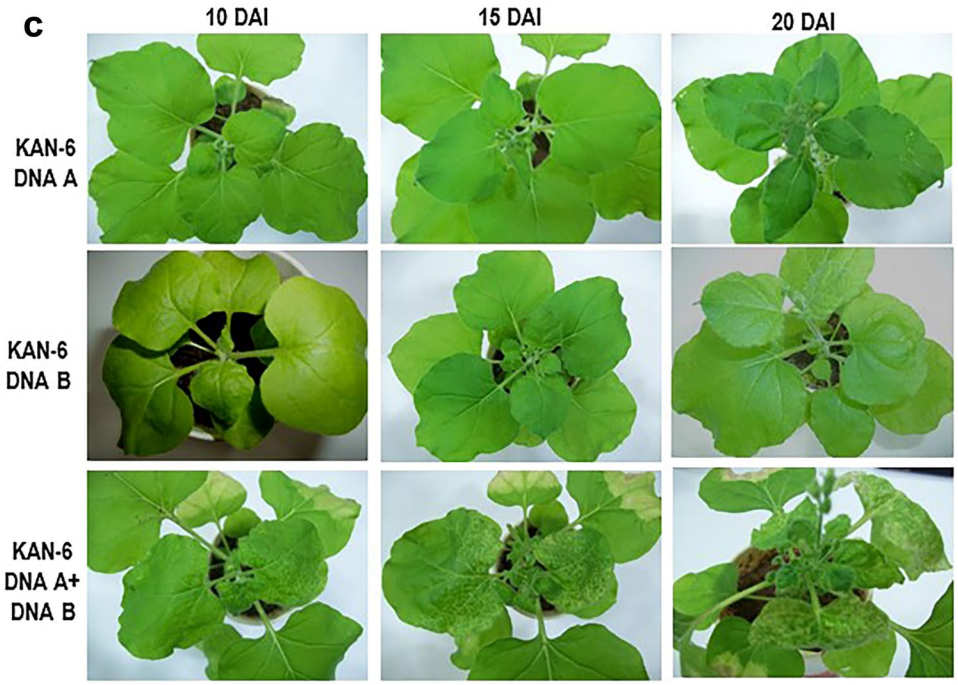


Fig. 2 Symptom expression in *N. benthamiana* plants inoculated with infectious clones as labeled at different time intervals. **a** FAI-19, **b** MOD-21, **c** KAN-6

20 DAP. However, later, mild symptoms appeared at 50 DAP in Kufri Bahar also. The presence or absence DNA A and DNA B components in young non-inoculated leaves of both tobacco and potato were confirmed by PCR analysis using DNA A- and DNA B-specific primers (data not-shown).

Fig. 2 (continued)



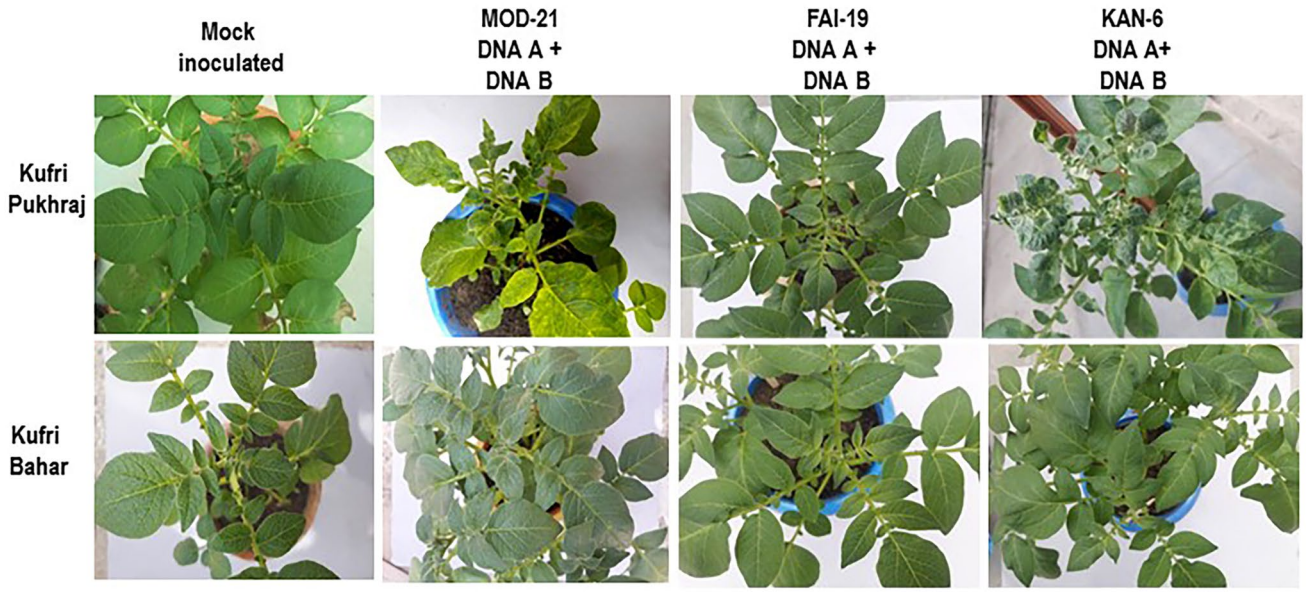


Fig. 3 Symptom expression in potato cultivars, Kufri Pukhraj and Kufri Bahar inoculated with infectious clones (photographed 30 DAP/ 30 days after first inoculation)

So far, reports are available on complete genome sequencing of bipartite begomovirus, ToLCNDV from potato (Jeevalatha et al. 2017a; Usharani et al. 2004), but Koch's postulate is not yet fulfilled. For the first time, in this study, we produced infectious clones of ToLCNDV isolates and studied symptom expression in potato to satisfy Koch's postulate. Initially we tried with agro-infiltration in leaves and then stem injection. But these methods failed to produce symptoms in potato. Therefore, another method involving germinating tuber was used and combined with stem injection (Supplementary Fig. 2) which led to infection with infectivity of about 66.66–83.33%. Earlier reports also suggest that two to three doses of inoculation are required to agro-infect potato (Salaria et al. 2020).

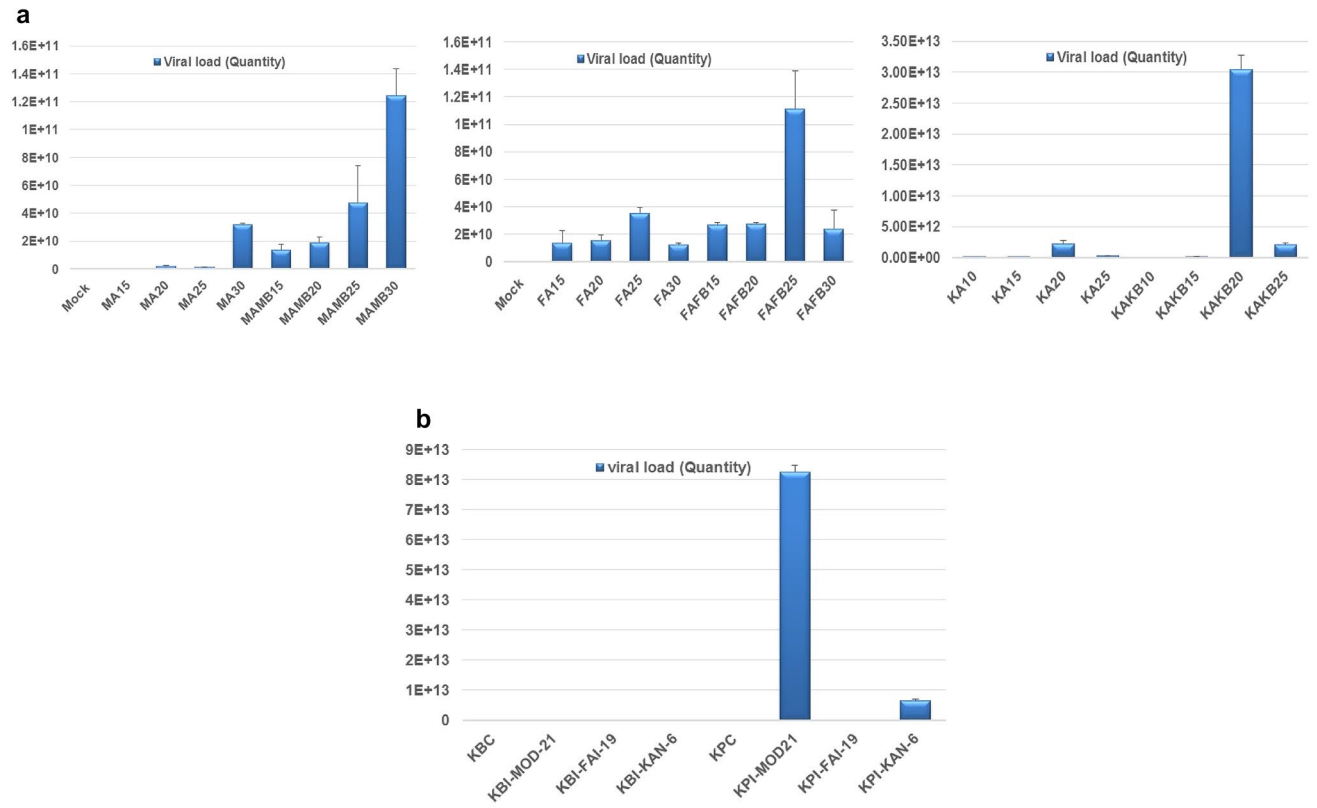


Fig. 4 Quantitative PCR analysis of viral DNA in agro-infiltrated *N. benthamiana* at 15, 20, 25 and 30 DAI (**a**); MA-MOD-21 DNA A, MB-MOD-21 DNA B, MAMB- MOD-21 DNA A + DNA B, FA-FAI-19 DNA A, FB-FAI-19 DNA B, FAFB- FAI19 DNA A + DNA B, KA-KAN-6 DNA A, KB-KAN-6 DNA B, KAKB-KAN-6 DNA A + DNA B and potato plants (**b**); Kufri Bahar and Kufri Pukhraj plants inoculated with both DNA A and DNA B components of MOD-21, FAI-19 and KAN-6 (quantified at 30 DAP or 30 days after first inoculation). The error bar represents the standard deviation of the three replicates

Viral DNA quantification

Differences in the levels of viral DNA were observed among the infectious clones. In *N. benthamiana*, an increase in viral DNA concentration with the progression of time was noticed in case of MOD-21. But in case of FAI-19 and KAN-6, the viral DNA concentration increased initially and then reduced at later stages (30 DAI in FAI-19 and 25 DAI in KAN-6). Viral DNA concentration was maximum at 25 DAI in FAI-19 and 20 DAI in KAN-6. The concentration of viral DNA was higher when both DNA A and DNA B constructs were co-inoculated and it was observed in all the three infectious clones (Fig. 4a). In potato, the viral DNA was very low (2.09×10^2 to 2.8×10^3 copies in 20 ng of total DNA) in Kufri Bahar inoculated with all three isolates (Fig. 4b). Maximum viral DNA was observed in Kufri Pukhraj inoculated with MOD-21 (8.25×10^{13}) followed by KAN-6 (6.64×10^{12}) and FAI-19 (4.8×10^6). The results of the study supports the earlier findings that Kufri Bahar produce no/mild symptom with very low virus load (Jeevalatha et al. 2017b).

Conclusion

Infectious clones of three ToLCNDV isolates (KAN-6, MOD-21 and FAI-19) produced dissimilar symptoms in both tobacco and potato, indicating the association of ToLCNDV isolates of varying symptomatology with apical leaf curl disease of potato. The infectious clones of this study could serve as study material to explore the changes in the genomic components that led to infection in potato and also the agro-inoculation method standardized in this study will be useful in screening of hybrids and germplasms for ToLCNDV resistance.

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Author contributions AJ—conceptualization, AJ and PK—sample processing, cloning and sequencing of KAN-6 isolate, AJ—development of infectious clones AJ and AK—agro-inoculation experiments in *N. benthamiana,* VG, SS, AJ and RK—agro-inoculation experiments in

potato, AJ, PK, SS and RK—qPCR analysis, AJ, SS, RK and SKC helped in preparing and editing the manuscript.

Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethical statement This article does not contain any studies with human participants or animals performed by any of the author.

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