Project ID 451

Competitive Research Grant

Sub-Project Completion Report

on

Molecular Identification of the Tomato Leaf Curl Virus (ToLCV) Resistant / Tolerant Tomato Plants

Project Duration

May 2016 to September 2018

Plant Breeding and Biotechnology Laboratory,
Department of Botany, University of Dhaka, Dhaka-1000

Submitted to
Project Implementation Unit-BARC, NATP 2
Bangladesh Agricultural Research Council
Farmgate, Dhaka-1215

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Molecular Identification of the Tomato Leaf Curl Virus (ToLCV) Resistant / Tolerant Tomato Line
Project Implementation Unit
National Agricultural Technology Program-Phase II Project (NATP-2)
Bangladesh Agricultural Research Council (BARC)
New Airport Road, Farmgate, Dhaka -1215
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Acronyms

The Following abbreviations have been used throughout the text:

BARI	:	Bangladesh Agricultural Research Institute
BBS	:	Bangladesh Bureau of Statistics
BLAST	:	Basic Local Alignment Search Tool
bp	:	Base pair
C	:	Centigrade/ Celsius
$CaCl_2$	:	Calcium chloride
cm	:	Centimeter (s)
CP	:	Coat Protein
CR	:	Common Region
DNA	:	Deoxyribo Nucleic Acid
e.g.	:	Example gratia, for example
et al.	:	*et alil* and others
etc.	:	et cetra, and the rest
FAOSTAT	:	Food and Agriculture Organization Statistics
Fig/s	:	Figure / Figures
Fwd	:	Forward
g/gm	:	gram (s)
GUS	:	β-glucoronidaseA
ha (s)	:	Hectare (s)
HCl	:	Hydrochloric acid
i.e	:	id est=which to say in other words
I.U.	:	International Units
IR	:	Intergenic region
Kb	:	Kilo base pair
Kg	:	Kilogram
Km	:	Kilometer
KNO_3	:	Potassium nitrate
LB	:	Luria Broth
LlR	:	Large intergenic region
m	:	Meter (s)
M	:	Molar
MW	:	Molecular Weight
mg / l	:	Milligram per liter
mg	:	Milligram
min (s)	:	Minute (s)
ml (s)	:	Milliliter (s)

mM	:	Millimolar
MT	:	Metric Ton
NaOH	:	Sodium hydroxide
NCBI	:	National Center for Biotechnology Information
NH_4NO_3	:	Ammonium nitrate
nm	:	Nanometer
No.	:	Number
NSP	:	Nuclear shuttle protein
nt	:	Nucleotides
OD	:	Optical density
PCR	:	Polymerase chain reaction
pH	:	Negative logarithm of hydrogen ion
RbCl	:	Rubidium chloride
RCA	:	Rolling circle amplification
Rev	:	Reverse
RFLP	:	Random fragment length polymorphism
RNA	:	Ribonucleic acid
SDS	:	Sodium dodecyl sulfate
SIR	:	Small intergenic region
SS	:	Single stranded
ToLCV	:	Tomato Leaf Curl Virus
ToLCD	:	Tomato Leaf Curl Disease
TYLCV	:	Tomato Yellow Leaf Curl Virus
UV	:	Ultraviolet
v/v	:	Volume by volume
w/v	:	Weight by volume
Wt.	:	Weight
%	:	Percentage
+ve	:	Positive
µ	:	Micron
µg	:	Microgram
µl	:	Microlitre
µM	:	Micromole

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Executive Summary

A number of tomato germplasm collected from Plant Genetic Resource Centre, BARI, were found to be resistant and the others were infected by ToLCV while being grown in the experimental field of the Department of Botany, University of Dhaka. These observations have provided some clue to the presence of resistance/tolerance genes in the genome which required further experimental validation. Molecular confirmation of the presence of ToLCV in the infected and asymptomatic tomato plants indicated their susceptibility and tolerance to the disease. However, to claim that asymptomatic germplasm are resistant required showing the presence of resistance gene/s (Ty genes) as well as maintaining disease free condition while infected with the pathogen. For this reason, the identification of resistance/tolerance genes (Ty) in their genome were carried out through Ty gene specific PCR and finally, by creating artificial disease condition to prove the resistance/tolerance potentiality both at phenotypic level as well as molecular level. The tomato plants, successfully identified as resistant/tolerant against ToLCV can be used as donor parents in conventional breeding programs towards the development of tomato plants conferring resistance/tolerance against ToLCD.

CRG Sub-Project Completion Report (PCR)

A. Sub-project Description

1. Title of the CRG sub-project: Molecular Identification of the Tomato Leaf Curl Virus (ToLCV) Resistant / Tolerant Tomato Plants
2. Implementing organization: Department of Botany, University of Dhaka, Dhaka-1000
3. Name and full address with phone, cell and E-mail of PI/Co-PI (s):

   Professor Dr. Mohammad Nurul Islam, Plant Breeding and Biotechnology Lab, Department of Botany, University of Dhaka, Dhaka-1000. Mobile No: 01911308364,

   Email: mnurul@du.ac.bd

   Professor Dr. Rakha Hari Sarker, Plant Breeding and Biotechnology Lab, Department of Botany, University of Dhaka, Dhaka-1000. Mobile No: 01711547429,

   Email: rhsarker@yahoo.co.uk
4. Sub-project budget (Tk):
   - 4.1 Total: 1500000.00
   - 4.2 Revised (if any): N/A
5. Duration of the sub-project:
   - 5.1 Start date (based on LoA signed): 15 May 2017
   - 5.2 End date: 30 September 2018

Justification of undertaking the sub-project: Tomato (*Solanum lycopersicum* L.) is the 3rd most important vegetable crop in Bangladesh and ranking forth in the world. In Bangladesh during the year 2013-14 net tomato production was recorded 251,000 tons and yield was 95,437 hectogram/hectare of land (FAOSTAT, 2015). The average production of tomato is remarkably low (3798 kg/acre) in Bangladesh compared to other countries (BBS, 2011-2012)such as India (8226.4 kg/acre), China (20196.24 kg/acre) and USA (37929.8 kg/acre) (FAOSTAT 2012) etc and this attributed production loss is due to its susceptibility to various pests and diseases caused by virus, bacteria, fungi and nematodes. Where an early infection of tomato plant by begomoviruses can cause 100% yield loss (Lapidot et al. 1997). Tomato yellow leaf curl virus (TyLCV) and Tomato leaf curl virus (ToLCV) are the two important begomoviruses responsible for this damage. Six genes responsible for TYLCV (Zamir et al. 1994; Ji and Scott 2006 and Hanson et al. 2006) and ToLCV (Maruthi et al. 2003) resistance/tolerance have been identified in tomato plants using SSR markers and named as Ty genes such as Ty1, Ty2, Ty3, Ty3a, Ty4 and Ty5 etc. Resistance to tomato infecting begomoviruses has been successfully introgressed from *Solanum pimpinellifolium, Solanum peruvianum, Solanum chilense and Solanum habrochaites* (Ji et al.

2007b). From these sources, a few resistance genes have been well characterized and mapped using molecular markers. A partially dominant major resistance gene, Ty-1, was mapped to the short arm of chromosome 6 *S. chilense* accession LA1969 (Zamir et al. 1994). A major resistance QTL derived from *S. pimpinellifolium* (Hirsute-INRA) was mapped to a different position on chromosome 6 (TG153-CT83; Chague et al. 1997). Hanson et al. (2000) mapped a dominant resistance gene, Ty-2, in *S. habrochaites* derived line H24, to the short arm of chromosome 11. A partially dominant major gene, Ty-3, derived from *S. chilense* (LA2779 and LA1932), was mapped to chromosome 6 (Ji et al. 2007a). Through fine mapping it was demonstrated that Ty-1 and Ty-3 are allelic and code for an RNA-dependent RNA polymerase (Verlaan et al. 2013). The Ty-4 gene was mapped to the long arm of chromosome 3. A recessive resistance gene (ty-5) was identified on chromosome 4 in the plants derived from cultivar Tyking (Hutton et al. 2012). Ty-6, a major begomovirus resistance gene on chromosome 10 (Gill et al. 2010). Most of these resistance sources are known to support virus replication. However, the level of virus accumulation is lower than the levels in susceptible cultivars (reviewed in Prasanna et al. 2014). Similarly, a low level of virus accumulation and a positive correlation between virus level and disease severity were found in Ty-2-carrying plants (Barbieri et al. 2010). So, confirming of the presence of different Ty genes linked to the resistance/tolerance against ToLCV might open a new avenue for the development of resistant/tolerant tomato lines.

6. Sub-project goal: Characterization of local tomato germplasm carrying ToLCV resistance/tolerance gene/s using advanced molecular techniques.
7. Sub-project objective (s):
    a) Screening of Tomato Leaf Curl Virus (ToLCV) resistant/tolerant germplasm through rolling circle amplification (RCA) analysis.
    b) Identification of the resistant/tolerant plants using molecular markers (SSR) linked to ToLCV resistance/tolerance.
    c) Molecular confirmation of the ToLCV resistance/tolerance by agro-inoculation of the ToLCV infectious clone into the promising tomato lines.
8. Implementing location (s): Plant Breeding and Biotechnology Lab, Department of Botany, University of Dhaka, Dhaka-1000.
9. Materials and Methodology in brief:

Materials: Germplasm of seven tomato accessions and 2 BARI tomato varieties (table 1) used in this experiment were obtained through the courtesy of Plant Genetic Resource Centre (PGRC), Bangladesh Agricultural Research Institute (BARI), Gazipur, Bangladesh. These tomato germplasm were maintained in the field of Botanic Garden, Department of Botany, University of Dhaka for subsequent analysis (Fig.

1 and 2). Natural environment was maintained with no chemical interference through fertilizers or pesticides but only composts were added. Chemical pesticides were avoided to allow the prevalence of whitefly population for transmission of the virus to the tomato leaves. The leaf samples were collected after their morphological observations and genomic DNA was isolated for subsequent analysis. Rolling Circle Amplification (RCA) experiment was conducted to confirm the ToLCV infection. Polymerase Chain Reaction (PCR) was carried out to confirm the presence of six different Ty genes; Ty-1, Ty-2, Ty-3, Ty-3a, Ty-4 and Ty-5 using gene specific primers (Table 2).

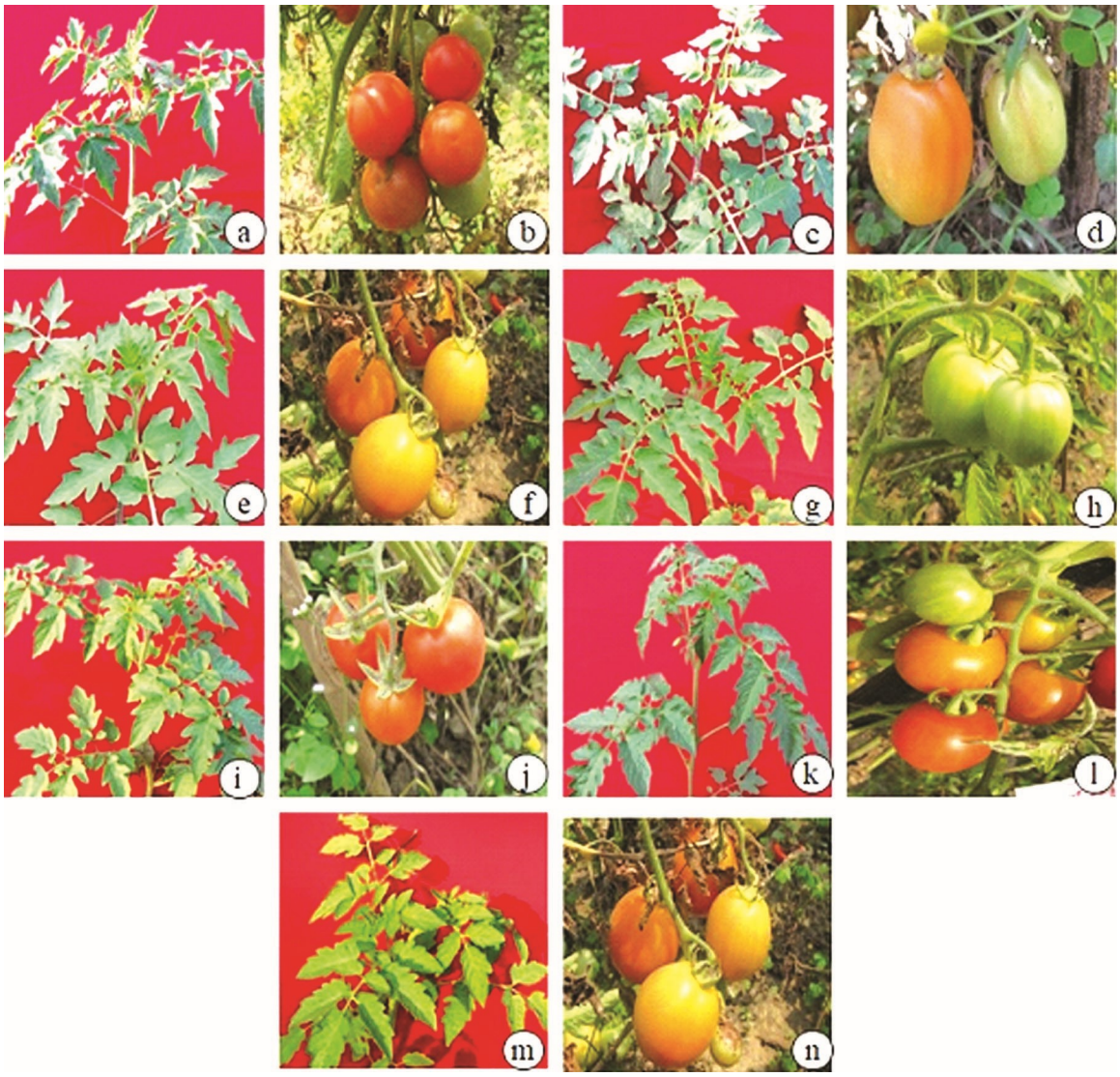

Figure 1. Tomato germplasm used in this study a) and b) showing plant and fruits of BD10124, respectively; c) and d) showing plant and fruits of BD10122, respectively; e) and f) showed plant and fruits of BD7755, respectively; g) and h) showing plant and fruits of Local Kushtia-1, respectively; i) and j) indicating plant and fruits of BD10125, respectively; k) and l) indicating plant and fruits of BD10123, respectively; m) and n) indicating plant and fruits of BD7761, respectively.

The plant/s showing resistant characters in the field with negative RCA results and presence of all six genes tested for, were selected for further analysis through sequencing of the PCR amplified products of three Ty genes. In addition, a second generation was raised from the selected plants to test for the stability of the Ty genes and their resistant characteristics.

Table 1. List of tomato germplasm cultivated for the present experiment.

Name/ Acc. No. of the germplasm	Origin	Number of plant plants/germplasm studied in 2016-17 session	Number of plant plants/germplasm studied in 2017-18 session
BD-10125	PGRC, BARI	33	48
BD-10124	PGRC, BARI	20	36
BD-10123	PGRC, BARI	47	60
BD-10122	PGRC, BARI	20	36
BD-7755	PGRC, BARI	19	60
BD-7761	PGRC, BARI	31	72
Local Khustia-1	PGRC, BARI	48	48
BARI tomato-8	HRC, BARI	16	24
BARI tomato-14	HRC, BARI	16	24
Total number of samples		250	408

PGRC = Plant Genetic Resource Centre, HRC= Horticulture Research Centre, BARI= Bangladesh Agricultural Research Institute

Cloning vector: pGreen0029, pBI121.

Rolling Circle Amplification Kit.

pGreen FWD primer: CTCTTCGCTATTACGCCAGC

pGreen Rev primer: GTGAGCGGATAACAATTTCAC

Methodology:

In order to achieve the proposed objectives following experiments were conducted during this research program-

i) **Selecting Primers**: Literature survey was conducted to select suitable primers for respective SSR markers (of the Ty genes)- The primers selected after literature survey are summarized in the table 2.

ii) **Collection of leaf samples**: Both symptomatic and asymptomatic tomato leaf samples were collected from the experimental field, where germplasm of seven different local tomato accessions and two varieties (BARI Tomato-8 & 14) were grown without the application of any insecticides.

iii) **DNA isolation:** Isolation of DNA from the collected leaf samples was conducted following modified CTAB method (Islam et al. 2012) and stored at -20 °C for future analysis.

Table 2. List of the primers of the respective Ty genes selected based on literature survey.

Name of the Marker	Primer Name	Sequence	Expected amplicon size	Reference
Ty-1	JB-1	*F 5'AACCATTATCCGGTTCACTC 3'* *R 5'TTTCCATTCCTTGTTTCTCTG 3'*	900 bp	De Castro et al. 2007
Ty2	TG0302F/TY2R1	*F 5'TGGCTCATCCTGAAGCTGATAGCGC 3'* *R 5'TGAT(T/G)TGATGTTCTC(T/A)TCTCT(C/A)GCCTG 3'*	450 bp	Brenda et al. 2007
Ty3	FLUW 25	*F 5'CAAGTGTGCATATACTTCATA(T/G)TCACC 3'* *R 5'CCATATATAACCTCTGTTTCTATTTCGAC 3'*	450 bp	Melinda et al. 2007
Ty3a	P6-25	*F 5' F 5'GGTAGTGGAAATGATGCTGCTC 3'* *R 5'GCTCTGCCTATTGTCCCATATATAACC 3'*	320bp	Jenseen et al. 2007
Ty4	C2_AT5g51110	*F 5' TGGTGGAAGGCACAGGGCAC 3'* *R 5' TCTTTACTTGATCTATTTTAGCAGC 3'*	325 bp	SGN 2008
Ty5	AVRDC-TM719/ AVRDC-TM273	*F 5' TCGATTTGGAATGAGTTTTC 3'* *R 5' TGAAATAGATTTGTCAGGTGTT 3'* *Or* *F 5' GGTGCTCATGGATAGCTTAC 3'* *R 5' CTATATAGGCGATAGCACCAC 3'*	237 bp Or 180/173 bp	Chen H M et al. 2015

IV) Rolling Circle Amplification (RCA) analysis: ToLCV is a monopartite of bipartite circular single stranded DNA virus, RCA analysis (Fire and Xu 1995) was performed to confirm the presence of ToLCV using TepmliPhi superscript kit manufactured by GE-Healthcare. The protocol is given below-

Protocol for Rolling Circle Amplification analysis

- 5 µl aliquots of sample buffer were transferred to an eppendorf tube.
- 1 µl sample was added to the dispensed sample buffer.
- The samples were denatured by heating at 95 ˚C for 3 min. and then cooled down to room temperature or 4˚C.
- By this time TempliPhi premix was prepared in a separate tube by combining 5µl of reaction buffer and 0.2 µl enzyme mix for each TempliPhi reaction. Master mixture was prepared for multiple samples.
- 5 µl of the TempliPhi premix was transferred to the cooled, denatured sample and incubated at 30 ˚C for 18 hrs.
- Heat-inactivation of the enzyme was done by incubating at 65˚C for 10 min and then cooled to 4˚C.

- The RCA products were digested with BamHI enzyme and separated in 1% agarose gel for visualization and documentation.

Figure 2. Cultivation of tomato germplasm in the field of Botanical garden, Department of Botany, University of Dhaka.

v) Molecular marker analysis: Polymerase chain reaction (PCR) experiments were carried out using isolated DNA as template, primers (mentioned in the selection of primers sections) specific for six different molecular markers (SSR) termed as Ty genes linked to ToLCV resistance/tolerance and GoTaq Green PCR master mixture manufactured by Promega.

Protocol for PCR-

a) Master mixture was prepared by mixing all of the PCR components for desired number of samples. The volume of the PCR reaction was set to 25 µl.

b) In the master mixture both forward and reverse primers (100 pmol/primer) along with 200 µM of dNTP and 0.5 unit of Taq DNA polymerase was used for PCR.

c) Master mixture was aliquoted in desired number of PCR tubes and 50ng DNA sample was added before starting the PCR.

d) PCR program was used here based on the Tm of particular primer pairs mentioned in the table 3.
e) PCR amplified DNA was separated under 1% agarose gel and the amplified product was visualized and documented in a gel documentation system.

Table 3. Molecular markers used for PCR and their conditions.

	PCR step	**Ty-1**	**Ty-2**	**Ty-3**	**Ty-3a**	**Ty-4**	**Ty-5**
	Initial denaturation	95°C for 5 minutes					
30 Cycles	Denaturation	94°C for 1 minute					
	Annealing	55°C for 30 seconds	56°C for 30 seconds	53°C for 30 seconds	53°C for 30 seconds	51°C for 1 minute	55°C for 1 minute
	Extension	72°C for 1 minute					
	Final extension	72°C for 10 minutes					

vi) DNA Sequencing of the cloned ToCV isolate genome and amplified Ty genes: Polymerase chain reaction using an proof reading DNA polymerase (Phusion, Thermo Scientific) and vector specific primers was performed to amplify the cloned 2.7 kb viral DNA. Similarly, Ty genes were also amplified using gene specific primers. PCR products were purified and sent for automated sequencing in MCLab USA.

vii) BLAST search analysis: The sequences obtained from the sequencing reaction for ToLCV geneome and Ty gene identification was analyzed though NCBI-BLAST program. BLAST is a well know similarity search program with different search options for analyzing query sequence, in this case nucleotide BLAST (BLASTn) was used.

viii) Construction of an infectious clone of a monopartite ToLCV genome: Before attempting the construction of the infectious clone the full-length genome of a monopartite ToLCV was cloned and sequence characterized earlier in the same research group. The construction of ToLCV infectious clone was attempted with some modifications of the protocol mentioned in the original research proposal. The following procedure was used for the construction of ToLCV infectious clone -

Step I: To develop the infectious clone at first a 300 bp part of the ToLCV genome containing the origin of replication and other elements required for replication initiation (also called Common Region or CR)

was cloned using BamHI and EcoRI restriction sites in the pGreen vector. This has generated CR/pGreen vector. Then the full genome of ToLCV was cloned using BamHI restriction site in the CR/pGreen vector. The expected orientation of the ToLCV genome in contrast to the already cloned CR was confirmed by restriction digestion of EcoRI enzyme, which was selected, based on the restriction map analysis of the ToLCV genome sequence using Bioedit software. The positive clone was used to maintain the infectious clone and sub-clone into the pBI121 vector.

Step-II: The infectious clone of the ToLCV was fished out using HindIII and SacI restriction enzymes from the pGreen background. The destination vector, pBI121 was also linearized with the same restriction enzymes and cloned in *E. coli* Top10 competent cells following ligation. Positive clones were confirmed though restriction digestion and PCR using coat protein gene specific primers, which yielded ToLCV infectious clone/pBI121 vector.

Step III: The LBA4404 strain of *Agrobacterium tumefacians* was transformed with ToLCV infectious clone/pBI121 vector for resistance/tolerance assay.

ix) Resistance/tolerance assay: The tomato plants identified based on phenotypic and molecular analysis as well as the presence of Ty genes linked to resistance/ tolerance was agro-inoculated using the LBA4404 strain of *Agrobacterium tumefacians* having no plasmid vector, carrying pBI121 vector or infectious clone/pBI121 vector. The protocol for agro-inoculation is given below-

1. For agro-inoculation, glycerol stocks were streaked on solid LB plates containing 50mg/ml of kanamycin and 25mg/ml of each of the two antibiotics (streptomycin and rifampicin) and incubated at 28 °C for 48 hours.
2. A single colony of bacterial cell was inoculated to 6 ml LB broth containing kanamycin and rifampicin and placed at 28 °C in shaker incubator for 48 hours and 1ml culture was diluted in 100 ml LB medium containing required amount antibiotics and incubated at 28 °C with continuous shaking.
3. Cells were harvested by centrifugation at 9500 rpm at 4°C for 15 min.
4. For infiltration, 3 volume of induction buffer (10 mM CaCl2 and 100µM acetosyringone) were added to the cells and incubated at 28 °C in shaker incubator till OD600 reached 1. Different dilution degrees must be tried in order to achieve the correct one for our purpose. An OD600 ~0.2 is enough to infect *N. benthamiana*.
5. The activated *Agrobacterium* -inoculum was used to infiltrate 2-3 fully extended leaves per plant at the 5 to 6 leaf stage using 5ml needless syringe

6. Followed the same procedure to infiltrate the control group

After agro-inoculation, plants were maintained in controlled condition in a plant growth chamber for proper growth and development. Care was taken so that the no spillage has happened during infiltration. Leaf samples were collected for 5, 10, 15 dpi with the observation of symptom development (if any) for subsequent analysis. DNA and RNA were isolated from the agro-inoculated leaf samples for a) RCA analysis to prove the multiplication rate of the virus and b) to analyze the Ty gene expression. Finally, the remaining agro-inoculated plants were burned to ash.

Results and Discussion

Tomato originated in Western South America from where it spread to Europe and Asia. Regardless of its origin, PGRC BARI, has collected some tomato plants growing in different areas of Bangladesh. Previous studies reported the presence of Ty genes in wild tomato species, that could be introgressed into cultivated tomato plants to enhance ToLCV resistance and tolerance (Zamir et al. 1994, Hanson et al. 2006, Ji et al. 2007, Anbinder et al. 2009, Verlaan et al. 2011, Hutton et al. 2012). With this information, this study was carried out to identify the tomato germplasm collected by PGRC, BARI towards the resistance or tolerance potentiality against tomato leaf curl virus (ToLCV) infection.

Objective 1: Screening for Tomato Leaf Curl Virus (ToLCV) resistant/tolerant germplasm through Rolling Circle Amplification (RCA) analysis

To achieve the goal of this objective various experiments were conducted and the results are described below-

1.1 Field Detection of Tomato Leaf Curl Virus infection

Field screening for tomato leaf curl disease of the tomato plants were carried out based on the appearance of typical tomato leaf curl disease (ToLCD) symptoms; inter-veinal chlorosis, stunting, leaf curling, flower drop and reduced fruit yield representing very low number of fruits (Fig. 3). These symptoms were then used to score the disease employing a modified arbitrary scale as described by Muniyappa et al. (1991). The scoring criteria are mentioned below-

Resistant (R) = No symptoms

Mild infection (M) = Slight yellowing, slow growth but no leaf curl

Moderate infection (Mo) = Yellowing, stunting and mild leaf curl

Susceptible (S) = Severe stunting, severe curling and no or very less yield

The results of disease scoring are summarized and out of the 658 plants observed, 20 were found to be resistant, 23 were mildly infected, 62 were moderately infected, 10 were susceptible and 9 showed wilting. Disease score for 124 plants have been presented in Table 4. Since, this score representing arbitrary results it needs further confirmation at molecular level because plants showing no symptoms within the same accessions are simply escapes. During the filed observation BARI Tomato -8 and BARI Tomato- 14 were found to be susceptible towards ToLCV infection as showing severe stunting and leaf curling symptoms, hence not included in the table.

Table 4. Disease scores of different Plants from 7 accessions of the tomato germplasm detected in the field.

Plant no.	Disease score in different germplasm						
	BD10124	BD10122	BD7755	Local Khushtia-1	BD10125	BD10123	BD7761
1	**R**	W	M	**R**	M	Mo	S
2	**R**	S	M	**R**	Mo	Mo	Mo
3	Mo	W	M	M	S	Mo	Mo
4	Mo	W	M	M	S	Mo	S
5	Mo	Mo	Mo	Mo	Mo	Mo	S
6	Mo	W	S	Mo	Mo	Mo	Mo
7	W	Mo	Mo	S	Mo	Mo	Mo
8	Mo	W	S	Mo	Mo	Mo	Mo
9	M	W	S	M	Mo	Mo	S
10	M	**R**	M	Mo	Mo	S	S
11	**R**	M	M	**R**	Mo	Mo	S
12	**R**	**R**	Mo	**R**	M	Mo	S
13	**R**	M	S	**R**	Mo	Mo	S
14	**R**	**R**	M	**R**	Mo	Mo	S
15	Mo	Mo	**R**	M	Mo	Mo	Mo
16	M	W	**R**	Mo	M	-	-
17	**R**	Mo	**R**	**R**	Mo	-	-
18	Mo	S	M	M	M	-	-
19	Mo	W	Mo	-	-	-	-
20	-	M	-	-	-	-	-

'R' Resistant 'M' Mild infection 'Mo' Moderate infection 'S' Susceptible 'W' Wilted

1.2 Molecular analysis of the ToLCV infection in seven accessions of tomato germplasm

During the field observation, 20 out of 250 Plants belonging to all seven accessions of tomato germplasm; BD10124, BD10122, BD7755, Local-Khushtia-1, BD10125, BD10123 and BD7761, analyzed during the study, were recorded with resistant characters and others showed mild, moderate or susceptible symptoms. So it was necessary to confirm if these disease symptoms were attributed to ToLCV infection. In order to confirm the ToLCV infection by the presence of viral genomic DNA in the infected leaf, rolling circle amplification (RCA) experiment was performed using genomic DNA isolated from the leaf samples of 124 plants belonging to the seven germplasm were subjected to RCA analysis in order to confirm the ToLCV infection. After digestion of the RCA products with BamHI restriction enzyme, 11 susceptible scored plants showed amplification of a DNA band of ~2.7 kb as expected.

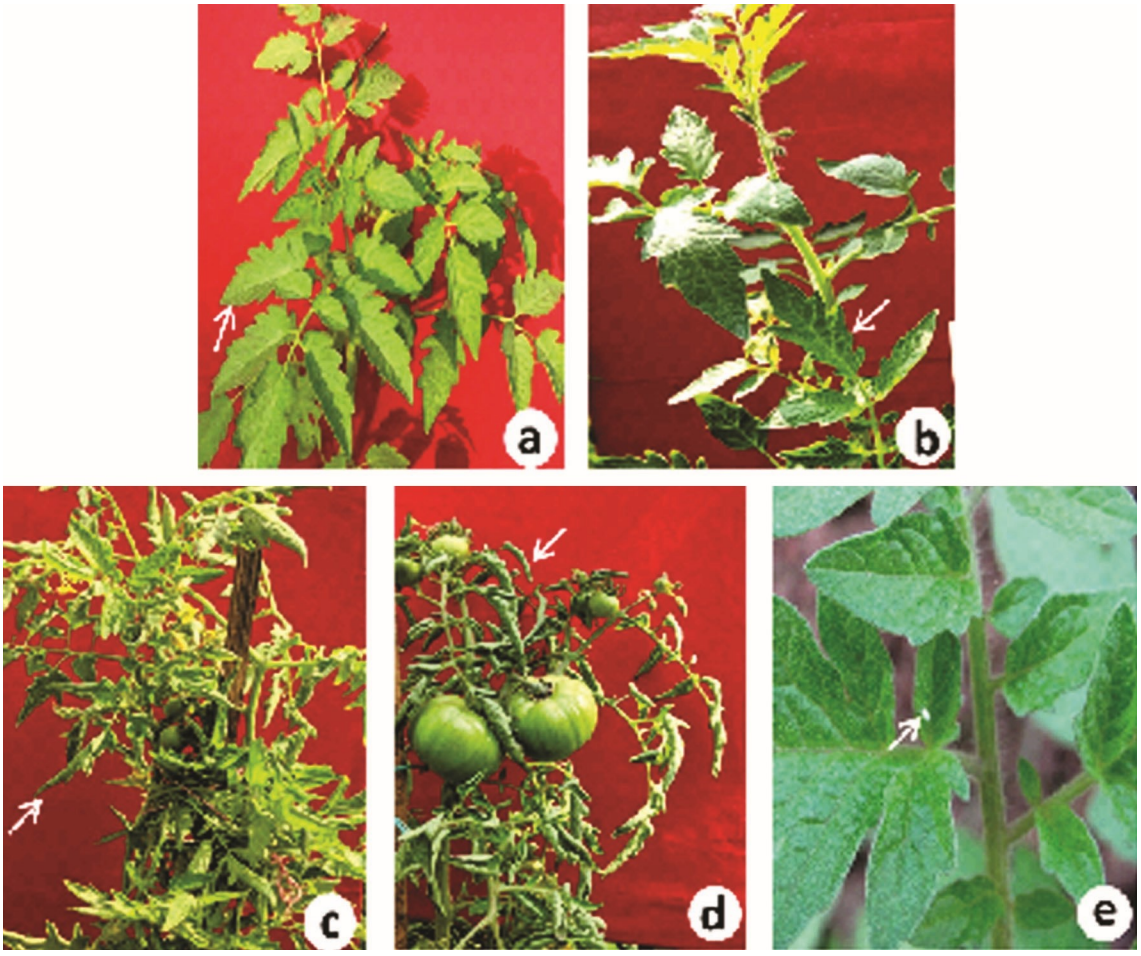

Figure 3. Tomato Plants showing different types of leaf curl disease symptom. a) Plant showing ToLCV resistant characters. Arrow shows healthy leaf, b) Plant showing mild infection symptoms. Arrow shows leaf with interveinal chlorosis, c) Plant showing moderate infection symptoms. Arrow shows leaf with slight curling, d) Plant showing susceptible infection symptoms. Arrow shows leaf with severe curling, e) Whitefly, *Bemisia tabaci* (arrow) on tomato leaf in the field.

The appearance of ~2.7 kb (in 1.0% agarose gel) DNA band indicated the presence of tomato leaf curl viral genome in the infected leaves. The plant number 18 from BD10122 scored as susceptible, showed a band of ~1.3 kb besides ~2.7 kb, indicating the presence of viral beta satellite molecule. Plant number 5 from BD7755 and 9 from Local-Khushtia-1, scored as moderate and mild, respectively, showed bands at ~2.1kb and ~600bp, totaling ~2.7kb which could be a different strain of ToLCV with multiple BamH1 restriction sites. In leaf samples from the plants scored as resistant, no band corresponding to ~2.7 kb was observed, as expected but should be validated with the presence of Ty genes (Fig. 4). Five representative BamHI digested RCA products of the plant samples including resistant, mildly infected, moderately infected and susceptible from each tomato Acc. were selected and electrophoresed for comparison. (Fig. 4 and 5).

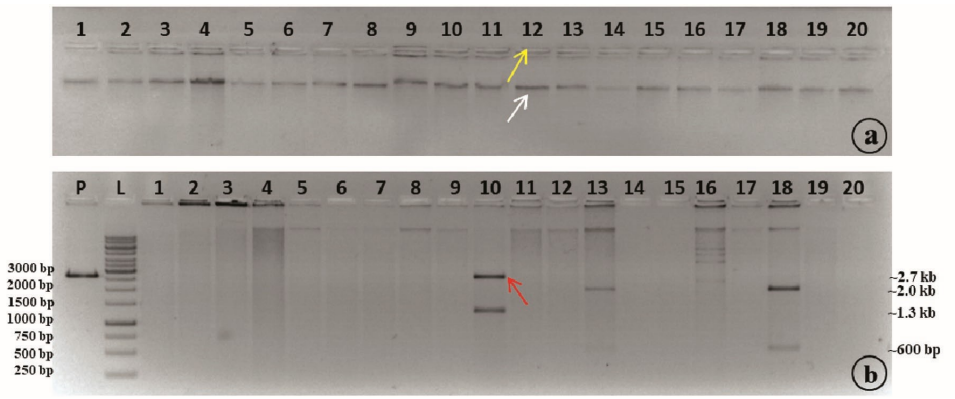

Figure 4. Agarose gel electrophoresis results of- a) Genomic DNA isolated from five plants each of the Acc. BD10124, BD10122, BD7755 and Local-Khushtia-1 respectively in 0.8 % agarose gel. Yellow arrow shows DNA loading well and white arrow shows genomic DNA band; b) RCA products were digested with BamHI and separated on 1.0% agarose gel. A representative gel electrophoresis photo from RCA results of 20 plants from four Acc. is shown here. Lane M is the marker. Lane P is the control & lane 1 to 20 represent the different plant number. Lane 10 for plant number 18 of BD10122 showed ~2.7 kb amplification indicating positive ToLCV infection (red arrow) along with ~1.3kb amplification of beta satellite. Lanes 13 and 18 for plant number 5 from BD7755 and 9 of Local-Khushtia-1, respectively, showed bands at ~2.1kb and ~600bp.

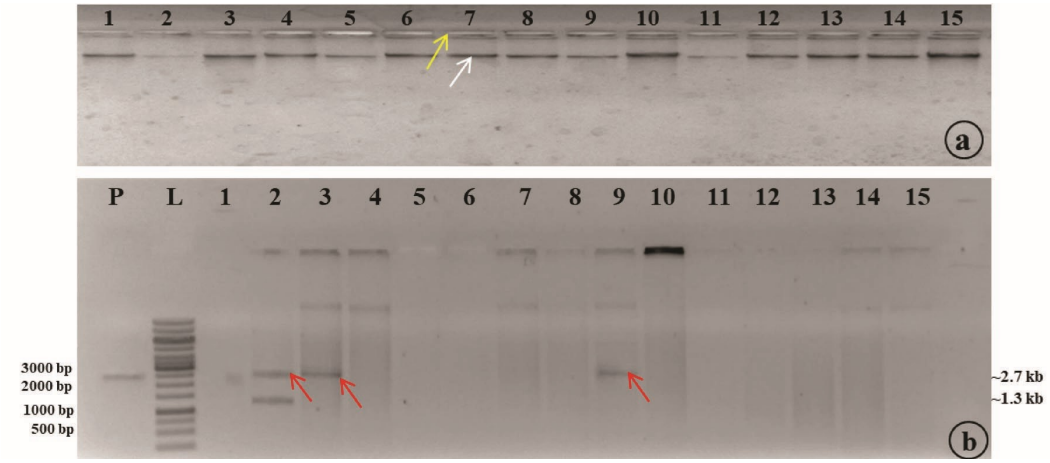

Figure 5. Agarose gel electrophoresis results of- a) Genomic DNA isolated from five plants each of the Acc. BD10125, BD10123 and BD7761 respectively in 0.8% agarose gel. Yellow arrow showed DNA loading well and white arrow showed genomic DNA band; b) RCA products were digested with BamHI and separated on 1.0% agarose gel. A representative gel electrophoresis photo from RCA results of 15 plants from three Acc. is shown here. Lane M is the marker, lane P is the control and lanes 1 to 15 represent the different plant number. Lanes 2, 3 and 9 for plant number 3 and 4 of BD10125 and 10 of BD10123 showed ~2.7 kb amplification indicating positive ToLCV infection (red arrows).

1.3 Cloning of Tomato Leaf Curl Viruses (ToLCVs) full genome into a suitable vector

Tomato leaf curl viruses (ToLCV) contain bipartite genome (DNA-A and DNA-B) or monopartite genome (DNA-A only) and sometimes may contain DNA-β. In order to amplify the ToLCV viral genome from the DNA of the infected tomato leaf samples TempliPhi™ DNA amplification kit based Rolling Circle Amplification (RCA) experiment was performed. The RCA products produced in the reactions were linearized by restriction digestion with fastdigest BamHI (Thermo Scientific) restriction enzyme. After gel electrophoresis the presence of ~2.7 kb band was observed in case of DNA samples

infected with ToLCV. Figure 6 represents the RCA analysis of the plant samples collected from BD10125 accession. The RCA amplified ~2.7 kb band from the plant number 7 (lane 1in Fig. 6) was gel purified by using Gene JET Gel Purification Kit (Thermo Scientific) and subjected to clone into pGreen0029 vector. The vector was digested with fastdigest BamHI (Thermo Scientific) restriction enzyme, which produced a ~4.5 kb band in gel electrophoresis and the linear vector DNA was dephosphorylated by Alkaline phosphatase enzyme (FastAP, Thermo Scientific) to prevent self-ligation.

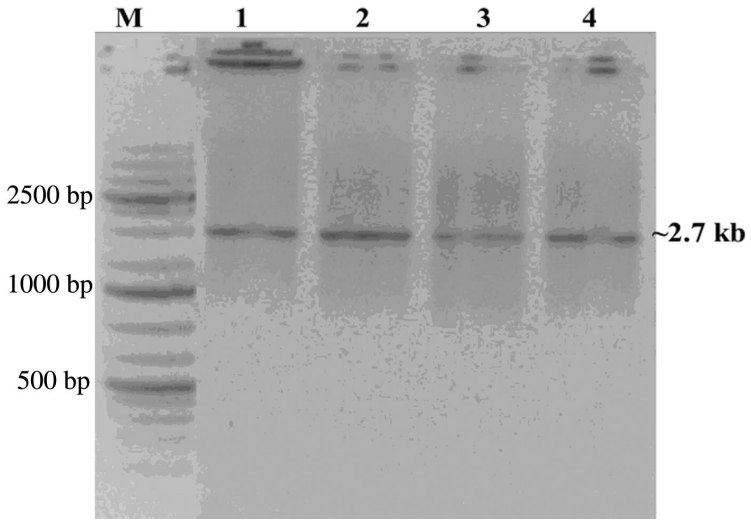

Figure 6. Restriction digestion of RCA products to confirm the presence of viral genome. RCA products of three samples were digested with BamH1 and separated on 1% agarose gel. Lane M is the marker and lanes 1 to 3 representing the DNA samples isolated from plants 7 to 9 of BD-10125 wild tomato variety and 4 is the RCA positive control. All bands are showing ~2.7 Kb amplification.

The ligation reaction was set for sample by mixing the vector and insert in an eppendorf tube along with buffer and ligase enzyme and incubated for 22 to 30 minutes. The *E. coli* top10 competent cells were transformed with ligation products. After transformation the *E. coli* cells were plated on kanamycin containing LB agar medium to select the bacterial cells as single colony transformed with the ligation products. The colonies were further subjected to colony PCR using vector specific primers and restriction digestion of the isolated plasmid DNA for cloning confirmation. Colony PCR and restriction digestion obtained a 2.7 kb size DNA band in agarose gel electrophoresis confirming cloning of the insert into the vector (Fig. 7a, b). Plasmid DNA from this colony was isolated for sequencing of the ToLCV genome.

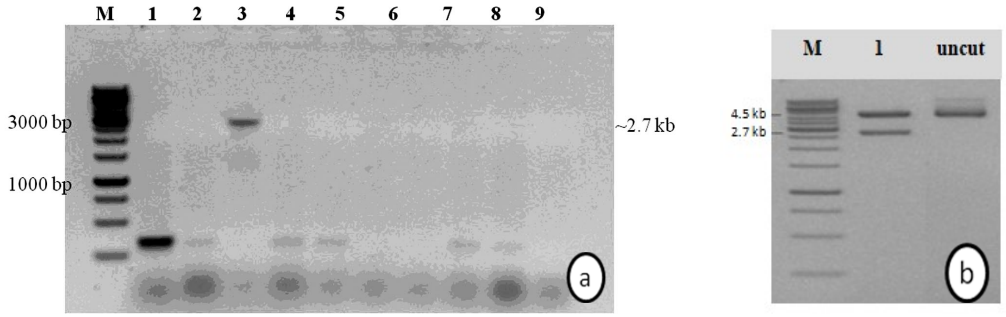

Figure 7. Molecular confirmation of cloning of the ToLCV genome into pGreen 0029 vectot. a) Colony PCR of the cloned isolate was performed using vector specific priemrs, where M= 1kb marker and Lane 3 showed ~2.7 kb amplification and b) restriction digestion with BamHI enzyme and lane 1 showed ~2.7 kb band.

1.4 Sequence analysis of Cloned DNA of the ToLCV isolate

The purified PCR products were sequenced by cycle sequencing method and data for both forward and reverse primer were obtained for the clone.

1.4.1 Sequences of ToLCV obtained by pGreen vector specific forward primer

GGGAAAGGGAAGTGCCTGGCACGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACG
ACGTTGtAAAACGACGGCCAGTGAGCGCGCGTAATACGACTCACTATAGGGCGAATTGGGTA
CCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCCTGCAGCCCGGG
GGATCCCACATTTTGTAATGTGGGAACTTGGGGACCAAGTTTATAGAGGGGACAAGGAAAC
AATTAAGCTTTGAGGGGTGATTTTCATTGGTCCACAGGTCTTTGTCAGTTAGTGCGGTGCGG
GGACCACTTTAAAAAATCGCGGCCATCCGGTAATATTATACGGATGGCCGCTTTGGGAATTT
TGAAATTTGAATTAAAGAACTTTTCAAAATTACATTTATGCCATTTGGAGTCTCCATATATAT
AGGACTCCAATACACCGATACATAAAAACAGTTGAGAGACTCCGATTGACCAAGTCAATAT
GGCAGCCCCCAATTGATTTAAAATAAATGCAAATAACCTCTTTCTCATTTATCCCAAGTGTTT
TCTTTTT

1.4.2 Analysis of the sequence through NCBI-BLAST program

The sequences obtained from the sequencing reaction was analyzed though NCBI- BLAST program. BLAST is a well know similarity search program with different search options for analyzing query sequence, in this case nucleotide BLAST (BLASTn) was used. After analysis of the query sequence BLAST presented a 'hit list' having significant similarity with begomoviruses (Table 4). From the hit list, it was observed that query sequence is having 93% similarity with already reported Tomato Leaf Curl Bangladesh Virus (ToLCBV) isolate, complete genome (Islam et al. 2019).

Table 4. Percent identities (nucleotides) of the isolate ToLCV with selected begomoviruses reported worldwide

Description	Maxs core	Total score	Query cover	E value	Identity	Accession
Tomato leaf curl Bangladesh virus isolate ToLCBV-[BD:Cox:01:15:Tom:06], complete genome	**326**	**595**	**66%**	**4e-85**	**93%**	**KM383765.1**
Tomato leaf curl Bangladesh virus isolate ToLCBV-[BD:Joy:01:05:Tom:06], complete genome	326	595	66%	4e-85	93%	KM383762.1
Tomato leaf curl Bangladesh virus isolate ToLCBV-[BD:Joy:03:08:Tom:07], complete genome	326	600	66%	4e-85	93%	KM383760.1
Tomato leaf curl Bangladesh virus isolate ToLCBV-[BD:Joy:04:09:Tom:07], complete genome	326	589	66%	4e-85	93%	KM383759.1
Tomato leaf curl Bangladesh virus isolate ToLCBV-[BD:Jam:01:39:Tom:10], complete genome	320	583	66%	2e-83	93%	KM383764.1

Previously different strains of tomato leaf curl virus genome were also successfully amplified using RCA based on DNA amplification during the study conducted for the identification of ToLCV (Pandey et al. 2010, Pratap et al. 2011 and Idris et al. 2007, Kamal et al.2015).

From the RCA analysis of the ToLCV infection it was evident that the symptomatic leaf samples were associated with the presence of viral genome and asymptomatic samples didn't contain viral DNA however, some asymptomatic samples were positive for RCA analysis. Absence/presence of viral genome in the healthy leaf samples were suspected towards the inheritance of Ty genes linked to ToLCV resistance/ tolerance. So, analysis of the inheritance of Ty genes in the tomato germplasm of the current study was conducted in the next phase.

Objective 2: Molecular confirmation of the presence of ToLCV resistance/tolerance genes

Genomic DNA isolated from 658 samples from all the nine-tomato germplasm covering both infected and no-infected condition were used in PCR analysis with the forward and reverse primer of specific Ty genes responsible for resistantce/tolerantce against ToLCV. PCR amplified samples were viewed in agarose gel and results for each gene have been presented below-

2.1.1 Molecular confirmation of Ty1 gene

After gel electrophoresis of the PCR products with forward and reverse primer of JB-1a positive ~900 bp were observed (Figure 8). From 658 samples of the 7 local and 2 varieties (BARI-8 and BARI-14) there were 44 samples showing positive band of ~ 900 bp.

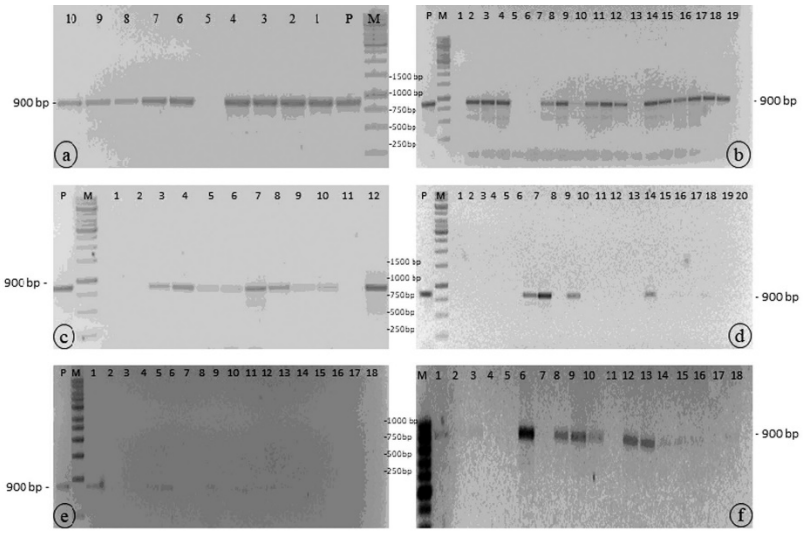

Figure 8. Representative gel electrophoresis photo from the PCR of 658 samples with the forward and reverse primer JB-1 to identify the Ty1 resistance/tolerance gene (M=marker)

2.1.2Molecular confirmation of Ty2 gene

The positive band was found in PCR with the forward and reverse primer of TG0302F/TY2R1. After gel electrophoresis a positive ~450 bp were observed (Figure 9). From 658 samples of the 7 natural and 2 cultivar varieties there are 85 samples showed positive band in ~ 450 bp.

2.1.3 Molecular confirmation of Ty3 gene

The positive band was found in PCR with the forward and reverse primer of FLUW 25. After gel electrophoresis a positive ~450 bp were observed (Figure 10). From 658 samples of 7 local and 2 cultivar varieties there are 90 samples showed positive band in ~ 450 bp.

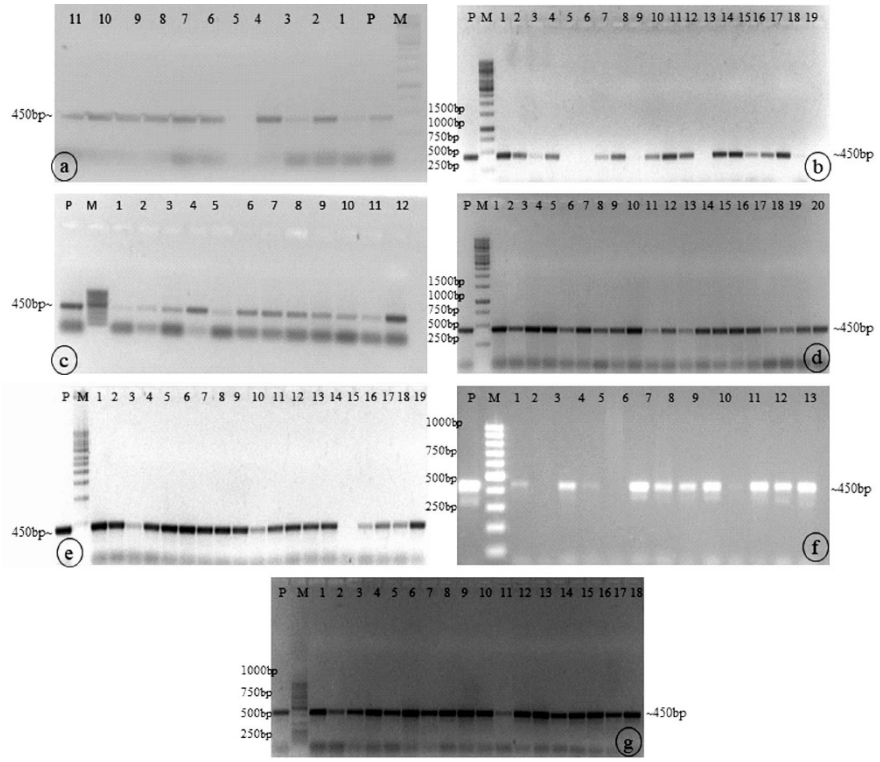

Figure 9. Representative gel electrophoresis photo from the PCR of 658 samples with the primer TG0302F/TY2R1 to identify the Ty2 resistance gene (M=marker)

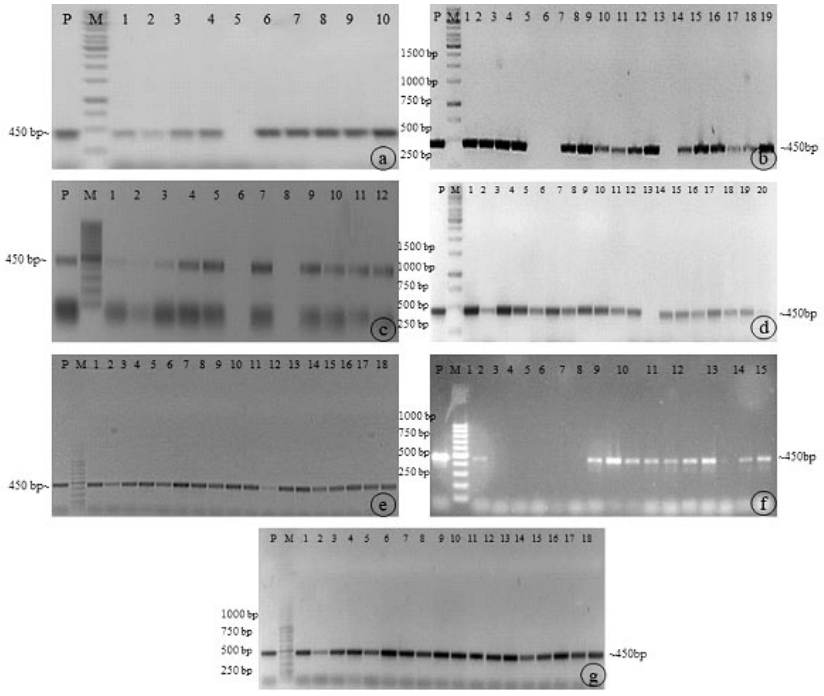

Figure 10. Representative gel electrophoresis photo from the PCR of 658 samples with the primer FLUW 25 to identify the Ty3 resistance/tolerance gene (M=marker)

2.1.4 Molecular confirmation of Ty3a gene

The positive band was found in PCR with the forward and reverse primer of P6-25. After gel electrophoresis a positive ~320 bp were observed (Figure 11). From 658 samples of 7 local and 2 cultivar varieties, there are 87 samples showed positive band in ~ 320 bp.

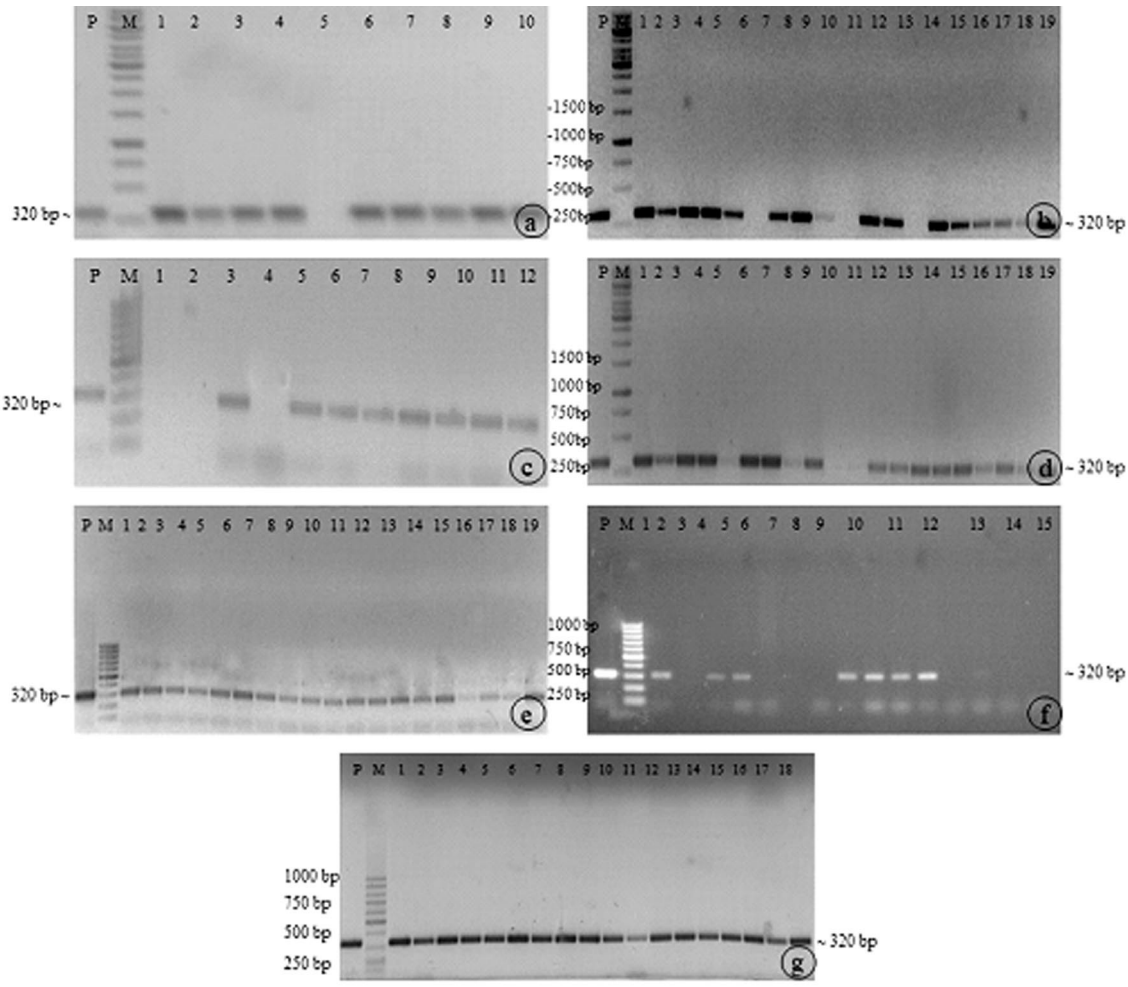

Figure 11. Representative gel electrophoresis photo from the PCR of 658 samples with the forward and reverse primer P6-25 to identify the Ty3a resistant gene (M=marker)

2.1.5 Molecular confirmation of Ty4 gene

The positive band was found in PCR with the forward and reverse primer of C2_At5g62390. After gel electrophoresis a positive ~325 bp were observed (Figure 12). From 658 samples of 7 local and 2 cultivar varieties there are 94 samples showed positive band in ~ 325 bp.

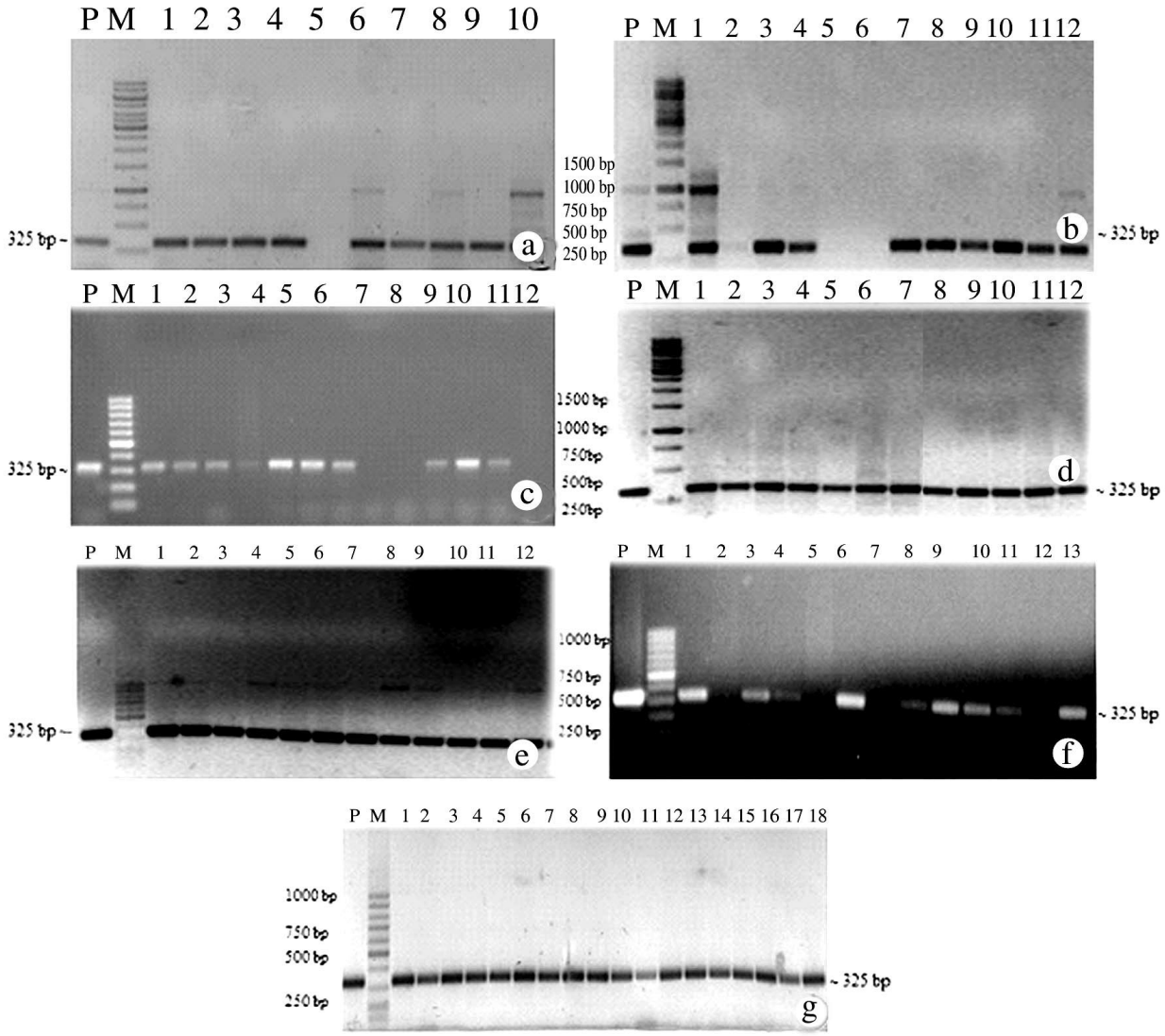

Figure 12. Representative gel electrophoresis photo from the PCR of 658 samples with the primer C2_At5g62390 to identify the Ty4 resistance/tolerance gene (M=marker)

2.1.6 Molecular confirmation of Ty5 gene

The positive band was found in PCR with the forward and reverse primer of AVRDC- TM 273andAVRDC-TM719. After gel electrophoresis a positive ~180 bp or ~ 237 bp were observed (Figure 13). From 658 samples of 7 local and 2 cultivar varieties there are 127 samples showed positive band in ~180 bp or ~237 bp size (Figures 13 and 14)

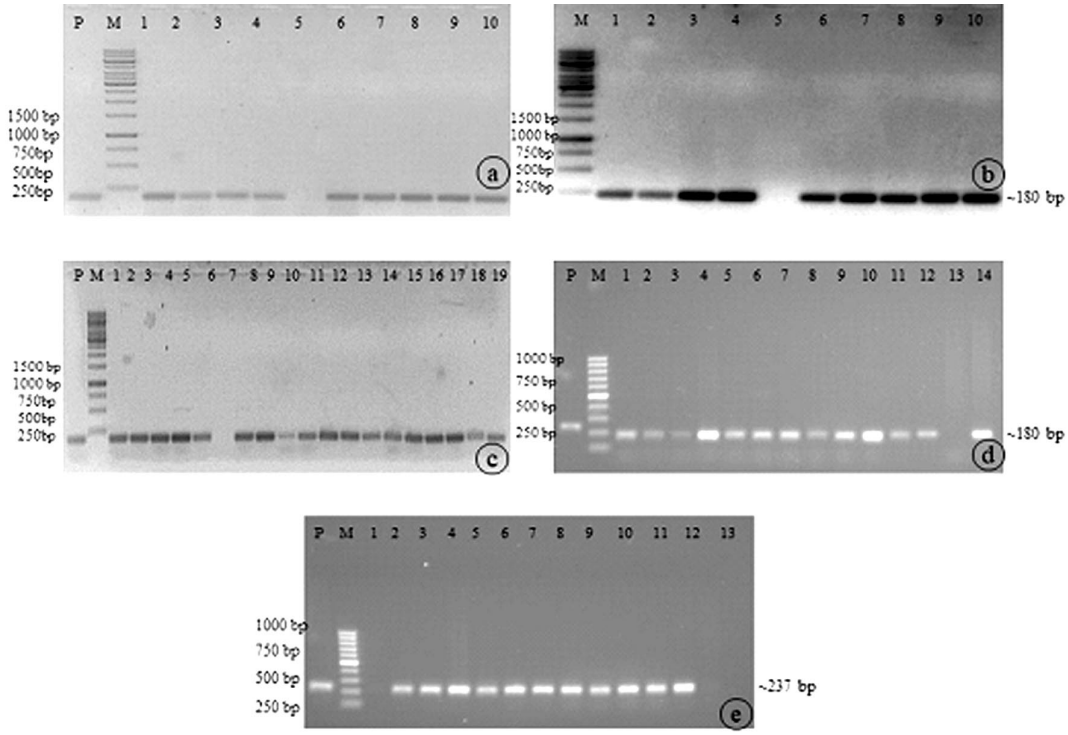

Figure 13.Representative gel electrophoresis photo from the PCR of 658 samples with the forward and reverse primer AVRDC-TM 273 and AVRDC-TM719 to identify the Ty5 resistance/tolerance gene (M=marker)

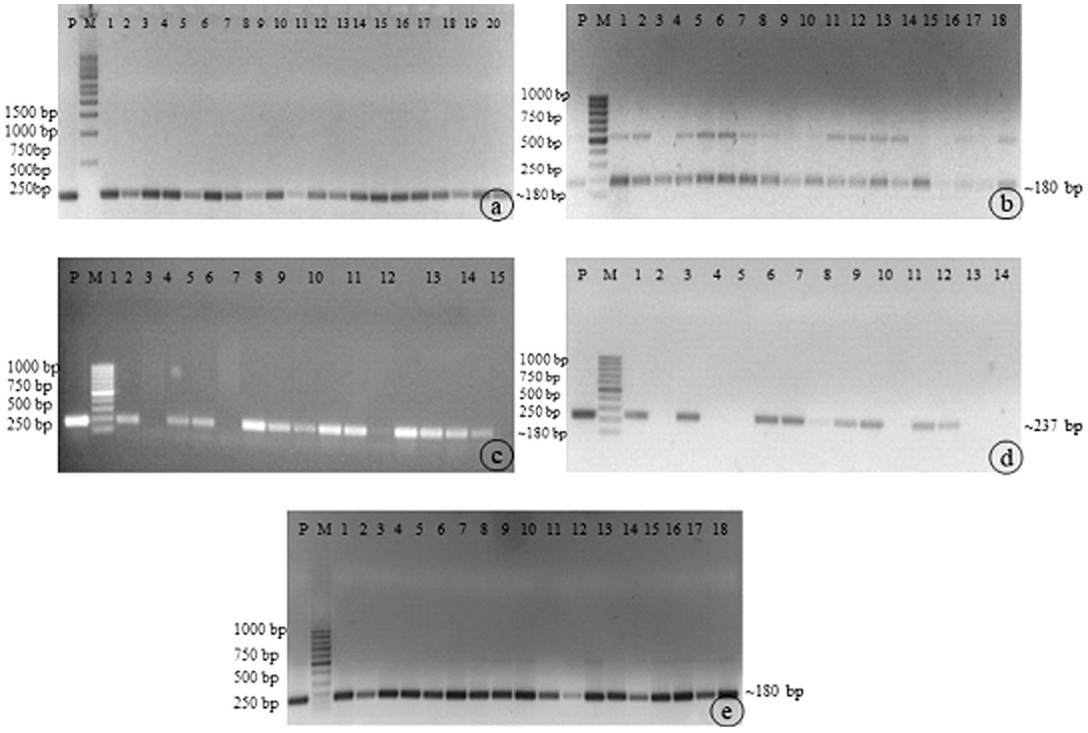

Figure 14.Representative gel electrophoresis photo from the PCR of 658 samples with the forward and reverse primer AVRDC-TM 273 and AVRDC-TM719 to identify the Ty5 resistance/tolerance gene (M=marker).

2.2 Comparative analysis of the presence/absence of Ty genes in different plants of the Seven germplasm

Based on the PCR analysis of the Ty genes and RCA analysis, all the plants of the seven germplasm were further compared with the field observation of their disease scores to relate the Ty gene's mode of action against the pathogen. The results have been summarized from Table 5-11 according to the germplasm. Data have been presented for 19 plants per germplasm in each table.

Table 5. Comparative analysis of resistance in BD10124 germplasm plants based on their disease score, RCA screening and Ty genes

BD10124	Disease score	RCA screening	Ty-1	Ty-2	Ty-3	Ty-3a	Ty-4	Ty-5
1	R	-	-	+	+	+	+	+
2	R	-	+	+	+	+	+	+
3	Mo	-	+	+	+	+	+	+
4	Mo	-	+	+	+	+	+	+
5	Mo	-	-	-	-	+	-	+
6	Mo	-	-	-	-	-	-	-
7	W	-	+	+	+	+	+	+
8	Mo	-	+	+	+	+	+	+
9	M	-	+	-	+	+	+	+
10	M	-	+	+	+	-	+	+
11	R	-	+	+	+	+	+	+
12	R	-	+	+	+	+	+	+
13	R	-	-	-	-	-	-	+
14	R	-	+	+	+	+	+	+
15	Mo	-	+	+	+	+	-	+
16	M	-	+	+	+	+	+	+
17	R	-	+	+	+	+	+	+
18	Mo	-	+	+	+	+	+	+
19	Mo	-	+	+	+	+	+	+

'+' Present and '-' Absent

'R' Resistant 'M' Mild infection 'Mo' Moderate infection 'S' Susceptible 'W' Wilted

Table 6. Comparative analysis of resistance in BD10122 germplasm plants based on their disease score, RCA screening and Ty genes

BD10122	Disease score	RCA screening	Ty-1	Ty-2	Ty-3	Ty-3a	Ty-4	Ty-5
1	W	-	-	+	+	+	+	+
2	R	+	-	+	+	+	+	+
3	W	-	-	+	+	+	+	+
4	W	-	+	+	+	+	+	+
5	Mo	-	-	+	+	+	+	+
6	W	-	-	+	+	+	+	+
7	Mo	-	+	+	+	+	+	+
8	W	-	-	+	+	+	+	+
9	W	-	+	+	+	+	+	+
10	R	-	-	+	+	-	+	+
11	M	-	-	+	+	-	+	+
12	R	-	-	+	-	+	+	+
13	M	-	-	+	+	+	+	+
14	R	-	+	+	+	+	+	+
15	Mo	-	-	+	+	+	+	+
16	W	-	+	+	+	+	+	+
17	Mo	-	-	+	+	+	+	+
18	R	+	+	+	+	+	+	+
19	W	-	-	+	+	+	+	+
20	M	-	-	+	-	+	+	+

'+' Present and '-' Absent
'R' Resistant 'M' Mild infection 'Mo' Moderate infection 'S' Susceptible 'W' Wilted

Table 7. Comparative analysis of resistance in BD7755 germplasm plants based on their disease score, RCA screening and Ty genes

BD7755	Disease score	RCA screening	Ty-1	Ty-2	Ty-3	Ty-3a	Ty-4	Ty-5
1	M	-	+	+	+	+	+	+
2	M	-	-	+	+	+	+	+
3	M	-	-	+	-	+	+	+
4	M	-	+	+	+	+	+	+
5	Mo	-	+	+	+	+	+	+
6	R	+	-	+	+	+	+	+
7	Mo	-	-	+	+	+	+	+
8	S	+	-	+	+	+	+	+
9	S	+	-	+	+	+	+	+
10	M	-	+	+	+	+	+	+
11	M	-	-	+	+	+	+	+
12	Mo	-	-	+	+	+	+	+
13	S	+	-	+	+	+	+	+
14	M	-	-	+	+	+	+	+
15	R	-	-	-	+	+	+	+
16	R	-	-	+	+	+	+	-
17	R	-	-	+	+	+	+	+
18	M	-	-	+	+	+	+	+
19	Mo	-	-	+	+	+	+	+

'+' Present and '-' Absent
'R' Resistant 'M' Mild infection 'Mo' Moderate infection 'S' Susceptible 'W' Wilted

Table 8. Comparative analysis of resistance in Local Khushtia-1 germplasm plants based on their disease score, RCA screening and Ty genes

Local Khushtia-1	Disease score	RCA screening	Ty-1	Ty-2	Ty-3	Ty-3a	Ty-4	Ty-5
1	R	-	+	+	+	+	+	+
2	R	-	-	+	+	+	+	+
3	M	-	+	+	+	+	+	+
4	M	-	-	+	+	+	+	+
5	Mo	-	-	+	+	+	+	+
6	Mo	-	+	+	+	+	+	+
7	R	+	-	+	+	+	+	+
8	Mo	-	+	+	+	+	+	+
9	M	-	+	+	+	+	+	+
10	Mo	-	+	+	+	+	+	+
11	R	-	-	+	+	+	+	+
12	R	-	+	+	+	+	+	+
13	R	-	+	+	+	+	+	+
14	R	-	+	+	+	+	+	+
15	M	-	+	+	+	+	+	+
16	Mo	-	-	+	+	+	+	+
17	R	-	-	+	+	+	+	+
18	M	-	+	+	+	+	+	+

'+' Present and '-' Absent
'R' Resistant 'M' Mild infection 'Mo' Moderate infection 'S' Susceptible 'W' Wilted

Table 9. Comparative analysis of resistance in BD10125 germplasm plants based on their disease score, RCA screening and Ty genes

BD10125	Disease score	RCA screening	Ty-1	Ty-2	Ty-3	Ty-3a	Ty-4	Ty-5
1	M	-	-	+	+	+	+	+
2	Mo	-	+	+	+	+	+	+
3	R	+	-	+	+	+	+	+
4	R	+	+	+	+	+	+	+
5	Mo	-	+	+	+	+	+	+
6	Mo	-	-	+	+	+	+	+
7	Mo	+	-	+	+	+	+	+
8	Mo	-	+	+	+	+	+	+
9	Mo	-	-	+	+	+	+	+
10	Mo	-	-	-	-	-	-	-
11	Mo	-	-	+	+	+	+	+
12	M	-	-	+	+	+	+	+
13	Mo	-	-	+	+	+	+	+
14	Mo	-	-	+	+	+	+	+
15	Mo	-	-	+	+	+	+	+
16	M	-	-	+	+	+	+	+
17	Mo	-	-	+	+	+	+	+
18	M	-	-	+	+	+	+	+

'+' Present and '-' Absent
'R' Resistant 'M' Mild infection 'Mo' Moderate infection 'S' Susceptible 'W' Wilted

Table 10. Comparative analysis of resistance in BD10123 germplasm plants based on their disease score, RCA screening and Ty genes

BD10123	Disease score	RCA screening	Ty-1	Ty-2	Ty-3	Ty-3a	Ty-4	Ty-5
1	Mo	-	-	+	-	-	+	+
2	Mo	-	-	+	-	+	+	+
3	Mo	-	-	+	-	+	+	+
4	Mo	-	+	+	+	+	+	+
5	Mo	-	+	+	+	+	+	+
6	Mo	-	+	+	+	+	+	+
7	Mo	-	+	+	+	+	+	+
8	Mo	-	-	+	+	+	+	+
9	Mo	-	-	+	+	+	+	+
10	R	+	-	+	+	-	+	-
11	Mo	-	-	+	+	-	+	+
12	Mo	-	-	+	+	+	+	+
13	Mo	-	+	+	+	+	+	+
14	Mo	-	-	+	+	-	+	+
15	Mo	-	-	+	+	+	+	+

'+' Present and '-' Absent

'R' Resistant 'M' Mild infection 'Mo' Moderate infection 'S' Susceptible 'W' Wilted

Table 11. Comparative analysis of resistance in Local Kushtia-1 germplasm plants based on their disease score, RCA screening and Ty genes

BD7761	Disease score	RCA screening	Ty-1	Ty-2	Ty-3	Ty-3a	Ty-4	Ty-5
1	S	+	-	-	-	+	+	+
2	Mo	-	+	+	+	+	+	+
3	Mo	-	+	-	+	+	+	+
4	S	+	-	-	+	+	+	+
5	S	+	-	-	-	-	-	-
6	Mo	-	+	-	+	+	+	+
7	Mo	-	+	+	+	+	+	+
8	Mo	-	+	+	+	+	+	+
9	S	+	-	-	-	-	-	-
10	S	+	-	-	-	-	-	-
11	S	+	-	-	+	+	+	-
12	R	+	-	+	+	+	+	+
13	S	+	-	-	-	-	-	-
14	S	+	-	-	-	-	-	-
15	Mo	-	+	+	+	+	-	+

'+' Present and '-' Absent

'R' Resistant 'M' Mild infection 'Mo' Moderate infection 'S' Susceptible 'W' Wilted

2.3 Multiplex PCR analysis of selected plants

Among the 658 plants of the 7 wild tomato germplasm, line numbers 12 and 14 of the germplasm BD10124, line number 14 of the germplasm BD10122, line number 1 of BD7755 germplasm and line number 1 of Local Khushtia-1 contained all 6 genes as revealed by the presence of positive band through PCR analysis with the gene specific primers. So, these plants were selected for multiplex PCR analysis for further confirmation and reproducibility of presence of these 6 genes.

The selected plants also showed resistant characters in field observation followed by negative RCA results. Hence, they were further analyzed through sequencing.

2.4 Sequencing and analysis of the sequences of the amplified Ty genes

After multiplex PCR confirmation of six Ty genes in the 5 selected resistant plants; plants 12 and 14 from BD10124, line 12 from BD10122, line 1 from BDD775 and line 1 from Local-Khushtia-1 germplasm, the plants were subjected to PCR amplification with primers specific to Ty-2, Ty-4 and Ty-5 genes separately as they were reported to be linked with resistance for ToLCV infection. On the other hand Ty1, Ty3, and Ty3a genes were amplified from selected plants from BD-10123, BD-10125, BD-7761 and Local-Khushtia-1 germplasm (Figure 16). Amplified DNA bands were gel purified and sent for sequencing to MCLAB, USA. The sequences obtained through automated sequencing using both forward and reverse primers were subjected to further analysis.

These sequences were analyzed through BLAST program in NCBI-database. BLAST analysis of all the Ty gene sequences showed similarity with previously reported Ty markers from tomato accessions.

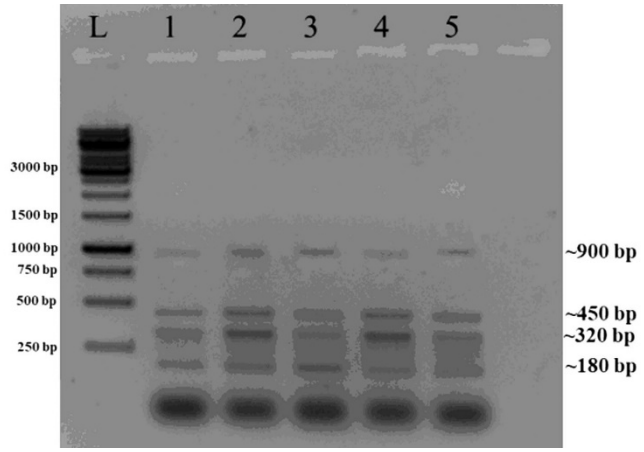

Figure 16. Multiplex PCR products run on 1% agarose gel electrophoresis. Lane L shows the ladder and lanes 1-5 are PCR products of plants 12 and 14 of BD10124, line 14 of BD10122, line 1 of BD7755 and line 1 of Local Khushtia-1 germplasm, respectively. All 5 plants showed multiple bands at ~900 bp, ~450 bp, ~320 bp and~180 bp conferring the PCR amplification of Ty gene specific markers, Ty-1, Ty-2, Ty-3, Ty-3a, Ty-4 and Ty-5, respectively.

1. Sequence analysis of Ty-2 gene amplified by using TG0302F/TY2R1 primer pair

The amplicons from all five plants were sequenced and analyzed through BLAST search and presented bellow-

1.1.1 Sequences obtained from Line 12 of BD10124 germplasm with the forward primer:

TTATGTGATTTTTTCGGAGGCTGTGTTTTCTTTGATTTGATGATTGCGGATGCATCCATTTTCA
TTCTGAGCTCTTTGGTCCTCCCCTTGACAATTTCTTGTTAATATTCAAGAAAATGAGAACATT
AGCACAATCTAGGCATCTAGAAACTTGTATTAGAGATTTAAACCATATTTGCGAAGACTTTG
GTCCCACTAGAGGGGGTGTGCATCATACAGTAATCTGACCCAAAAGATTGAGAGAGCAAGT
GTCAGCAAGGGAGAGAGAAGTGCAAATATGAGTGAGAAAGAAAACTTAATCCTACAGTCAG
GAGAATAAAGTTAAGAAGTAAATTTGGTGTTGGCAAAATTTGTTGCGGATGTTCCCTATTGT
TTACAGGCGAGAGAAGAGAANNTT

The NCBI-BLAST database search output showed that the partial sequence obtained from the forward TG0302F/TY2R1 primer has 100% nucleotide similarities with *Solanum lycopersicum* chromosome 11 from accessions (CP023767.1) and (HG975523.1), confirming its presence in chromosome 11 as per previous reports. It also shares 100% nucleotide similarities with *Solanum lycopersicum* cultivar Gh13, isolate M82-1-8 and isolate from breeding line H24marker, from the accessions (KF887341.1), (EU046613.1) and (EU046610.1), respectively for the RFLP marker T0302.

Table 13. Per cent identities of the amplicon at the nucleotides level obtained using the forward primer of Ty-2 gene with reported Ty-2 gene

Accession	Description	Max score	Total score	Query cover	E value	Identity
EU046613.1	***Solanum lycopersicum* isolate M82-1-8 T0302 RFLP marker genomic sequence**	726	726	92%	0.0	**100%**
CP023767.1	***Solanum lycopersicum* cultivar I-3 chromosome 11**	726	726	92%	0.0	**100%**
HG975523.1	***Solanum lycopersicum* chromosome ch11, complete genome**	726	726	92%	0.0	**100%**
KF887341.1	***Solanum lycopersicum* cultivar Gh13 marker TG0302 genomic sequence**	726	726	92%	0.0	**100%**
EU046610.1	***Solanum lycopersicum* isolate breeding line H24 T0302 RFLP marker genomic sequence**	398	692	92%	1e-108	**92%**

1.1.2 Sequences obtained from the Line 12 of BD10124 germplasm with the reverse primer:

GGNNNTCCGCACAAATTTTNCCAACACCAAATTTACTTCTTAACTTTATTCTCCTGACTGTAG
GATTAAGTTTTCTTTCTCACTCATATTTGCACTTCTCTCTCCCTTGCTGACACTTGCTCTCTCA
ATCTTTTGGGTCAGATTACTGTATGATGCACACCCCCTCTAGTGGGACCAAAGTCTTCGCAA
ATATGGTTTAAATCTCTAATACAAGTTTCTAGATGCCTAGATTGTGCTAATGTTCTCATTTTC
TTGAATATTAACAAGAAATTGTCAAGGGGAGGACCAAAGAGCTCAGAATGAAAATGGATGC
ATCCGCAATCATCAAATCAAAGAAAACACAGCCTCCGAAAAAATCACATAATACCTGAGTC
ACAGTTTAGCGCGCTATCAGCTTCNNNAAATGAANCCAAA

The NCBI-BLAST database search output showed that the partial sequence obtained from the reverse TG0302F/TY2R1 primer has 99% nucleotide similarities with *Solanum lycopersicum* chromosome 11 from accessions (CP023767.1) and (HG975523.1), confirming its presence in chromosome 11 as per previous reports. It also shares 99% nucleotide similarities with *Solanum lycopersicum* isolate M82-1-8 and cultivar Gh13 from the accessions (EU046613.1) and (KF887341.1), respectively for the RFLP marker T0302. It further shares 92% nucleotide similarity with isolate from breeding line H24 (EU046610.1) for the T0302 RFLP marker.

1.2.1 Sequences obtained from the Line 14 of BD10124 germplasm with the forward primer:

TNTTCGGAGGCTGTGTTTTCTTTGATTTGATGATTGCGGATGCATCCATTTTCATTCTGAGCTC
TTTGGTCCTCCCCTTGACAATTTCTTGTTAATATTCAAGAAAATGAAAACATTAGCACAATCT
AGGCATCTAAAAACTTGTATTATAGATTTAAACCATATTTGCGAAGACTTTGGTCCCACTAA
AGGGGGTGTGCATCATACAGTAATCTGACCCAAAAGATTGAGAGAGCAAGTGTCAGCAAGG
GAGAGAGAAGTGCAAATATGAGTGAGAAAGAAAACTTAATCCTACAGTCAGGAGAATAAA
GTTAAAAAGTAAATTTGGTGGTGGCAAAATTTGTCGCGGATGTACCCTATTGATTACATGCG
AGCAAAAGAGAAAAAACAATTACAA

Table 14. Per cent identities of the amplicon at the nucleotides level obtained using the reverse primers of Ty-2 gene with reported Ty-2 gene

Accession	Description	Max score	Total score	Query cover	E value	Identity
EU046613.1	***Solanum lycopersicum* isolate M82-1-8 T0302 RFLP marker genomic sequence**	717	717	92%	0.0	**99%**
CP023767.1	***Solanum lycopersicum* cultivar I-3 chromosome 11**	712	712	92%	0.0	**99%**
HG975523.1	***Solanum lycopersicum* chromosome ch11, complete genome**	712	712	92%	0.0	**99%**
KF887341.1	***Solanum lycopersicum* cultivar Gh13 marker TG0302 genomic sequence**	688	688	89%	0.0	**99%**
EU046610.1	***Solanum lycopersicum* isolate breeding line H24 T0302 RFLP marker genomic sequence**	350	696	92%	3e-94	**92%**

The NCBI-BLAST database search output showed that the partial sequence obtained from theforwardTG0302F/TY2R1 primer has 97% nucleotide similarities with *Solanum lycopersicum* chromosome 11 from accessions (CP023767.1) and (HG975523.1), confirming its presence in chromosome 11 as per previous reports. It also shares 97% nucleotide similarities with *Solanum lycopersicum* cultivar Gh13 (KF887341.1) and isolate M82-1-8 (EU046613.1)for the RFLP marker T0302. It further shares 89% nucleotide similarity with isolate from breeding line H24 (EU046610.1) for the same RFLP marker.

Table 15. Per cent identities of the amplicon at the nucleotides level obtained using the forward primer of Ty-2 gene with the reported Ty-2 gene

Accession	Description	Max score	Total score	Query cover	E value	Identity
EU046613.1	***Solanum lycopersicum* isolate M82-1-8 T0302 RFLP marker genomic sequence**	638	638	89%	0.0	**97%**
CP023767.1	***Solanum lycopersicum* cultivar I-3 chromosome 11**	638	638	89%	0.0	**97%**
HG975523.1	***Solanum lycopersicum* chromosome ch11, complete genome**	638	638	89%	0.0	**97%**
KF887341.1	***Solanum lycopersicum* cultivar Gh13 marker TG0302 genomic sequence**	638	638	89%	0.0	**97%**
EU046610.1	***Solanum lycopersicum* isolate breeding line H24 T0302 RFLP marker genomic sequence**	348	609	89%	1e-93	**89%**

1.2.2 Sequences obtained from the Line 14 of BD10124 germplasm with the reverse primer:

GNNNNATNNNCGCNACANTTTTNCCAACACCAAATTTACTTCTTAACTTTATTCTCCTGACTG
TAGGATTAAGTTTTCTTTCTCACTCATATTTGCACTTCTCTCTCCCTTGCTGACACTTGCTCTC
TCAATCTTTTGGGTCAGATTACTGTATGATGCACACCCCCTCTAGTGGGACCAAAGTCTTCGC

AAATATGGTTTAAATCTCTAATACAAGTTTCTAGATGCCTACATTGTGCTAGTGTTCTCATTT
TCTTGAATATTAACAAGAAGAGATCGTGGGGAGGACCAACAAACACATAATAAAGATGGAG
GCATCCTCCNTCATCATAACATAAAAAAAAACNCCGCCGCCAAAAAAATCTCATACTTGCTT
AACCACTTATAACCCCGCTAGCTATACNAAAAAAAAAAANAAA

The NCBI-BLAST database search output showed that the partial sequence obtained from the reverseTG0302F/TY2R1 primer has 91.0% nucleotide similarities with *Solanum lycopersicum* chromosome 11 from accessions (CP023767.1) and (HG975523.1), confirming its presence in chromosome 11 as per previous reports. It also shares 91.0% nucleotide similarities with *Solanum lycopersicum* cultivar Gh13 (KF887341.1) and isolate M82-1-8 (EU046613.1)for the RFLP marker T0302. It further shares 93% nucleotide similarity with isolate from breeding line H24 (EU046610.1) for the T0302 RFLP marker.

Table 16. Per cent identities of the amplicon at the nucleotides level obtained using the reverse primer of Ty-2 gene with reported Ty-2 gene

Accession	Description	Max score	Total score	Query cover	E value	Identity
CP023767.1	***Solanum lycopersicum* cultivar I-3 chromosome 11**	484	484	81.0%	8e-135	**91.0%**
HG975523.1	***Solanum lycopersicum* chromosome ch11, complete genome**	484	484	81.0%	8e-135	**91.0%**
KF887341.1	***Solanum lycopersicum* cultivar Gh13 marker TG0302 genomic sequence**	484	484	81.0%	8e-135	**91.0%**
EU046613.1	***Solanum lycopersicum* isolate M82-1-8 T0302 RFLP marker genomic sequence**	484	484	81.0%	8e-135	**91.0%**
EU046610.1	***Solanum lycopersicum* isolate breeding line H24 T0302 RFLP marker genomic sequence**	333	493	57%	3e-89	**93%**

1.3.1 Sequences obtained from the Line 14 of BD10122 germplasm with the forward primer:

TNNGNANNNNNCNNNNNCNNATGTGATTTTTTCGGAGGCTGTGTTTTCTTTGATTTGATGAT
TGCGGATGCATCCATTTTCATTCTGAGCTCTTTGGTCCTCCCCTTGACAATTTCTTGTTAATAT
TCAAGAAAATGAGAACATTAGCACAATCTAGGCATCTAGAAACTTGTATTAGAGATTTAAAC
CATATTTGCGAAGACTTTGGTCCCACTAGAGGGGGTGTGCATCATACAGTAATCTGACCCAA
AAGATTGAGAGAGCAAGTGTCAGCAAGGGAGAGAGAAGTGCAAATATGAGTGAGAAAGAA
AACTTAATCCTACAGTCAGGAGAATAAAGTTAAGAAGTAAATTTGGTGTTGGCAAAATTTGT
TGCGGATGTTCCCTATTGTTTACAGGCGAGAGAAGAGAACATCACATCAA

The NCBI-BLAST search output showed that the partial sequence obtained with the forward primer TG0302F/TY2R1 has 99% nucleotide similarities with *Solanum lycopersicum* chromosome 11 from

accessions (CP023767.1) and (HG975523.1), confirming its presence in chromosome 11 as per previous reports. It also shares 99% nucleotide similarities with *Solanum lycopersicum* Gh13 cultivar (KF887341.1) and isolate M82-1-8 ((EU046613.1) for the RFLP marker, T0302. It also showed 93% nucleotide similarity with isolate from breeding line H24 (EU046610.1) for the T0302 RFLP marker.

Table 17. Per cent identities of the amplicon at the nucleotides level obtained using the forward primer of Ty-2 gene with reported Ty-2 gene

Accession	Description	Max score	Total score	Query cover	E value	Identity
CP023767.1	***Solanum lycopersicum* cultivar I-3 chromosome 11**	737	737	93%	0	**99%**
HG975523.1	***Solanum lycopersicum* chromosome ch11, complete genome**	737	737	93%	0	**99%**
KF887341.1	***Solanum lycopersicum* cultivar Gh13 marker TG0302 genomic sequence**	737	737	93%	0	**99%**
EU046613.1	***Solanum lycopersicum* isolate M82-1-8 T0302 RFLP marker genomic sequence**	737	737	93%	0	**99%**
EU046610.1	***Solanum lycopersicum* isolate breeding line H24 T0302 RFLP marker genomic sequence**	418	709	93%	9e-115	**93%**

1.3.2 Sequences obtained from the Line 14 of BD10122 germplasm with the reverse primer:

TNNGNANNNNNCNNNNNCNNATGTGATTTTTTCGGAGGCTGTGTTTTCTTTGATTTGATGAT
TGCGGATGCATCCATTTTCATTCTGAGCTCTTTGGTCCTCCCCTTGACAATTTCTTGTTAATAT
TCAAGAAAATGAGAACATTAGCACAATCTAGGCATCTAGAAACTTGTATTAGAGATTTAAAC
CATATTTGCGAAGACTTTGGTCCCACTAGAGGGGGTGTGCATCATACAGTAATCTGACCCAA
AAGATTGAGAGAGCAAGTGTCAGCAAGGGAGAGAGAAGTGCAAATATGAGTGAGAAAGAA
AACTTAATCCTACAGTCAGGAGAATAAAGTTAAGAAGTAAATTTGGTGTTGGCAAAATTTGT
TGCGGATGTTCCCTATTGTTTACAGGCGAGAGAAGAGAACATCACATCAA

The NCBI-BLAST search output showed that the partial sequence obtained with the reverse primer TG0302F/TY2R1 has 99% nucleotide similarities with *Solanum lycopersicum* chromosome 11 from accessions (CP023767.1) and (HG975523.1), confirming its presence in chromosome 11 as per previous reports. It also shares 99% nucleotide similarities with *Solanum lycopersicum* Gh13 cultivar (KF887341.1) and isolate M82-1-8 ((EU046613.1) for the RFLP marker, T0302. It also showed 93% nucleotide similarity with isolate from breeding line H24 (EU046610.1) for the T0302 RFLP marker.

Table 18. Per cent identities of the amplicon at the nucleotides level obtained using the reverse primer of Ty-2 gene with reported Ty-2 gene

Accession	Description	Max score	Total score	Query cover	E value	Identity
CP023767.1	***Solanum lycopersicum*** **cultivar I-3 chromosome 11**	737	737	93%	0.0	**99%**
HG975523.1	***Solanum lycopersicum*** **chromosome ch11, complete genome**	737	737	93%	0.0	**99%**
KF887341.1	***Solanum lycopersicum*** **cultivar Gh13 marker TG0302 genomic sequence**	737	737	93%	0.0	**99%**
EU046613.1	***Solanum lycopersicum*** **isolate M82-1-8 T0302 RFLP marker genomic sequence**	737	737	93%	0.0	**99%**
EU046610.1	***Solanum lycopersicum*** **isolate breeding line H24 T0302 RFLP marker genomic sequence**	418	709	93%	7e-115	**93%**

1.4.1 Sequences obtained from the Line 1 of BD7755 germplasm with the forward primer:

TTTNTTTTNGGGGCTGTGTTTTCTTTGATTTGGGAATGAGATGCATCCTTTTCTGTCCGAGCTC
TTTGGTCCTCCCCTTGACAATTTCTTGTTAATATTCAAGAAATGANAAATTGACACAATCTAG
GCATCTAAAAACTTGTATTAAAAATTTAAACCATATTTGCGAAAAACTTTGGTCCCACTAAA
GGGGGGGGGCATCATACATAATTCTGACCCANAGTTTGAGAGAGCAAGTGTCACCNGGGAG
AGAAAAATGCACATATGAGTGAGAAAAAAAACTTTATCACACACTCTCGAGAATATTATTA
AAAAATTTTTGTTGTGTGTTAAATTTTTTTTTCAAATATTCA

The NCBI-BLAST search output showed that the partial sequence obtained with the reverse primer TG0302F/TY2R1 has 86% nucleotide similarities with *Solanum lycopersicum* chromosome 11 from accessions (CP023767.1) and (HG975523.1), confirming its presence in chromosome 11 as per previous reports. It also shared 86% nucleotide similarities with *Solanum lycopersicum* Gh13 cultivar (KF887341.1) and isolate M82-1-8 ((EU046613.1) for the RFLP marker, T0302.

1.4.2 Sequences obtained from the Line 1 of BD7755 germplasm with the reverse primer:

GGNANNCNNNNNACANTTTNGCCGACACCNNGTTTACTTCTTAACTTTATTCTCCTGACTGT
AGGATAAGTTTCCTTTACCACTCTATTTGCACTTCTCTCTCCCTTGCTGACACTTGCTCTCTCA
ATCTTTTGGGTCAAATGACTGTATGATGCGCACCCCCTCAAATGGGACCCATATCTTCGCAA
ACTTGGTTTAAATTTCTAGTAGGAGTTTCTAGATGCCTTNATTGTGCTAGGGTTCTCATGTTC
TTGGATATCAAGGAAAAGGTAACAGGGAAATGANAGAGAACTAANATTAATCCTGCATTCA
TGAGAAAAATCAAATAAATAACTCCGCNGTTGGCAAAATAAGTTGCNTAATTACNCCTATTG
TATACGAGCNAG

Table 19. Per cent identities (nucleotides) of the amplicons obtained using the forward primer of Ty-2 gene with reported Ty-2 gene

Accession	Description	Max score	Total score	Query cover	E value	Identity
CP023767.1	***Solanum lycopersicum* cultivar I-3 chromosome 11**	337	337	79%	2e-90	**86%**
EU046613.1	***Solanum lycopersicum* isolate M82-1-8 T0302 RFLP marker genomic sequence**	638	638	89%	0.0	**97%**
HG975523.1	***Solanum lycopersicum* chromosome ch11, complete genome**	337	337	79%	2e-90	**86%**
KF887341.1	***Solanum lycopersicum* cultivar Gh13 marker TG0302 genomic sequence**	337	337	79%	2e-90	**86%**

The NCBI-BLAST search output showed that the partial sequence obtained with the reverse primer TG0302F/TY2R1 has 88% nucleotide similarities with *Solanum lycopersicum* chromosome 11 from accessions (CP023767.1) and (HG975523.1), confirming its presence in chromosome 11 as per previous reports. It also shares 88% nucleotide similarities with *Solanum lycopersicum* Gh13 cultivar (KF887341.1) and isolate M82-1-8 ((EU046613.1) for the RFLP marker, T0302.

Table 20. Per cent identities of the amplicon at the nucleotides level obtained using the reverse primers of Ty-2 gene with reported Ty-2 gene

Accession	Description	Max score	Total score	Query cover	E value	Identity
CP023767.1	***Solanum lycopersicum* cultivar I-3 chromosome 11**	292	292	60%	5e-77	**88%**
EU046613.1	***Solanum lycopersicum* isolate M82-1-8 T0302 RFLP marker genomic sequence**	638	638	89%	0.0	**97%**
HG975523.1	***Solanum lycopersicum* chromosome ch11, complete genome**	292	292	60%	5e-77	**88%**
KF887341.1	***Solanum lycopersicum* cultivar Gh13 marker TG0302 genomic sequence**	292	292	60%	5e-77	**88%**

1.5.1 Sequences obtained from the Line 1 of Local-Khushtia-1 germplasm with the forward primer:

TNNGANNNTNCNNNNNCNNNTATGTGATTTTTTCGGAGGCTGTGTTTTCTTTGATTTGATGAT
TGCGGATGCATCCATTTTCATTCTGAGCTCTTTGGTCCTCCCCTTGACAATTTCTTGTTAATAT
TCAAGAAAATGAGAACATTAGCACAATCTAGGCATCTAGAAACTTGTATTAGAGATTTAAAC
CATATTTGCGAAGACTTTGGTCCCACTAGAGGGGGTGTGCATCATACAGTAATCTGACCCAA
AAGATTGAGAGAGCAAGTGTCAGCAAGGGAGAGAGAAGTGCAAATATGAGTGAGAAAGAA
AACTTAATCCTACAGTCAGGAGAATAAAGTTAAGAAGTAAATTTGGTGTTGGCAAAATTTGT
TGCGGATGTTCCCTATTGTTTACAGGCGAGAGAAGAGAACAATCACATCA

The NCBI-BLAST search output showed that the partial sequence obtained with the reverse primer TG0302F/TY2R1 has 99% nucleotide similarities with *Solanum lycopersicum* chromosome 11 from accessions (CP023767.1) and (HG975523.1), confirming its presence in chromosome 11 as per previous reports. It also shares 99% nucleotide similarities with *Solanum lycopersicum* Gh13 cultivar (KF887341.1) and isolate M82-1-8 ((EU046613.1) for the RFLP marker, T0302. It also showed 92% nucleotide similarity with isolate from breeding line H24 (EU046610.1) for the T0302 RFLP marker.

Table 21. Per cent identities of the amplicon at the nucleotides level obtained using the forward primers of Ty-2 gene with reported Ty-2 gene

Accession	Description	Max score	Total score	Query cover	E value	Identity
CP023767.1	***Solanum lycopersicum*** **cultivar I-3 chromosome 11**	732	732	93%	0.0	**99%**
HG975523.1	***Solanum lycopersicum*** **chromosome ch11, complete genome**	732	732	93%	0.0	**99%**
KF887341.1	***Solanum lycopersicum*** **cultivar Gh13 marker TG0302 genomic sequence**	732	732	93%	0.0	**99%**
EU046613.1	***Solanum lycopersicum*** **isolate M82-1-8 T0302 RFLP marker genomic sequence**	732	732	93%	0.0	**99%**
EU046610.1	***Solanum lycopersicum*** **isolate breeding line H24 T0302 RFLP marker genomic sequence**	411	703	93%	1e-112	**92%**

1.5.2 Sequences obtained from the Line 1 of Local-Khushtia-1 germplasm with the reverse primer:

ACNNCNNNCNNCNNATTTTNGCCAACAGCAAATTTANTTGCTTAACTTTATTCTCCTGGACT
GTAGGATTAAGTTTTCTTTCTCACTCATATTTGCACTTCTCTCTCCCTTGCTGACACTTGCTCT
CTCAATCTTTTGGGTCAAATTACTGTATGATGCACACCCCCTCTAGTGGGACCAAAGTCTTCG
CAAATATGGTTTAAATCTCTAATACAAGTTTCTAGATGCCTAGATTGTGCTAATGTTCTCATT
TTCTTGAATATTAACAAGAAATTGTCAAGGGGAGGACCAAAGAGCTCAGAATGAAAATGGA
TGCATCCGCAATCATCAAATCAAAGAAAACACAGCCTCCGAAAAAATCACATAATACCTGA
GTCACAGTTTAGCGCGCTATCAGCTTCAGGATGAGCCAA

The NCBI-BLAST search output showed that the partial sequence obtained with the reverse primer TG0302F/TY2R1 has 98% nucleotide similarities with *Solanum lycopersicum* chromosome 11 from accessions (CP023767.1) and (HG975523.1), confirming its presence in chromosome 11 as per previous reports. It also shares 98% nucleotide similarities with *Solanum lycopersicum* Gh13 cultivar (KF887341.1) and isolate M82-1-8 ((EU046613.1) for the RFLP marker, T0302. It further shares 92% nucleotide similarity with isolate from breeding line H24 (EU046610.1) for the T0302 RFLP marker.

Table 22. Per cent identities of the amplicon at the nucleotides level obtained using the reverse primers of Ty-2 gene with reported Ty-2 gene

Accession	Description	Max score	Total score	Query cover	E value	Identity
EU046613.1	***Solanum lycopersicum*** **isolate M82-1-8 T0302 RFLP marker genomic sequence**	701	701	93%	0.0	**98%**
CP023767.1	***Solanum lycopersicum*** **cultivar I-3 chromosome 11**	695	695	93%	0.0	**98%**
HG975523.1	***Solanum lycopersicum*** **chromosome ch11, complete genome**	695	695	93%	0.0	**98%**
KF887341.1	***Solanum lycopersicum*** **cultivar Gh13 marker TG0302 genomic sequence**	651	651	87%	0.0	**98%**
EU046610.1	***Solanum lycopersicum*** **isolate breeding line H24 T0302 RFLP marker genomic sequence**	361	687	93%	1e-97	**89%**

2. Sequence analysis of Ty-4 gene amplified by using C2_AT5g51110 primer pair

2.1.1 Sequences obtained from the Line 14 of BD10124 germplasm with the forward primer:

ANTNNCNACAGNNNCNAATCAAGTTCATGCTGAGCTGTGGACTCCTTCGATTGGTGAGTACG
CTTCCTCAGTCACTCCTTNNCCTCTAGCATTATTTAAGAATTCATCTGTAGCGCGTTCTATGT
GATAACTGTACTGACTTACAGCAAGATTGGGTAAAACACTTGGCAAATTAAGTTCATTTATT
CTCAATCTCTTTTACTTGTGTTGGAATTCTTTAATGATCATAGTGGTACATTCTAATTGCAGG
AGGTTTGAGCATGAATGATTTCATCATAGCTGCTAAAATAGATCAAGTAAAGAATTCCAACA
CAAGTAAAAGAGATTGAGAATAAATGAACTTAATTTGCCAAGTGTTTTACCCAATCTTGCTG
TAAGTCAGTACAGTTATCACATAGAACGCGCTACAGATGAATTCTTAAATAATGCTAGAGGA
AAGGAGTGACTGAGGAAGCGTNCCAACGAATCCCAGGAGNCCNCAGCTTTATTATGATGTT
GNTNTTAATGAAGAAT

The NCBI-BLAST search output showed that the partial sequence obtained with the forward primer C2_AT5g51110 has 100% nucleotide similarities with previous records of *Solanum lycopersicum* putative pterin-4-alpha-carbinolamine dehydratase (LOC101265812), mRNA (XM 004253276.3) and cDNA clone of LEFL1034AG11 in *Solanum lycopersicum* leaf (AK322159.1). It also shares 99% nucleotide similarity with clone HBa0065A14 in chromosome 3 of *Solanum lycopersicum* (AC246924.1), confirming its presence in chromosome 3 as per previous records. It further shares 99% nucleotide similarities with *Solanum lycopersicum* cultivar I-3 chromosome 00 (CP023756.1).

Table 23. Per cent identities of the amplicon at the nucleotides level obtained using the forward primers of Ty-4 gene with reported Ty-4 gene

Accession	Description	Max score	Total score	Query cover	E value	Identity
AC246924.1	*Solanum lycopersicum* chromosome 3 clone HBa0065A14, complete sequence	520	1655	89%	3e-145	**99%**
CP023756.1	Solanum lycopersicum cultivar I-3 chromosome 00	514	816	89%	1e-143	**99%**
XM 004253276.3	PREDICTED: *Solanum lycopersicum* putative pterin-4-alpha-carbinolamine dehydratase (LOC101265812), mRNA	102	173	17%	1e-19	**100%**
AK322159.1	*Solanum lycopersicum* cDNA, clone: LEFL1034AG11, HTC in leaf	102	173	17%	1e-19	**100%**

2.1.2 Sequences obtained from the Line 14 of BD10124 germplasm with the reverse primer:

GNATCTTNNNGCTCNANCTNCTGCATTAGAATGTACCACTATGATCATTAAAGAATTCCAAC
ACAAGTAAAAGAGATTGAGAATAAATGAACTTAATTTGCCAAGTGTTTTACCCAATCTTGCT
GTAAGTCAGTACAGTTATCACATAGAACGCGCTACAGATGAATTCTTAAATAATGCTAGAGG
AAAGGAGTGACTGAGGAAGCGTACTCACCAATCGAAGGAGTCCACAGCTCAGCATGAACTT
GATTAGACTGTTGTAATTGATGAAGAGTGGGGAGGTGCCCTGTGCCT

The NCBI-BLAST search output showed that the partial sequence obtained with the forward primer C2_AT5g51110 has 100% nucleotide similarities with previous records of *Solanum lycopersicum* putative pterin-4-alpha-carbinolamine dehydratase (LOC101265812), mRNA (XM 004253276.3) and cDNA clone of LEFL1034AG11 in *Solanum lycopersicum* leaf (AK322159.1). It also shares 99% nucleotide similarity with clone HBa0065A14 in chromosome 3 of *Solanum lycopersicum* (AC246924.1), confirming its presence in chromosome 3 as per previous records. It further shares 99% nucleotide similarities with *Solanum lycopersicum* cultivar I-3 chromosome 00 (CP023756.1).

Table 24. Per cent identities of the amplicon at the nucleotides level obtained using the reverse primers of Ty-4 gene with reported Ty-4 gene

Accession	Description	Max score	Total score	Query cover	E value	Identity
AC246924.1	***Solanum lycopersicum* chromosome 3 clone HBa0065A14, complete sequence**	503	1006	92%	2e-140	**99%**
CP023756.1	***Solanum lycopersicum* cultivar I-3 chromosome 00**	497	497	92%	7e-139	**99%**
XM 004253276.3	**PREDICTED: *Solanum lycopersicum* putative pterin-4-alpha-carbinolamine dehydratase (LOC101265812), mRNA**	150	150	27%	2e-34	**100%**
AK322159.1	***Solanum lycopersicum* cDNA, clone: LEFL1034AG11, HTC in leaf**	150	150	27%	2e-34	**100%**

2.2.1 Sequences obtained from the Line 12 of BD10124 germplasm with the forward primer:

TNNACAGTCTAATCAAGTTCATGCTGAGCTGTGGACTCCTTCGATTGGTGAGTACGCTTCCTC
AGTCACTCCTTTCCTCTAGCATTATTTAAGAATTCATCTGTAGCGCGTTCTATGTGATAACTG
TACTGACTTACAGCAAGATTGGGTAAAACACTTGGCAAATTAAGTTCATTTATTCTCAATCTC
TTTTACTTGTGTTGGAATTCTTTAATGATCATAGTGGTACATTCTAATTGCAGGAGGTTTGAG
CATGAATGATTTCATCATAGCTGCTAAAATAGNNAAGTAAAGAATTCCAACACAGGTAGAA
GAGATTGGGAATTAATGAACTTAATTTGTCAAGTGTTTTGCCCCATCTTGCTGTAAGTCAGTC
CGGTTATCCCGTAGACCGCGCTATTGATCTTATGTTATATTAAAGAGAGAGGACTGGGAGAG
NNTCTTCCCCCCTTCCAAAGGAATCCCCACCCCCCCTGAGCTTGATTANAATGTTGTAATTGA
TGAAAAAAGGGGGAGGTGCCCTCNCCCNTCCCCCAA

The NCBI-BLAST search output showed that the partial sequence obtained with the forward primer C2_AT5g51110 has 95% nucleotide similarities with previous records of *Solanum lycopersicum* putative pterin-4-alpha-carbinolamine dehydratase (LOC101265812), mRNA (XM 004253276.3) and cDNA clone of LEFL1034AG11 in *Solanum lycopersicum* leaf (AK322159.1). It also shares 99% nucleotide similarity with clone HBa0065A14 in chromosome 3 of *Solanum lycopersicum* (AC246924.1), confirming its presence in chromosome 3 as per previous records. It further shares 99% nucleotide similarities with *Solanum lycopersicum* cultivar I-3 chromosome 00 (CP023756.1).

Table 25. Per cent identities of the amplicon at the nucleotides level obtained using the forward primers of Ty-4 gene with reported Ty-4 gene

Accession	Description	Max score	Total score	Query cover	E value	Identity
AC246924.1	***Solanum lycopersicum* chromosome 3 clone HBa0065A14, complete sequence**	527	1054	53%	2e-147	**99%**
CP023756.1	***Solanum lycopersicum* cultivar I-3 chromosome 00**	521	521	53%	8e-146	**99%**
XM 004253276.3	**PREDICTED: *Solanum lycopersicum* putative pterin-4-alpha-carbinolamine dehydratase (LOC101265812), mRNA**	87.9	172	18%	4e-15	**95%**
AK322159.1	***Solanum lycopersicum*cDNA, clone: LEFL1034AG11, HTC in leaf**	87.9	172	18%	4e-15	**95%**

2.2.2 Sequences obtained from the Line 12 of BD10124 germplasm with the reverse primer:

TCTTCNNGGCTCAACCTCCTGCATTAGAATGNACCACTATGATCATTAAAGAATTCCAACAC
AAGTAAAAGAGATTGAGAATAAATGAACTTAATTTGCCAAGTGTTTTACCCAATCTTGCTGT
AAGTCAGTACAGTTATCACATAGAACGCGCTACAGATGAATTCTTAAATAATGCTAGAGGA
AAGGAGTGACTGAGGAAGCGTACTCACCAATCGAAGGAGTCCACAGCTCAGCATGAACTTG

ATTAGACTGTTGTAATTGATGAAGAGTGGGGAGGTGCCCTGTGCCTTCCACCAANNTNNAAN
ANNNTGTTGTTTATGAATTTTTTTTTTAAGATTTGTTCCCTCCTGCGNGTGCCCGTTCCTGANC
AANANAACGCCCCTAAGGGTTTCNTTTAAATC

The NCBI-BLAST search output showed that the partial sequence obtained with the forward primer C2_AT5g51110 has 100% nucleotide similarities with previous records of *Solanum lycopersicum* putative pterin-4-alpha-carbinolamine dehydratase (LOC101265812), mRNA (XM 004253276.3) and cDNA clone of LEFL1034AG11 in *Solanum Lycopersicum* leaf (AK322159.1). It also shares 99% nucleotide similarity with clone HBa0065A14 in chromosome 3 of *Solanum lycopersicum* (AC246924.1), confirming its presence in chromosome 3 as per previous records. It further shares 99% nucleotide similarities with *Solanum lycopersicum* cultivar I-3 chromosome 00 (CP023756.1).

Table 26. Per cent identities of the amplicon at the nucleotides level obtained using the reverse primers of Ty-4 gene with reported Ty-4 gene

Accession	Description	Max score	Total score	Query cover	E value	Identity
AC246924.1	***Solanum lycopersicum* chromosome 3 clone HBa0065A14, complete sequence**	525	1051	70%	4e-147	**99%**
CP023756.1	***Solanum lycopersicum* cultivar I-3 chromosome 00**	520	520	70%	2e-145	**99%**
XM 004253276.3	**PREDICTED: *Solanum lycopersicum* putative pterin-4-alpha-carbinolamine dehydratase (LOC101265812), mRNA**	163	163	21.0%	4e-38	**100%**
AK322159.1	***Solanum lycopersicum* cDNA, clone: LEFL1034AG11, HTC in leaf**	163	163	21.0%	4e-38	**100%**

2.3.1 Sequences obtained from the Line 14 of BD10122 germplasm with the forward primer:

ANAGTCTAATCAAGTTCATGCTGAGCTGTGGACTCCTTCGATTGGTGAGTACGCTTCCTCAGTCAC
TCCTTTCCTCTAGCATTATTTAAGAATTCATCTGTAGCGCGTTCTATGTGATAACTGTACTGACTT
ACAGCAAGATTGGGTAAAACACTTGGCAAATTAAGTTCATTTATTCTCAATCTCTTTTACTTGTGT
TGGAATTCTTTAATGATCATAGTGGTACATTCTAATTGCAGGAGGTTTGAGCATGAATGATTTCAT
CATAGCTGCTAAAATAGATCAAGTAAAGAATTCCAACACAAGTAAAAGAGATTGAGAATAAATG
AACTTAATTTGCCAAGTGTTTTACCCAATCTTGCTGTAAGTCAGTACAGTTATCACATAGAACGCG
CTACAGATGAATTCTTAAATAATGCTAGAGGAAAGGAGTGACTGAGGAAGCGTACTCACCAATC
GAAGGAGTCCACAGCTCAGCATGAACTTGATTAGACTGTTG

The NCBI-BLAST search output showed that the partial sequence obtained with the forward primer C2_AT5g51110 has 100% nucleotide similarities with previous records of *Solanum lycopersicum* putative pterin-4-alpha-carbinolamine dehydratase (LOC101265812), mRNA (XM 004253276.3) and

cDNA clone of LEFL1034AG11 in *Solanum lycopersicum* leaf (AK322159.1). It also shares 99% nucleotide similarity with clone HBa0065A14 in chromosome 3 of *Solanum lycopersicum* (AC246924.1), confirming its presence in chromosome 3 as per previous records. It further shares 99% nucleotide similarities with *Solanum lycopersicum* cultivar I-3 chromosome 00 (CP023756.1).

Table 27. Per cent identities of the amplicon at the nucleotides level obtained using the forward primers of Ty-4 gene with reported Ty-4 gene

Accession	Description	Max score	Total score	Query cover	E value	Identity
AC246924.1	***Solanum lycopersicum* chromosome 3 clone HBa0065A14, complete sequence**	538	1862	96%	7e-151	**99%**
CP023756.1	***Solanum lycopersicum* cultivar I-3 chromosome 00**	532	920	96%	3e-149	**99%**
XM 004253276.3	**PREDICTED*: Solanum lycopersicum* putative pterin-4-alpha-carbinolamine dehydratase (LOC101265812), mRNA**	102	271	28%	1e-19	**100%**
AK322159.1	***Solanum lycopersicum* cDNA, clone: LEFL1034AG11, HTC in leaf**	102	271	28%	1e-19	**100%**

2.3.2 Sequences obtained from the Line 14 of BD10122 germplasm with the reverse primer:

TCTGCTCAACCTCCTGCATTAGAATGTACCACTATGATCATTAAAGAATTCCAACACAAGTA
AAAGAGATTGAGAATAAATGAACTTAATTTGCCAAGTGTTTTACCCAATCTTGCTGTAAGTC
AGTACAGTTATCACATAGAACGCGCTACAGATGAATTCTTAAATAATGCTAGAGGAAAGGA
GTGACTGAGGAAGCGTACTCACCAATCGAAGGAGTCCACAGCTCAGCATGAACTTGATTAG
ACTGTTGTAATTGATGAAGAGTGGGGAGGTGCCCTGNGCCTTCCACCAAANCTNNAAAANN
ATGTNNTCATTTGCTCTTTTTTTTCNAGTTTTTTTACCCCCCCCCGANTNNNTCTTTCTTANCAA
AAAAACNTCCTAGGAAAACTTTTTAAATTCTGGTGAGAAAAGGNGGGGGGAGGCANCTCCC
CCCNAC

The NCBI-BLAST search output showed that the partial sequence obtained with the forward primer C2_AT5g51110 has 99% nucleotide similarities with previous records of *Solanum lycopersicum* putative pterin-4-alpha-carbinolamine dehydratase (LOC101265812), mRNA (XM 004253276.3) and cDNA clone of LEFL1034AG11 in *Solanum lycopersicum* leaf (AK322159.1). It also shares 99% nucleotide similarity with clone HBa0065A14 in chromosome 3 of *Solanum lycopersicum* (AC246924.1), confirming its presence in chromosome 3 as per previous records. It further shares 99% nucleotide similarities with *Solanum lycopersicum* cultivar I-3 chromosome 00 (CP023756.1).

Table 28. Per cent identities of the amplicon at the nucleotides level obtained using the reverse primer of Ty-4 gene with reported Ty-4 gene

Accession	Description	Max score	Total score	Query cover	E value	Identity
AC246924.1	***Solanum lycopersicum* chromosome 3 clone HBa0065A14, complete sequence**	527	1054	64%	1e-147	**99%**
CP023756.1	***Solanum lycopersicum* cultivar I-3 chromosome 00**	521	521	64%	6e-146	**99%**
XM 004253276.3	**PREDICTED: *Solanum lycopersicum* putative pterin-4-alpha-carbinolamine dehydratase (LOC101265812), mRNA**	159	159	19%	6e-37	**99%**
AK322159.1	***Solanum lycopersicum*cDNA, clone: LEFL1034AG11, HTC in leaf**	159	159	19%	6e-37	**99%**

2.3.2 Sequences obtained from the Line 1 of BD7755 germplasm with the forward primer:

GTNNAATCAAGTTCATGCTGAGCTGTGGACTCCTTCGATTGGTGAGTACGCTTCCTCAGTCA
CTCCTTTCCTCTAGCATTATTTAAGAATTCATCTGTAGCGCGTTCTATGTGATAACTGTACTG
ACTTACAGCAAGATTGGGTAAAACACTTGGCAAATTAAGTTCATTTATTCTCAATCTCTTTTA
CTTGTGTTGGAATTCTTTAATGATCATAGTGGTACATTCTAATTGCAGGAGGTTTGAGCATGA
ATGATTTCATCATAGCTGCTAAAATAGNNAAGTAAAGAATTCCAACACAAGTAAAAGAGAT
TGAGAATAAATGAACTTAATTTGCCAAGTGTTTTACCCTATCTTGCTGTTGGTCAGTACAGTT
ATCCCGTCAAACGCGCAAATGATGATTTAGTAATTATTGAGGGAGGAATGGAGNAGCCTTGT
TTTCCCNATTCAAGGAGTCCCAGCTTCCCATGAAATTGATTAGAATGTTTGTAATTGATGAA
AAAAAGGGGGGGGGNGCCTGNNCCTTTCCCCAAANNCAAN

The NCBI-BLAST search output showed that the partial sequence obtained with the forward primer C2_AT5g51110 has 95% nucleotide similarities with previous records of *Solanum lycopersicum* putative pterin-4-alpha-carbinolamine dehydratase (LOC101265812), mRNA (XM 004253276.3) and cDNA clone of LEFL1034AG11 in *Solanum Lycopersicum* leaf (AK322159.1). It also shares 99% nucleotide similarity with clone HBa0065A14 in chromosome 3 of *Solanum lycopersicum* (AC246924.1), confirming its presence in chromosome 3 as per previous records. It further shares 99% nucleotide similarities with *Solanum lycopersicum* cultivar I-3 chromosome 00 (CP023756.1).

Table 29. Per cent identities of the amplicon at the nucleotides level obtained using the forward primer of Ty-4 gene with reported Ty-4 gene

Accession	Description	Max score	Total score	Query cover	E value	Identity
AC246924.1	***Solanum lycopersicum* chromosome 3 clone HBa0065A14, complete sequence**	514	1382	88%	1e-143	**99%**
CP023756.1	***Solanum lycopersicum* cultivar I-3 chromosome 00**	508	685	88%	6e-142	**99%**
XM 004253276.3	**PREDICTED: *Solanum lycopersicum* putative pterin-4-alpha-carbinolamine dehydratase (LOC101265812), mRNA**	87.9	159	16%	4e-15	**95%**
AK322159.1	***Solanum lycopersicum*cDNA, clone: LEFL1034AG11, HTC in leaf**	87.9	159	16%	4e-15	**95%**

2.4.1 Sequences obtained from the Line 1 of BD7755 germplasm with the reverse primer:

GCTCAACCTCCTGCATTAGAATGNACCACTATGNTCATTAAAGAATTCCAACACAAGTAAAA
GAGATTGAGAATAAATGAACTTAATTTGCCAAGTGTTTTACCCAATCTTGCTGTAAGTCAGT
ACAGTTATCACATAGAACGCGCTACAGATGAATTCTTAAATAATGCTAGAGGAAAGGAGTG
ACTGAGGAAGCGTACTCACCAATCGAAGGAGTCCACAGCTCAGCATGAACTTGATTAGACT
GTTGTAATTGATGAAGAGTGGGGAGGTGCCCTGTGCCTTCCACCAANNGCTCANAATGAANT
GGATGCTTCGGCTTCTTCAAATCAAAGATNACACAGCCTCCGAAGAAATCACATACTACCTG
AGTCACAAAAAACCTCGCTATCAGCTTCGTTAATGAACCCAGGCCAAAA

The NCBI-BLAST search output showed that the partial sequence obtained with the forward primer C2_AT5g51110 has 100% nucleotide similarities with previous records of *Solanum lycopersicum* putative pterin-4-alpha-carbinolamine dehydratase (LOC101265812), mRNA (XM 004253276.3) and cDNA clone of LEFL1034AG11 in *Solanum lycopersicum* leaf (AK322159.1). It also shares 99% nucleotide similarity with clone HBa0065A14 in chromosome 3 of *Solanum lycopersicum* (AC246924.1), confirming its presence in chromosome 3 as per previous records. It further shares 98% nucleotide similarities with *Solanum lycopersicum* cultivar I-3 chromosome 00 (CP023756.1).

Table 30. Per cent identities of the amplicon at the nucleotides level obtained using the reverse primers of Ty-4 gene with reported Ty-4 gene

Accession	Description	Max score	Total score	Query cover	E value	Identity
AC246924.1	***Solanum lycopersicum* chromosome 3 clone HBa0065A14, complete sequence**	521	1043	67%	6e-146	**99%**
CP023756.1	***Solanum lycopersicum* cultivar I-3 chromosome 00**	516	516	67%	3e-144	**98%**
XM 004253276.3	**PREDICTED: *Solanum lycopersicum* putative pterin-4-alpha-carbinolamine dehydratase (LOC101265812), mRNA**	163	163	20%	4e-38	**100%**
AK322159.1	***Solanum lycopersicum* cDNA, clone: LEFL1034AG11, HTC in leaf**	163	163	20%	4e-38	**100%**

2.5.1 Sequences obtained from the Line 1 of Local-Khushtia-1 germplasm with the forward primer:

ACAGNNCTAATCAAGTTCATGCTGAGCTGTGGACTCCTTCGATTGGTGAGTACGCTTCCTCA
GTCACTCCTTTCCTCTAGCATTATTTAAGAATTCATCTGTAGCGCGTTCTATGTGATAACTGT
ACTGACTTACAGCAAGATTGGGTAAAACACTTGGCAAATTAAGTTCATTTATTCTCAATCTCT
TTTACTTGTGTTGGAATTCTTTAATGATCATAGTGGTACATTCTAATTGCAGGAGGTTTGAGC
ATGAATGATTTCATCATAGCTGCTAAAATAGTNAAGTAAAGAATTCCAACACAAGTAAAAG
AGATTGAGAATAAATGAACTTAATTTGCCAAGTGTTTTACCCAATCTTGCTGTAAGTCAGTA
CAGTTATCACATAGAACGCGCTACAGATGAATTCTTAAATAATGCTAGAGGAAAGGAGTGA
CTGNGGAAGCGTACTCACCGATCGAAGGAGCCCCCAGCCCTGATTGAACTGTTGTNATACTG
NAAAAATGGNTGAAGAGCCTGCCCCTTCCCCAAANNNAAANN

The NCBI-BLAST search output showed that the partial sequence obtained with the forward primer C2_AT5g51110 has 96% nucleotide similarities with previous records of *Solanum lycopersicum* putative pterin-4-alpha-carbinolamine dehydratase (LOC101265812), mRNA (XM 004253276.3) and cDNA clone of LEFL1034AG11 in *Solanum Lycopersicum* leaf (AK322159.1). It also shares 99% nucleotide similarity with clone HBa0065A14 in chromosome 3 of *Solanum lycopersicum* (AC246924.1), confirming its presence in chromosome 3 as per previous records. It further shares 99% nucleotide similarities with *Solanum lycopersicum* cultivar I-3 chromosome 00 (CP023756.1).

Table 31. Per cent identities of the amplicon at the nucleotides level obtained using the forward primers of Ty-4 gene with reported Ty-4 gene

Accession	Description	Max score	Total score	Query cover	E value	Identity
AC246924.1	***Solanum lycopersicum* chromosome 3 clone HBa0065A14, complete sequence**	521	1692	86%	8e-146	**99%**
CP023756.1	***Solanum lycopersicum* cultivar I-3 chromosome 00**	516	835	86%	4e-144	**99%**
XM 004253276.3	**PREDICTED: *Solanum lycopersicum* putative pterin-4-alpha-carbinolamine dehydratase (LOC101265812), mRNA**	91.6	166	16%	3e-16	**96%**
AK322159.1	***Solanum lycopersicum* cDNA, clone: LEFL1034AG11, HTC in leaf**	91.6	166	16%	3e-16	**96%**

2.5.2 Sequences obtained from the Line 1 from Local Khushtia-1 germplasm with the reverse primer:

GCTCAAACCTCCTGCATTAGAATGTACCACTATGATCATTAAAGAATTCCAACACAAGTAAA
AGAGATTGAGAATAAATGAACTTAATTTGCCAAGTGTTTTACCCAATCTTGCTGTAAGTCAG
TACAGTTATCACATAGAACGCGCTACAGATGAATTCTTAAATAATGCTAGAGGAAAGGAGT
GACTGAGGAAGCGTACTCACCAATCGAAGGAGTCCACAGCTCAGCATGAACTTGATTAGAC
TGTTGTAATTGATGAAGAGTGGGGAGGTGCCCTGTGCCTTCCACCAACTCNTANGAAAATGG
ATGCGTCCTGATCTCNTNGCCCGAGGGTTAGCCTCCNAGNGGTCATCACTCCTGATCACATA
AAACCCCCCTCCCACTAANNTTTATAATGTTNGAGGGAAGGAAGGGNGAGGNGGAACCTCC
CACCCAAGAA

The NCBI-BLAST search output showed that the partial sequence obtained with the forward primer C2_AT5g51110 has 99% nucleotide similarities with previous records of *Solanum lycopersicum* putative pterin-4-alpha-carbinolamine dehydratase (LOC101265812), mRNA (XM 004253276.3) and cDNA clone of LEFL1034AG11 in *Solanum lycopersicum* leaf (AK322159.1). It also shares 99% nucleotide similarity with clone HBa0065A14 in chromosome 3 of *Solanum lycopersicum* (AC246924.1), confirming its presence in chromosome 3 as per previous records. It further shares 99% nucleotide similarities with *Solanum lycopersicum* cultivar I-3 chromosome 00 (CP023756.1).

Table 32. Per cent identities of the amplicon at the nucleotides level obtained using the reverse primers of Ty-4 gene with reported Ty-4 gene

Accession	Description	Max score	Total score	Query cover	E value	Identity
AC246924.1	***Solanum lycopersicum* chromosome 3 clone HBa0065A14, complete sequence**	534	1069	64%	8e-150	**99%**
CP023756.1	***Solanum lycopersicum* cultivar I-3 chromosome 00**	529	529	64%	4e-148	**99%**
XM 004253276.3	**PREDICTED: *Solanum lycopersicum* putative pterin-4-alpha-carbinolamine dehydratase (LOC101265812), mRNA**	163	163	20%	5e-38	**99%**
AK322159.1	***Solanum lycopersicum* cDNA, clone: LEFL1034AG11, HTC in leaf**	163	163	20%	5e-38	**99%**

3. Sequence analysis of Ty-5 gene amplified by using AVRDC-TM273 primer pair

3.1.1 Sequences obtained from the Line12 of BD10124 germplasm with the forward primer:

GTGTGNGTNNNGATGATGACGACGACGACGACGACGACGATGNTGATGACGATGAAGGTGN
NGNTGCTAGTGTCTGTACACTAGTGGAGGTGATGATGGTGNNGGTGGTGCTATCGCCTATAT
AGAACAATTGGCTNNATATTTAGAAGCTTGTNTTNNATATTCAAACTCTCTCCTACCTCGATT
TTGGTCCCGTAGAGGGTGGGCGTGCACCNGNATGTCCTCGGNGCCTTAGGATTCACGACAAC
CAGNGNCGTGGN

The NCBI-BLAST search output showed that the partial sequence obtained with the forward primer AVRDC-TM273 shows 94% and 93% nucleotide similarities with *Solanum lycopersicum* chromosome 4 from accessionsCP023760.1 and HG975516.1, respectively, confirming its presence in chromosome 4 as per previous reports.

Table 33. Per cent identities of the amplicon at the nucleotides level obtained using the forward primer of Ty-5 gene with reported Ty-5 gene

Accession	Description	Max score	Total score	Query cover	E value	Identity
CP023760.1	***Solanum lycopersicum* cultivar I-3 chromosome 4**	189	189	41.0%	3e-46	**94%**
HG975516.1	***Solanum lycopersicum* chromosome ch04, complete genome**	183	183	41.0%	1e-44	**93%**

3.1.2 Sequences obtained from the Line 12 of BD10124 germplasm with the reverse primer:

CNAGTGNNNAGACACTAGCACCACCACCTTCATCGTCATCATCATCGTCGTCGTCGTCGTCG
TCGTCATCANNANTNNCATNATCATCATCAAAACCTTTAGTATCACCACCACCGGTAAGCTA
TCCATGAGCACCAGGAAAGATTACTGTATGATGCCTCCCCCCTCTCCCCGGGACCAGAGTCT
TCGCAAATATGGTTTAAATCTCTAATACAGGTTCCTAGATGCCTAGAAGGTGCTAATGTTCTC
ATTTTCTTGAATATAAACAANAAATTGTGAGGGGGAGGACCAAAGAACTCAAAATGATAAT
GGATGCCG

The NCBI-BLAST search output showed that the partial sequence obtained with the reverse primer AVRDC-TM273 shows 94% nucleotide similarities with *Solanum lycopersicum* chromosome 4 from accessions CP023760.1 and HG975516.1 confirming its presence in chromosome 4 as per previous reports.

Table 34. Per cent identities of the amplicon at the nucleotides level obtained using the reverse primer of Ty-5 gene with reported Ty-5 gene

Accession	Description	Max score	Total score	Query cover	E value	Identity
CP023760.1	***Solanum lycopersicum* cultivar I-3 chromosome 4**	207	207	38%	1e-51	**94%**
HG975516.1	***Solanum lycopersicum* chromosome ch04, complete genome**	198	198	38%	6e-49	**94%**

3.2.1 Sequences obtained from the Line 14 of BD10124 germplasm with forward primer:

TGTGNGTGNNGNNGANGATGACGACGACGACGACGACGACGATGATGACGACGANGGTGG
TGNTGCTNCTNNCGGCAGTCTACTGGAGGAGATGATGATGGNGNTGGTGNTGTTNTCTATAA
AGAAGANNGANGNTATCTTACTCGCNTGTCNTCTCNNATGAGACGCTACCGCCGCCTCCTCG
TTCGTCGTCATGATGGTGGTGGTGGTNGTAGTAGTNCCCTCGACACCCTNCAGTAACAGACA
CCANTGTTNAGACCACTGCCGCTAAGAGCAATAAGAAACCTAATTNTACACCCGGGAAA

The NCBI-BLAST search output showed that the partial sequence obtained with the forward primer AVRDC-TM273 shows 98% nucleotide similarities with *Solanum lycopersicum* chromosome 4 from accessionsCP023760.1 and HG975516.1, confirming its presence in chromosome 4 as per previous reports.

Table 35. Per cent identities of the amplicon at the nucleotides level obtained using the forward primer of Ty-5 gene with reported Ty-5 gene

Accession	Description	Max score	Total score	Query cover	E value	Identity
CP023760.1	***Solanum lycopersicum* cultivar I-3 chromosome 4**	307	367	36%	2e-81	**98%**
HG975516.1	***Solanum lycopersicum* chromosome ch04, complete genome**	307	367	36%	2e-81	**98%**

3.2.2 Sequences obtained from the Line 14 of BD10124 germplasm with the reverse primer:

CNNGTNNACAGACACTAGCACCACCACCTTCATCGTCATCATCATCGTCGTCGTCGTCGTCG
TCGTCATCATNATCATCATCATCATCATAACCATTATTATCATCACCACCGCCGGGCTATTAT
TGATGANCACCAGNNTCAGATTACTGTATGATGCCCNGCATCTCCCCCCCANAACCAAAGTC
TTCGAAAATATGGTTTAATCTCCAAAAAGAGGNTCTANGAGNCNNAAATGACCCTAATGTTC
TCATTTTCTTGNATAACAACGANAATGTGNNNAGGAAAACAAAAAAGATCTCAAANTGATN
NTGGANGCNTCAGTAATCAACTNANAAAANACNC

The NCBI-BLAST search output showed that the partial sequence obtained with the forward primer AVRDC-TM273 shows 99% nucleotide similarities with *Solanum lycopersicum* chromosome 4 from accessions CP023760.1 and HG975516.1, confirming its presence in chromosome 4 as per previous reports.

Table 36. Per cent identities of the amplicon at the nucleotides level obtained using the reverse primers of Ty-5 gene with reported Ty-5 gene

Accession	Description	Max score	Total score	Query cover	E value	Identity
CP023760.1	**Solanum lycopersicum cultivar I-3 chromosome 4**	322	322	40%	6e-86	**99%**
HG975516.1	**Solanum lycopersicum chromosome ch04, complete genome**	322	322	40%	6e-86	**99%**

3.3.1 Sequences obtained from the Line 14 of BD10122 germplasm with the forward primer:

GNGTGNGNGTNANGATGATGACGACGACGACGACGACGATGATGATGACGATGAAGGTGG
TGGTGCTAGTGTCTGTACACTAGTGGAGGTGATGATGGTGATGGTGGTGCTATCGCCTATAT
AGAGACCACACTCTAGGCATCTACANATCTGTTCTAGTGTTAGACCCTACCGCGACAACGTT
TTTCGTCTGTTGTTGGTGGTGGTCGTCGTTCTTNNATTCCCCCGAAAACTTTGAGAGAACCAG
TGTCTCAACGGAGAGAGATNTGCTATTATGGATGACAAAGANACTTNTCCTACATCAG

The NCBI-BLAST search output showed that the partial sequence obtained with the forward primer AVRDC-TM273 shows 100% and 99% nucleotide similarities with *Solanum lycopersicum* chromosome 4 from accessionsHG975516.1and CP023760.1, respectively, confirming its presence in chromosome 4 as per previous reports.

Table 37. Per cent identities of the amplicon at the nucleotides level obtained using the forward primer of Ty-5 gene with reported Ty-5 gene

Accession	Description	Max score	Total score	Query cover	E value	Identity
HG975516.1	***Solanum lycopersicum* chromosome ch04, complete genome**	206	206	34%	4e-51	**100%**
CP023760.1	***Solanum lycopersicum* cultivar I-3 chromosome 4**	200	200	34%	2e-49	**99%**

3.3.2 Sequences obtained from the Line 14 of BD10122 germplasm with the reverse primer:

CNANGTGNACAGACACTAGCACCACCACCTTCATCGTCATCATCATCGTCGTCGTCGTCGTC
GTCATCATCATCATCATCATCATCATCAAAACCTTTAGTATCACCACCACCGGTAAGCTATCC
ATGANCACCA

The NCBI-BLAST search output showed that the partial sequence obtained with the forward primer AVRDC-TM273 shows 98% and 96% nucleotide similarities with *Solanum Lycopersicum* chromosome 4 from accessionsHG975516.1and CP023760.1, respectively, confirming its presence in chromosome 4 as per previous reports.

Table 38. Per cent identities of the amplicon at the nucleotides level obtained using the reverse primers of Ty-5 gene with reported Ty-5 gene

Accession	Description	Max score	Total score	Query cover	E value	Identity
HG975516.1	***Solanum lycopersicum* chromosome ch04, complete genome**	233	233	86%	6e-60	**98%**
CP023760.1	***Solanum lycopersicum* cultivar I-3 chromosome 4**	219	219	86%	2e-55	**96%**

3.4.1 Sequences obtained from the Line 1 of BD7755 germplasm with the forward primer:

TNTNTGNGNGTGACGACGACGACGACGACGATGATGATGACGATGATGGTGCTTTCNCTGCT
CTCTGNTCATCCCTNACTGTCGCTGGTTATGATGCGCGAACTGGCGTTCCTGACCACAATATA
GGTTTCTNAAAACTCGTGTNTGATATTTTTATCCTTTTGCCATTTCNTTGGGTTGNTAGAGGG
GGTGTGCGTCGTCGTAGTGTTCCGACTCAAGAGATTGAGAGANCGAGTGTCAGCGAGGN

The NCBI-BLAST search output showed that the partial sequence obtained with the forward primer AVRDC-TM273 shows 98% nucleotide similarities with *Solanum lycopersicum* chromosome 4 from accessionsCP023760.1 and HG975516.1, confirming its presence in chromosome 4 as per previous reports.

Table 39. Per cent identities (nucleotides) of the amplicons obtained using the forward primer of Ty-5 gene with reported Ty-5 gene

Accession	Description	Max score	Total score	Query cover	E value	Identity
CP023760.1	***Solanum lycopersicum*** **cultivar I-3 chromosome 4**	325	413	56%	5e-87	**98%**
HG975516.1	***Solanum lycopersicum*** **chromosome ch04, complete genome**	325	411	56%	5e-87	**98%**

3.4.2 Line 1 from BD7755 germplasm with the reverse primer (150 bp)

ATNNNNGNCACTAGCACCACCACCTTCATCGTCATCATCATCGTCGTCGTCGTCGTCGTCATC
ATCATNANCNTCANNANNATCAAAACCTTTAGGATCACCACCACCGNNNNAGCTAAAATGN
NNACCAANA

The NCBI-BLAST search output showed that the partial sequence obtained with the forward primer AVRDC-TM273 shows 81.0%nucleotide similarities with *Solanum lycopersicum* chromosome 4 from accessionsCP023760.1 and HG975516.1, confirming its presence in chromosome 4 as per previous reports.

Table 40. Per cent identities of the amplicon at the nucleotides level obtained using the reverse primer of Ty-5 gene with reported Ty-5 gene

Accession	Description	Max score	Total score	Query cover	E value	Identity
CP023760.1	***Solanum lycopersicum*** **cultivar I-3 chromosome 4**	131	131	23%	5e-87	**81.0%**
HG975516.1	***Solanum lycopersicum*** **chromosome ch04, complete genome**	131	131	23%	5e-87	**81.0%**

3.5.1 Sequences obtained from the Line 1 from Local-Khushtia-1 germplasm with the forward primer of Ty-5 gene:

TNTNNNNNTNNNGTNTNNNGATGATGACGACGACGACGACGACGATGATGACGACGAT
GGTGGTGGTGCTGCTGTTGGCTGTCTACTGGAGGTGGTGATGATGNTGATGGTGCTCTGTCC
TGACTGACGCCAAGATTGNNTAAAACACTAGCCACACTAAGCGCATTCAGTCTCCAGCCCTT
TTACTTGCGTCGTAGTGCTGTTGTGGGCGGTGGTCGTCGTCGTAATCGCAGTAGGCTTCATCA
TGATCGTCACCGTTATTGCTGTAACGGTTGGTNGGGTAAAGAAGACCAACT

The NCBI-BLAST search output showed that the partial sequence obtained with the forward primer AVRDC-TM273 shows 97% nucleotide similarities with *Solanum lycopersicum* chromosome 4 from accessionsCP023760.1 and HG975516.1, confirming its presence in chromosome 4 as per previous reports.

Table 41. Per cent identities of the amplicon at the nucleotides level obtained using the forward primer of Ty-5 gene with reported Ty-5 gene

Accession	Description	Max score	Total score	Query cover	E value	Identity
CP023760.1	***Solanum lycopersicum*** **cultivar I-3 chromosome 4**	331	331	53%	8e-89	**97%**
HG975516.1	***Solanum lycopersicum*** **chromosome ch04, complete genome**	331	331	53%	8e-89	**97%**

3.5.2 Sequences obtained from the Line 1 of Local-Khushtia-1 germplasm with reverse primer of Ty-5 gene:

CNCTAGCACCACCACCTTCATCGTCATCATCATCGTCGTCGTCGTCGTCGTCATCATCAT
CATCATCATCATCATAACCTTTATTATCATCACCACCGGNGGGNTATTATTGATGANCACCA
NTNNTCATCGTGTTTGGGTNTGTCCCCTTATACGGATCGTCCCNCANNTCAAGTTGNGTACGT
ACGAGAAAACCGGNNTCGCG

The NCBI-BLAST search output showed that the partial sequence obtained with the forward primer AVRDC-TM273 shows 98% nucleotide similarities with *Solanum lycopersicum* chromosome 4 from accessionsCP023760.1 and HG975516.1, confirming its presence in chromosome 4 as per previous reports.

Table 42. Per cent identities of the amplicon at the nucleotides level obtained using the reverse primer of Ty-5 gene with reported Ty-5 gene

Accession	Description	Max score	Total score	Query cover	E value	Identity
CP023760.1	***Solanum lycopersicum*** **cultivar I-3 chromosome 4**	313	313	32%	3e-83	**98%**
HG975516.1	***Solanum lycopersicum*** **chromosome ch04, complete genome**	313	313	32%	3e-83	**98%**

4. Sequence Analysis of the Ty 1 gene amplified by using JB-1 primer from different germplasm

4.1.1 Sequences obtained from the line 7 of germplasm BD-10123 by JB-1 forward primer:

CNAATNCTNCTNCACTAACTTCTCATTCCATCCCACTTTCTTAACGTACTCATCACTCGCCCT
CGTAAAGAACCCCGCATTAATAGCTGAACCACCACCTAAGAGCGAGCACGGTGGTTGAAGA
CACCGTCTGTAGAGATGAAAAGTTGCGAGGGAGATGACGGAAAAATGTTAGCTAAGTTGCT
TGAGAAACCGTTAATGTTTGTGATGTTTGGGTTTCCATAGGGTAAGCACCTCTTTCTAATAAC
AGAACATTGAACGATTGTGAGAGTGTTGCTGCTAATGCACAACCAGCAGTTCCTCCTCCTAT
TATGATGTAATCGAACssGAAATAACCTTTGGTGATGACGTANCATCTTCGCAAACGTCGAAT
ATGGGGCTACATTGCATCAAACAATTATTAATATTTTAATTTTATTTATTTGACAAAAACTCT
TTTTTTTTTCTAAATATTCTATGATCTTCATAGAATTTGTGACCCTTTTTTGATTTTGAATTTTT
GATAGTAAATTTTTTTTTTTTAACTTCAATACATGTTAACTTGTCATAGCAAGTTATTGTCTTA
TTTTGAATTAACCAATTCTATTTACCTTCATTTGACTTTTACCTAATAACTTGAACATGGNAA
AACTAATTCCTTTTTCTTACCCCCTCTTATTATTTTTTTTTTTTTAAAAAAAAAAAAT

The NCBI-BLAST database search output showed the partial sequence obtained from the JB-1 forward primer has 96% nucleotides similarities with *Solanum lycopersicum*, chromosome ch06, complete genome (HG975518.1). Here it is sharing maximum 99% nucleotides identities with *Solanum lycopersicum*, Protein HOTHEAD (LOC101244002), mRNA, (NM 001317332.1), *Solanum lycopersicum,* c DNA, clone: LEFL2029D08, HTC in fruit, (AK327423.1). This gene contains GMC oxidoreductase domain. This domain found to be associated with pfam00732 (Table 43).

In case of *Solanum pennellii* chromosome ch06, complete genome (HG975445.1) and it is sharing maximum 95% nucleotides identities with *Solanum pennellii, Solanum pennellii* Protein HOTHEAD – like (LOC 107021617), transcript variant X1, mRNA, (XM 015222307.1) and *Solanum pennellii* Protein HOTHEAD –like (LOC 107021617), transcript variant X1, mRNA, (XM 015222306.1).

Table 43. Per cent identities of the amplicon at nucleotides level obtained using the forward primer of Ty-1 gene with reported Ty-1 gene

Description	Max Score	Total Score	Query cover	E value	Ident	Accession
Solanum lycopersicum, cultivar 1-3 chromosome 6	1105	1105	95 %	0.0	96%	CP023762.1
Solanum lycopersicum chromosome ch06, complete genome	1105	1105	95 %	0.0	96%	HG975518.1
Solanum lycopersicum Protein HOTHEAD (LOC101244002),mRNA	675	675	51 %	0.0	99%	NM 001317332.1
Solanum lycopersicum c DNA ,clone : LEFL2029D08, HTC in fruit	675	675	51 %	0.0	99%	AK327423.1
Solanum pennellii chromosome ch06, complete genome	597	597	54 %	2e-166	95%	HG975445.1

5 Sequence Analysis of the Ty 3 gene amplified by using Fluw-25 primer pair

5.1.1 Sequence obtained from the line 7 of germplasm BD-10123 obtained by forward primer of Ty 3 gene

CNTNNANCCCTTACCNTNCGTATTTAGCATAGGGTGAGTGAAATGTAGGATTATACATGGGG
TATTCNNTCTTAAAAACGTGTTATAAAGGCTAAAAGGGAATTCTACTTCTTGTAAAATATAA
AGGTAGTGGAAATGATGCTGCTCAAATTATTGTGTGAACATATTATGAGAGGTAGGATTAAG
AATGAAGTTATATAAGATAAAGTGGAAGTTACTTTTCGAAAAAAAAGAAAGACGAAAAAAA
TGAGATTGAAATGGATTGAATACGTGAAGAAGAGATGCATGGGTTCACCAATAAAAAGGTT
TGAGAGTTTGACTTAAGAAGAGGTAGAAGTAGGTTGAAAAACAACTAGTAAAGTTTTACTTT
TAGTTTTGTTTTGATTGCACATTTTTTTAGTCGAAATAGAAACAGAGGTCCNTATATGGGGN

The NCBI-BLAST database search output showed the partial sequence obtained from the forward Fluw-25 primer has 98% nucleotides similarities with Solanum lycopersicum, chromosome ch06, complete genome (HG975518.1). In case of *Lycopersicum esculentum*, clone 56B23 chromosome 6, genome sequence (AY678298.1) here it is sharing maximum 98% nucleotides identities similarities with *Lycopersicum esculentum.*

Lastly, *Solanum pennellii* chromosome ch06, complete genome**,** it is sharing maximum 92% nucleotides identities similarities with *Solanum pennellii*, (HG975445.1).

Table 44. Per cent identities of the amplicon at nucleotides level obtained using the forward primer of Ty-3 gene with reported Ty-3 gene

Description	Max Score	Total Score	Query Cover	E value	Ident	Accession
Solanum lycopersicum cultivar 1-3 chromosome 6	732	732	92%	0.0	98%	CP023762.1
Solanum lycopersicum chromosome ch06, complete genome	732	732	92%	0.0	98%	HG975518.1
Lycopersicum esculentum clone 56B23 chromosome6,genomic sequence	732	732	92%	0.0	98%	AY678298.1
Solanum pennellii chromosome ch06, complete genome	468	572	86%	8e-128	92%	HG975445.1

5.1.2 Sequence obtained from the line 13 of germplasm BD-10123 obtained by forward primer of Ty 3 gene:

TCCAAACCTTNNAACCTTNNCNTNGTTATTAGCATAGGGTGAGTGAAATGTAGGATTATACATGG
GGTATTCANNCGTAAGAACGTGTTATAAAGGCTAAAAGGGAAGTTCACTTCTTGTAAAATATAAA
GGTAGTGGAAATGATGCTGCTCAAATTATTGTGTGAACATATTATGAGAGGTAGGATTAAGAATG
AAGTTATATAAGATAAAGTGGAAGTTACTTTTCGAAAAAAAAAAAAGACGAAAAAAATGAGATT
GAAATGGATTGAATACGTGAAGAAGAGATGCATGGGTTCACCAATAAAAAGGTTTGAGAGTTTG
ACTTAAGAAGAGGTAGAAGTAGGTTGAAAAACAACTAGGTAAGTTTTACTTTTAGTTTTGTTTTG
ATTGCACATTTTTTTAGTCGAAATAGAAACAGAGGTTATATATGGA

The NCBI-BLAST database search output showed the partial sequence obtained from the forward Fluw-25 primer has 98% nucleotides similarities with Solanum lycopersicum, chromosome ch06, complete

genome (HG975518.1). In case of *Lycopersicum esculentum*, clone 56B23 chromosome6, genome sequence (AY678298.1) here it is sharing maximum 98% nucleotides identities similarities with *Lycopersicum esculentum.* Lastly, *Solanum pennellii* chromosome ch06, complete genome**,** it is sharing maximum 93% nucleotides identities similarities with *Solanum pennellii*, (HG975445.1).

Table 45. Per cent identities of the amplicon at nucleotides level obtained using the forward primer of Ty-3 gene with reported Ty-3 gene

Description	Max Score	Total Score	Query Cover	E value	Ident	Accession
Solanum lycopersicum cultivar 1-3 chromosome 6	767	767	96%	0.0	98%	CP023762.1
Solanum lycopersicum chromosome ch06, complete genome	767	767	96%	0.0	98%	HG975518.1
Lycopersicum esculentum clone 56B23 chromosome6,genomic sequence	767	767	96%	0.0	98%	AY678298.1
Solanum pennellii chromosome ch06, complete genome	481	481	72%	1e-131	93%	HG975445.1

5.2.1 Sequence obtained from the line 8 of germplasm BD-10125 by forward primer of Ty 3 gene:

TTTTTTTCCAAACCCTTCAAACCTTACCATCGTTATTAGCATAGGGTGAGTGAAATGTAGGATTATACA
TGGGGTATTCAGTCGTAAGAACGTGTTATAAAGGCTAAAAGGGAAGTCTACTTCTTGTAAAATATAAA
GGTAGTGGAAATGATGCTGCTCAAATTATTGTGTGAACATATTATGAGAGGTAGGATTAAGAATGAAG
TTATATAAGATAAAGTGGAAGTTACTTTTCGAAAAAAAAGAAAGACGAAAAAAATGAGATTGAAATG
GATTGAATACGTGAAGAAGAGATGCATGGGTTCACCAATAAAAAGGTTTGAGAGTTTGACTTAAGAA
GAGGTAGAAGTAGGTTGAAAAACAACTAGGTAAGTTTTACTTTTAGTTTTGTTTTGATTGCACATTTTT
TTAGTCGAAATAGAAACAGAGTNATATATGGAGCCCTAAAAAAATGTGCAATCAAAACAAAACTAAA
AGTAAAACTTTACCTAGNNGTTTTCAACCTACTTCTACCTCCTTTGAGNNCACCCCCCTTTTTATTGGG
GGGACCCTGCATCTCTTCTTCCGTATTCATCCTTTTCATCNCATTTTTTTCGCCTTCTTTTTTTTTCNANA
GGNNATNCNTTATCTTATATAAATTCTNTTAATCTNCNNNCCTNNTATGNNCACATAATTGGGGCGCTC
TTTCCTTACTTTATATTTTTTC

The NCBI-BLAST database search output showed the partial sequence obtained from the forward Fluw-25 primer has 99% nucleotides similarities with Solanum lycopersicum, chromosome ch06, complete genome (HG975518.1). In case of *Lycopersicum esculentum*, clone 56B23 chromosome 6, genome sequence (AY678298.1) here it is sharing maximum 99% nucleotides similarities with *Lycopersicum esculentum.*

Table 46. Per cent identities of the amplicon at nucleotides level obtained using the forward primer of Ty-3 gene with reported Ty-3 gene

Description	Max Score	Total Score	Query cover	E value	Ident	Accession
Solanum lycopersicum cultivar 1-3 chromosome 6	782	782	61%	0.0	99%	CP023762.1
Solanum lycopersicum chromosome ch06, complete genome	782	782	61%	0.0	99%	HG975518.1
Lycopersicum esculentum clone 56B23 chromosome 6, genome sequence	782	782	61%	0.0	99%	AY678298.1

6. Sequence Analysis of the Ty 3 gene amplified by using P6-25 primer pair

6.1.1 Sequence obtained from the line 7 of germplasm BD-10123 obtained by forward primer of Ty 3a gene:

GAGAGGNNGGATNAGATGANGTTATATAAGATAAAGCGGAAGTTACTTTTCGAAAAAAAAA
GAAANANGAAAAAAATGAGATTGAAATGGATTGAATACGTGAAGAAAGATGCATGGGTTC
ACCAATAAAAAGGTTTGAGAGTTTGACTTAAGAAGAGGTAGAAGTAGGTTGAAAAACAACT
AGGTAAAGTTTTACTTTTAGTTTTGTTTTGATTGCACATTTTTTTAGTCGAATAGAAACAGAG
GTTATATATGGGACAATAGGCAGAGCA

The NCBI-BLAST database search output showed the partial sequence obtained from the forward P6-25 primer has 97% nucleotides similarities with Solanum lycopersicum, chromosome ch06, complete genome (HG975518.1). In case of *Lycopersicum esculentum*, clone 56B23 chromosome6, genome sequence (AY678298.1) here it is sharing maximum 97% nucleotides identities similarities with *Lycopersicum esculentum.*

Table 47. Per cent identities of the amplicon at nucleotides level obtained using the forward primer of Ty-3a gene with reported Ty-3a gene

Description	Max Score	Total Score	Query Cover	E value	Ident	Accession
Solanum lycopersicum cultivar 1-3 chromosome 6	470	470	93%	1e-128	97%	CP023762.1
Solanum lycopersicum chromosome ch06, complete genome	470	470	93%	1e-128	97%	HG975518.1
Lycopersicum esculentum clone 56B23 chromosome6,genomic sequence	470	470	93%	1e-128	97%	AY678298.1

6.2.1 Sequence obtained from the line 8 of germplasm BD-10125 obtained by forward primer of Ty 3a gene:

GATNAGATGAAGTTATATAAGATAAAGTGGAAGTTACTTTTCGAAAAAAAAAGAAAGACGA
AAAAAATGAGATTGAAATGGATTGAATACGTGAAGAAGAGAGCATGGGTTCACCAATAAAA
AGGTTTGAGAGTTTGACTTAAGAAGAGGTAGAAGTAGGTTGAAAAACAACTAGGTAAAGTT
TTACTTTTAGTTTTGTTTTGATTGCACATTTTTTTAGTCGAAATAGAACAGAGGTTATATATG
GGACAATAGGCAGAGCA

The NCBI-BLAST database search output showed the partial sequence obtained from the forward P6-25 primer has 100% nucleotides similarities with *Solanum lycopersicum,* chromosome ch06, complete genome (HG975518.1). In case of *Lycopersicum esculentum*, clone 56B23 chromosome6, genome sequence (AY678298.1) here it is sharing maximum 100% nucleotides identities similarities with *Lycopersicum esculentum.* Lastly, *Solanum pennellii* chromosome ch06, complete genome**,** it is sharing maximum 90% nucleotides identities similarities with *Solanum pennellii*, (HG975445.1).

Table 48. Per cent identities of the amplicon at nucleotides level obtained using the forward primer of Ty-3a gene with reported Ty-3a gene

Description	Max Score	Total Score	Query Cover	E value	Ident	Accession
Solanum lycopersicum cultivar 1-3 chromosome 6	479	479	89%	2e-131	100%	CP023762.1
Solanum lycopersicum chromosome ch06, complete genome	479	479	89%	2e-131	100%	HG975518.1
Lycopersicum esculentum clone 56B23 chromosome6,genomic sequence	479	479	89%	2e-131	100%	AY678298.1
Solanum pennellii chromosome ch06, complete genome	202	202	55%	5e-48	90%	HG975445.1

6.3.1 Sequence obtained from the line 9 of germplasm BD-7761 obtained by forward primer of Ty 3a gene:

GGGNNNNTTTNNNAGAGGNAGGANNAGATGAANTTATATAAGATAAAGTTGGAAGTTACTT
TTCGAAAAAAAAAGAAAGACGAAAAAAATGAGATTGAAATGGATTGAATACGTGAAGAGA
GATGCATGGGTTCACCAATAAAAAGGTTTGAGAGTTTGACTTAAGAAGAGGTAGAAGTAGG

TTGAAAAACAACTAGGTAAAGTTTTACTTTTAGTTTTGTTTTGATTGCACATTTTTTTAGTCG
AATAGAAACAGAGGTTATATATGGGACAATAGGCAGAGCA

The NCBI-BLAST database search output showed the partial sequence obtained from the forward P6-25 primer has 97% nucleotides similarities with Solanum lycopersicum, chromosome ch06, complete genome (HG975518.1). In case of *Lycopersicum esculentum*, clone 56B23 chromosome6, genome sequence (AY678298.1) here it is sharing maximum 97% nucleotides identities similarities with *Lycopersicum esculentum.*

Table 49. Per cent identities of the amplicon at nucleotides level obtained using the forward primer of Ty-3a gene with reported Ty-3a gene

Description	Max Score	Total Score	Query Cover	E value	Ident	Accession
Solanum lycopersicum cultivar 1-3 chromosome 6	475	475	92%	3e-130	97%	CP023762.1
Solanum lycopersicum chromosome ch06, complete genome	475	475	92%	3e-130	97%	HG975518.1
Lycopersicum esculentum clone 56B23 chromosome6,genomic sequence	475	475	92%	3e-130	97%	AY678298.1

Objective 3: Molecular confirmation of the ToLCV resistance/tolerance by agro-inoculation of the ToLCV infectious clone into the promising tomato plants.

The tomato plants which have been confirmed to carry different Ty genes (Ty1, Ty2, Ty3, Ty3a, Ty4 and Ty5) are expected to be resistant / tolerant. The plant plants which carry the respective Ty genes responsible for resistance or tolerance were identified based on field screening and the presence of Ty genes. These plants will further subjected to confirm their resistance tolerance inheritance using Agro-inoculation of an infectious clone of the ToLCV. For this purpose, attempts were made to develop an infectious clone of the ToLCV and followed by phenotypic as well as molecular analysis of the promising plants.

3.1 **Construction of infectious clone**

The results of the step-by-step cloning procedure of the infectious clone of the monopartite ToLCV has been described bellow-

Step I: The insertion of the 300 bp Common Region or CR of the monopartite ToLCV was done using BamHI and EcoRI restriction sites in the pGreen vector. This has generated CR/pGreen vector. Then the

full genome of ToLCV was cloned using BamHI restriction site in the CR/pGreen vector. The expected orientation of the ToLCV genome in contrast to the already cloned CR was confirmed by restriction digestion of EcoRI enzyme. The positive clone was used to maintain the infectious clone and sub-clone into the pBI121 vector (Fig. 17).

Step-II: The infectious clone of the ToLCVs was fished out using HindIII and SacI restriction enzymes from the pGreen background. The destination vector, pBI121 was also linearized with the same restriction enzymes and cloned in *E. coli* Top10 competent cells following ligation. Positive clones were confirmed though restriction digestion and PCR using coat protein gene specific primers, which yielded ToLCV infectious clone/pBI121vector (Fig. 18).

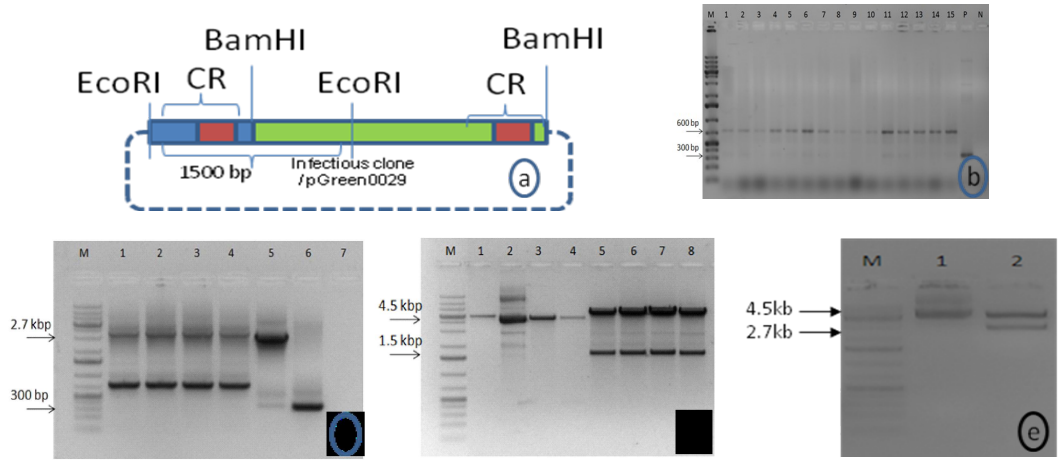

Figure 17. Cloning confirmation of ToLCV infectious clone in pGreen vector: a) Diagrammatic presentation of the infectious clone showing enzyme position used to confirm the orientation of different fragments, b) Colony PCR using vector specific primers showing the cloning of CR in pGreen0029 vector. Lane M is the marker and Lanes 1-15 all are positive, c) PCR of the plasmids of 300bp+2.7kbp/pGreen with vector specific primers. Lane M is the marker, lanes 1-4 representing the mixed amplification, lane 5 showing 3.3 kb amplification, lane 6 shows 300bp of only pGreen vector and lane 7 is the negative control, d) Restriction digestion of Cloned plasmid of 300bp+2.7kb/pGreen with EcoRI. Lane M is the Marker. Lanes 1–4 shows the undigested plasmids and lane 5 – 8 shows digested cloned plasmid of 300bp+2.7kbp/pGreen and e) Restriction digestion of the infectious clone with BamHI enzyme to show the presence of viral genome in the infectious clone.

Step III: The LBA4404 strain of *Agrobacterium tumefacians* was transformed with the infectious clone. Colony PCR using CP forward and reverse primers showed amplification of a 300 bp confirming the transformation of LBA4404 strain of *Agrobacterium tumefacians* (Fig. 18d)

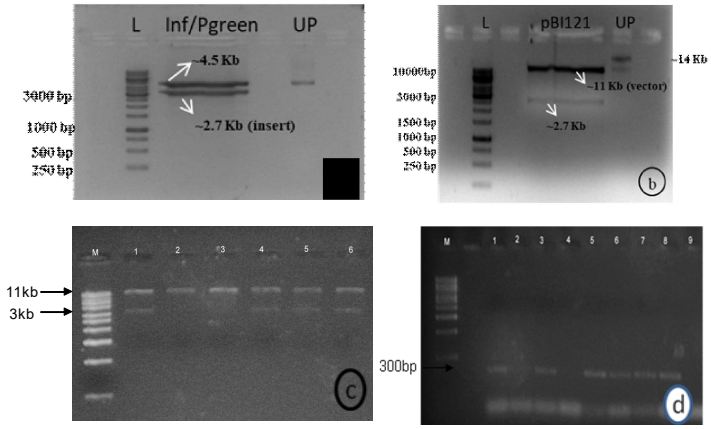

Fig. 18. Results of HindIII and SacI digestion of Inf/pGreen and pBI121. UP- indicating bands for uncut plasmids and L shows 1Kb ladder- a) Inf/pGreen showing ~2.7 Kb fallout (viral insert), b) pB1121 showing ~3.0 Kb GUS gene and some part of the vector as fallout and ~11 Kb DNA fragment and c) Restriction with BamHI enzyme confirming the sub-cloning of infectious clone into pBI121 vector and d) Colony PCR with coat protein specific primers of ToLCV-JB showing positive amplification in the lanes 2, 4, 6, 7, 8 respectively representing the transformed *Agrobacterium* colonies.

3.2 Agro-inoculation of the resistant and susceptible plants of tomato and tobacco plant with infectious clone of ToLCV isolate:

The tomato plants identified based on phenotypic and molecular analysis as well as the presence of Ty genes linked to resistance/ tolerance was agro-inoculated using the LBA4404 strain of *Agrobacterium tumefacians* having no plasmid vector, carrying pBI121 vector or infectious clone/pBI121 vector (Fig 19a). Simultaneously tobacco plants were also agro-inoculated with similar manner (Fig. 19d). Plants were maintained in controlled condition in the growth camber for proper growth and development. Care was taken so that the no spillage has happened during infiltration. Leaf samples were collected for 5, 10, 15 dpi with the observation of symptom development for subsequent analysis.

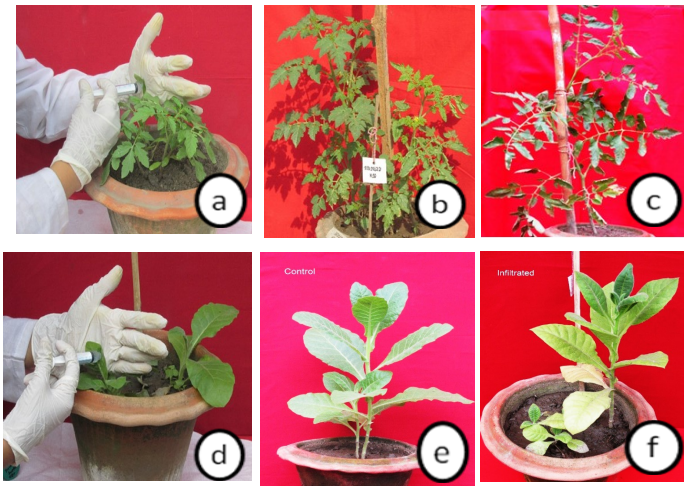

Fig. 19. Agro-inoculation in both tomato and tobacco plants; a, b, and c) are showing inoculation in tomato and d, e, and f) are showing inoculation in tobacco plants at 4-6 leaves stages by 5ml needleless syringe.

Tomato: Experimental plants/seedlings inoculated with tomato leaf curl virus infectious clone started to show symptoms with 15-17 days post inoculation, with increased upward leaf curling, a greater reduction in leaf size, followed by severe stunting in the susceptible plants (Fig. 19b) but resistant line did not develop such symptom (Fig. 19c).

Tobacco: Symptoms appeared within 15-25 days post inoculation in agro inoculated plants but there was no symptom seen in mock and healthy control plants (Fig. 19e). However, upward leaf curling, yellowing and reduced leaflet size, as well as stunted growth was observed in case of the inoculation with infectious clone (Fig. 19f).

3.3 Molecular confirmation of resistance

Genomic DNA isolated from the leaf samples of tobacco as well as putative resistant and susceptible plants after agro-inoculation were subjected to RCA analysis in order to confirm the ToLCV infection. After digestion of the RCA products with BamHI restriction enzyme, tobacco and susceptible H10 line of the accessions BD101223 showed amplification of a DNA band of ~2.7 kb as expected. The appearance of ~2.7 kb (in 0.8% agarose gel) DNA band indicated the presence of tomato leaf curl viral genome in the infected leaves. The putative resistant line A12 of the accessions BD10124 showed no band corresponding to ~2.7 kb was observed, as expected (Fig. 20b, c).

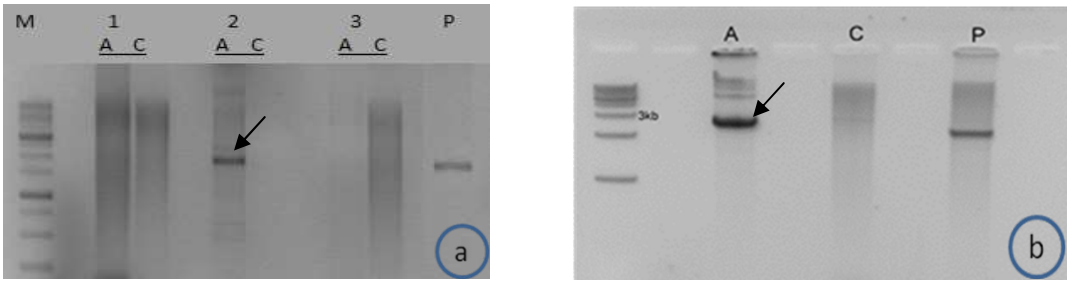

Fig. 20. RCA analysis of the agro-inoculated samples of the tobacco plant, resistant and susceptible plants of tomato plant. a) RCA analysis in tomato where lane 1A is resistant plant (A12) and Lanes 2A and 3A are susceptible plant (H10) inoculated with *Agrobacterium* carrying infectious clone (lane 1A and 2A), Agrobacterium carrying pBI121 vector (3A) and only *Agrobacterium* (Lanes 1c, 2c, and 3c) respectively, b) RCA analysis in tobacco plant where Lane A means *Agrobacterium* carrying infectious clone of ToLCVs and C is only *Agrobacterium*.

4.1 Summary on Ty-2, Ty-4 and Ty-5 sequence results:

The presence of three genes, Ty-2, Ty-4 and Ty-5 reported to confer resistance against ToLCV have been confirmed in twenty plants belong to different tomato germplasm. BLAST analysis results showed that Ty-2 gene showed 86%-100% nucleotide similarities with loci in chromosome 11 of previously studied tomato accessions and thus confirms its presence in the selected plants. For Ty-4 gene, BLAST analysis results showed 95%-100% similarity with loci in chromosome 3 of previously studied tomato accessions, further confirming its presence in the selected tomato plants. BLAST analysis results for Ty-5 gene showed 81.0%-100% similarity with loci in chromosome 4 of previously studied tomato accessions and hence, this also confirms its presence in the selected tomato plants. The Ty1, Ty3 and Ty3a genes linked to the tolerance phenotype of ToLCV infection have been confirmed in three plants.

4.2 Testing for stability of Ty genes in the five selected plants

The selected plants were further analyzed for the stability of their Ty genes in their progeny plants by raising plants from their seeds. Their symptoms were recorded and scored on the same disease scale described earlier (Muniyappa et al. 1991). It is shown that the progeny plants carrying the Ty genes did not develop any leaf curl disease symptom where as the susceptible plants lacking the Ty genes develops characteristic symptom for ToLCV infection (Fig. 21a). Leaves collected during fruiting stage were subjected to genomic DNA isolation followed by their molecular analysis for the presence or absence of the Ty genes. Three progeny plants from each parental line have been selected and analyzed in Table 49 for the presence of the Ty genes. A representative gel photo for Ty4 gene is also shown (Fig. 21b).

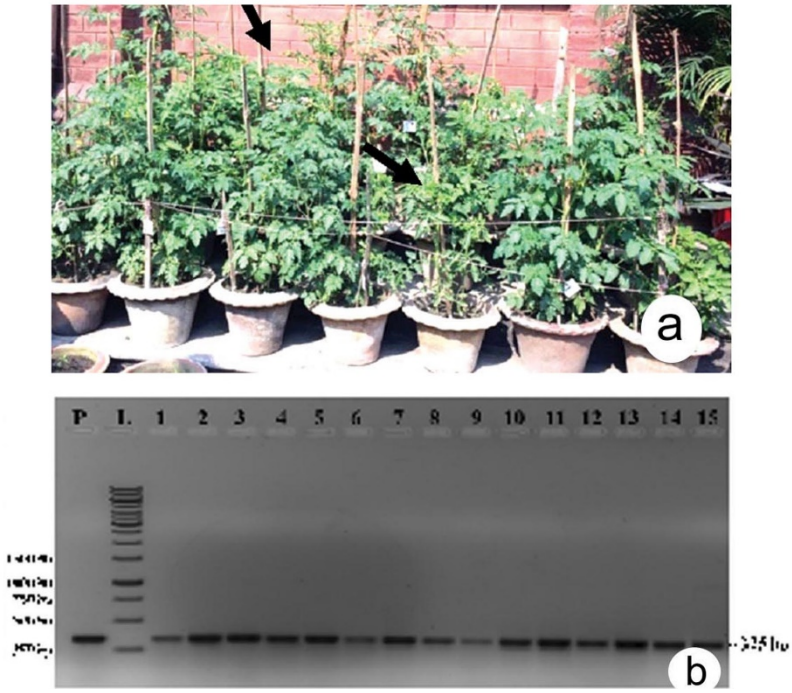

Fig. 21. Phenotypic and molecular analysis of the selected progeny plants of tomato. a) Phenotypic observation showed susceptible plants developed leaf curl symptom (arrow), and b) Result of electrophoresis of PCR products of Ty-4 gene marker on 1% agarose gel. P is the positive control and L is the 1 kb ladder. Lanes 1-3 are the progeny plants of line 12 of BD10124, lanes 4-6 are progeny plants of line 14 of BD10124, lanes 7-9 are progeny plants of line 14 of BD10122, lanes 10-12 are progeny plants of line 1 of BD775 and lanes 13-15 are progeny plants of line 1 of Local-Khushtia-1. All plants showed positive amplification of ~325 bp DNA band indicating the presence of Ty-4 gene.

Table 49. Comparison of progeny plants of selected plants for disease scores and presence or absence of Ty genes

Parent line	Progeny plants	Ty-1	Ty-2	Ty-3	Ty-3a	Ty-4	Ty-5
Plant no. 12 from BD10124	1	+	+	+	+	+	+
	2	+	+	+	+	+	+
	3	+	+	+	+	+	+
Plant no. 14 from BD10124	1	+	+	+	+	+	+
	2	+	+	+	+	+	+
	3	+	+	+	+	+	+
Plant no. 14 from BD10122	1	+	+	+	+	+	+
	2	+	+	+	+	+	+
	3	+	+	+	+	+	+
Plant no. 1 from BD7755	1	+	+	+	+	+	+
	2	+	+	+	+	+	+
	3	+	+	+	+	+	+
Plant no. 1 from Local Khushtia-1	1	+	+	+	+	+	+
	2	+	+	+	+	+	-
	3	+	+	+	+	+	-

'+' present and '-'absent

Discussion

The experiments were carried out with 7 tomato accessions of Bangladesh; BD10124, BD10122, BD7755, Local-Khushtia-1, BD10125, BD10123 and BD7761 as well as two varieties released by BARI such as BARI Toamto-8 and BARI Toamto-14. These were cultivated in a natural environment without the application of fertilizers or insecticides. Hence, whitefly populations prevailed, increasing the chances of ToLCV transmission in the field.

Disease symptoms were recorded and a modified arbitrary scale as described by Muniyappa et al. (1991) was employed to score the plants as resistant, mildly infected, moderately infected and as susceptible. From these results spanning 124 plants from 7 accessions mentioned earlier, 16.1% of the plants were scored as resistant, 18.5% were scored as mildly infected, 42.7% were scored as moderately infected and just 15.3% were found to be susceptible. Among the plants, 7.3% wilted and died at early stages of growth. These plants were taken from the field and the release of exudates from the cut end of stem in water confirmed bacterial infection that caused the wilting. It may be mentioned here that all the plants from both the BARI Toamto varieties showed susceptibility towards ToLCVs infection.

BD10124 consisted of 36.1% of resistant plants with no susceptible plants and only one plant showed wilting. It had 15.8% mildly infected plants and 40% of moderately infected plants. BD10122 consisted of 15% resistant plants with a few susceptible plants and most of the wilted plants were from this germplasm. The confinement of wilted plants among these two plants further confirms their cause as bacterial infection through soil as these plants were adjacently cultivated in the field. BD7755 also consisted of 31.6% mildly infected and 15% resistant plants with a few susceptible ones. Local-Khushtia-1 consisted of 27.7% each of resistant plants and mildly infected plants and only one susceptible line. BD10125, BD10123 and BD7761 did not show any resistant plants and consisted of mostly moderately infected plants with the highest number of susceptible plants in BD7761.

Overall, though BD10124 showed the most number of resistant individuals, it had a high percentage of moderately infected plants whereas Local-Khushtia-1 showed 27.7% resistant plants with a lower number of mildly or moderately infected plants compared to BD10124.

Sometimes physiological leaf roll symptoms in the tomato plants may appear due to environmental factors. So, to identify and confirm the disease symptoms caused by ToLCVs, molecular level analysis of the presence of ToLCV was conducted. Since, tomato leaf curl viruses may contain a circular single stranded DNA genome (DNA-A of 2.7-2.8Kb) in case of monopartite or two circular single stranded DNA genome (DNA-A and DNA-B), Rolling Circle Amplification (RCA) technique could be used to identify its presence in the leaf samples from infected plants. RCA technique is an isothermal, enzymatic

process which synthesizes numerous copies of long ssDNA using short circular ssDNA templates (Zhao et al. 2008). Total DNA isolated from the leaf samples of both healthy/asymptomatic and symptomatic plants were subjected to RCA reaction. The RCA products from susceptible plants upon digestion with BamHI restriction enzyme, produced bands of ~2.7kb, as expected, suggesting the presence of a begomovirus in the infected leaves. In non-susceptible leaves, no bands corresponding to ~2.7kb was observed, as expected.

Some of the susceptible plants showed a second band of ~1.3kb indicating the presence of DNA-β satellite molecules. These beta satellite molecules can substitute DNA-B in monopartite begomoviruses like ToLCVs for systemic movement (Saeed et al. 2007) and intensify disease symptoms in a host dependent manner (Li et al. 2005). This is in conformity with results of this experiment where plants showing appearance of betasatellite bands were found to be susceptible despite containing all six Ty genes, especially Ty-2 gene which is a dominant resistant gene, suggesting that beta satellite compromises Ty-2 gene resistance, consistent with previous studies (Al-Shihi et al. 2018).

Few of the plants showing mild or moderate infection showed two bands of ~2.1 kb and ~600 kb, totaling ~2.7kb. This could indicate the presence of a different or mutated strain of the virus with multiple recognition sites for BamHI restriction enzyme. These plants showed the presence of all six Ty genes which means that the expression of these gene may not occured. Sequencing of these viral genomes could indicate the specific species and further confirm it as a member of ToLCVs.

Six major begomovirus resistance genes, Ty-1 to Ty-6, originating from wild species have been identified and mapped through molecular markers (Zamir et al. 1994, Hanson et al. 2006, Ji et al. 2007, Anbinder et al. 2009, Hutton et al. 2013). The 124 plants from seven tomato accessions were subjected to PCR reaction with CAPS and SSR markers linked to these Ty genes (Ty-1 to Ty-5 in this study) in order to confirm the presence of these genes in them.

Ty-1 and Ty-3 are partially dominant resistance genes mapped to chromosome 6 (de Castro et al. 2007, Ji et al. 2007). Recent studies by Verlaan et al. (2013) indicated that Ty-1 and Ty-3 are allelic and encode an RNA dependent RNA polymerase; this resistance is likely part of the host RNA silencing defense against foreign nucleic acids. RNA silencing, known as post-transcriptional gene silencing (PTGS) in plants is a host defense mechanism targeted against invasive or mobile RNA elements such as viruses leading to sequence specific RNA degradation. Silencing can be triggered by replicating viruses that allow the production of high levels of aberrant mRNA which become double-stranded (ds) RNA by the host-encoded RNA dependent RNA polymerase activity (encoded by Ty-1/ Ty-3 against ToLCVs). The dsRNA are then cleaved by Dicer-like enzymes into short interfering RNAs leading to RNA degradation.

Consistent with this idea, Butterbach et al. (2014) showed increased cytosine methylation of viral genomes in Ty-1 containing tomato plants. In this study, Ty-1 gene was found to be absent in 50% of the resistant plants and Ty-3 was absent in 10% of the resistant plants. These results indicated better resistance conferred by Ty-3 than Ty-1 and their partial dominance though the other Ty genes may be involved. Zamir et al. (1994) referred Ty-1 as a tolerance gene that allow for viral replication without symptom development in plants but viral replication was absent in Ty-1 containing plants most probably due to the presence of the other Ty resistant genes.

Ty-2 is a dominant resistance gene introgressed from a *S. habrochaites* accession into tomato line H24, mapped to the short arm of chromosome 11 (Hanson et al. 2000). The Ty-2 gene was present in almost all the resistant plants observed in this study, except 2 plants which indicated its genetic dominance and high resistance properties. The gene was sequenced from selected plants and BLAST analysis was carried out, that showed 86%-100% nucleotide similarities with loci in chromosome 11 of previously studied tomato accessions which are in conformity with previous studies (Hanson *et al*. 2000). Expression analysis of its sequence in previous studies produced results for CCT motif family proteins, chaperone proteins and pentatricopeptide repeat protein where chaperone proteins showed the most expression, highest being in the seeds during the late fruiting stage. Tomato leaf curl causing begomoviruses have been recently found to be vertically transmitted through seed in addition to whitefly-mediated transmission (Kil et al. 2016). According to recent studies by Yamaguchi *et al.* 2018, a nucleotide binding domain and Leucine rich repeat containing (NB-LRR) gene, TYNBS1, is responsible for resistance mediated by the Ty-2 begomovirus resistance locus of tomato. From the expression data of this study, CCT family protein encoded by Ty-2 best matches this report. However, it has very low expression with the highest being during the green equatorial stage in the inner epidermis of fruit. Previous results suggest that Ty-3a, an allele derived from *S. chilense* accession LA1932 (whereas Ty-3 is derived from LA2779) (Scott et al.1996, Ji et al. 2007) is less efficient at countering virus symptoms than Ty-3.In the present study, Ty-3a was found to be absent in a few of the resistant plants but its correlated presence with Ty-3 in almost all of the plants, evades any chances of assessing its resistance properties.

Ty-4 gene showed the most presence in all the plants tested for, especially in the resistant plants. In the plants where Ty-4 gene was absent, all other genes were also absent except one line scored as moderately infected, where Ty-3a and Ty-5 were present only. Expression data of Ty-4 gene from selected plants included 4-pterin-alpha-carbinoleamine dehydratase which correlated with the BLAST analysis results that was predicted by automated computational analysis. The record was derived from a genomic sequence (NW_004194527.1) annotated using gene prediction method: Gnomon, supported by mRNA and EST evidence. The protein shows highest expression in the breaker equatorial stage in the inner

epidermis of fruit. Ty-5 gene was present in all of the resistant plants and also in resistant plants lacking all other Ty genes. Therefore, Ty-5 can be regarded as having high resistance properties though its presence in susceptible plants with RCA positive results negates high resistance chances or it could be attributed to its recessive properties. Expression data from Ty-5 sequence in selected plants produced results for NAC protein family which is in conformity with previous reports that state that Ty-5 encodes an NAC domain 1 protein (NAC 1) (Anbinder et al. 2009).

Ty-6 gene, a newly discovered ToLCV resistant gene was not tested for in this study due to unavailability of markers specific to the Ty-6 gene. Nevertheless, the presence or absence of this gene in the plants could further explain their resistant/susceptible properties.

Among the 124 plants from 7 tomato accessions, 5 promising plants from 4 accessions; BD10124, BD10122, BD7755 and Local Khushtia-1 were selected based on their resistant characters, negative RCA results and presence of all six genes. Three major Ty resistant genes (Ty-2, Ty-4 and Ty-5) were sequenced from these plants for further confirmation through BLAST analysis producing positive results.

To assess the stability of the Ty genes in the selected plants, their progeny plants were raised in the same way as the parent plants. On testing of the Ty gene status of these progeny plants, similar results were obtained for all the Ty genes except for Ty-5 gene which was absent in two of the progeny plants of line 1 from Local-Khushtia-1.

The aforementioned plants could be further assessed for their resistance properties by agroinfiltration with infectious clones of specific ToLCV strains prevailing in Bangladesh. For this purpose, the construction of an infectious clone of ToLCV-JB have been attempted where a reconstituted ToLCV-JB viral genome have been cloned into pBI121 vector.

In conclusion, it was seen that in all cases, the Ty-containing plants showed lower disease symptoms and lower viral loads than tomato plants lacking Ty genes. Nevertheless, there were distinct differences between the plants, with the plants bearing all six Ty genes performing the best. However, in the long term, plants with only a single resistance gene would seem undesirable – a resistance based on a single gene (a single mechanism) being, at least in theory, less durable than multiple genes. The best example of this being the resistance to cotton leaf curl disease in Pakistan introduced in the late 1990s, the resistance likely based on multiple genes was, as far as has so far been determined, based on a single mechanism (targeting the avirulence gene C2 of begomoviruses), and was broken within the space of 5 years (reviewed by Sattar et al. 2013).

Five promising plants have been identified in this study that could be used as donor parents in breeding programs for pyramiding ToLCV resistant genes into cultivated tomato plants. Further studies are warranted to confirm the findings here, to see how the plants stand-up over multiple (more than 2) years to assess whether growing tomato with a specific resistance may lead to virus/ betasatellite variants that may overcome the resistance. Moreover, the presence of Ty-6 gene in these plants could also be tested to assess its resistant properties. Finally, it can be said that presence of more than one Ty gene/locus is generally not possible unless there is a guided gene pyramiding program is followed, however, in the present study we ended up with several lines containing all the Ty genes. It was also observed that some plants were RCA positive indicating that the Ty genes might not expressed which is our future focus on this research.

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10. Research highlight/findings (Bullet point – max 10 nos.):

- The results indicate that multiple Ty genes are more effective against ToLCV infection.
- Many line have been identified with resistance/tolerance properties. Five promising plants carrying all the Ty genes were sequence characterized in this study that could be used as donor parents in future breeding programs.
- The use of local tomato accessions carrying resistant/tolerant genes in breeding program is an effective way to avoid the use of transgenic plants that pose biosafety problems.

B. Implementation Position

1. Procurement: N/A

Description of equipment and capital items	PP Target		Achievement		Remarks
	Phy (#)	Fin (Tk)	Phy (#)	Fin (Tk)	
(a) Office equipment					
(b) Lab & field equipment					
(c) Other capital items					

2. Establishment/renovation facilities:

Description of facilities	Newly established		Upgraded/refurbished		Remarks
	PP Target	Achievement	PP Target	Achievement	

3. Training/study tour/ seminar/workshop/conference organized:

Description	Number of participant			Duration (Days/weeks/ months)	Remarks
	Male	Female	Total		
(a) Training					
(b) Workshop					

C. Financial and physical progress

Fig in Tk

Items of expenditure/activities	Total approved budget	Fund received	Actual expenditure	Balance/ unspent	Physical progress (%)	Reasons for deviation
A. Contractual staff salary	530710	530710	513850	16860	96.82	fund utilized in other cases
B. Field research/lab expenses and supplies	749290.00	740841	737290	3551	98.40	-do-
C. Operating expenses	90000	86953	89230	-2277	99.14	shortage of fund
D. Vehicle hire and fuel, oil & maintenance	00	00	00	00	00	--
E. Training/workshop/seminar etc.	00	00	00	00	00	--
F. Publications and printing	75000	00	15000	-15000	20.00	shortage of fund
G. Miscellaneous	55000	52250	55000	-2750	100.00	-
H. Capital expenses	00	00	00	00	00	-

D. Achievement of Sub-project by objectives: (Tangible form)

Specific objectives of the sub-project	Major technical activities performed in respect of the set objectives	Output (i.e. product obtained, visible, measurable)	Outcome(short term effect of the research)
a) Screening of Tomato Leaf Curl Virus (ToLCV) resistant/tolerant germplasm through rolling circle amplification (RCA) analysis.	**Literature survey:** To identify the genes linked to ToLCV infection resistance tolerance and to select of primers for each gene.	Different types of Ty genes(Ty1, 2, 3, 3a, 4 and 5) identified based on the literature survey to analyze the tomato germplasm	Primers for 6 Ty genes have been selected and purchased to analyze the germplasm
	Collection of germplasm: Plant genetic resource centre has collection of different tomato accession of Bangladesh and BARI has developed high yielding tomato varieties. So, germplasm will be collected from both PGRC and BARI.	Seven different germplasm of local tomato accessions and two cultivar tomato varieties were collected from PGRC and BARI.	Seeds of the Tomato germplasm are in hand.
	Raising seedling and cultivation of tomato for field observation.	The seedlings of tomato germplasm were raised and cultivated in the field of botanical garden maintaining the natural condition. Morphological data were recorded based on disease score.	Both ToLCD symptomatic and asymptomatic leaf samples from 250 plant plants were collected and preserved in -80 °C freezer for DNA isolation.
	DNA extraction: DNA will be extracted for further molecular analysis.	DNA was extracted using modified CTAB method and preserved successfully for further analysis.	Isolated DNA was preserved in both 4°C refrigerator and -20 °C freezer for future analysis.
	Rolling Circle amplification using the isolated DNA as template.	Infected and non-infected leaf samples will be screened through RCA analysis to identify the presence or absence of tomato leaf curl virus genome.	Majority of the infected and some asymptomatic leaf samples were positive in RCA analysis confirming the ToLCV infection or presence of the viral genome in case of asymptomatic samples.

b) Identification of the resistant/tolerant plants using molecular markers (SSR) linked to ToLCV resistance/tolerance.	Polymerase chain reaction using the primers of respective Ty gene using DNA of the each line/germplasm.	Polymerase chain reaction (PCR) experiment was conducted using isolated DNA as template and primers specific for six different molecular markers (SSR) termed as Ty genes linked to ToLCV resistance/tolerance.	More than 20 plants of the collected germplasm showed the presence of Ty genes but negative for RCA analysis and RCA positive asymptomatic samples.
	Sequencing of the Ty genes for confirmation.	Sequences obtained for the respective Ty genes from different plants were confirmed through bioinformatics analysis	**Five plants** carrying all the Ty genes were finally selected for future use as donor parent.
c) Molecular confirmation of the ToLCV resistance/tolerance by agro-inoculation of the ToLCV infectious clone into the promising tomato plants.	Construction of infectious clone of the ToLCV	Infectious clone was made, transferred into *Agrobacterium* strain LBA4404 and preserved in -87 °C degree freezer for further use.	A tool for ToLCV infection has been developed.
	Agro-infiltration of the infectious clone	Both susceptible and resistant plants were used for agro-inoculation using the only *Agrobacterium* strain LBA4404, carrying pBI121 plasmid or ToLCV infection clone/pBI121 plasmid.	One tomato line containing Ty genes line was confirmed to show the resistance through agro-inoculation of infectious clone. But to confirm other lines as well as to do more research required more fund.
	Phenotypic observation of agro-infiltrated tomato plants	The Ty gene carrying as well as mock inoculated tomato plant did not develop any disease symptom but susceptible one developed	
	Molecular identification of the resistant/tolerant tomato plants	No viral genome was amplified in the Ty gene carrying as well as mock inoculated tomato plant but susceptible one inoculated with infectious clone showed amplification of 2.7 kb DNA band in RCA analysis corresponding to the size of the viral genome.	

E. Materials Development/Publication made under the Sub-project:

Publication	Number of publication		Remarks (e.g. paper title, name of journal, conference name, etc.)
	Under preparation	Completed and published	
Technology bulletin/ booklet/leaflet /flyer etc.			
Journal publication	01		
Information development			
Other publications, if any		**02**	1. Oral presentation in Annual Plant Tissue Culture and Biotechnology Conference 2017 held in 6-7 April 2018: Molecular characterization of Tomato Leaf Curl Virus (ToLCV) resistance in tomato (*Solanum lycopersicum*) germplasm 2. Poster presented in 6th SABP conference 2018: Molecular Characterization of Tomato Leaf Curl Virus Resistance in Wild Tomato (*Lycopersicon esculentum* Mill.) Germplasm of Bangladesh

F. Technology/Knowledge generation/Policy Support (as applied):

i. Generation of technology (Commodity & Non-commodity)

ToLCV resistant/tolerant Tomato germplasm identified and confirmed using molecular techniques.

ii. **Generation of new knowledge that help in developing more technology in future**

The resistant/tolerant Tomato germplasm identified and confirmed in response to Tomato leaf curl disease (ToLCD) caused by ToLCV could be utilized as desired gene source for future breeding program as well as to isolate the candidate gene/s.

iii. Technology transferred that help increased agricultural productivity and farmers' income

No.

iv. Policy Support

No

G. Information regarding Desk and Field Monitoring

i) Desk Monitoring [description & output of consultation meeting, monitoring workshops/seminars etc.):

ii) Field Monitoring (time& No. of visit, Team visit and output):

Field monitoring team visited once.

I. Lesson Learned/Challenges (if any)

i) Team work is very important.

ii) Constant support to carry out the research.

iii) Should follow the standard guideline for fund management.

J. Challenges (if any)

i) Fund disbursement procedure killed too much of time!

ii) Too much paper work!!

Signature of the Principal Investigator
Date …….

Seal

Counter signature of the Head of the organization/authorized representative
Date ……..

Seal