Molecular characterization of Begomovirus infecting sweet pepper in Oman

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Whitefly transmitted tomato yellow leaf curl is one of the most devastating viral disease of cultivated sweet pepper (Capsicum frutescens grossum) and other vegetables in Oman. Infected sweet pepper plants showed typical begomovirus symptoms as upward leaf curling, interveinal and leaf chlorosis, and growth stunting. Begomovirus infecting sweet pepper in Oman was detected by polymerase chain reaction (PCR) using begomovirus specific degenerate primers (PAL1v1978/PAR1c496 and AV494/AC1048). Core region (74-604 bp) of coat protein gene of the begomovirus was amplified by PCR with tomato yellow leaf curl virus (TYLCV) specific degenerate primers (TycpV369/TycpC1023). Core region of coat protein gene contains highly conserved regions and is used to identify the begomovirus infecting sweet peppers. Virus identification was performed by percent sequence identity and parsimony analysis using core coat protein gene sequences of sweet pepper virus with complete genome, core region of coat protein and coat protein gene sequences from reference begomoviruses. The core region sequence identity of coat protein gene of sweet pepper virus from Oman was 92.2, 96.5, 94.0, 93.8, and 96.5% with TomGV-Lebanon, TYLCV-Guadeloupe, TYLCV-Israel, TYLCV-Kuwait, and TYLCV-Mexico, respectively. Phylogenetic trees and percent sequence identity with reference to begomoviruses permitted the identification of sweet pepper tomato yellow leaf curl virus clustered with its closest relatives from Middle East regions but formed a separate strain.

Keywords: sweet pepper, begomovirus, core region of coat protein gene

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Introduction

Diseases of many vegetables, including sweet pepper, caused by whitefly-transmitted geminivirus are known worldwide. The typical symptoms of sweet pepper disease caused by begomovirus are leaf curl, leaf crumple, foliar mosaic, and mottle among others. However, symptoms of a given virus-host combination may vary depending on cultivars, environmental conditions and virus strain. Infection of a single host by two different viruses causing similar symptoms is often noticed, which can complicate the diagnosis of the disease and make the phenotype symptoms an unreliable criteria for viral identification. While DNA sequence comparison and phylogenetic analysis with sequences of known begomoviruses can evidently provide a reliable viral identification.

In Oman, exotic cultivars of sweet pepper are cultivated each year during October-March in the northern coastal region of Al-Batinah. The crop is grown to meet the high domestic demand for fresh produce. However, the major constraint in sweet pepper cultivation is a disease, resulting in leaf curling, mottling and growth stunting of plants, caused by whitefly-transmitted geminivirus. Geminiviruses are a group of plant DNA viruses characterized by the geminate shape and particle size of 18-25 nm. Whitefly transmitted geminiviruses are classified into the genus Begomovirus of the family Geminiviridae, and are transmitted by Bemisia tabaci. The majority of the Begomoviruses are known to have bipartite genome but single genome (DNA-A) monopartite viruses are being reported, in increasing numbers, mainly from the old world. Identification of Begomoviruses using serology is not suitable, because high titre antisera are difficult to prepare and lack sufficient specificity. Consequently, DNA based molecular diagnostic techniques, such as polymerase chain reaction (PCR) amplification, using universal or specific primers and DNA sequencing has supplemented serology for detection and identification of begomoviruses. So far, classification and phylogenetic relationships of begomoviruses have been based upon complete monopartite viral genomes.
or the DNA-A component of the bipartite viruses, coat protein (CP) and/or replication association protein gene (Rep) trees. Although complete genome sequences are desirable for identification and classification of geminiviruses, this requires considerable time and expense. Full-length CP gene sequences are accepted by the International Committee on the Taxonomy of Viruses (ICTV) for the provisional classification of begomoviruses when complete genome (monopartite virus) or DNA-A component (bipartite virus) sequences are unavailable. For sequences to be useful for identification at both the species and strain/variant levels, regions of the genome that contain both conserved and variable elements should be targeted. So far, CP gene has been proven useful for plant virus detection and identification. PCR based studies have established the diversity of tomato yellow leaf curl virus in Sudan, showing that there are two distinct, yet closely related, Begomovirus species associated with tomato leaf curl disease. In this paper, authors describe the molecular characterization of a tomato yellow leaf curl virus (TLCV), a begomovirus infecting sweet pepper in Oman.

Materials and Methods

Sample Collection

To identify the causal agent(s), sweet pepper samples showing typical leaf curl disease symptoms were collected from different fields in Al-Batinah region during 2004-2005. Samples were brought in cool boxes from field and stored at -80°C until used for DNA extraction.

Total DNA Extraction

Total DNA was extracted from symptomatic and asymptomatic leaves of sweet pepper using Dellaporta extraction buffer with some modifications.

PCR

DNA samples extracted from symptomatic sweet pepper plants were used as template for PCR. DNA extracted from asymptomatic plants and sterile water was used as negative control. Begomovirus specific degenerate primer pairs PAL1v1978 (5'-GCATCTGCAGGCCCACATYGTCTTYCCNGT-3') and PAR1c496 (5'-AATACTGCAGGGCTTYCTR-TACATRGG-3') were used to prime the amplification of 1.1-1.4 kb and 500-600 bp product, respectively. TYLCV core CP gene specific degenerate primer pair TyCpV369 (5'-ACGCCGTYCTCGAAGGGTTTCG-3') and TyCpC1023 (5'-GTACAWGCATATACATAA-CAAGGC-3') was used to amplify 500-650 bp core CP region of the virus genome. Fifty ng of each DNA preparation in 1 µL TE were added to ‘Ready-To-Go’ PCR beads (Pharmacia Biotech, Sweden), followed by 1 µL of each primer (10 pmole) and 22 µL of sterile deionized water for a final reaction mixture volume of 25 µL. The DNA was amplified by 35 cycles, consisting of denaturation at 94°C for 30 sec (2 min for cycle 1 and 45 sec for cycle 35), annealing at 55°C for 1 min, and primer extension at 72°C for 2 min (5 min for cycle 35). After amplification, 4 µL aliquot from each sample was electrophoresed in a 1% agarose gel and visualized by staining with ethidium bromide and UV illumination.

Cloning of PCR Amplified DNA

The PCR amplified DNA fragments using CP gene specific primer pairs TyCpV369/TyCpC1023 were purified using the Wizard PCR DNA purification system (Promega Corp., Wisconsin, USA). The purified DNA was cloned using Promega pGEM-T Easy Vector System II cloning kit according the manufacturer’s instructions (Promega Corp., Wisconsin, USA). Recombinant plasmids were screened by blue/white color screening on indicator plates and PCR amplification using TyCpV369/TyCpC1023 primers.

Sequencing and Phylogenetic Analysis

A cloned TyCpV369/TyCpC1023 fragment from infected pepper virus was sequenced to verify phylogenetic relationships; the sequence was deposited in NCBI Gene Bank under the Accession Number DQ132859.

Automatic sequencing was performed on an ABI Prism Model 377 (PE Applied Biosystem, CA, USA) with TyCpV369/TyCpC1023 primers using Big Dye terminator chemistry at DNA sequencing facility of Sultan Qaboos University.

DNA sequence of sweet pepper virus CP gene was analyzed in BLAST on NCBI nucleotide database to obtain information on sequence similarity. CP and complete genome sequences of 31 geminiviruses (Table 1) were aligned by the CLUSTAL W method with MegAlign suite of LaserGene 6.0 software.
The sequences were visually inspected for logical placement of gaps and manually adjusted where necessary. Fifteen CP sequences belonging to geminivirus were separately aligned including Oman strain to analyze CP sequence identity using MegAlign suit of Lasergene 5.01 (Table 2). Cladistic analyses were performed with PAUP (Phylogenetic Analysis Using Parsimony, version 4.0b10)\(^{17}\). Uninformative characters were excluded from analyses. A phylogenetic tree was constructed by a heuristic search via random stepwise addition, implementing the tree bisection and reconnection algorithm to find the optimal phylogenetic tree(s). Beet curly top virus (Curtovirus), a geminivirus was designated as outgroup to root the tree. The analysis was replicated 1000 times. Bootstrap analysis was performed to estimate stability and support for the inferred clades\(^{18}\).

**Results**

**Survey of Disease**

Ten sweet pepper grown agricultural farms, averaging 10-15 acres in size, were surveyed in Al-Batinah, northern region of the Sultanate of Oman. Mainly green cultivars of Sweet peppers were grown on farms either under shade house or open field during September to February. All the farms surveyed were 5-10 km apart from each other and had varying degree of leaf curl disease incidence. The disease incidence ranged from 5 to 45% in older crops (data not shown). Further, the incidence on sweet peppers was high on field grown plants as compared to those grown under shade house.

**Symptoms in Diseased Plants**

Typical leaf curl symptoms were observed on sweet peppers infected with TYLCV. Peppers exhibited upward curling of leaf margins with interveinal and marginal chlorosis, and thickening of veins (Fig. 1). In severe infection, yellow of the leaves was evident. Infected leaves were often found thicker than the normal ones.

**Detection of TYLCV by PCR Assays**

A total of sixty samples (6 sample from each farm) collected from infected sweet pepper yielded
Table 2—Comparison of nucleotide sequence of coat protein gene of begomovirus infecting sweet pepper from Oman with other begomoviruses

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Gemini virus strains

1. SACMV-SA
2. TomBV-MT
3. TomBV-FR
4. TomGV-DR
5. TomGV-LB
6. TomGV-SD
7. TYLCV-FR
8. TYLCV-GD
9. TYLCV-IS
10. TYLCV-KU
11. TYLCV-MX
12. TYLCV-OM
13. TYLCV-RU
14. TYLCV-RUSG

*Abbreviations of virus strains and descriptions are given in Table 1

were aligned using Bio Edit program and analyzed in BLAST. NCBI BLAST results revealed that sequence from symptomatic sweet pepper showed 98% identity with TYLCV reported from Egypt and Kuwait; 97% identity to TYLCV from Lebanon, Israel, and Jordan; 95% identity to TYLCV strains from Puerto Rico and Iran; 92% identity to TYLCV strain from Mali; 94% identity to Sudan strains; 85% identity to Cassava mosaic virus from South Africa; and 87% identity to tomato begomovirus from many countries (Table 2). Comparison of CP gene sequences of many begomoviruses from different parts of the world showed that TYLCV sequence from Oman is 96.5% identical to those reported from Guadeloupe and Mexico; 94% identical to strains from Israel; 93.8% identical to Kuwait; and more than 90% identical to tomato begomovirus strains from Dominique Republic, Lebanon, and Sudan (Table 2).

Phylogenetic Analyses

Results of phylogenetic analyses on core CP gene of TYLCV from sweet pepper in Oman (Fig. 3) revealed that sweet pepper virus clusters with TYLCV strains from Kuwait, and Israel and TomGV from Lebanon but formed a separate clade indicating a new strain. The closest relative of TYLCV from Oman was tomato begomovirus from Lebanon and Sudan. These results support the conclusion that sweet pepper TYLCV from Oman is closely related to other TYLCV strains from Middle East region, but forms a separate strain and can be named as TYLCV-Oman strain.

begomovirus specific 1.2 kb products when amplified by PCR using the primer pair PAL1v1978/PAR1c496 and 650 bp with primer pair AV494 and AC1048 (Figs 2A & B). A 602 bp product was amplified by PCR using the TYLCV core CP gene specific primer pairs TyCPV369 and TyCP1023 (Fig. 2C). No PCR products were amplified from asymptomatic plant samples unless otherwise mentioned.

CP Gene Sequence Analysis

PCR amplified DNA fragments of 602 bp, using TYLCV CP gene specific primer pairs, were sequenced. One PCR positive symptomatic sample from each farm, i.e. a total of 10 samples, was sequenced for phylogenetic analysis. The sequences
Discussion

Geminiviruses constitute an important group of pathogen characterized by a circular single stranded DNA genome and unique geminate particle morphology\(^\text{19}\). The TYLCV, a member of whitefly-transmitted Geminiviruses, is a worldwide threat for cultivated tomatoes and other agricultural crops\(^\text{3,20-22}\).

Al-Batinah region is located on northern part of the Sultanate of Oman along the coastal line and comprises more than 80% of the total arable land. During the survey in Al-Batinah region, 5-10% incidence of leaf curl disease was found on sweet peppers grown under shade house, while 35-45% on those grown under open field. The low disease incidence on plants under shade house could be due to restricted movement of whiteflies, whereas in field population of whiteflies was found in great abundance. In addition, most of the field grown sweet pepper crops were located adjacent to tomato field, which showed more than 80% incidence of leaf curl disease.

TYLCV was first recorded in Israel as a whitefly-transmitted virus in tomato (Lycopersicon esculentum) crops\(^\text{23-25}\). It has been identified on the basis of molecular data and placed in the Genus Begomovirus, Family Geminiviridae. TYLCV occurs in most eastern Mediterranean countries\(^\text{26}\) and parts of sub-Saharan Africa, Asia, Australia, and the Caribbean\(^\text{27}\). In Europe two species of TYLCV are present\(^\text{21}\), comprising tomato yellow leaf curl virus (TYLCV) syn TYLCV-IL, and tomato yellow leaf curl Sardinia virus (TYLCSV) (using the most recent taxonomic nomenclature described\(^\text{3}\)).

All fragments obtained with TYLCV coat protein gene specific PCR primers had a 90% or greater nucleotide identity with their respective viruses. The begomovirus specific primers yielded expected product confirming the presence of virus in infected sweet pepper samples\(^\text{14,28-31}\). The CP gene is the most highly conserved gene in the genus Begomovirus\(^\text{14}\). The CP gene sequence is used to predict strains, species and taxonomic lineages of begomoviruses\(^\text{32}\). International Committee on Taxonomy of Viruses (ICTV) accepts the classification of begomoviruses based on CP sequence when full length genomic sequences are not available\(^\text{9,33}\).

Alignment of core CP sequences of different begomoviruses with CP sequence of the present Oman isolate and phylogenetic analysis of aligned sequences clearly indicates that virus strain isolated
from sweet pepper in Oman is a TYLCV strain. CP sequence analysis and phylogenetic tree reconstruction permits a prediction of relatedness among begomoviruses that is highly similar to the outcome from alignment of begomovirus CP gene sequences\(^8,10\). The core CP region has highly conserved amino acid sequences interrelated with variable bases in all members of *Begomovirus*. These features provide useful molecular tool to perform preliminary identification of begomoviruses. The core CP sequence contains sufficient conserved and variable regions that virtually mimic the overall composition of the complete CP sequence\(^3\). Padidam *et al*\(^8\) have used highly variable 5' end of the CP gene sequence (~ 200 nt) as a molecular marker to reveal differences between closely related strains of begomoviruses. However, complete reliance on highly variable region may complicate the begomovirus identification that otherwise can be done by the conserved region. Present results from core CP gene sequence alignment and construction of phylogenetic tree using CP sequences and complete genome sequences have clearly established that the virus infecting sweet pepper from Oman is a discrete strain of TYLCV belonging to the genus *Begomovirus* and the name *tomato yellow leaf curl virus-Oman* (TYLCV-OM) is proposed for the same.

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**References**


