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Occurrence of *Agrobacterium Vitis* Carrying Two Opine-Type Plasmids in Tunisian Vineyards

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Abstract

Fifty DNA samples were recovered from infected wood tissue of 1 and 2-year-old grapevine (*Vitis vinifera* L.) cultivars collected from several vineyards located in the Center of Tunisia. The strains were differentiated using a Multiplex PCR assay with combination of *pehA*, *virF* and *virD2* gene-specific primers which amplified the corresponding fragments from nearly all strains and distinguished clearly between *Agrobacterium vitis* and A. tumefaciens strains carrying octopine or nopaline pTis and A. vitis vitopine strains. The isolates were segregated into three main groups, the first group carries octopine type Ti plasmids; the second vitopine type Ti plasmids and the third group both octopine and vitopine type Ti plasmids. The polygalacturonase gene sequence from 10 isolates showed 94-97% identity to the sequences of *A. vitis* previously deposited in NCBI GenBank database. Obtained nucleotide sequences were submitted to Genbank under accession numbers from JX946285 to JX946294.

Keywords: *Agrobacterium vitis*; Crown gall; Multiplex PCR; Opine-type; *Vitis vinifera*

Introduction

Crown gall is one of the most important bacterial diseases of grapevine worldwide and is especially debilitating on cultivars of *Vitis vinifera* [1]. It is widespread, mainly in temperate areas, and especially in Mediterranean countries [2]. *Agrobacterium vitis* is the predominant species that causes the disease, although *A. tumefaciens* is occasionally isolated from infected vines [3]. Basically, the taxonomy of bacteria belonging to Agrobacterium genus has been determined relying on their phytopathogenic abilities. However, the loss or acquisition of the tumour-inducing (Ti) or root-inducing (Ri) plasmid could lead to a change of species status. Recently, on the basis of biochemical features, agrobacteria were divided into 3 biovars, but this classification did not correspond to the classification based on phytopathogenic abilities. Further studies, mostly based on ribosomal genes, confirmed that classification into biovars reflected phylogenetic relationships and, consequently, agrobacteria were classified into five species: *A. radiobacter* (biovar 1 strains), *A. rhizogenes* (biovar 2), *A. vitis* (biovar 3), *A. rubi* and *A. larrymoorei* [4].

Typically, infections are initiated at wound sites on trunks and canes caused by freezing temperatures or other cultural practices. In addition to crown gall disease, *A. vitis* induces electrolyte leakage and strong necrotic lesions on the roots of grapevine [1,5]. Transmission of *A. vitis*, that is adapted to living in the vascular system of grapevine plants, occurs by vegetative propagation of infected cuttings [6]. In fact, members of *Agrobacterium* spp. are known as soil-borne plant pathogens, and soils are generally considered as sources for infection. However, in the case of grapevine the systemically infected propagating material is the main source of infection [7]. Since vineyard soils have been excluded as a source of infection with *A. vitis*, spreading of the disease can be prevented by utilization of pathogen-free propagation material [6].

Four major T-DNA structures have been characterized in strains of *A. vitis*. They differ by their numbers of T-DNAs (delineated by characteristic border sequences) and by gene composition [8]. Strains are often referred to by the type of opine synthase gene or genes carried on their Ti plasmids (i.e., nopaline [N], vitopine [V], or octopine/cuccumopine [OCD]). Since the OC strains carry two T regions in their T-DNA, TA and TB, they are further grouped depending on whether TA region is large (OL) or small (OS). The TA region is highly homologous to the TL region of the octopine Ti plasmids of the biovar 1 strains and has the same overall organization; the TB region id unique for o/c Ti plasmids and carries a set of auxin genes and cucumopine synthase gene [9]. Phylogenetic models of *A. vitis* Ti plasmids have been developed by Otten et al. [10] that are based on T-DNA structure, homology of oncogenes to those on other Ti plasmids, and the pattern of insertion by various insertion sequence (IS) elements. It was discovered that the type of Ti plasmid carried by an *A. vitis* strain is highly correlated with the restriction fragment fingerprints derived from its intergenic spacer region (ITS) which lays between the 16s and 23s rRNA genes [11]. Similar correlation was shown between Ti plasmid type and restriction fragment fingerprints generated from the five region of the 23 gene [12].

Recent studies using specific primers designated to identify *Agrobacterium* species isolated from grapevine showed failure of some primers to distinguish clearly between *A. tumefaciens* and *A. vitis* and between pathogenic and non-pathogenic isolates [13]. For example, virC primers can detect pathogenic *A. tumefaciens* [14] but cannot detect *A. vitis* [15]. Similarly, virD2 specific primers [16] which amplify corresponding sequence of *A. tumefaciens*, failed to identify *A. vitis* [17]. The aim of this study was to detect *A. vitis* and *A. tumefaciens* and to distinguish between them using novel primers set [17] under Multiplex PCR conditions and to assay the reliability of this method.

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Material and Methods

Sampling and DNA extraction

Several vineyards located in the region of "Regueb" in the center of Tunisia were inspected for grapevine crown gall occurrence. Samples of newly developed galls from the infected *Vitis vinifera* cultivars were collected, placed in plastic bags and transported to the laboratory. Plant samples were washed under running tap water to remove adhering soil particles, surface-sterilized by dipping into 0.5% (v/v) sodium hypochlorite for 2 min, rinsed three times with sterile distilled water (SDW). Bacterial DNA was extracted from vascular tissue of galls, crown and canes. 1 g of wood tissue was homogenized at room temperature in a plastic mesh bags (provided by Sediag) with a ball-bearing device in 10 ml of CTAB buffer (3% CTAB,1 M Tris-HCl pH 8, 20 mM EDTA, 1.4 M NaCl) with the addition of 0.2% 2-mercaptoethanol; 1 ml was transferred to an Eppendorf tube and incubated in a water bath at 65°C for 20 min. After extraction with 1 ml of chloroform, nucleic acids were precipitated from the aqueous phase with an equal volume of isopropanol, collected by centrifugation, washed with 70% ethanol, dried, dissolved in 100 µl of water and stored at -20°C until use.

Oligonucleotide primers

A combination of $VIFF_1$/$FIRFR_2$ and $VIRD2S4F_{716}$/$VIRD2S4R_{1036}$ primers [17] was used in a multiplex PCR to detect the *virF* and *virD2* regions in the most common opine types of *A. vitis* which are nopaline, octopine and vitopine types [1,18]. In order to identify *A. vitis* strains and differentiate them from the *A. tumefaciens* strains, PGF/PGR [15], a polygalacturonase specific primer set was added to the mixture of the multiplex PCR. PCR amplication of *virF, virD2* and pehA genes would produce 382, 320 and 466 pb bands, respectively.

PCR amplification

The reaction were carried out in a reaction volume of 25 µl containing 1X buffer, 3 mM $MgCl_2$, 200 µM each dNTP, 0.3 µM for primers VIRFF1/VIRFR2 and $VIRD2S4F_{716}$/$VIRD2SRR_{1036}$, 0.4 µM for primers PGF/PGR, 5% DMSO, 0.02 U/µl DNA Polymerase (Go Taq Flexi DNA polymerase, PROMEGA) and 5 µl of template DNA. PCR experiments were performed in a thermal cycler "Gene Amp PCR System 9700" (Applied Biosystems). The amplification was started with an initial denaturation step at 94° for 1 min, followed by 40 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. Finally the reaction was completed with an extension step at 72°C for 5 min. Samples were analyzed after electrophoretic separation in ethidium-bromide stained 2.5% (w/v) agarose gels (10 µl of 1% ethidium bromide solution in 100 ml of 2.5% agarose). The PCR products gel was visualized under UV light. Considering the PCR inhibitors possibly present in each DNA sample, amplification with dilute DNA sample was performed if there were no PCR products from the starting template.

Sequencing of *pehA* gene

Ten randomly selected isolates were subjected to PCR assay targeting polygalacturonase gene with specific primers PGF/PGR. The amplicons of *pehA* polygalacturonase gene resulted from PCR amplification were sequenced. DNA sequencing was performed by Genomic Platform service of PTP (Parco Tecnologico Padano, Lodi, Italy). The nucleotide sequence data were assembled by employing the Contig Assembling program of the sequence analysis software BIOEDIT, version 7.0.0 [19] and blasted to compare their identity with the sequence of *A. vitis* previously deposited in Gene Bank database (Accession No.CP000633.1gb).

Nucleotide sequence accession numbers

Polygalacturonase gene *pehA* partial sequences were checked manually and nucleotide arrangements at ambiguous positions were clarified using both primer direction sequences. All sequences were deposited in GenBank database under the accession numbers JX946285 (strain TUNAv1), JX946286 (TUNAv6), JX946287 (TUNAv7), JX946288 (TUNAv13), JX946289 (TUNAv14), JX946290 (TUNAv30), JX946291 (TUNAv34), JX946292 (TUNAv36), JX946293 (TUNAv39) and JX946294 (TUNAv40).

Results

Multiplex PCR with primers combination $VIRFF_1$/$VIRFR_2$, designated to detect octopine/nopaline isolates of *A. vitis*, $VIRD2S4F_{716}$/$VIRD2S4R_{1036}$, which amplified the corresponding sequences from vitopine isolates only and PGF/PGR, involved to amplify *pehA* gene of *A. vitis*, showed that all of the analyzed isolates amplified a product of 466 bp with polygalacturonase gene-specific primers except one isolates designated TUNAv37 corresponding on *A. tumefaciens*. Furthermore, the results of Mutiplex PCR (Figure 1) revealed that all isolates except

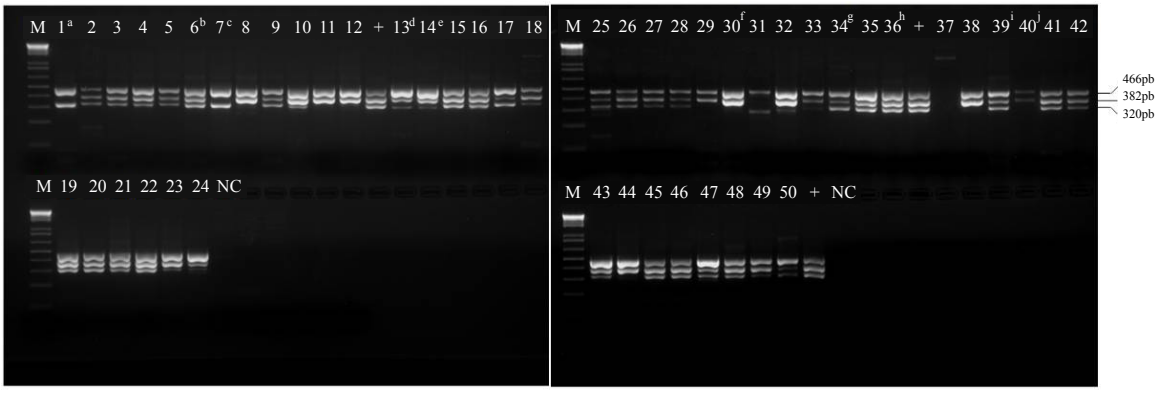

Figure 1: Multiplex PCR with primer pairs PGF/PGR (466 bp), $VIRFF_1$/$VIRFR_2$ (382 bp) and $VIRD2S4F_{716}$/$VIRD2S4R_{1036}$ (320 bp) using DNA extracted from plants. 1: isolates TUNAv1 (O); 2-6: isolates TUNAv2-6 (OV) ; 7: isolates TUNAv7 (V); 8: isolates TUNAv8 (O); 9: isolates TUNAv9 (OV); 10: isolates TUNAv10 (OV); 11: isolates TUNAv11 (O); 12: isolates TUNAv12 (O); 13-28: isolates TUNAv13-28 (OV) ; 29 : isolates TUNAv29 (O) ; 30 : isolates TUNAv30 (O) ; 31 : isolates TUNAv31 (V) ; 32-36 : isolates TUNAv32-36 (OV) ; 37 : isolates TUNAt37 , *A. tumefaciens* (O); 38 : isolates TUNAv38 (O) ; 39 : isolates TUNAv39 (OV); 40 : isolates TUNAv40 (O); 41-43 : isolates TUNAv41-43 (OV); 44 : isolates TUNAv44 (O); 45-50 : isolates TUNAv45-50 (OV). +: positive control; NC: Negative Control. M: Invitrogen 1kb plus (cat. 10787-018). a-j: sequenced *pehA* gene strains.

TUNAv37 carry *vir* gene sequence. Among of the 49 *vir* positive isolates, eight isolates had octopine-type and three isolates designated TUNAv 1, TUNAv7 and TUNAv31 had vitopine-type. Interestingly, thirty-eight isolates were positive for the presence of both *virD2* and *virF* sequences (Figure 1) as well as for both octopine and vitopine synthases. None of all the isolates had nopaline type indicating that Tunisian isolates probably do not carry Bivar 3 nopaline type Ti plasmids.

Discussion

In this study, we collected 50 bacterial DNA samples from affected plants collected from different vineyards located in the region of "Regueb" in the center of Tunisia. All of the isolates except one were found to be virulent, and 49 of them were assigned to *A. vitis*. Early studies to identify *Agrobacterium* with PCR used pure bacterial cultures to determine the suitability of primers which were usually designed on the basis of Ti plasmid *vir* region or T-DNA sequences [16]. The *pehA* specific PGF and PGR primers [15] were combined with the primers designed for *virF* and *virD2* sequences [17]. The *pehA*-specific sequences allowed distinguishing *A. vitis* from *A. tumefaciens* [15]. The *virF* is a host range factor that occurs on the octopine-type Ti plasmid of *A. tumefaciens* and octopine and nopaline-type Ti plasmid of *A. vitis* [20]. Multiplex PCR with these primers revealed to be suitable to unambiguously detect all the assayed *A. vitis* strains and to partially discriminate among their different pTi plasmids, since *virF*-specific primers detected all octopine and nopaline strains whereas *virD2* gene primers detected all vitopine strains.

Agrobacterium vitis strains are classified into three taxonomical groups based on Ti plasmid-encoded opine markers, as octopine, nopaline and vitopine-type strains. Of these, octopine strains are found most commonly in grapevine accounting for 60-70% of isolates [1]. PCR results with opine synthase-specific primers showed that eight (16.33%) of the *A. vitis* isolates belong to the octopine groups and three (6.12%) to the vitopine group. Nopaline type isolates were not found. Interestingly, thirty-eight (77.55%) of the *A. vitis* isolates were found to be positive for both *virF* and *virD2* genes, as well as for vitopine and octopine synthase gene. Bini et al. [17] reported similar results with the strain IBV-BO 5372 and they hypothesized that this strain might harbor two distinct Ti plasmids, one coding for vitopine and the other for octopine-markers. Szegedi et al. [21] explained the dual opine character of these isolates by the different incompatibility properties of octopine and vitopine pTis.

The polygalacturonase gene sequence from 10 isolates designated TUNAv 1, 6, 7, 13, 14, 30, 34, 36, 39 and TUNAv 40 showed 94-97% identity to the sequences of *A. vitis* previously deposited in NCBI GenBank database (GenBank Accession No.CP000633.1gb). Based on this preliminary study, we found that Tunisian vineyards occurs *A. vitis* Biovar 3 strains octopine and vitopine utilizing. Although, only one isolate of *A. tumefaciens* Biovar 1 was found in the present work among the fifty isolates, *A. tumefaciens* Biovar 1 or 2 as well as 3 (*A. vitis*) was recovered from the same host in Greece, Hungary, etc [22]. Further research is needed to determine whether causal agents of grapevine crown gall other than Biovar 3. Further research, by bacteria isolating and examining additional strains, can also determine if strains that carry Ti plasmids encoding the utilization of opine other than octopine and vitopine also exist in the region.

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