J. Crop Prot. 2019, 8 (4):379-388

Research Article

Detection and molecular characterization of tomato big bud disease in Qazvin province

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Abstract: Some samples were collected from tomato fields in Qazvin from tomato plants with big bud symptoms such as plant droop and purplish vein under the leaf, enlarged and sac-like pistils and malformed buds. DNA was extracted from the veins and vascular tissues of the plant with CTAB-based methods. In symptomatic plants, DNA fragments of 1800 and 1200bp were amplified by PCR using P1/P7, R16F2n/R16R2 primers. Restriction fragment length polymorphism (RFLP) analysis of nested R16F2n/R16R2 primed PCR product (1200bp) showed that the tomato big bud phytoplasma from Qazvin (TOM-Qazvin) is a member of clover proliferation (16SrVI). Phylogenetic analysis of 16SrRNA and putative restriction site analysis of the R16F2n/R16R2 primed sequence classified TOM phytoplasma in clover proliferation (16SrVI) group and belonged to subgroup 16SrVI-A. Virtual RFLP by using 1200bp sequencing of 16SRNA and 17 restriction enzymes confirmed that TOM-Qazvin belonged to the subgroup 16SrVI-A and16SrVI group. To our knowledge, this is the first report of tomato big bud disease in Qazvin province.

Keywords: big bud, iphyclassifier, RFLP, tomato, phytoplasma

Introduction

Phytoplasmas are plant pathogenic (previously called MLOs) un-culturable and wall-less bacteria, they are associated with a wide variety of economically important plants (McCoy *et al.,* 1989; Seemuller *et al.,* 2002). Tomato big bud is a phytoplasma diseases reported from some countries in the world (Shaw *et al.,* 1993; Dale and Smith, 1975; Del Serrone *et al.,* 2001; Anfoka *et al.,* 2003; Ciccarone, 1951; Zimmermann-Gries and Klein, 1978; Vibio *et al.,* 1996). Affected tomato plants reveal stunting, purpling, dwarfed, enlargement and elongation of stems and pedicels and enlarged, malformed buds accompanied by enlarged, malformed sepals and virescent petals (Ghandi *et al*., 2003). Detection and identification of phytoplasmas is necessary for proper management of these diseases (Maixner, 2010). Unfortunately, phytoplasma diseases are increasing (Baghaee *et al.,* 2016). Significant progress has been made by the use of DNA-based methods for detection, identification and classification of phytoplasmas (Ghandi *et al*., 2003). In particular, by restriction site and sequence analysis of 16SrDNA many phytoplasmas have been identified and phylogenetically classified (Anfoka *et al.,* 2003; Lee *et al.,* 2000; Schneider *et al.,* 1993).The introduction of the polymerase chain reaction

Handling Editor: Masoud Shams-Bakhsh

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Received: 18 February 2019, Accepted: 14 July 2019
Published online: 3 August 2019

[DOR: 20.1001.1.22519041.2019.8.4.5.1]

(PCR) to amplify conserved genes has greatly improved the detection and identification of a broad range of phytoplasmas (Gundersen and Lee, 1996). Phytoplasmas are present in low titer in plant tissues, so the nested PCR method is used to improve phytoplasma detection (Olmos *et al.,* 1999). Symptoms of tomato big bud have been previously reported from different provinces of Iran: Fars (Salehi and Izadpanah, 1992), Isfahan, Ardabil, Western Azarbaijan (Rashidi *et al.,* 2006), Khorasan (Jamshidi *et al.,* 2010), Lorestan (Dehghani and Salehi, 2011) and Karaj (Moslemkhani *et al.,* 2014) but there is a little information about phytoplasma group in Iran. This study was performed for detection and characterization of big bud disease of tomato from naturally symptomatic plants in Qazvin province by using PCR and RFLP assay with specific primer.

Materials and Methods

Plant sample

Tomato plants that showed big bud symptoms such as shoot proliferation and swollen, virescent buds were collected from tomato fields in Qazvin province. Samples were placed in a plastic bag with a moist towel and stored at 4 °C.

Nucleic acid extraction

DNA was extracted from 300 grams of fresh veins tissues of naturally symptomatic plants, using CTAB methods (Doyle *et al.,* 1990). Total DNA of healthy tomato plants were used as negative controls.

PCR analysis

Detection and characterization of phytoplasma contamination was performed using direct PCR by two primer pairs P1/P7 to amplify 1800bp ribosomal operon. It consists part of the 16SrRNA gene, the 16S-23S spacer region and a portion of the 5' region of 23SrRNA gene. A 1:40 dilution of the direct PCR product amplified by P1/P7 primer pair were used as template for nested PCR, using primer pair R16F2n/R2, which amplifies an internal DNA fragment of 1200bp from the 16SrRNA gene based on Gunderson and Lee method (Gunderson and Lee, 1996). PCR was conducted in 20µl using 2µl of extracted DNA, 0.5µM of each primer P1/P7, 200mM of each dNTP, 1 unit of Taq DNA polymerase (CinnaGen, Iran) and lX PCR buffer and 2mM $MgCl_2$. PCR of performed in a thermal cycler (Eppendorf, Germany) using denaturation step at 94 °C for 5min and the second step, 35 cycles containing denaturation at 94 °C for 1min. Annealing at 57 °C and at 72 °C for 1.5min the third step, at 72 °C for 10min. Nested PCR were performed in a thermal cycler using a denaturation step at 94 °C for 5min and the second step, 35 cycles containing denaturation at 94 °C for 1min annealing at 57 °C and at 72 °C for 1.5min. The third step, at 72 °C for 10min. PCR products were analyzed by electrophoresis in a 1% agarose gel in 1X TBE buffer (67mM Tris-HCl, 22mM boric acid, 10mM EDTA, pH 8.0) together with 100bp DNA markers. DNA band were stained with ethidiuim-bromide and visualized with a UV transilluminator.

Restriction fragment length polymorphism and Virtual RFLP

Nested-PCR products (1200bp) of phytoplasma were separately digested with 8restriction endonucleases: *Rsa*I, *Mse*I, *Taq*I, *Alu*I, *Cfo*I, *Hinf*I, *Hae*III and *Hpa*II (Lee *et al.,* 1998) in restriction fragment length polymorphism (RFLP) analysis. The RFLP products were conducted by electrophoresis of digested DNA through 2% agarose gel, staining with ethidium-bromide and visualization with a UV transilluminator (Lee *et al.*1998).Virtual RFLP analysis of 16SrDNA fragment was carried out using the software iPhyclassifier to determine subgroup association of big bud in tomato and selected phytoplasmas (Zhao *et al.,* 2009a,b). The 1200bp R16F2n/R16R2 fragment of 16SrRNA gene from phytoplasma isolate were separately digested with 17 restriction enzymes *Rsa*I, *Mse*I, *Taq*I, *Alu*I, *Cfo*I, *Hinf*I, *Hae*III, *Hpa*II, *Bam*HI, *Bfa*I, *Bst*UI, *Dra*I, *Eco*RI, *Hha*I,

[DOR: 20.1001.1.22519041.2019.8.4.5.1]

*Kpn*I, *Rsa*I, *Sspi and Sau3*AI. Then the putative restriction site maps were compared with the patterns of isolates that were deposited in GenBank (Lee *et al.,* 1998). Digested fragments of nested-PCR products were separated on 1% agarose gel and visualized under UV transilluminator.

DNA sequencing and phylogenetic analyses

Based on the Tamura *et al*. method after comparing the RFLP patterns, direct sequence was performed and the intended isolate was selected to determine its nucleotide sequence (Macrogen Biosystems, South Korea) (Tamura *et al*. 2007). Each selected sequence was deposited in the GenBank database and compared with other sequences (Table 1) by CLUSTALW program. A phylogenetic tree was constructed by the neighbour-joining method (Saitou and Nei, 1987). *Acholeplasma laidlawii*, a culturable mollicute phylogenetically related to phytoplasmas was used as outgroup to root the tree. The resulted phylogram was printed using TREEVIEW.

Table 1 Phytoplasma group designations and GenBank accession numbers of 1200bp of 16SrRNA gene sequences examined in this study.

Phytoplasma/disease common name	GenBank (Acc. No.)	16SrDNA (group-subgroup)
Tomato big bud phytoplasma	JF508507	16SrI
Willow proliferation phytoplasma	JX123321	16SrVI-A
Tomato big bud phytoplasma	JF508511	16SrVI-A
Tomato big bud phytoplasma	JF508512	16SrVI-A
Tomato big bud phytoplasma	JF508509	16SrVI
Tomato big bud phytoplasma Yazd	MG788318	16SrII
Behshahr perinwinkle	KC661072	16SrVI
Tomato big bud phytoplasma	KR150879	16SrVI
Candidatus phytoplasma	KY321932	16SrVI
Tomato big bud phytoplasma Yazd	MG788318	16SrII
Iranian cabbage	EF592606	16SrVI-A
Candidatus phytoplasma	AY390261	16srVI
Elm phytoplasma	AF268895	16SrVI-C
Potato purple top	GU004369	16srVI
Ash yellow phytoplasma	AF268895	16SrVI-A
Tomato big bud phytoplasma	JF508513	16SrIX-E
Tomato big bud phytoplasma	AY863192	16SrIII
Tomato big bud phytoplasma	EF193359	16srVI
Stolbur phytoplasma	AF248959	16SrXII-A
Oenthera phytoplasma	M30790	16SrI

Results

Plant sample

Tomato plants collected from Qazvin province showed disease symptoms similar to tomato big bud phytoplasma such as swollen green buds that fail to develop normally and do not set fruit and had purple veins, proliferated leaves of lateral shoots, hypertrophic calyxes and greening of flower petals (Fig. 1).

PCR analysis

Target DNA fragments of approximately 1800 and 1200bp were amplified using two universal primer pairs P1/P7 and R16f2n/R16R2, respectively (Fig. 2). No DNA band observed from similarly processed sample of healthy plant. Nested-PCR assays with the primer pair R16F2/R2 showed the strong b and of approximately 1200bp (Fig. 2).

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Actual and *in silico* RFLP analysis

Digestion of nested PCR products of TOM phytoplasma showed bands of approximately 1200bp that amplified by using primer pair R16F2n/R2with *Rsa*I, *Mse*I, *Taq*I, *Alu*I, *Cfo*I, *Hinf*I, *Hae*III and *Hpa*II restriction enzymes (Fig. 3). Digestion with VIII restriction enzymes were shown in a considerably similar RFLP profile of 16SrDNA with each enzyme (Fig. 3). TOM- Qazvin phytoplasma identified in this study was identical with patterns previously published for subgroup 16SrVI-Amembers of 16SrVI group (Shaw *et al.,* 1993). Virtual RFLP patterns using iphyclassifire program after digestion with 17 different endonocleases (Fig. 4) confirmed actual RFLP were most similar to members of 16SrVI group. Virtual RFLP pattern of TOM-Qazvin and Kermanshah tomato big bud (KTBB) (JF508507) that related to sub-group A in 16SrVI group was identical (Jamshidi *et al.,* 2014).

Figure 1 Big bud symptoms in naturally infected tomato. Respectively left to Right: (1) Purplish leaves in infected plants. (2) Floral phyllody on infected tomato plants. (3) Big bud symptom in infected tomato plants.

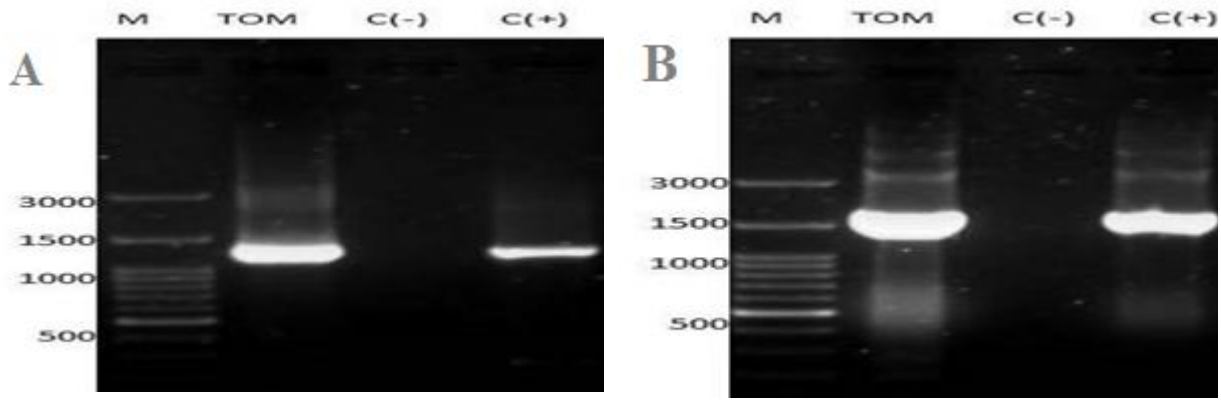

Figure 2 A- Electrophoresis pattern of 1200bp of 16SrRNA operon amplified by nested-PCR using primer pairs P1/P7 and R16F2n/R16R2n. **B-** Electrophoresis pattern of 1800bp of rRNA operon amplified by direct PCR using primer pairs P1/P7, Lane M: DNA ladder (100 bp). C (-), Healthy plant. C (+) *Candidatus* Phytoplasma asteris.

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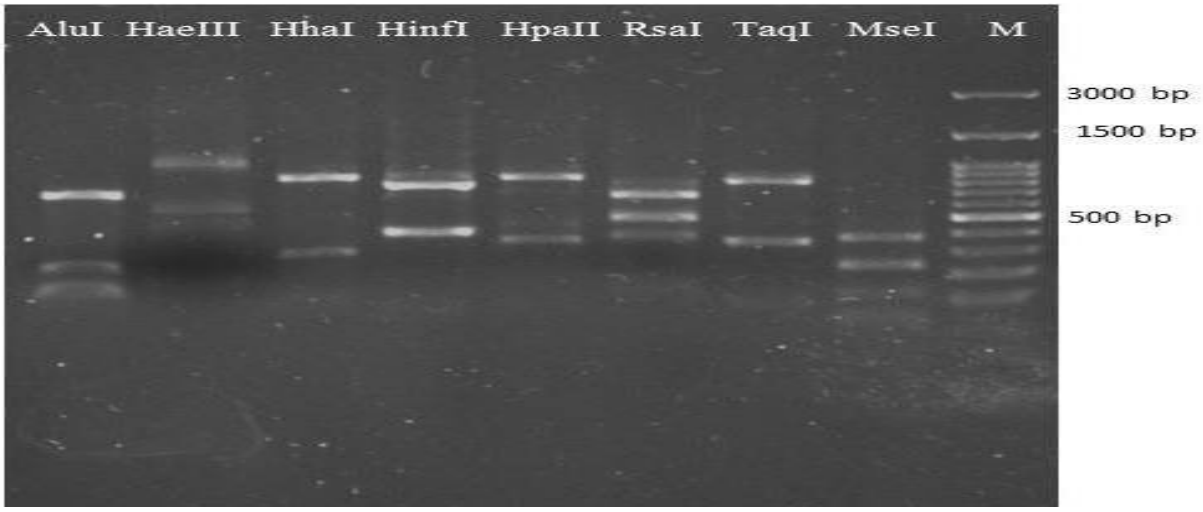

Figure 3 Restriction fragment length polymorphism of 16S rDNA amplified by nested-PCR using P1/P7 followed by R16F2n/R2 primer pairs from infected tomato plant. Lane M, DNA ladder. DNA products digested using*Hpa*II, *Taq*I, *Rsa*I, *Hinf*I, *Alu*I, *Rsa*I, *Cfo*I, *Mse*I separated through a 1%agarose gel.

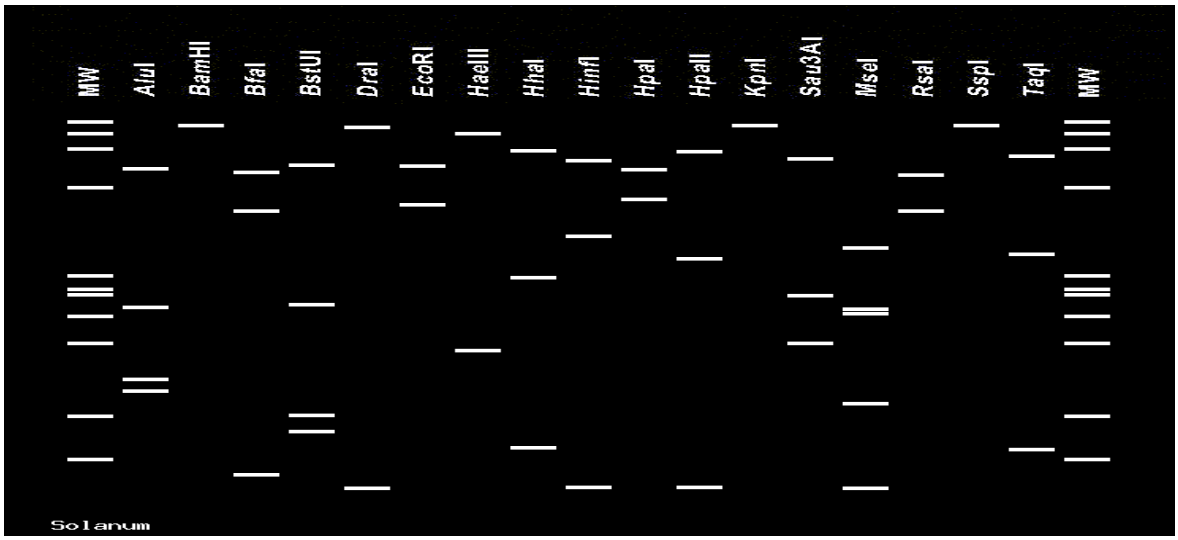

Figure 4 Virtual restriction fragment length polymorphism (RFLP) pattern of R16F2n/R2 PCR product sequence recognition sites for the following 17 restriction enzymes were used in the simulated digestions: *RsaI, MseI,TaqI*, *Alu*I, CfoI, *HinfI*, *Hae*III, *Hpa*II, *Bam*HI, *Bfa*I, *Bst*UI, *Dra*I, *Eco*RI, *Hha*I, *Kpn*I ,*Rsai, Sspi and Sau3*AI.

Sequence analyses

A phylogenetic tree was created by neighbour-joining analyses of nearly identical lengths of 16SrRNA gene from 23 isolates and *Acholeplasma laidlawii* as outgroup (Fig. 5). Based on the results of blast searches by using P1/P7 primed sequence of 23 TBB phytoplasma isolates showed that TOM-Qazvin was closely related to Clover proliferation (16SrVI) group (GenBank No. JF508507) with 99% identity. This isolate also was closely related to the phytoplasmas that infect willows trees in China (Zhang *et al.,* 2012).

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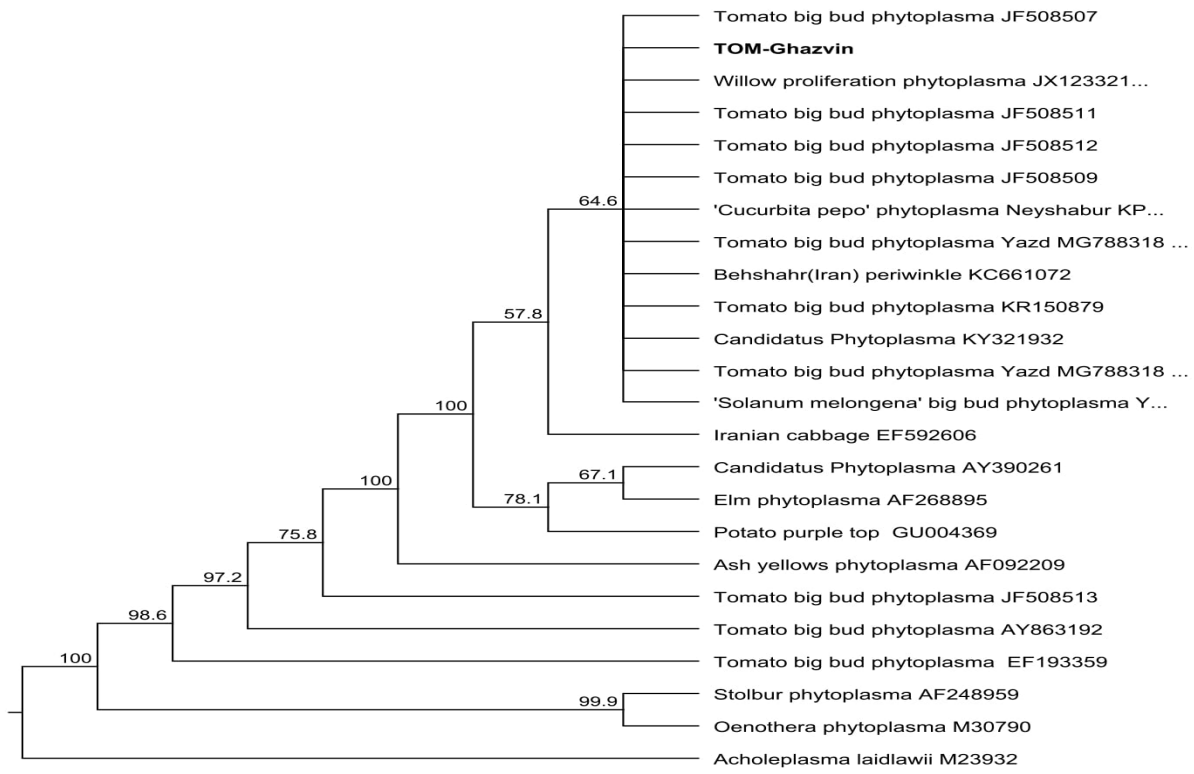

Figure 5 A neighbour-joining tree created of the 16SrRNA inter-genic spacer region of 23 phytoplasma strains associated with tomato big bud disease from GenBank and A*choleplasma laidlawii* as an out-group and position of TOM- Qazvin was showed in phylogram. Numbers above the branches bootstrap support (100 replicates).

Discussion

Tomato is a very important crop grown in Iran for fresh eating, industry, seed production and export. Disease symptom of tomato plants in Qazvin province from different area were similar to tomato big bud symptoms that have been reported from different areas in the world (Anfoka *et al*., 2003; Serrone *et al.*, 2001). Phytoplasma disease of tomato plant was reported previously in different countries such as China (Xu *et al.,* 2013), Mexico (Tapia-Tusell *et al.,* 2012), Egypt (EL-Banna *et al.,* 2007), Brazil (Amaral Mello *et al.,* 2008). In previous studies data showed that phytoplasma belonged to groups *I*, *II*, *III*, *V*, *VI* and *XII* of 16SrRNA, indicating phytoplasma disease of the tomato is genetically diverse (Santos-Cervantes *et al.*, 2008). besides, tomato big bud was associated with phytoplasma group 16SrI in the USA (Lee *et al*. 1993, 1998), with phytoplasma group 16SrI, 16SrV, 16SrXII in Italy (Serrone *et al*., 2001) also 16SrVI group was associated with tomato plants with symptoms of big bud disease in Jordan (Anfoka *et al.* 2003). In Australia, 16SrII group belonging to phytoplasma was associated with tomato big bud (Davis *et al*. 1997). Shaw *et al.* (1993) showed the beet leafhopper transmitted virescence agent (BLTVA) that caused tomato big bud in California which belonged to group 16SrVI.Del Serrone *et al.* showed that tomato plants in central Italy showing big bud-like disease symptoms were infected with phytoplasmas belonging to four different groups (I, III, V and XII) (Del Serrone *et al.,* 2001). It was also reported that the tomato big bud phytoplasma from Arkansas was affiliated with genetic subgroup 16SrI-A (Lee *et al.*, 1993). Different phytoplasmas associated with tomato diseases from 16SrDNA groups have been characterized all over the world using

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DNA-based techniques and sequence analysis (Anfoka *et al.,* 2003; Santos-Cervantes *et al.,* 2007, 2008). Presence of tomato big bud previously reported in provinces of Iran (Dehghani and Salehi, 2011). Symptom of tomato big bud in region of Iran is different. Tomato big bud disease was reported from provinces such as Khorasan, Western Azerbaijan, Eastern Azerbaijan, Kermanshah, Kurdistan and Fars for the first time, with except of Fars (Salehi *et al*. 2005) and Western Azerbaijan (Rashidi *et al.*, 2006). Association of a 16SrII group related phytoplasma with big bud disease had been previously reported from Fars and Yazd provinces (Salehi *et al.* 2005). Actual and putative RFLP and sequence analyses of AGTB (JF508509), KETBB (JF508507), KRTBB (JF508509), FTBB (JF508508) and ASTBB (JF508511) isolates were almost identical and related to the 16SrVI group. Also KTBB (JF508509) and KSTBB (JF508513) isolates were similar and belonged to the 16SeIX group. Based on the same analyses, clover proliferation group related TBB phytoplasma that belonged to 16SrVI-A subgroup, and pigon pea witches' broom phytoplasma group related to TBB phytoplasma belongs to 16SrIX-E subgroup (Jamshidi *et al*., 2014). Another study in Karaj vicinity on different cultivars of tomato indicated that 89% of phytoplasmas present in tomato samples belonged to 16SrVI; but less than 11% belonged to 16SrI group which were observed only in the Pardis and Mateen cultivars (Moslemkhani *et al.* 2014). In Iran, cabbage *Brassica oleraceavar*. capitata and safflower *Carthamus tinctorius* L. were reported as hosts for 16SrVI group (Salehi *et al*., 2007, 2009). In this study, based on the results of RFLP and sequence analyses TOM-Qazvin phytoplasma belongs to 16SrVI-A subgroup and it is the first report of tomato plant as a host for 16SrVI in Qazvin province.

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دریافت: ۲۹ بهمن ۱۳۹۷؛ پذیرش: ۲۳ تیر ۱۳۹۸

چکیده: در بررسی‌های انجام شده از مزارع گوجه‌فرنگی استان قزوین از بوته‌های دارای علائم تورم جوانه شامل کوتولگی، ارغوانی‌شدن رگبرگ‌ها در سطح زیرین برگ، تبدیل کاسه گل به یک جسم کیسه مانند و جوانه‌های بدشکل نمونه‌برداری صورت‌گرفت. برای شناسایی عامل بیماری از نمونه‌های دارای علائم تیپیک، DNA از رگبرگ‌ها و بافت آوندی گیاه با روشی مبتنی بر CTAB استخراج شد. ردیابی احتمالی آلودگی فیتوپلاسمایی با آزمون PCR مستقیم با استفاده از جفت آغازگرهای P1/P7 و آزمون PCR دو مرحله‌ای با استفاده از جفت آغازگرهای P1/P7 و R16F2n/R16R2 انجام شد و به‌ترتیب باندهای مورد انتظار ۱۲۰۰ و ۱۸۰۰ جفت باز از نمونه گوجه‌های آلوده دیده شد. طی آنالیز محصول مرحله دوم PCR (۱۲۰۰ جفت باز) با استفاده از آزمون چندشکلی طولی قطعات برشی (RFLP) عامل فیتوپلاسمایی متعلق به زیرگروه A از گروه 16SrVI بود. نتایج بلاست توالی تکثیر شده در NCBI و آنالیز آن توسط نرم‌افزار آنلاین iphyclassifier نشان داد که عامل بیماری تورم جوانه گوجه‌فرنگی متعلق به گروه نامبرده است. RFLP مجازی با استفاده از ترادف (۱۲۰۰ جفت باز) ژن 16SrRNA و ۱۷ آنزیم برشی علاوه بر تأیید نتایج، نشان داد که عامل فیتوپلاسمایی متعلق به گروه 16SrVI و زیرگروه 16SrVI-A است.

واژگان کلیدی: تورم جوانه، iphyclassifier، RFLP، گوجه‌فرنگی، فیتوپلاسما

[DOR: 20.1001.1.22519041.2019.8.4.5.1]