柿树带化病相关植原体的形态及分子鉴定

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摘要: 柿树为我国重要的经济林果树, 柿果、柿叶和柿霜等包含丰富的生物活性成分, 具有重要的医学价值。从山东省邹城市采集到表现带化并伴随节间缩短和叶片变小的柿树枝条样品, 利用透射电子显微镜进行植原体细胞检测, 结果显示染病的柿枝条韧皮部筛管细胞内存在直径为200~600 nm的圆形或者椭圆形植原体粒子。提取染病柿树枝条和健康枝条的韧皮部总DNA, 利用植原体16S rRNA基因及延伸因子*tuf*基因通用引物进行PCR扩增, 从染病组织DNA中得到了长度分别约为1.2和0.8 kb的片段, 健康柿树枝条没有扩增条带。序列一致率分析表明, 柿树带化植原体16S rRNA基因和*tuf*基因与枣疯植原体相应基因的核苷酸一致率分别高达99.8%和99.9%; *i*PhyClassifier在线分析表明, 柿树带化植原体与16SrV-B亚组代表株系的16S rRNA基因具有相同的酶切图谱, 相似系数为1.0; 基于两个基因构建的系统进化树均表明, 柿树带化植原体与16SrV-B亚组成员聚为一簇。生物学和分子生物学鉴定结果表明, 柿树带化病存在植原体, 且该植原体属于榆树黄化组(16SrV) B亚组, 与候选种‘*Candidatus* Phytoplasma ziziphi’相关。
关键词: 植原体; 柿树; 柿树带化病; 榆树黄化组

柿树为双子叶植物纲(Magnoliopsida)柿目(Ebenales)柿科(Ebenaceae)柿属(*Diospyros*)植物。原产于我国, 是地中海及亚洲的许多国家重要的经济果树, 柿果、柿蒂和柿霜等包含丰富的生物活性成分, 如黄酮类低聚物、单宁、酚和类胡萝卜素, 具有重要的药疗效用。目前, 国内对该树种的研究多集中于遗传育种、脱涩机理、果实营养成分及采后生理学等方面(袁录霞等2011)。在果树栽培过程中普遍对真菌病害的研究较多(余贤美等2015; 丁向阳等2015; 姚昇华等2015; 张敬泽和徐同2005), 对其他病原类型的病害则关注较少。

植原体(phytoplasma)是一类不能人工培养、无细胞壁、专性寄生于植物筛管细胞和介体昆虫细胞内的原核生物。主要通过菟丝子、人工嫁接和介体昆虫传播。已经发现其与植物上发生的1 000多种病害有关系, 常引起丛枝、小叶、花变绿(叶)等典型症状(Bai等2006)。目前16S rDNA序列的鉴定结合电镜技术或其他基因的鉴定是国际上通用的植原体检测和鉴定方法(Lee等1993; Zhao等2009, 2010; Lai等2014)。2015年, 本课题组在山东省果树研究所柿树科研基地发现了当年生和二年生柿树枝条呈带化症状, 与已报到的植原体引起的带化症状相似。调查发现, 这种病害主要侵染植株的枝条, 引起树体生长缓慢且畸形, 导致后期果实产量下降, 阻碍了柿产业的发展, 为了有效预防其大面积发生, 迫切需要查明发病原因, 从而制定相应的防治策略。本研究采用组织形态学及分子生物学两种技术, 进一步对柿树带化植原体进行了形态和分子鉴定。

材料与方法

1 材料

表现带化症状的柿树(*Diospyros kaki* Thunb.)样品和健康样品均采自山东省邹城市香城镇; 克隆载体pMD18-T、PCR产物回收试剂盒、*Taq* DNA聚合酶等分子生物学试剂产品均购自TaKaRa公司; 引物由上海铂尚生物技术有限公司合成。

2 方法

2.1 电子显微镜观察

参照汪跃和金开璇(1992)的方法, 取感病枝条的韧皮部组织(2 mm×2 mm)观察是否存在植原体, 样品经3%的戊二醛和1%锇酸双重固定, 乙醇逐级脱水, 环氧丙烷置换, Epon812渗透包埋, 切片后经饱和醋酸双氧铀和柠檬酸铅双重染色, 在JEM-1200 EXII型透射电镜下观察, 记录并拍摄电镜照片。

2.2 分子检测

2.2.1 基因扩增

采用CTAB法(漆艳香等2004)分别提取表现带化的柿树和表现健康的柿树组织总DNA, 以总

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DNA为模板, 进行16S rRNA基因和延伸因子(TUF)基因*tuf*的扩增。利用植原体16S rRNA基因通用引物R16mF2/R16mR1 (Lee等1993)进行PCR扩增, 再以此扩增产物为模板并稀释10倍后用R16F2n/R16R2 (Gundersen和Lee 1996)进行巢式PCR。利用植原体*tuf*基因通用引物fTuf1/rTuf1 (Schneider等1997)对延伸因子基因进行PCR扩增, 再以此扩增产物按1:20比例稀释后为模板用fTufu/rTufu进行巢式PCR扩增, 引物序列见表1。

表1 用于植原体16S rRNA和*tuf*基因扩增的引物序列

Table 1 Primers used in this research to amplify 16S rRNA and *tuf* genes

引物名称	引物序列	T_m/°C
R16mF2	5′-CATGCAAGTCGAACGGA-3′	52
R16mR1	5′-CTTAACCCCAATCATCGAC-3′	52
R16F2	5′-ACGACTGCTAAGACTGG-3′	55
R16R2	5′-TGACGGGCGGTG TGTACAAACCCCG-3′	55
fTuf1	5'-CACATTGACCACGGTAAAAC-3'	53
rTuf1	5'-CCACCTTCACGAATAGAGAAC-3'	53
fTufu	5'-CCTGAAGAAAGAGAACGTGG-3'	55
rTufu	5'-CGGAAATAGAATTGAGGACG-3'	55

2.2.2 PCR产物纯化、克隆及序列测定

PCR产物经凝胶电泳检测, 目标条带纯化采用MiniBEST Agarose Gel DNA Extraction Kit (TaKaRa公司), 具体操作过程参照试剂盒说明进行。基因的克隆参照pMD™18-T Vector Cloning Kit载体说明书进行。采用Cohen等(1972)的方法制备大肠杆菌DH5α的感受态细胞, 采用热激法将连接产物转化感受态细胞, 经蓝白斑初步筛选, 挑取重组子菌落进行培养, 利用碱裂解法提取质粒(蔡红等2005), PCR及酶切鉴定为阳性的重组质粒委托上海铂尚生物技术有限公司进行测序。

2.2.3 序列分析

将所得DNA序列输入GenBank进行BLAST检索, 参照Fránová等(2014)下载植原体不同组及亚组的代表株系相关序列, 采用DNAMAN version 6和DNASTAR软件对所得到的核苷酸序列进行相似性比对。利用MEGA 5.2软件(Tamura等2011)构建系统进化树。

2.2.4 16S rRNA基因虚拟RFLP分析

将克隆得到的16S rRNA基因序列通过植原体在线分类软件*i*PhyClassifier (http://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi)进行包含17种限制性内切酶的虚拟RFLP分析及相似系数计算, 并分析确定其分类地位(Zhao等2009)。

实验结果

1 柿树带化病病株症状

感病柿树植株表现为典型的带化症状, 多在一至二年生苗木或者新枝上发生。新发枝条在春季常伴随着新叶变小和簇生、节间变短等症状, 部分枝条随着植株的生长除带化之外的其他症状变得不明显(图1-A和B)。

2 电镜观察结果

通过透射电子显微镜观察, 在感病植株韧皮组织的筛管细胞中发现有典型的植原体细胞存在

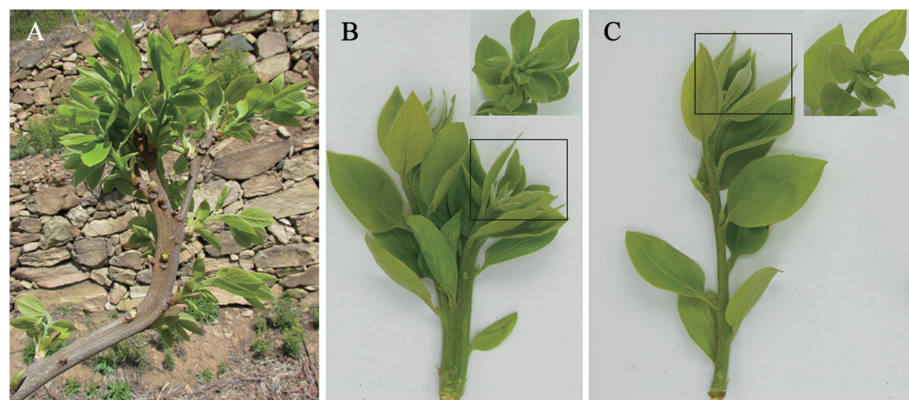

图1 表现带化症状和健康的柿树枝条

Fig.1 Stem fasciation of persimmon branch and healthy-looking branch

A: 二年生带化症状枝条; B: 当年生带化症状枝条; C: 当年生健康枝条。

(图2), 直径大小在200~600 nm之间, 形状多为圆形和椭圆形, 说明植株中存在植原体。

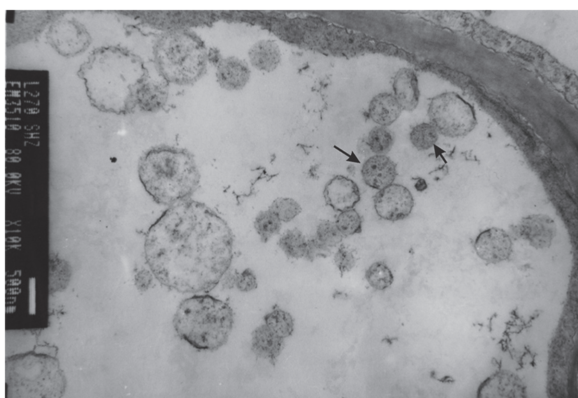

图2 柿树带化植原体的透射电镜观察结果
Fig.2 Transmission electron micrograph of phytoplasma in infected persimmon plant

3 16S rRNA基因克隆及序列分析

以染病的柿树总DNA为模板, 经巢式PCR扩增, 得到长度约为1.2 kb的片段, 以表现健康的柿树组织为模板未扩增到任何条带(图3)。将该片段克隆到T载体, 选取2个重组质粒进行测序, 结果显示, 扩增得到的片段长度为1 248 bp, G+C的含量为45.8%。BLAST搜索表明此序列为植原体16S rRNA基因序列(GenBank登录号为KP174135), 证明柿树带化是由植原体引起的, 并将此植原体暂时命名为柿带化植原体(persimmon fasciation, PF)。经同源性分析表明, PF与中国的两个枣疯病植原体分离物JWB-FJP1 (KF017279)和JWB-Xch (JQ675716)的相似性均为99.8%, 与16SrV组中其他分离物的核苷酸一致率均在99%以上。同源进化分析结果也表明, PF聚类到16SrV组中, 与16SrV-B亚组植原体聚集为一簇(图4)。

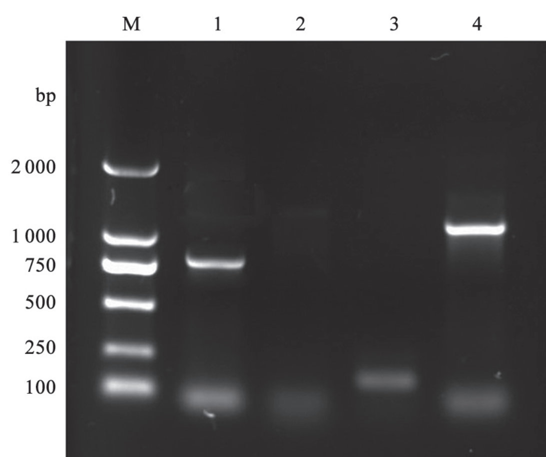

图3 柿树带化病植原体16S rRNA基因和*tuf*基因扩增产物的电泳图
Fig.3 The gel electrophoresis of PCR product of 16S rDNA and *tuf* genes from persimmon fasciation phytoplasma

M: DL2000 DNA Marker; 1: 感病柿树组织*tuf*基因; 2: 健康柿树组织*tuf*基因; 3: 健康柿树组织16S rRNA基因; 4: 感病柿树组织16S rRNA基因。

4 虚拟RFLP分析及相似系数分析

利用在线工具*i*PhyClassifier对PF进行虚拟RFLP分析, 计算并比较了用17种限制性内切酶进行酶切后不同植原体株系间的相似系数和酶切图谱, 结果表明: PF的酶切图谱与16SrV-B亚组枣疯植原体代表株系JWB-G1 (AB052876)酶切图谱完全一致(图5和表2), 相似系数为1.00。进一步明确了柿树带化中检测到的植原体的分类地位。

5 *tuf*基因克隆及序列分析

经测序分析, 确定本研究获得的柿树带化植原体*tuf*基因部分片段长度为823 bp, 可编码274个氨基酸, GenBank登录号为KT968866。核苷酸一致率分析表明, 柿树带化植原体*tuf*基因与河北唐县酸枣(JWB-TXSZ)的一致率最高为99.9%, 与枣疯植原体TXDO株系、BJLZ株系及XJJ1株系的*tuf*基因序列相似性分别为99.8%、99.8%和99.6%。与同属于16SrV组的A亚组成员‘*Candidatus* (*Ca.*) Phytoplasma ulmi’ EY1株系(FN561879)、E亚组成员‘*Ca*. Phytoplasma rubi’ (KR233474)及C亚组的‘*Ca.* Phytoplasma vitis’ (AM939565)的序列一致率分别为91.7%、92.6%和92.7%。而与其他组植原体*tuf*基因核苷酸一致率均小于90%。系统发育树也表明, 本研究的PF-*tuf*基因序列与枣疯植原体聚为一枝(图6)。进一步表明柿树带化植原体PF分离物属于16SrV-B亚组。

讨　论

植原体侵染植物, 常引起黄化、丛枝和绿变等症状, 带化并不是植原体侵染后的典型症状, 国内外仅有少量植物带化病的报道, 例如百合带化病、紫穗槐带化病、国槐带化病等(汪跃和金开璇1992; 刘小勇等1999; Bertaccini等2005)。迄今, 关于

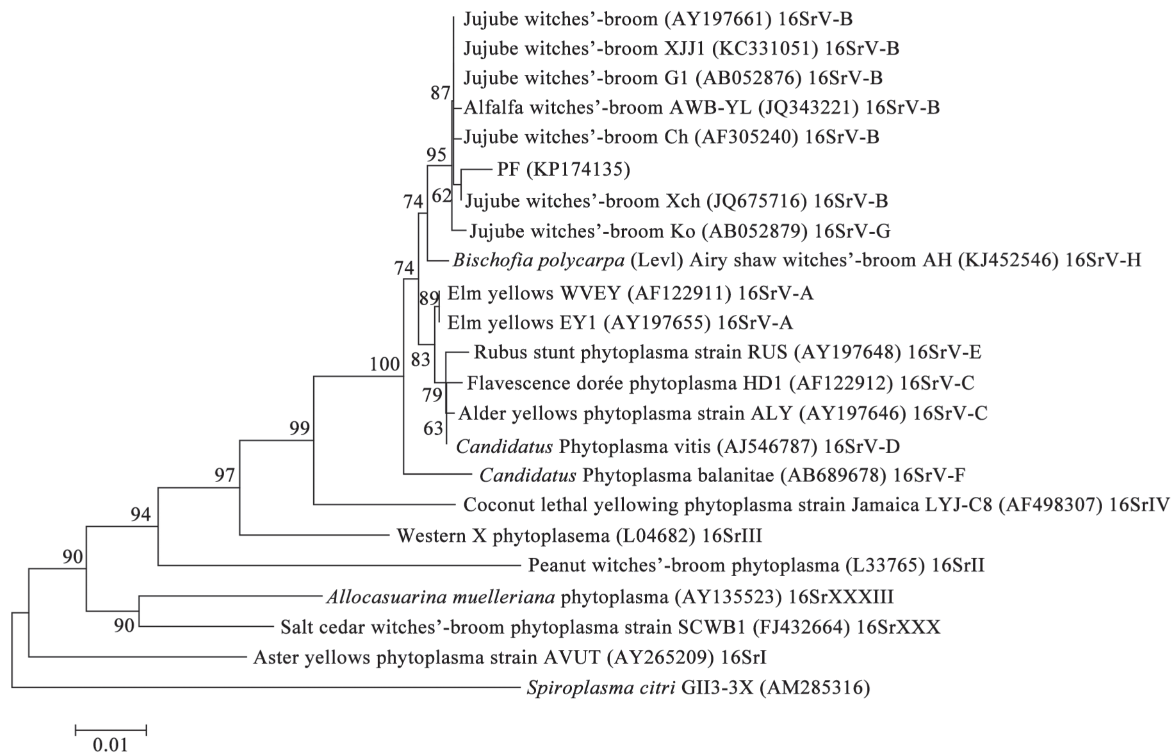

图4 利用邻接法构建的基于16S rRNA基因的系统发育树

Fig.4 Phylogenetic tree constructed using the neighbour-joining method based on 16S rRNA gene sequences

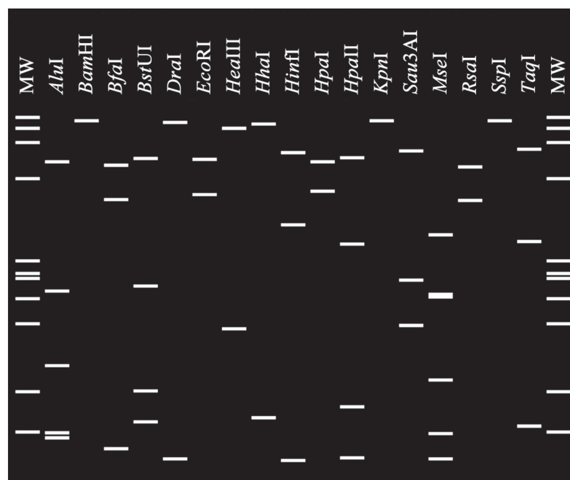

图5 柿树带化植原体16S rRNA基因F2nR2片段的虚拟RFLP图谱

Fig.5 Virtual RFLP patterns derived from *in silico* digestions of 16S rRNA gene F2nR2 fragments of persimmon fasciation phytoplasma

柿树带化病的报道仅限于我国。刘小勇等(1999)从表现带化的柿树中扩增到植原体16S rDNA片段, 利用8种限制性内切酶对其进行酶切, 酶切图谱与国槐带化、柳树丛枝和榆树丛枝的酶切图谱相似, 并未明确指出柿树带化病植原体的分类地位。

已有的报道表明欧洲的榆树丛枝植原体属于16SrV组(Mäurer等1993), 而我国山东和新疆榆树丛枝植原体分别与'*Ca*. Phytoplasma asteris'株系OAY和'*Ca.* Phytoplasma ziziphi'同源性最高, 即分别属于翠菊黄化组B亚组(16SrI-B) (Gao等2011)。国槐带化病最早报道于1992年(汪跃和金开璇1992), 后来本课题组成员利用16S rDNA序列将其鉴定为16SrI-B组成员。柳树丛枝植原体归属于三叶草丛生组(16SrVI) (Khadhair和Hiruki 1995)。这些研究表明国槐带化、柳树丛枝和榆丛枝植原体具有不同的分类地位, 仅依据少量的限制性内切酶图谱无法明确柿带化植原体的分类地位。

近年来的研究表明植原体可以离体培养(Contaldo等2016), 但是限于特殊的仪器需求, 多数实验室很难获得植原体的纯培养, 因此, 不能像其他的原核生物一样进行对植原体进行系统的鉴定和分类研究, 早期的植原体鉴定方法主要依靠寄主植物的种类、病症、传播昆虫及抗菌素实验等

表2 柿树带化植原体及16SrV组的各亚组代表株系16S rRNA基因的相似系数

Table 2 Similarity coefficients of 16S rRNA gene from persimmon fasciation phytoplasma and typical strains of each 16SrV subgroup

	植原体代表株系	1	2	3	4	5	6	7	8	9	10	11	12
1	KP174135-PF3	1.00											
2	AF122911-16SrV-A	0.90	1.00										
3	AB052876-16SrV-B	1.00	0.90	1.00									
4	AY197661-16SrV-B	1.00	0.90	1.00	1.00								
5	JQ343221-16SrV-B	1.00	0.90	1.00	1.00	1.00							
6	JQ675716-16SrV-B	1.00	0.90	1.00	1.00	1.00	1.00						
7	AY197646-16SrV-C	0.87	0.90	0.87	0.87	0.87	0.87	1.00					
8	AJ548787-16SrV-D	0.90	0.93	0.90	0.90	0.90	0.90	0.97	1.00				
9	AY197648-16SrV-E	0.87	0.90	0.87	0.87	0.87	0.87	0.94	0.91	1.00			
10	AB689678-16SrV-F	0.91	0.82	0.91	0.91	0.91	0.91	0.79	0.76	0.79	1.00		
11	AB052879-16SrV-G	0.97	0.89	0.97	0.97	0.97	0.97	0.84	0.81	0.84	0.88	1.00	
12	KJ452546-16SrV-H	0.88	0.78	0.88	0.88	0.88	0.88	0.78	0.74	0.78	0.86	0.85	1.00

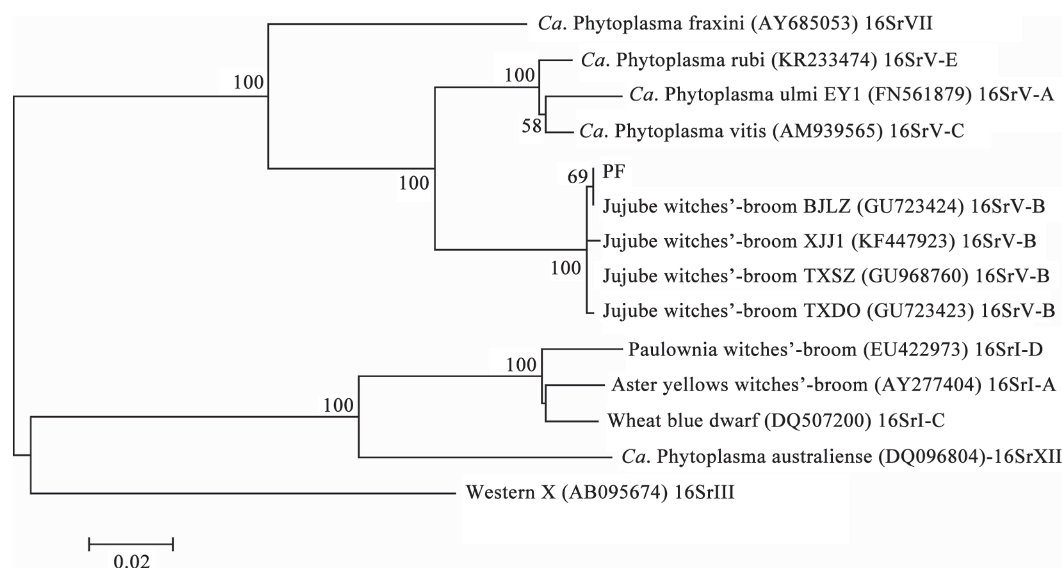

图6 柿树带化植原体及13个相关植原体基于*tuf*基因构建的系统发育树

Fig.6 Phylogenetic tree based on *tuf* gene nucleotide sequence of phytoplasmas associated with persimmon fasciation and other 13 phytoplasma *tuf* gene sequences

(金开璇和汪跃1996), 这些检测鉴定过程不仅费时、费力, 还易受多种外界环境因素的干扰。随着分子杂交、DNA克隆、PCR以及RFLP等技术的广泛应用(王海荣等2015), 植原体分子生物学信息的不断积累, 也推动了植原体的系统分类研究。16S rDNA序列的同源进化及*i*PhyClassifier分析可以作为区分和鉴定植原体的简便、可靠、实用的方法(Lee等1993; Zhao等2009), 与植原体的保守基因16S rDNA相比, 16S~23S rDNA间区, *rp*和*tuf*基因序列可变性增强, 更适合研究亲缘关系比较近的植原体亚组及株系的划分(Streten和Gibb 2005; Fránová等2014)。因此, 同时对16S rDNA基因和其他基因序列进行分析能为植原体的鉴定和分类提供更准确的信息支持, 利用该方法已成功鉴定了棣棠丛枝病相关植原体、花生丛枝植原体和重阳木丛枝植原体等(常文程等2012; 万琼莲等2014; Lai等2014)。植原体细胞专性寄生于植物筛管, 大小50~1 000 nm, 具有细胞结构, 但是无细胞壁, 仅有单位膜包裹, 根据植原体细胞的这些特点, 本研究利用电镜技术初步确定柿树带化枝条中存在植

原体, 并利用植原体16S rRNA基因和*tuf*基因的通用引物, 分别扩增了柿树带化植原体的16S rRNA基因和*tuf*基因, 经基因序列分析和系统进化分析, 明确了山东邹城柿树带化枝条中检测到的植原体归属于榆树黄化植原体16SrV-B亚组。

植原体引起的叶片黄化、生长衰退和带化等症状的感病组织中病菌的浓度往往低于表现典型丛枝症状的植原体感病组织中的浓度(Berges等2000)。直接PCR方法的灵敏度受植物组织总DNA提取液浓度及其他抑制剂物质的影响, 往往会出现假阴性结果(Lee等1994)。巢式PCR技术增加了PCR扩增的灵敏性和特异性, 是非典型症状植原体病害诊断、病原检测和鉴定的有效方法(Lee等1994; Gundersen和Lee 1996; 王洁等2010; 常文程等2012)。本研究前期试验中, 通过直接PCR扩增, 未获得明显目的条带, 推测可能的原因是植原体浓度含量低, 且柿树组织中含有大量的多酚和不溶性单宁抑制了PCR的扩增, 因此, 采用巢式PCR对16S rRNA基因和*tuf*基因进行扩增, 为避免巢式PCR和极易发生污染出现假阳性的问题, 本研究同时应用电子显微镜技术为带化柿树组织中存在植原体细胞提供直接证据。

柿树带化病多发生于当年生新枝上, 造成枝条生长缓慢, 部分枝条生长3~4年后带化症状变得不明显甚至恢复, 这在葡萄黄花病害和苹果丛枝病病害中也曾发现, 这与染病植株的品种以及植株内部过氧化氢的积累有关(Bellomo等2007; Musetti等2004)。虽然目前柿树带化病仅有零星发病, 但因其具有可传播性, 需要对病原种类、传播方式等进行研究, 以预防柿树带化病在适宜条件下大面积流行。调查发现, 在采集样本的资源圃周围存在大量枣疯病病株, 研究结果也表明, 柿树带化植原体归属于16SrV-B亚组, 与枣疯植原体具有较近的亲缘关系, 柿带化组织中的植原体是否由枣疯病植原体传播而来还有待更多基因信息及传毒介体等方面的深入研究。因此, 在制定防治策略时除及时铲除柿树带化枝条外, 还应重点关注周边枣疯病株, 切断一切可能的传染源。

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Morphological and molecular detection of phytoplasma associated with persimmon fasciation disease

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Abstract: Persimmon (*Diospyros kaki*) is an important economic forest fruit tree species in China. Fruit, leaves and powder on the surface of a dried persimmon contain several biologically active compounds, used traditionally for many medicinal purposes. Persimmon plants with stems fasciation, internodes shorten, and little leaf were sampled in Zoucheng, Shandong, China. Transmission electron microscope technology was used to detect phytoplasmal cells from the tissue of symptomatic plants and a great quantity of structures resembling phytoplasmas, about 200–600 nm, circular or elliptical, were observed in phloem sieve elements. Total DNA was extracted from symptomatic plants as well as asymptomatic plants via the modified CTAB method. PCR was conducted to detect putative phytoplasma using universal primers for phytoplasmal 16S rRNA gene and *tuf* gene, and specific fragments of circa 1.2 kb and 0.8 kb in length were amplified from diseased persimmon tissue, respectively. Sequence analysis indicated that 16S rRNA gene and *tuf* fragments shared 99.8% and 99.9% identity with that of jujube witches'-broom phytoplasma, respectively. *i*PhyClassifier analysis for 16S rRNA gene showed that persimmon fasciation phytoplasma has the same restriction enzyme pattern with the typical isolate of subgroup 16SrV-B, and the similarity coefficient was 1.0. Phylogenetic tree based on 16S rRNA or *tuf* gene revealed that persimmon fasciation phytoplasma strain formed a sublineage with members of subgroup 16SrV-B. In conclusion, phytoplasma associated with persimmon fasciation in Zoucheng, Shandong, China was classified to the subgroup B of the elm yellows group (16SrV) and related to '*Candidatus* Phytoplasma ziziphi'.

Key words: phytoplasma; persimmon; persimmon fasciation disease; elm yellows group

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