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Amplification of *virD1* and opine synthase genes from tumorigenic rose and aster strains of *Agrobacterium* in Taiwan by polymerase chain reaction

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ABSTRACT

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We tried to amplify the *virD1* gene and opine synthase genes from tumorigenic strains of *Agrobacterium* in Taiwan by polymerase chain reaction (PCR). The *virD1* gene was amplified from tumorigenic strains of *Agrobacterium* isolated from rose gall tissues; but could not be amplified from tumorigenic strains of *Agrobacterium* isolated from aster gall tissues. In the PCR analysis of three opine synthase genes, the segment of agrocinopine synthase gene was amplified from the tumorigenic rose strains whereas the octopine synthase gene was amplified from tumorigenic aster strains. None of these tumorigenic strains could generate PCR product with oligonucleotide primers for nopaline synthase gene. These results implicate that the sequence of *virD1* virulence gene may be distinct among different groups of phytopathogenic *Agrobacterium*. In addition, the type of tumor-inducing plasmid may be quickly determined by PCR amplification of opine synthase gene(s) from the agrobacterial cell.

Key words : polymerase chain reaction (PCR), *virD1*, agrocinopine synthase gene, nopaline synthase gene, octopine synthase gene.

INTRODUCTION

Crown gall disease, caused by phytopathogenic *Agrobacterium*, was found on several plants in Taiwan in recent years. The infected plants included small-leaved banyan (*Ficus microcarpa* L. F.) [9], rose (*Rosa hybrida*), and aster (*Aster ericoides* L.). An influence of the disease on the propagation of seedlings was observed in the rose nursery. It is well known that the presence of tumor-inducing (Ti) plasmid is required for the bacterial cell to induce tumorous growth of plant tissues [18]. Two DNA regions on the Ti plasmid are essential for bacterial pathogenicity. One region contains the transferred DNA (T-DNA) and the other contains the virulence (*vir*) genes [10,19,21].

Infection of plants by pathogenic *Agrobacterium* usually starts from wounded sites of plants. Phenolic compounds released from wounded tissues of plants are necessary for the induction of *vir* gene expression that is required for the transfer of T-DNA into plant cells [10,19,21]. After T-DNA integration into the plant nuclear DNA, the oncogenes on T-DNA are expressed to induce tumorous growth of plant tissues. Two of the oncogenes, *iaaM* and *iaaH*, located on T-DNA, encode enzymes for auxin biosynthesis. The third oncogene, *ipt*, is required for cytokinin biosynthesis [19]. Expression of opine synthase genes on T-DNA in plant cells results in the production of opines which are utilized by agrobacteria as carbon and nitrogen sources and play a role in horizontal transfer of Ti plasmid among different agobacteria [4]. Various opines are synthesized in different *Agrobacterium*-infected plant tissues and used in categorizing plasmids carried in *Agrobacterium* cells [4,19].

Polymerase chain reaction (PCR) has been used to

identify pathogenic strains of *Agrobacterium* with oligonucleotide primers designed according to the sequences of *virA*, *virB2*, *virC1*, *virC2* and *virD2* [6,8,15,17] on *vir* region. In addition, oligonucleotide primers for *iaaH* and *iaaM* on T-DNA region were reported for the detection of grapevine strains [5] and the primers for *ipt* sequence on T-DNA region were used for the detection of rose strains [14]. In this study, we examined the efficacy of PCR amplification of *virD1* gene on *vir* region and opine synthase gene(s) on T-DNA region from tumorigenic rose and aster strains of *Agrobacterium* in Taiwan and found that these tumorigenic strains from different plants displayed distinct patterns in the PCR amplification.

MATERIALS AND METHODS

Bacterial strains

Agrobacteria were isolated from the gall tissues of rose and aster plants and purified on medium 523 [11] (Table 1). The bacteria were cultured on medium 523 at 28℃ and stored at -80℃ in 523 broth containing 15% glycerol.

Agrobacterium tumefaciens strains C58 and B6 from Cultural Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, Taiwan were used as control strains. *A. tumefaciens* strain C58 carries Ti plasmid that was characterized as nopaline and agrocinopine-types; *A. tumefaciens* strain B6 carries Ti plasmid of octopine-type [19]. Strain LBA4301 lacks the Ti plasmid and is avirulent [13].

Pathogenicity assay

Overnight cultures of agrobacteria were sprayed onto the surface of carrot discs and incubated in moist chamber [1]. Tomato (*Lycopersicum esculentum* Mill.), rose (*Rosa hybrida*), and aster (*Aster ericoides* L.) plants were inoculated with bacteria by puncturing the stem or twig tissues with toothpicks. Three wounded sites were inoculated for each strain on each kind of plants. The inoculated plants were kept in a plant growth chamber (25℃). Tumor formation was examined at ten-day intervals.

Preparation of Ti plasmid for PCR

Ti plasmid was isolated from bacterial cells of agrobacteria as described by Kado and Liu (1981) [12]. The cell lysate was incubated at 55℃ for 60 min to denature the chromosomal DNA and extracted with an equal volume of phenol-chloroform solution. After centrifugation, the supernatant containing Ti plasmid was used for PCR. Alternatively, bacterial cells (about 10^8 cfu/ml) were boiled for 10 min, immediately incubated on ice and used directly as the template in PCR without further treatment.

Table 1. Pathogenicity assay of *Agrobacterium* strains

Strain	Pathogenicity [1]				Source (plant and place)
	R	A	T	C	
Rose strain					
AR3	+[2]	—	+	+	Our lab (rose; Taichung, Taiwan)
AR14	+	—	+	+	Our lab (rose; Nantou, Taiwan)
AR32	+	—	+	+	Our lab (rose; Hualien, Taiwan)
R007	+	—	+	+	Provided by S. H. Hseu (rose; Central Taiwan)
R022	+	—	+	+	Provided by S. H. Hseu (rose; Central Taiwan)
Aster strain					
AASW2-1	—	+	+	+	Our lab (aster; Pingtung, Taiwan)
AASR16	—	+	+	+	Our lab (aster; Central Taiwan)
AT2	—	+	+	+	Provided by T. C. Huang, (aster; Pingtung, Taiwan)
AT5	—	+	+	+	Provided by T. C. Huang, (aster; Pingtung, Taiwan)
AT6	—	+	+	+	Provided by T. C. Huang, (aster, Pingtung, Taiwan)

1. Pathogenicity of bacterial strains tested on the twigs or stems of rose (R), aster (A), and tomato (T) plants and the carrot discs (C).
2. "+" = gall formation on three sites of inoculation, "—" = no visible gall appeared on the inoculation sites.

PCR

The primer sequences for *virD1* and three opine synthase genes were listed in Table 2. PCR was performed in a mixture (50μl) containing 5μl of 300-fold diluted cell lysate or 5μl of boiled bacterial cell suspension. Other ingredients were 0.8μM each of oligonucleotide primers, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.3 mM $MgCl_2$, 50μM each of deoxynucleoside triphosphates, 0.01% gelatin, and 1 U Taq DNA polymerase. The reaction mixture was first heated at 95℃ for 2 min and then performed 40 PCR cycles of denaturation at 95 ℃ for 1 min, primer annealing at 55 ℃ for 2 min, and DNA extension at 72 ℃ for 2 min in a RoboCycler temperature cycler (Stratagene, La Jolla, CA). At the end of PCR, the reaction mixture was incubated at 72℃ for 10 min and then stored at 4℃.

Table 2. Oligonucleotide primers for PCR

Amplified sequence and expected size [1]		Primer sequence	Source of primer sequence	Reference
virD1	444 bp	5'-ATGTCGCAAGGCAGTAGGCCCACCT-3' 5'-CTACAAGGCGTCTTTCAGCAGCGAGC-3'	*virD1* on *vir* region	16
acs	1,071 bp	5'-ATTCAAGAATGCACCGCGAG-3' 5'-TATATTAAGATCCAAGTGTGG-3'	Agrocinopine synthase gene (*acs*) on T-DNA	3
nos	1,242 bp	5'-ATGGCAATTACCTTATCCGCA-3' 5'-TTACTCCACCATCTCGTCC-3'	Nopaline synthase gene (*nos*) on T-DNA	2
ocs	1,077 bp	5'-ATGGCTAAAGTGGCAAT-3' 5'-TCAAACTCCATTGAGAGCCC-3'	Octopine synthase gene (*ocs*) on T-DNA	7

[1.] Full-length sequence: *virD1*, nopaline synthase gene (*nos*), octopine synthase gene (*ocs*); part of sequence: agrocinopine synthase gene (*acs*).

Agarose gel electrophoresis

PCR products (20μl) were analyzed at 100 V for 25 min in 1.5% or 2% agarose gel depending on the size of DNA fragments. Gel electrophoresis was performed in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). One-kb DNA ladder (Gibco/BRL, Gainthersburg, MD) was used as molecular size standard. After electrophoresis, gel was stained with ethidium bromide (0.5μg/ml) for 10-20 min. DNA bands were visualized and photographed over a UV transilluminator (300 nm).

RESULTS

Symptom development in plants after infection of rose and aster strains of *Agrobacterium*

In all the tested strains, tumors appeared on the cut surface of carrot discs and the wounded sites of tomato seedlings within 10 days after inoculation. The pathogenicity assay showed that the rose strains could infect rose twigs but could not infect the aster flower twigs. On the other hand, the aster strains could infect the aster flower twigs but could not infect the rose twigs. Tumors appeared on the rose and aster plants within 10 days after inoculation and gradually increased the size after further incubation. The leaves of infected rose plants frequently became yellowing and then dropped. The results for the pathogenicity assay of agrobacterial strains were shown in Table 1.

Detection of *virD1* sequence

Using the Ti plasmid extract as PCR template, the *virD1* primers could amplify a 444-bp DNA fragment from *A. tumefaciens* strain C58 which is a virulent strain. However, DNA fragment of similar size could not be amplified from the virulent strain B6 and the avirulent strain LBA4301. When the *virD1* primers were tested on the pathogenic agrobacteria isolated from plants in Taiwan, the tumorigenic rose strains were able to generate the *virD1* copies. However, as in *A. tumefaciens* strain B6, our *virD1* primers could not amplify the *virD1* gene from tumorigenic aster strains. An unexpected DNA fragment (about 800 bp) was frequently generated after PCR amplification with *virD1* primers from all the test strains, including the avirulent strain, LBA4301 (Fig. 1).

Amplification of acs, *nos*, and *ocs* genes

With the *acs* primers, we could amplify 1,071-bp DNA fragment from *A. tumefaciens* strain C58. We also amplified

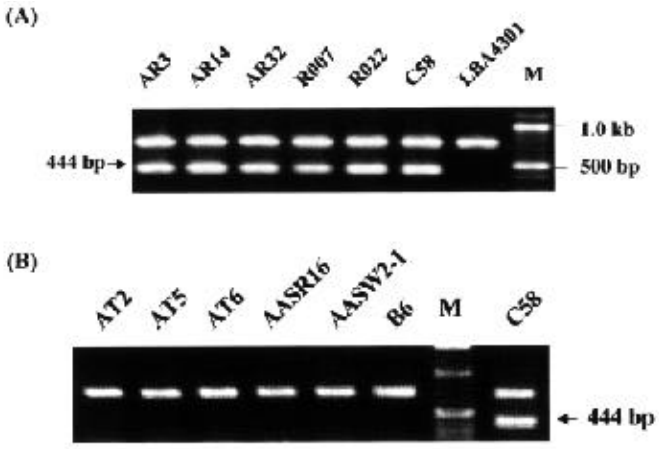

Fig. 1. PCR products amplified from tumorigenic rose and aster strains of *Agrobacterium* with primers for *virD1*. (A) rose strains; (B) aster strains. *Agrobacterium tumefaciens* strains C58 and B6 are control strains. LBA4301 is an avirulent strain without Ti plasmid. One-kb DNA ladder is the molecular size standard.

DNA fragments of simlar size from tumorigenic rose strains. However, similar PCR product was not obtained from tumorigenic aster strains. The *nos* primers could amplify 1,242-bp DNA fragment from *A. tumefaciens* strain C58. However, none of our tumorigenic strains produced this PCR product. With the *ocs* primers, we could amplify 1,077-bp DNA fragments from strain B6 and tumorigenic aster strains. However, similar PCR product was not obtained from tumorigenic rose strains. All of these primer pairs could not amplify the predicted DNA fragments from the avirulent strain, LBA4301 (Table 3, Fig. 2). The amplification with Ti plasmid extract or boiled bacterial suspension as PCR template displayed similar patterns of amplified DNA fragments on agarose gel.

Table 3. PCR detection of *virD1* and opine synthase genes in tumorigenic strains of *Agrobacterium*

Strain	*virD1*	Opine synthase gene [1]		
		acs	*nos*	*ocs*
Rose strain				
AR3	+ [2]	+	—	—
AR14	+	+	—	—
AR32	+	+	—	—
R007	+	+	—	—
R022	+	+	—	—
Aster strain				
AASW2-1	—	—	—	+
AASR16	—	—	—	+
AT2	—	—	—	+
AT5	—	—	—	+
AT6	—	—	—	+
References:				
C58	+	+	+	—
B6	—	—	—	+
LBA4301 (avirulent strain)	—	—	—	—

1. Opine synthase genes: *acs*, agrocinopine synthase gene; *ocs*, octopine synthase gene; *nos*, nopaline synthase gene.
2. "+" = DNA fragment with expected size was amplified. "—" = DNA fragment with expected size was not amplified.

Discussion

In the PCR analysis, our *virD1* primers could not amplify *virD1* gene from tumorigenic aster strains, indicating that the sequence of *virD1* virulence gene might be different among different groups of phytopathogenic *Agrobacterium*. A comparison of sequences of our two *virD1* primers with *virD1* sequence of pTiA6NC showed that the sequences of

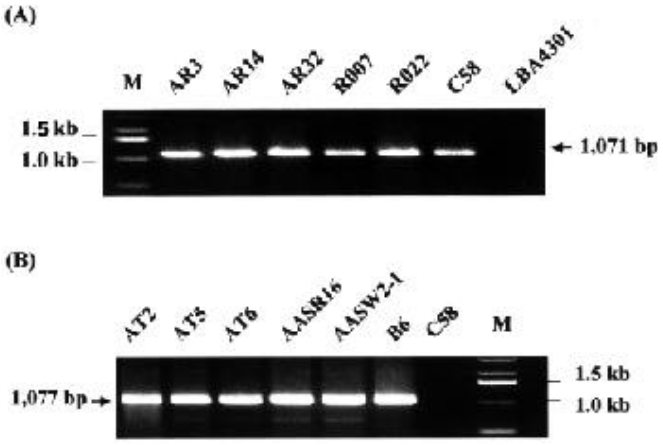

Fig. 2. PCR products amplified from tumorigenic rose and aster strains of *Agrobacterium* with primers for opine synthase genes. (A) rose strains; (B) aster stiains. Arrows indicate the amplified DNA fragments of agrocinopine synthase gene from rose strains and octopine synthase gene from aster stiains. *Agrobacterium tumefaciens* strains C58 and B6 are control strains. LBA4301 is an avirulent strain without Ti plasmid. One-kb DNA ladder is the molecular size standard.

virD1 primers share 61% and 85% homology to the corresponding regions of *virD1* in pTiA6NC [20]. Therefore, our *virD1* primers might not anneal efficiently with the *virD1* sequence of some agrobacteria.

Since a nonspecific DNA fragment larger than 444 bp was amplified from tumorigenic rose and aster strains and the avirulent strain, LBA4301, appearance of this PCR product should not indicate a trait of virulence. When different thermal cycler was used, another extra band displayed in all the amplification reaction with *virD1* primers (data not shown). The primer sequence of *virD1* might affect the efficiency and specificity of PCR amplification. The *virD1* gene of tumorigenic rose and aster strains will be sequenced to explain the different results in the amplification of *virD1* from these pathogenic agrobacteria.

Since the opine synthase genes are located on the T-DNA and transferred into plant cells after infection of pathogenic agrobacteria [4, 19], the type of opine synthase gene in pathogenic agrobacteria should be correlated with the type of opine synthesized in the transformed plant cells. We tested the feasibility of PCR in the identification of opine synthase genes carried by the pathogenic agrobacteria. The results indicate that the tumorigenic rose strains in Taiwan contain agrocinopine synthase gene and the tumorigenic aster strains in Taiwan contain octopine synthase gene. The types of opine in plant cells transformed by these tumorigenic strains will be

examined to show the correlation between PCR detection and the types of opine synthesized in transformed plant cells.

The pathogenicity assay showed that the rose strains could not infect the aster flower twigs and the aster strains could not infect the rose twigs. However, both of rose and aster strains could infect carrot discs and tomato plants. Thus, the host range of rose and aster strains may include the common and diverse host plants. In addition, the PCR amplification results showed that the rose strains were distinct from the aster strains. Presumably, the types of Ti plasmids in the tested rose and aster strains in Taiwan are different.

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摘　要

陳昭瑩[1,2]、廖惠玲[1]、鍾瑞洲[1]. 1999. 臺灣玫瑰及紫花宿苑癌腫病菌*virD1*及opine合成酵素基因之聚合酵素連鎖反應擴增分析. 植病會刊8:143-148. ([1.] 臺北市 國立臺灣大學植物病理學系；[2.] 聯絡作者：電子郵件cychen@ccms.ntu.edu.tw，傳真02-23657735)

本研究測試以聚合酵素連鎖反應(polymerase chain reaction, PCR)擴增玫瑰及紫花宿苑癌腫病菌*virD1*及opine合成酵素基因的效果。在供試臺灣菌株中，僅分離自玫瑰病株的癌腫病菌可以獲得*virD1*基因的PCR產物,而分離自紫花宿苑的癌腫病菌則無法擴增出預期的PCR產物。在opine合成酵素基因的擴增試驗，分離自玫瑰病株的癌腫病菌可以獲得 agrocinopine合成酵素基因片段的PCR產物；分離自紫花宿苑的癌腫病菌則可擴增得到octopine合成酵素基因的PCR產物。根據本研究結果推測癌腫病菌*virD1*基因的核酸序列可能在不同群的菌系呈現某種程度的差異性。此外，癌腫病菌 opine合成酵素基因的類型可能藉由聚合酵素連鎖反應即可以快速地鑑定。

關鍵詞： 聚合酵素連鎖反應、*virD1*、agrocinopine合成酵素基因、nopaline合成酵素基因、octopine合成酵素基因