EARLY DETECTION OF *AGROBACTERIUM TUMEFACIENS* IN SYMPTOMLESS ARTIFICIALLY INOCULATED CHRYSANTHEMUM AND PEACH PLANTS USING PCR

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SUMMARY

A PCR-based procedure has been developed to detect *Agrobacterium tumefaciens* in chrysanthemum and peach plants that had been inoculated in the roots but were symptomless. The procedure was simple and rapid, and discriminated tumorigenic from non-tumorigenic forms of *Agrobacterium*. It was highly sensitive since the target sequence was detected in spiked samples containing only 10±5 cfu/g fresh tissue of *Agrobacterium*. The PCR protocol detected the bacteria in the stems of both plant species while bacteria were not always detected by dilution plating, the current method for detecting *A. tumefaciens* in plants. The protocol needs further studies using naturally infected plant material, but once tested and validated might be used in laboratories that certify plant propagation material. It could then also be suitable for ecological and epidemiological studies on crown gall. In our work some evidence was obtained that *A. tumefaciens* may be translocated to the stem of root-inoculated peach plants.

Key words: crown gall, diagnosis, peach, systemic infection.

INTRODUCTION

Crown gall disease is very damaging to the nursery industry, as infected plants often cannot be sold. The disease may occasionally also cause serious losses during plant production due to tumour growth which interferes with xylem and phloem function. The causal agent of crown gall is *Agrobacterium tumefaciens*, ubiquitous in soil and infectious to over ninety dicotyledonous and three monocotyledonous families (De Cleene and De Ley, 1976). Here we refer to tumorigenic agrobacteria as *A. tumefaciens* even though a new nomenclature for bacteria belonging to the genus *Agrobacterium* has been proposed (Young *et al.*, 2001).

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Host range and oncogenic traits of *A. tumefaciens* are encoded by the Ti plasmid (pTi) a circular extrachromosomal DNA element. This plasmid contains 22 virulence genes (*Vir* region) that mediate the transfer of a portion of the Ti plasmid (T-DNA) into plant cells. The T-DNA is integrated into the plant genome, where subsequent expressions of its genes induce cell proliferation and tumour development (Ream, 1989).

Symptomless survival of pathogenic agrobacteria in plants has been demonstrated in grape, rose and weeping fig (Tarbah and Goodman, 1987; Martì *et al.*, 1999; Zoina *et al.*, 2001). This has important implications in nurseries, since the pathogen may be transmitted via vegetative propagation and even micropropagation systems (Cooke *et al.*, 1992; Poppenberger *et al.*, 2002). Currently, detection of pathogenic *Agrobacterium* strains is mostly done using traditional microbiological isolation on selective media followed by inoculation into herbaceous plants for pathogenicity testing. This method is space and time consuming and is less appropriate for the diagnosis of latent infections, given the relatively low sensitivity of the method (10^2 cfu/g). Early accurate detection of *A. tumefaciens* cells or of latent infections in plant propagation material is important in order to prevent crown gall outbreaks.

There have been several reports regarding the detection of pathogenic agrobacteria by serological and molecular techniques (Bishop *et al.*, 1989; Burr *et al.*, 1990; Cubero *et al.*, 1999). PCR using specific primers designed on pTi regions could be powerful for detecting tumorigenic bacteria in plant tissues (Cubero *et al.*, 1999) but PCR analysis is affected by the presence of polyphenolics and other compounds released by plant tissues that may inhibit DNA polymerase (John, 1992) and may yield false negatives (Wilson, 1997; Louws *et al.*, 1999). A procedure that provides high recovery of bacterial DNA and reduction of the effects of PCR-inhibiting compounds is urgently needed.

In this work, a reliable PCR protocol for detection of tumorigenic agrobacteria in artificially inoculated chrysanthemum and peach plants has been developed by adapting available techniques and reagents. This method could be further evaluated using naturally-infected material.

MATERIAL AND METHODS

Plant material, bacterial strains and experimental design. Protocols were set up using chrysanthemum plants (cv Vesuvio), a hybrid of *Dendrathema grandiflora* × *Chrysanthemum morifolium*, and peach seedlings (cv Montclair).

Chrysanthemum plants were inoculated with the tumorigenic *A. tumefaciens* strain C58, marked for rifampicin resistance, while *A. rhizogenes* strain B49C/83 selected for rifampicin resistance was used for peach seedlings inoculation. Both host plants were inoculated using *A. tumefaciens* strain C58 pTi-less derivative strain NT1, resistant to streptomycin. Plants were inoculated by dipping their roots for 5 min in bacterial suspensions containing both the tumorigenic and non-tumorigenic strains at different ratios ($10^3/10^7$; $10^5/10^7$; $10^7/10^7$ cfu ml^{-1}).

The two forms of agrobacterium were co-inoculated in order to mimic the natural presence of agrobacteria in soil where non-tumorigenic forms are usually more abudant than tumorigenic ones (Bouzar and Moore, 1987). Plants were transplanted in individual pots containing sterile soil and maintained in a greenhouse for one year. Each treatment included three groups of 20 plants randomly distributed in the greenhouse. Twenty uninoculated chrysanthemum and peach plants were used as controls.

Concentrations of inoculated bacteria in roots and stems was determined by dilution plating. Schroth crown gall medium (Schroth *et al.,* 1964) amended with 100 mg l^{-1} rifampicin and 500 mg l^{-1} streptomycin was used for isolation of C58 and NT1 marked strains, respectively; 2E medium (Brisbane and Kerr, 1983) amended with 100 mg l^{-1} rifampicin was used for isolation of strain B49c/83 Rif^+.

Preparation of plant samples. Fifteen days after inoculation three plants from each treatment were uprooted and tested for *A. tumefaciens*. Roots were shaken to remove soil and then washed thoroughly under running tap water. Leaves and shoots were excised and from each stem two samples (one gram each) were obtained from pieces cut at 5-10 cm and 15-20 cm from the plant crown. One gram of the thinner roots was cut from each plant. Root and stem samples were placed in flasks containing 0.5% NaClO, shaken at room temperature for 5 min, washed three times in sterile distilled water and then dried in a laminar flow cabinet. Samples were then placed in 50 ml Falcon tubes containing 9 ml of saline solution and homogenized for 3 min in a Polytron homogenizer Heidolph DIAX 600.

DNA extraction. The protocol of Llop *et al.* (1999) was used for isolating DNA from tissues of the first groups of chrysanthemum plants; later the procedure was modified to improve the yield and quality of the extracted DNA. Ground tissues were centrifuged (10,000*g*, 10 min); the pellets were resuspended in extraction buffer (200 mM Tris HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS, 2% PVP), incubated for 1 h at room temperature, and then centrifuged (5000*g*, 5 min). Supernatants were collected in 1.5 ml microfuge tubes and boiled for 5 min, then transferred to ice for 3 min and centrifuged at 10,000*g* for 3 min. The next steps were as described by Llop *et al.* (1999). The protocol used for chrysanthemums was also used for DNA extraction from peach plants.

PCR protocol. Primers used were $FGPvirB_{11+21}$ (TGCCGCATGGCGCGTTGTAG) and FGPvirG15' (GAACGTGTTTCAACGGTTCA) designed from the intercistronic region between vir B and vir G of the vir region of pTi (Nesme *et al.*, 1995). Reactions were performed in a volume of 50 µl containing: 10 mM PCR buffer 1X, 1.5 mM $MgCl_2$, 200 µM each dNTPs, 0.1 µM each primer, 2.5 U Taq DNA Polymerase Recombinant (Invitrogen, Carlsbad, CA, USA), 5 µl extracted DNA. To improve the effectiveness of the protocol in detecting the target sequence in roots, the procedure was modified as follows: the $MgCl_2$ concentration was increased to 5 mM and the extracted DNA was diluted 100, 500 and 1000 fold. After 5 min denaturation at 94°C, the profile of amplification cycle was: 94°C × 30 sec, 57°C × 45 sec, 71°C × 1 min, performed 35 times, and a final extension step at 71°C × 3 min. The PCRs were done using a Peltier Thermal Cycler PTC-200.

Evaluation of PCR sensitivity and comparison between molecular and dilution plating methods. The sensitivity of the procedure was determined by analyzing ground plant tissue spiked with various concentrations of bacterial suspensions. Suspensions of B49c/83 Rif^+ and C58 Rif^+ were each spectrophotometrically adjusted to 1×10^8 cfu ml^{-1} measuring the absorbance at 530 nm, then were serially diluted. The concentration of living cells in the suspensions was checked by dilution plating.

Stem and root tissues of control chrysanthemum and peach plants were prepared as described above. One ml of each dilution of B49c/83 Rif^+ suspension was mixed with one gram of peach tissue and one ml of each dilution of C58 Rif^+ suspension was mixed with one gram of chrysanthemum tissue. DNA was extracted using 500 µl of each sample and 5 µl of the extracted DNA was used as PCR template. Amended Schroth medium was used for isolation of C58 and NT1 marked strains; amended 2E medium was used for enumeration of strain B49c/83 Rif^+ (Brisbane and Kerr, 1983) as described earlier. Results obtained from the PCR were compared with those from dilution plating on selective media.

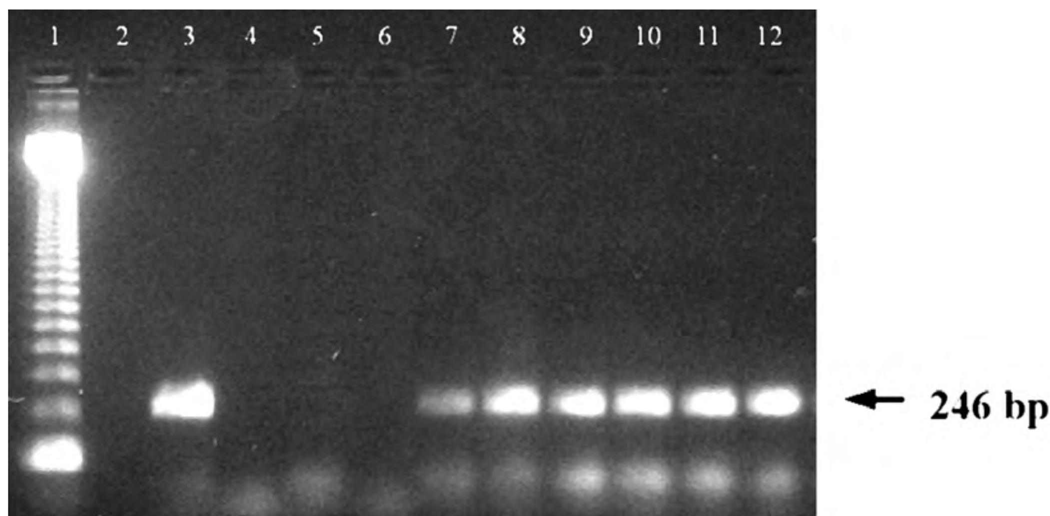

Fig. 1. PCR results on samples from chrysanthemum plants inoculated with the suspension containing 1×10^3 cfu ml^{-1} of C58 Rif^+ strain. Lane 1, marker; lane 2, uninoculated tissues (negative control); lane 3, C58 suspension (positive control); lanes 4-6 roots; lanes 7-9 stem (5-10 cm); lanes 10-12 stem (15-20 cm).

RESULTS

Disease development on chrysanthemum and peach. Tumors developed only on peach plants inoculated with the 1×10^7 cfu ml^{-1} suspension of the tumorigenic strain B49c/83 Rif^+. No tumors were observed on chrysanthemum with any concentration used.

Sensitivity of PCR. Sensitivity of PCR was evaluated by analyzing samples prepared by mixing serial dilutions of B49c/83 and C58 strain suspensions with 1 g of chrysanthemum and peach tissues respectively. Target sequences were detectable down to a concentration of 15 cfu ml^{-1} of agrobacteria in chrysanthemum and 5 cfu ml^{-1} in peach.

Detection of *A. tumefaciens* in chrysanthemum. The presence of endophytic tumorigenic bacteria was checked on all symptomless chrysanthemum and peach plants analysed both by PCR and by dilution plating. An average of 5-10 ng of total DNA was isolated from 1 g of both stem and root tissues, sufficient for PCR amplification. The 246 bp intercistronic region between *vir* B and *vir* G was amplified in all the stem sections collected from plants treated with the three different concentrations of virulent bacteria and in the roots of plants inoculated with 1×10^7 cfu ml^{-1} bacteria. Roots from the plants treated with the two less concentrated bacterial suspensions led to negative results when the Llop protocol (Llop *et al.* 1999) was followed (Fig. 1). However, the target sequence was amplified from all chrysanthemum root samples when the concentration of $MgCl_2$ in the PCR mixture was doubled.

All samples tested by PCR were also analysed by classical enumeration of bacteria on selective media. Use of a rifampicin-marked strain for plant inoculations allowed the recovery of introduced agrobacteria on the medium plates. Strain C58 was isolated from the first 10 cm of chrysanthemum stems but not from the upper sections. Comparison among the concentrations of agrobacteria recovered from the roots and stems of the plants treated with the three different bacterial suspensions showed no significant differences. The amount of recovered agrobacteria seemed to be not always related to the concentration of the starting inoculum. Only the avirulent strain NT1 was re-isolated from all stem sections of plants analyzed (Table 1).

Table 1. Concentrations of strains C58 Rif^+ and NT1 Str^+ (cfu/g fresh tissue) in roots and stems of chrysanthemum plants detected by the dilution plating method.

Plant organs	C58			NT1		
	T1[a]	T2	T3	T1[a]	T2	T3
Roots	0.4×10^3 b	19×10^3 a	$2,7\times10^3$ b	0.3×10^6 a	0.1×10^6 a	4.0×10^3 b
Stem (5-10 cm)	0.3×10^3 a	0.7×10^3 a	0.2×10^3 a	1.4×10^6 a	0.07×10^6 b	3.7×10^3 c
Stem (15-20 cm)	0	0	0	30×10^4 a	2×10^4 b	0.06×10^4 b

[a]T1, T2 and T3: plants coinoculated with C58 Rif^+ (10^7, 10^5 and 10^3 cfu ml^{-1} respectively) and with NT1 (10^7 cfu ml^{-1}). Numbers followed by the same letter are not significantly different (P = 0.01) in the Duncan test.

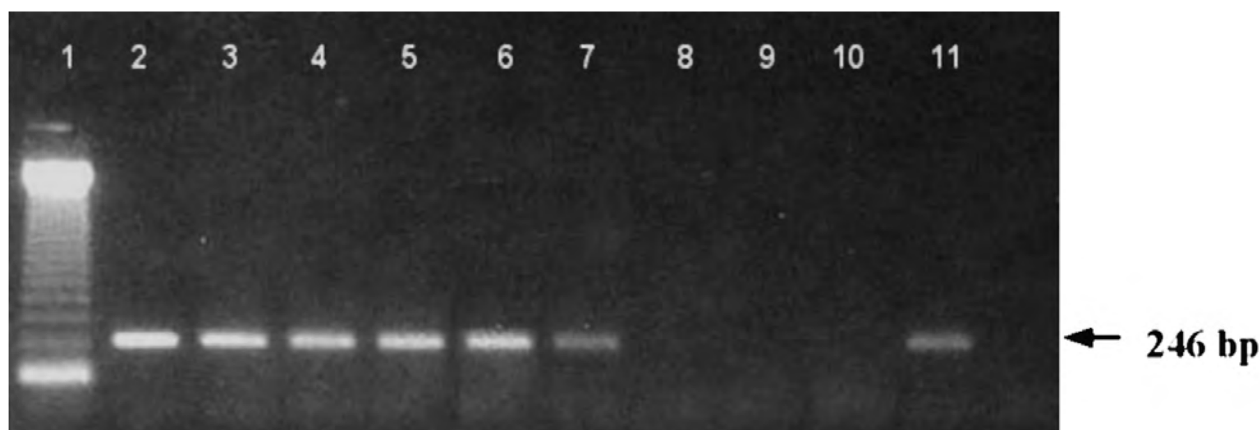

Fig. 2. PCR results on peach plants inoculated with 1x10^3 cfu ml^{-1} B49c Rif$^+$ strain. Lane 1, marker; lanes 2-4, stem 5-10 cm; lanes 5-7, stem 15-20 cm; lanes 8-10, roots; 11, B49c suspension (positive control).

Detection of *A. tumefaciens* in peach seedlings. The protocol by Llop *et al.* (1999) allowed the detection of tumorigenic agrobacteria by amplification of the 246 bp region from peach stems collected from plants inoculated with the three concentrations (10^3; 10^5; 10^7 cfu ml^{-1}) of B49c/83 Rif$^+$. No amplification was obtained from root tissues of plants inoculated with 10^3 and 10^5 cfu ml^{-1} of B49c/83 Rif$^+$ (Fig. 2), while the target sequence was amplified from root tissues of plants inoculated with 10^7 cfu ml^{-1} of the marked strain.

Amplification from roots inoculated with the two lower bacterial concentrations was achieved by diluting the extracted DNA at least 100 fold. Tumorigenic bacteria were recovered by dilution plating from the stems and roots of peach plants inoculated with 10^5 cfu ml^{-1} and from the roots of plants inoculated with 10^7 cfu ml^{-1}. No bacteria were recovered from plants inoculated with 10^3 cfu ml^{-1} (Table 2).

Comparison of PCR and dilution plating. Comparison of these two methods showed that the target sequence was detected from all chrysanthemum root and stem samples (Table 3). The marked strain was always isolated from roots and from some of the 5-10 cm stem samples but never from the upper part of the chrysanthemum stems (15-20 cm).

The PCR analysis on peach was always successful on samples collected from the stems, while some of the root samples were not amplified probably because of the strong inhibiting activity of some compounds in the root extracts. However, dilution of the extracts was effective in eliminating the inhibition. Isolation of bacteria from the stems was mostly unsuccessful; moreover the marked strain was isolated only from some of the root samples collected from the plants inoculated with 1 × 10^7 and 1 × 10^5 cfu ml^{-1}.

DISCUSSION

Viral, bacterial and fungal diseases of plants in the greenhouse or in the field may arise through use of contaminated but symptomless plants (Burr and Katz, 1984; Moore and Allen, 1986). Indexing is basic for the production of certified pathogen-free propagation material and thus specific, sensitive and rapid pathogen detection methods are needed. The pathogen can be detected directly or by using serological or molecular methods. PCR can be useful for detection (Henson and French, 1993) when specific primers (preferably targeting known pathogenicity genes) are combined with highly sensitive DNA extraction and detection procedures (Louws *et al.*, 1999).

Table 2. Concentrations of strains B49c/83 Rif+ and NT1 Str$^+$ (cfu/g fresh tissue) in roots and stems of peach plants detected by dilution plating method.

Plant organs	B49c/83			NT1		
	T1[a]	T2	T3	T1[a]	T2	T3
Roots	2.8×10^4 a	0.3×10^3 b	0 b	32×10^4 a	0.89×10^4 b	3.7×10^4 b
Stem (5-10 cm)	0 a	0.1×10^3 a	0 a	21×10^3 a	7.1×10^3 ab	2.1×10^3 b
Stem (15-20 cm)	0	0	0	0.6×10^4 b	54×10^3 a	72×10^3 a

[a]T1, T2 and T3: plants coinoculated with C58 Rif$^+$ (10^7, 10^5 and 10^3 cfu ml^{-1} respectively) and with NT1 (10^7 cfu ml^{-1}). Numbers followed by the same letter are not significantly different (P = 0.01) in the Duncan test.

Table 3. Comparison of the PCR-based protocol and the dilution plating used for agrobacteria detection.

Samples	Bacterial concentrations (cfu ml^{-1}) used for inoculation 10^7		10^5		10^3	
	PCR	Isolation	PCR	Isolation	PCR	Isolation
Chrysanthemum						
Roots	12/12[a]	12/12	12/12	12/12	12/12	12/12
Stem 5-10 cm	12/12	5/12	12/12	8/12	12/12	4/12
Stem 15-20 cm	12/12	0/12	12/12	0/12	12/12	0/12
Peach						
Roots	12/12	8/12	8/12	4/12	9/12	0/12
Stem 5-10 cm	12/12	0/12	12/12	4/12	12/12	0/12
stem 15-20 cm	12/12	0/12	11/12	0/12	12/12	0/12

[a] number of positive samples/number of analyzed samples.

The DNA extraction method used in this work was based on the procedure of Llop *et al.* (1999) that has been developed to be used in PCR protocols, for detecting quarantine bacteria in potato, rosaceous plants and citrus. We modified Llop's protocol by boiling and freezing the suspension containing the lysed cells in order to obtain better purification of the extracted DNA. Our procedure was applied to plants inoculated in the roots with a mixture of tumorigenic and non-tumorigenic marked strains of bacteria. Since most of the plants did not develop tumours, the protocol was set up for detecting tumorigenic bacteria in root and stem tissues of symptomless plants only.

The extraction procedure gave a clean product when applied to stem tissues, and the target DNA was always amplified. Apparently the presence of disturbing compounds in extracts from roots frequently inhibited the PCR in both plant species. Successful PCRs from chrysanthemum root extracts were achieved by increasing the $MgCl_2$ concentration in the amplification mix, suggesting that some compounds extracted with the DNA sequestered Mg^{2+} ions required as co-factor of DNA polymerase. However, increasing Mg^{2+} concentration did not improve detection in peach roots. But in this case, the two lower bacterial concentrations were detectable when the DNA extraction products were diluted at least 100 fold, suggesting that the effects of some inhibiting compounds extracted with the DNA could be removed by simple dilution.

Our protocol allows discrimination between pathogenic and non-pathogenic forms since the use of primers specific for the *vir* region of the *A. tumefaciens* Ti plasmid allows specific detection of tumorigenic agrobacteria. The procedure can be completed within 24 h, as compared with traditional dilution plating and pathogenicity tests which take 20-25 days. The sensitivity was very high in our artificial system since the target sequence was often detected even in DNA extracts obtained from plant tissues mixed with bacterial suspensions containing only 10±5 cfu ml^{-1}. Our procedure could possibly be suitable for detection of tumorigenic agrobacteria in asymptomatic symptomless plants, but this has still to be tested with naturally infected material and confirmed by other methods, including further plant inoculations, to see if PCR-positive plants develop tumours during later growth.

No toxic reagent (phenol, chloroform) is needed for the DNA extraction, the whole procedure is easy to perform and is suitable for routine analyses in laboratories charged for certification of plant propagation material. In our hands, the dilution plating method was almost ineffective for detection of agrobacteria from stems, given the small number of pathogenic agrobacteria introduced into the stem. Bacteria were never detected in the upper portion (15-20 cm) of chrysanthemum stems while they were detected only in the lower portion (5-10 cm) of the stems of peach plants inoculated with 1×10^5 cfu ml^{-1}. These results showed that the PCR allowed detection of low amounts of target DNA from dead or living bacterial cells that were undetectable by dilution plating.

In rare cases, multiplication of plants by micropropagation does not eliminate pathogenic bacteria from tissues, and persistence of *A. tumefaciens* has occasionally been reported (Cooke *et al.*, 1992; Poppenberger *et al.*, 2002).

After further studies as mentioned above, the protocol developed in this work may be useful for studying systemic and endophytic behaviour of tumorigenic agrobacteria in different host plants. Other recent evidence (Cubero *et al.*, 2006) on movement of these bacteria in woody plants further shows that the possibility of systemic infection must be considered when designing strategies for controlling crown gall disease.

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