ATTI Giornate Fitopatologiche
1996, 2, 499-506

DETECTION OF *AGROBACTERIUM TUMEFACIENS* FROM ROSE, CHRYSANTHEMUM, AND DAISY BY POLYMERASE CHAIN REACTION (PCR).

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Summary

Crown gall is a widespread plant tumor disease in temperate regions of the world caused by virulent strains of *Agrobacterium* . An important increase in the frequency of crown gall has been noticed recently on three floral species : rose, chrysanthemum and daisy. In order to control the crown gall propagation, the selection of healthy plants appears to be necessary. As a matter of fact, it seems that *Agrobacterium* strains have systemic activity and may be transmitted by vegetative propagation. For the prophylactic control it is imperative to have a reliable detection method of pathogenic strains of *Agrobacterium* .
The real cause of pathogenicity is the Ti plasmid (Tumor Inducing), located in its cytoplasm. During infection, part of this plasmid, the T-DNA, is transferred to the genome of a plant cell and causes the proliferation of the host cells and the formation of galls. Therefore, a method has been established for the analysis of bacterial strains isolated from galls of various origins by Polymerase Chain Reaction. Amplification was performed on three plasmidic sequences, located in the conserved virulence genes, and in the T-DNA (on *tmr* and *tms* genes). After analysis, the banding patterns of different strains corresponded to the biological tests for the determination of pathogenicity and the biochemical tests for the determination of the genus. Therefore, PCR can be considered as a valid tool for the isolation of pathogenic *Agrobacterium* strains from galls, and is now currently used in the laboratory.

Key words : floral cultures, crown gall, *Agrobacterium tumefaciens*, polymerase chain reaction.

Riassunto

IDENTIFICAZIONE DI *AGROBACTERIUM TUMEFACIENS* DA ROSA, CHRISANTEMO E MARGHERITA MEDIANTE AMPLIFICAZIONE GENICA (PCR)

Il tumore batterico, causato da ceppi virulenti di *Agrobacterium*, è ampiamente diffuso nelle regioni temperate del mondo. Recentemente si è riscontrato un forte incremento di tale malattia su tre importanti specie floricole quali rosa, crisantemo e margherita. La selezione di piante sane risulta necessaria al fine di contenere il tumore batterico : infatti i ceppi di *Agrobacterium* sembra abbiano un'attività sistemica e che possano essere trasmessi con la propagazione vegetativa.
Il principale requisito di una corretta profilassi è quello di avere a disposizione un metodo sicuro di identificazione dei ceppi patogeni di *Agrobacterium*. La causa effettiva della patogenicità è il plasmide Ti, localizzato nel citoplasma del batterio, parte del quale durante l'infezione è trasferita al genoma delle cellule della pianta che di conseguenza tendono a proliferare in modo anomalo e a formare galle. A tale proposito è stata messa a punto la tecnica di amplificazione genica (PCR) per l'analisi dei ceppi batterici da galle di diversa origine che si basa sull'amplificazione di tre sequenze plasmidiche localizzate nei geni deputati alla virulenza e nel T-DNA (sui geni tmr e tms). Dopo l'analisi con PCR, osservando le bande dei differenti ceppi batterici, si è potuta constatare una corrispondenza tra i risultati ottenuti con quelli forniti sia dai test biologici effettuati per la determinazione della patogenicità, che dai test biochimici necessari per la determinazione del genere. Alla luce di queste prove si può affermare che la PCR è una valida tecnica di determinazione dei ceppi patogeni di *Agrobacterium*, facilmente applicabile dai laboratori operanti nel settore.

Parole chiave : coltura floricole, tumore batterico, *Agrobacterium tumefaciens*, amplificazione genica.

Introduction

Agrobacterium species are gram-negative and soil-inhabiting. They are capable of saprophytic or parasitic growth and are responsible for the crown gall and hairy root diseases on dicotyledonous plants, especially on *rosaceae* (De Cleene M. and De Ley J., 1976). Concerning roses, crown gall is a real problem, particularly since the development of new cultivation techniques, and commercial practices. In order to evaluate the effect of the disease on the vigor of rose trees, a representative affected plantation has been analysed. 18 months after planting, a 63% difference of stem diameter between healthy and affected plants was observed. Furthermore, a plot was set up at INRA Antibes to quantify the effect of crown gall on nursery stock and on the yield of rose trees after artificial inoculation during grafting. The mortality rates of contaminated and control plants during the growing phase were 5.5% and 0.6%, respectively. Plant performances were studied for 8 months in the greenhouse. The controls produced a mean of 9.7 flowers per plant while contaminated plants produced only 6.2 flowers during the same period.

Concerning marguerite, the Regional Institute of san Remo has assessed a frequency of crown gall between 5 and 20% on mother plants for five years. In Albenga, an important increase of the disease has been noticed last year with a contamination of 100% in some cases.

In the case of chrysanthemum, some cultivars seemed to be very contaminated and the disease induced dwarf plants.

Crown gall problem is enhanced by the genetic infection mode : the pathogenic *Agrobacterium* strains have a large Ti (tumor-inducing) plasmid part of which called the T-DNA is transferred from the bacterium to the nuclear genome of host plant cells. Genes located on the T-DNA are expressed in transformed plant cells. The compounds they induce are responsible for their uncontrolled proliferation (Winans S.C., 1992).

Because this disease is of a genetic-type and caused by a bacterium, chemical control of crown gall (soil fumigation, inhibitory chemical substances,...) is not a practical solution (Lippincott J.A., et al., 1981). The discovery of a non-pathogenic *Agrobacterium* - K84- which produces a bacteriocin called agrocin, has allowed a reduction of crown gall incidence in Australia and New Zealand (Kerr A., 1980). However, most of the pathogenic rose and marguerite*Agrobacterium* are resistant to agrocin. Moreover, research to obtain plants resistant to crown gall is in progress, but it is long term work. The only means of rapidly decreasing crown gall incidence is to set up a sanitary selection of the plant material. In order to do that, we needed a molecular tool : the Polymerase Chain Reaction (PCR), which consists of amplifying a specific DNA fragment, with the help of a thermostable DNA polymerase. It is based on the annealing and extension of two oligonucleotide primers which flank a target DNA. Repeated amplification cycles (including denaturation, primer binding and primer extension) generate an exponential accumulation of the target DNA, which can be visualised (Saiki R.K., et al., 1988).

PCR was chosen for several reasons. First, it can directly detect a specific sequence of the real pathogenic agent i.e. the Ti plasmid (Nesme X., et al., 1989) (Dong L.C., et al, 1992). Secondly, this tool is already used for the detection of several

pathogenic microorganisms, and research has shown its reliability, rapidity and sensitivity. Thirdly, PCR enables a study of molecular diversity of the rose strains by analysing amplified DNA sequences which are less conserved than those used for specific detection.

In this work, we test the reliability of the PCR technique in specifically detecting pathogenic *Agrobacterium* among bacterial populations of rose, chrysanthemum and marguerite galls.

Material and methods

- *Agrobacterium* strains : we had a collection of 13 reference strains from either the CFPB ("Collection française de Bactéries Phytopathogènes", Angers, france), X. Nesme, or our collection and 109 different pathogenic strains isolated from rose, chryanthemum and marguerite galls in our laboratory since 1990.

- oligonucleotides : we used 3 pairs of primers supplied by X. Nesme (Nesme X., et al., 1989), (Nesme X. et al, in press). They flank Ti plasmid sequences called *vir*, *tmr*, *tms*, of 246 bp, 170 bp and 587 bp respectively (see fig. 1) .

- Isolation of *Agrobacterium* strains from galls : galls were washed, then ground with sterile water and macerated for 30 minutes. Several microliters of supernatant were put on LPGA medium on petri dishes (for 1 liter : 5g of yeast extract, 5g of peptone, 10g of glucose, 15g of agar). Cultures were grown from 2 to 7 days at 28°c. Strains were recovered using visual criteria and tested with biochemical and biological tests. They were then purified on LPGA.

- Determination of the genus *Agrobacterium* : biochemical tests (Kerr A. and Panagopoulos C.G., 1977) were used.

- Biological tests : The pathogenicity of isolated *Agrobacterium* were tested on tobacco : the stems were wounded and inoculated with the bacterium. The tobacco plants were then grown at 24°c for a month.

- Amplification of the target sequences : the PCR experiments were done in 1X PCR buffer (supplied with the *taq* DNA polymerase by Appligene), 200 µM of each dNTP, 0.1 µM of each primer, 1 µl of template bacteria (about 10^8 bacteria per ml in sterile water) and 0.125 units *taq* DNA polymerase Appligene. We used a standard amplification protocole of 35 cycles, with denaturation at 95°c, annealing at 58°c and extension at 72°c. PCR amplified DNAs were visualised by using 1.2% horizontal agarose gel electrophoresis run in TBE buffer (0.089 M Tris borate, 0.089 M boric acid and 0.002 M EDTA, pH 8.0) at 10 $V.cm^{-1}$ for 30 mn. The gels were stained in ethidium bromide (0.5 mg l^{-1}), destained in distilled water and photographed with a 312 nm ultraviolet source.

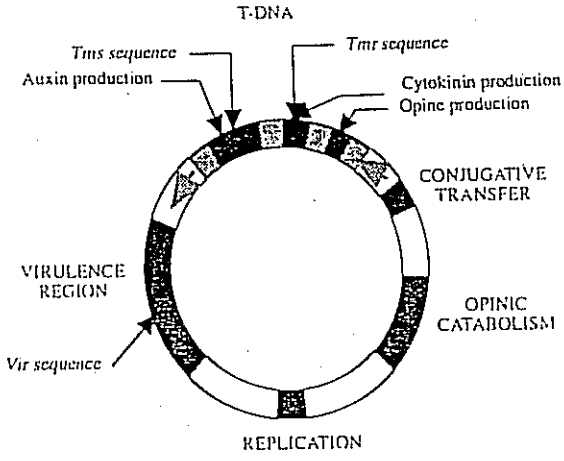

Fig 1 : genetic card of the Ti plasmid (from Beijersbergen & Hooykass, 1992). The amplified sequences are localized in the virulence region and in the T-DNA.

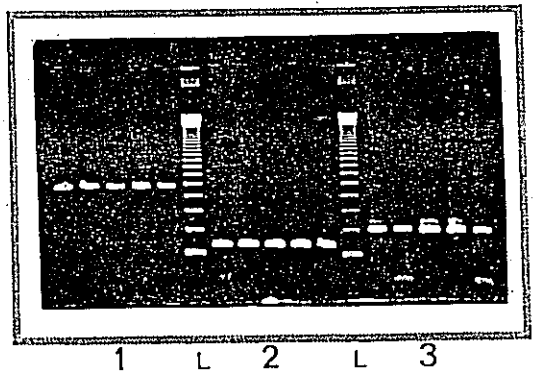

Fig 2 : visualisation of the specific amplification of the Ti plasmid sequences.
L : 123 bp ladder
1 : Tms sequence
2 : Tmr sequence
3 : Vir sequence

Results and discussion

Preliminary PCR of reference strains

Table 1 reports the amplification results of reference *Agrobacterium* strains with each pair of primers. The positive amplifications of the pathogenic strains confirmed that *tmr* and *vir* primers flank a conserved DNA region, while *tms* flanks a less conserved fragment which is not amplified for 2 out of the 11 pathogenic reference strains. So the *tms* sequence could not be used for the detection of all the pathogenic *Agrobacterium* from roses, and therefore we used only *tmr* and *vir* primers in our study.
Note that the non pathogenic strains were not positive with the PCR test, except for C58C1, from which the *tmr* sequence was amplified. C58C1 is Ti plasmid cured-C58; part of the *tmr* gene should probably have been duplicated on the chromosome, or on another plasmid of the strain.

Amplification of the rose and chrysanthemum *Agrobacterium* strains

First, we wanted to check that the Ti plasmid of all the rose and chrysanthemum strains isolated since 1990 could be amplified with our primers. All these strains belong to the genus *Agrobacterium*, according to biochemical tests, and induced the formation of galls on tobacco. The strains were amplified twice with each primer pair. Table 2 shows the results of this work : for 88 rose strains, the PCR results are in complete agreement with the biological and the biochemical results. In 8 cases, the *tmr* sequence was not amplified; In the case of chrysanthemum, for 10 strains only 2 give an amplification with the *tmr* sequence. This can be explained by different events such as molecular reorganizations, or mutations (punctual, deletion, insertion) modifying the hybridization primer sites. The T-DNA region had probably been less conserved during the evolution than the *vir* region, which is essential to the genetic transfer. Our preliminary results on margherite show an amplification of both *tmr* and *vir* sequences . Thus, this experiment showed that *vir* primers are valuable for detecting the Ti plasmid. Our *Agrobacterium* collection constitutes a good sampling of the diversity of the strains which cause crown gall on rose, chrysanthemum and marguerite ; thus PCR is a reliable method for detecting pathogenic *Agrobacterium*, by amplifying the *vir* sequence and by confirming the results with the amplification of the *tmr* sequence, which is positive in most cases.

Amplification of diverse rose bacteria

In the second phase of this work, we wanted to show that only the pathogenic *Agrobacterium* could be amplified from a population of various bacteria isolated from rose-galls. So we isolated 61 strains from 6 rose galls using the same methodology as for the isolation of *Agrobacterium*. The difference lies in the choice of the strains grown on the LPGA medium : we tried to recover a representative sampling of the visual diversity that we found on the plates. All these strains were purified and tested according to molecular, biological and biochemical criteria (Table 3). In all cases, biological tests confirmed the PCR amplification : of the 61 strains, 22 were found to be pathogenic on tobacco and positive with PCR. On the other hand, the biochemical test results differed from the others. Concerning the 39 non pathogenic strains, 37 did not belong to the *Agrobacterium* genus (this confirmed the visual differences that had been noted). The other 2 were non pathogenic *Agrobacterium*. One strain was pathogenic and did not seem to belong to the genus *Agrobacterium* according to the biochemical test. We consider that the biochemical test is not completely reliable, because in all the bacterial genus, there are some atypical strains. To complete this work, we tried to amplify some fluorescent *Pseudomonas*, which are often present in the rose galls : there was no amplification (data not shown). Following this experiment, we consider that PCR enables the detection of specifically pathogenic *Agrobacterium*, because the primers are specific.

Conclusion

We are convinced that PCR is a very effective technique for detecting the pathogenic *Agrobacterium*. It has been integrated as a determination tool in our protocol of isolation of pathogenic *Agrobacterium* from galls. We have now to adapt the technique to direct detection *in planta*, based on our observations of the dissemination of the bacteria by the plant. It involves 3 steps : finding a possibly preferencial localization of *Agrobacterium* in the plant, increasing the concentration of the bacteria, which is probably at very low levels in the plant and solving the problem of vegetal compounds that inhibit the amplification reaction (phenolic compounds, polysaccharids, tanins, plant DNA in excess...).

In the same way, we need to know these strains better from an epidemiological point of view, in particular so that some *Agrobacterium* characteristics can be associated with geographical origin; this work is justified because natural soilless populations would be homogeneous in a geographic place (Kerr A., 1974). So PCR is now being used to amplify less conserved regions of the plasmid - like the *tms* sequence- or the bacterial chromosome. This is followed by their analysis using RFLP (Restriction Fragment Lengh Polymorphism). Then the biochemical (opine type, biovar) characterization of the rose-strains will be completed. The data, associated with the effective detection of the pathogenic *Agrobacterium* in the plant, will help us to control crown gall on roses in the short term.

Table 1 : Amplification of pathogenic and non-pathogenic reference strains.
+ : amplification
− : no amplification

NON PATHOGENIC STRAINS	*vir* AMPLIFICATION	*tmr* AMPLIFICATION	*tms* AMPLIFICATION
CFPB 1937 (K84)	-	-	-
CFPB 2456	-	-	-
CFPB 2883 (C58C1)	-	+	-
PATHOGENIC STRAINS	***vir*** **AMPLIFICATION**	***tmr*** **AMPLIFICATION**	***tms*** **AMPLIFICATION**
CFPB 1903 (C58)	+	+	+
BO542	+	+	+
Ach5	+	+	+
A6	+	+	+
B6	+	+	-
T37	+	+	+
EU6	+	+	+
CFPB 1935	+	+	+
CFPB 1936	+	+	+
CFPB 2409 (A4)	+	+	-

Table 2 : Comparison of the results of the *Agrobacterium* amplification with the biological and biochemical tests.

	STRAIN NUMBER	AMPLIFICATION *vir* *tmr*	PATHOGENICITY	*AGROBACTERIUM* GENUS
rose	88	+ +	+	+
	8	+ -	+	+
chrysanth.	2	+ -	+	+
	8	+ -	+	+

Table 3 : Results of molecular, biological and biochemical tests carried out on 61 various strains isolated from rose galls.

STRAIN NUMBER	AMPLIFICATION *vir* *tmr*	PATHOGENICITY	*AGROBACTERIUM* GENUS
22	+ +	+	21 + 1 -
39	- -	-	2 + 37 -

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