SHORT COMMUNICATION

CROWN AND CANE GALL OF A BLACKBERRY-RASPBERRY HYBRID CAUSED BY *AGROBACTERIUM RHIZOGENES* IN NORTHERN ITALY

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SUMMARY

During a serious epidemic of crown and cane gall on the blackberry-raspberry (*Rubus occidentalis-Rubus idaeus*) hybrid Lockness in a specialized crop in the province of Treviso (northern Italy) Gram-negative bacteria were found associated with tumours. Following experimental inoculation these bacteria caused tumours on tomato stems and on pot-grown hybrid canes in the greenhouse. These bacteria were found to possess a Ti plasmid, common to *Agrobacterium*. The resulting fatty acid profile did not correspond to any known *Agrobacterium* species, but did indicate an affinity with the genus *Agrobacterium*. Partial 16S rRNA sequencing revealed that the bacteria were closely related to three strains of *Agrobacterium rhizogenes*, in particular with a strain isolated from a peach tumour.

Key words: Ti-plasmid, Ri-plasmid, blackberry, raspberry, crown gall, canegall, *Agrobacterium tumefaciens*, *Agrobacterium rhizogenes, Agrobacterium rubi*.

INTRODUCTION

Agrobacterium spp. are ubiquitous in nature (Bouzar and Moore, 1987) and belong to the *Proteobacteria* group. Pathogenicity in *Agrobacterium* is determined by the presence of a Ti- or Ri-plasmid. Strains harbouring a Ti-plasmid (pTi) are the causal agent of crown gall, a disorder known to affect at least 600 plant species worldwide. Ri-plasmid (pRi) harbouring strains are associated with hairy root disease. Disease symptoms initiate following the transfer of a DNA fragment (T-DNA) located on such plasmids into the genome of the recipient plant cell.

The taxonomy of *Agrobacterium* is confused. Strains were originally given epithets based on distinct pathological characters caused by their plasmids. Thus pTi strains were termed *A. tumefaciens*, pRi strains were termed *A. rhizogenes*, and avirulent strains, which do not harbour pTi or pRi, were termed as *A. radiobacter*. Strains can lose or gain pTi and pRi which implies, under this taxonomic system, that they would then change species, a clearly untenable classification. It is now recognised that there are at least five species, which can carry plasmids. Of these *A. rubi* and *A. larrymoori*, are pathogens of *Rubus* and *Ficus*, respectively, for which only a few strains exist in culture collections. The other three correspond to biovars 1, 2 and 3 (Holmes and Roberts, 1981; Kersters and De Ley, 1984; Young *et al.*, 1996). Biovar 1 contains many *A. radiobacter* and *A. tumefaciens* strains, biovar 2 includes many *A. rhizogenes* strains and biovar 3 corresponds to *A. vitis* (Ophel and Kerr, 1990). Although a proposal has recently been made to incorporate all *Agrobacterium* species into the genus *Rhizobium* (Young *et al.*, 2001), we have retained the established nomenclature in this report.

In August 2000 numerous severe tumours appeared on a blackberry-raspberry (*Rubus occidentalis-Rubus idaeus*) hybrid of cv Lochness in a specialized crop in the province of Treviso (northern Italy). Irregular, cerebriform, brownish tumours with diameters of 3-15 cm, were clearly visible on the crown of the plants beneath the superficial layer of soil (Fig. 1). Small, globular tumours were also observed on the aerial parts in correspondence with some pruning scars (Fig. 2). The galled canes showed stunted growth. The nursery material, imported from France, was planted in soil where no *Rubus* species had previously been cultivated. In the months following transplanting, a field survey of 650 plants revealed that 9% of the plants had developed crown galls. The subsequent year, approximately 90% of the plants had galls.

This study identified an unusual, tumourogenic strain of *A. rhizogenes* as causal agent of the epidemic of crown and cane gall observed in the province of Treviso.

Tumours with whitish parts were washed with tap water and rinsed with distilled water. A 1-2 mm layer was then removed from the blot-dried tumour surface and ground in 1-2 ml of sterile distilled water. The resulting suspension was streaked on YDC-agar plates (Lelliott and Stead, 1987). After three days of incuba-

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Fig. 1. Large tumour on the crown of a blackberry-raspberry hybrid in the field.

Fig. 2. Tumour on a cane in correspondence with a pruning wound.

tion (27°C), whitish, mucoid colonies were observed on the plates.

The pure cultures were determined to be Gram-negative rods, with average dimensions of 2 x 0.95 mm. After 4 days at 28°C on YDC-agar these formed circular colonies, 2 mm in diameter, which were mucoid, whitish, with entire edge and convex elevation.

Tomato plantlets (cv Money-maker), at the 5-6 leaf stage, were inoculated through a vertical scalpel slit (1 cm), at 2-5 cm from the crown, with a drop of bacterial suspension (10^8 cells ml^{-1}). The wound was then covered with moist cotton wool and wrapped with aluminium foil. Plantlets were kept at 25°C and 14 h light day^{-1}, and the wrapping was removed after 3 days. On day 15-20 post inoculation, whitish pearls were observed at the edges and/or the centre of the wounds. These pearls developed in the following weeks to form typical tumours. Control tomato plants, inoculated with water, remained symptomless and the wounds healed.

The virulence of three tumourogenic cultures on tomato plantlets was tested on cv Lochness rooted canes, transplanted at the end of the winter and grown in individual pots in a greenhouse using special vertical trellises, approximately 150 cm high. Immediately before inoculation, the plants were pruned to a height of 100 cm. Plants were inoculated by injecting 20 ml of a bacterial suspension (10^8 cells ml^{-1}) into a 1 cm vertical slit made with a scalpel just above the crown. The wounds were wrapped for 3 days, as before, and plants kept at 18-28°C. Twenty-four days after inoculation, shiny, pearly spherical masses, 1-2 mm in diameter, had appeared on the edges and in the centre of the wounds. Over the following weeks, these masses produced whitish mamelons, which developed over 2-3 months into large, partially brown, tumours. Wounds in control plants, inoculated with water, healed and did not form tumours. At the end of August, about 4 months after inoculation, an attempt was made to re-isolate the pathogen from the experimental tumours using the same technique as for natural tumours. Many colonies selected for their morphological features caused tumours on tomato plantlets. These cultures were shown to possess the Ti-plasmid and were phenotypically indistinguishable from the original strain inoculated.

The observed symptoms suggested that the causal agent of the disorder was possibly an *Agrobacterium* sp. harbouring a pTi (Moore, 1991). The PCR protocols of Haas *et al.* (1995) were used to determine whether the strain identified as being tumourogenic on tomato possessed pTi. Primers in the first protocol correspond to sequences from a highly conserved region of a *vir* gene (*virD2*) present on both pTi and pRi (virD2 A, 5'-ATGCCCGATCGAGCTCAAGT-3'; virD2 C, 5'-TCGTCTGGCTGACTTTCGTCATAA-3'). Primers in the second protocol correspond to sequences of the *ipt* oncogene only present on pTi (CYT, 5'-GATCG(G/C)GTCCAATG(C/T)TGT-3'; CYT', 5'-GATATCCATCGATC(T/C)CTT-3'). DNA was extracted from a bacterial strain shown to induce tumours on tomato using a genomic DNA extraction kit (Wizard Genomic DNA Purification Kit; Promega Corporation, Madison, WI, USA), and used as template in both of the protocols. Both PCRs generated bands of the expected sizes (*virD2* PCR, 224 bp; *ipt* PCR 427 bp) indicating that the strain possessed pTi and not pRi.

The presence of pTi suggested the strain involved was an *Agrobacterium* sp. To confirm this, an initial identification of the tumourogenic bacterium was attempted with fatty acid profiling (Stead *et al.*, 1992). The resulting profile did not conform to any known *Agrobacterium* strain profile, but indicated that the bacterium was closely related to the *Agrobacterium* genus.

The DNA extract, used as template in the Haas *et al.* (1995) PCR assay, was used for partial 16S rRNA sequencing. Sequencing was done directly from a 1,100 bp PCR product amplified by the primers fA (5'-CGAGAGTTAGATCTTGGCCAG-3') and rG (5'-CCCCACCTTCCTCTCGGCTATC-3') (Bala *et al.*, 2002). The PCR product was purified using a PCR purification kits (QIAquick PCR purification kit, QIAGEN GmbH, Hilden, Germany) and sequenced from the forward primer (fA). Approximately 700 bp of the PCR product was sequenced. A BLAST search of the GenBank database indicated that the partial 16S rRNA sequence of the strain causing crown gall in blackberry was closely related to three strains of *Agrobacterium rhizogenes* (Fig. 3) and in particular to the *A. rhizogenes* strain 163C, reported to cause tumours in peach (Paloma *et al.*, 2003).

A series of biochemical tests were conducted to confirm that the *Agrobacterium* sp. isolated did belong to biovar 2. As described by Moore *et al.* (2001), growth at 35°C, growth in 2% NaCl, and growth on Medium 2E (Brisbane and Kerr, 1983) was measured, as was acid production from carbohydrates, and 3-ketolactose production. The results of these tests showed an exact correlation with the expected results from a known *A. rhizogenes* (*Agrobacterium* biovar 2) strain (Table 1).

The symptoms observed on the blackberry-raspberry hybrid, both in the field and following experimental inoculation, make it possible to attribute the case as crown gall (Moore, 1991). In crown and cane gall of *Rubus* species and their hybrids, the galls on the crown and roots can be associated with pTi harbouring *A. tumefaciens* and *A. rubi* strains, with galls on aerial parts mostly associated with *A rubi*. In Treviso, crown galls collected in the field were associated with a tumourogenic *Agrobacterium* sp. identified, on the basis of partial 16S rRNA sequencing, as a strain very similar to *A. rhizogenes,* but harbouring pTi and not pRi. In *Rubus*, *A. rhizogenes* is generally associated with the rare, pRi-induced, hairy root syndrome (Moore, 1991) and thus the isolation of a pTi- harbouring strain from *Rubus* is unusual.

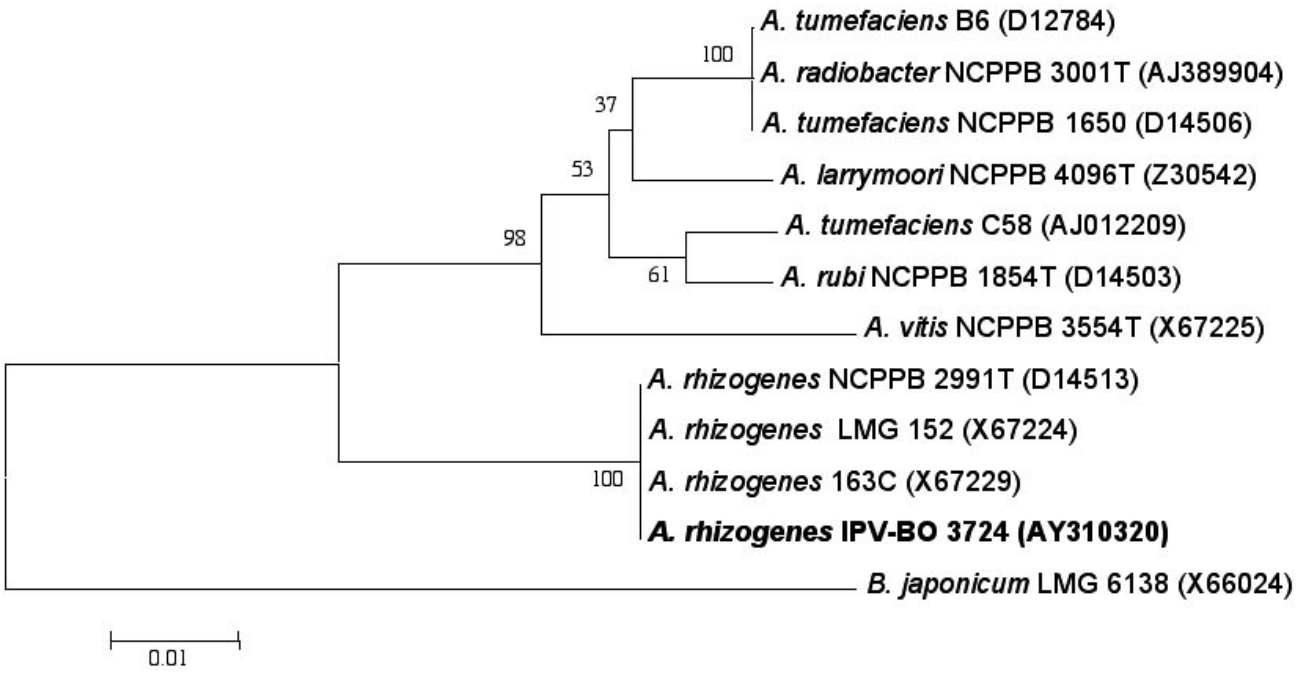

Fig. 3. Neighbour-joining phylogeny for partial 16S rRNA sequences (alignment length 500bp), of relationships of the tumourogenic *A. rhizogenes* strain IPV-BO 3724 and a panel of representative *Agrobacterium* strains. Numbers on the dendrogram represent the percentages of grouping confidence calculated by bootstrap analysis (100 bootstrap replicates).

Table 1. Biovar determination by biochemical tests as described by Moore *et al.* (2001) on putative *Agrobacterium rhizogenes* strain IPV-BO 3724.

Strain [a]	Growth on medium 2E [b]	Growth at 35°C	Growth on 2% NaCl	3-ketolactose production	Oxidase reaction	Acid from:	
						Sucrose	Melizitose
Expected results [c]							
Biovar 1	-	+	+	+	+	+	+
Biovar 2	+	-	-	-	-	-	-
Biovar 3	-	V	+	V	V	V	-
IPV-BO 3724	**+**	**-**	**-**	**-**	**-**	**-**	**-**

[a] Italian collection.
[b] Medium 2E for biovar 2 strains (Brisbane and Kerr, 1983).
[c] + = positive reaction, - = negative reaction, V = variable results. As observed by Moore *et al.* (2001).

The epidemic on the industrial crop in Treviso can therefore be attributed to crown and cane gall caused by an uncommon strain of *A. rhizogenes*. The assignment of the strain to biovar 2 is in agreement with the majority of the *A. tumefaciens* strains associated with the same syndrome (Hobolth, 1973; Zurowski *et al.,* 1985; Burr, 1997).

Crown gall is a damaging disease of *Rubus* sp. with infected canes showing stunted growth and producing less fruit. The 16S rRNA sequence data generated from the *A. rhizogenes* strain identified as the raspberry pathogen in this report showed closest homology to 16S rRNA data generated from a *A. rhizogenes* strain reported to induce crown gall on Peach. This suggests that the pathogen may have more than one host, though it is not known whether or not the same pTi is involved in both cases. *A. rhizogenes* is known to be spread via nursery propagation material (Garrett, 1978). With the reported failure of the *Agrobacterium* K84 biocontrol strain to prevent crown gall in raspberry (Burr *et al.*, 1983), reliable screening methods to guarantee the pathogen free status of nursery stock and also crop beds are likely to be the most effective form of control.

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