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Characterization of *Agrobacterium tumefaciens* strains isolated from grapevine

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Summary

Grapevine crown gall is predominantly induced by *Agrobacterium vitis*, but rarely, *Agrobacterium tumefaciens* may also cause this disease. We have collected 18 grapevine isolates of *A. tumefaciens* from distinct geographical regions and various grapevine varieties. Characterization of these strains showed that 7 of the 18 strains carried *A. vitis*-type octopine/cucumopine Ti plasmids, utilized tartrate, were avirulent on pea and did not respond to the *virC*-specific PCR primers like *A. vitis*. Eleven of the 18 strains carried *A. tumefaciens*-type Ti plasmids, did not grow on tartrate, were virulent on pea and reacted positively to *virC*-specific PCR primers. Two of these strains induced octopine/agropine-, and two strains induced agropine producing tumors on grapevine and/or *Kalanchoe tubiflora* plants. The remaining 7 strains showed the characteristic properties of *A. tumefaciens* nopaline strains and were sensitive to agrocin 84.

K e y w o r d s : *Agrobacterium vitis*, crown gall, opines, tartrate-utilization, Ti plasmids, *Vitis vinifera.*

A b b r e v i a t i o n s : bp = base pair, pTi = Tumor inducing plasmid, pTr = Tartrate utilization plasmid, PCR = polymerase chain reaction.

Introduction

The crown gall disease of grapevine is predominantly caused by *Agrobacterium vitis* strains that may have octopine-, nopaline-, or vitopine Ti plasmids (Burr *et al*. 1998, Burr and Otten 1999, Ridé *et al*. 2000). Occasionally, *Agrobacterium tumefaciens* with octopine-, or nopaline Ti plasmids (Knauf *et al*. 1983, Salomone *et al*. 1996, Ridé *et al*. 2000, Argun *et al*. 2002) may also occur in grapevine. Most of the *A. vitis* isolates have a more limited host range than *A. tumefaciens* strains (Panagopoulos *et al*. 1978, Szegedi 1985, Bien *et al*. 1990). The host range in virulent strains is determined by the Ti plasmid (Thomashow *et al*. 1980). *A. tumefaciens* strains with nopaline-, or succinamopine-type Ti plasmids are sensitive to agrocin 84 produced by the antagonistic strain *Agrobacterium radiobacter* K84 (van Larebeke *et al*. 1975, Watson *et al*. 1975, Chilton *et al*. 1984). *A. tumefaciens* strains carrying octopine Ti plasmids, as well as all *A. vitis* isolates irrespective of the type of their Ti plasmids are resistant to agrocin 84 (Engler *et al*. 1975, Kerr and Roberts 1976, Kerr and Panagopoluos 1977, Panagopoulos *et al*. 1978, Burr and Katz 1983, Knauf *et al*. 1983, Bien *et al*. 1990).

Utilization of L(+)tartrate is a characteristic feature both of biotype 2 (*Agrobacterium rhizogenes*) and 3 (*A. vitis*) strains, but biotype 1 (*A. tumefaciens*) strains do not grow on tartrate as sole carbon source (Kerr and Panagopoulos 1977, Sule 1978, Ophel and Kerr 1990). Plasmid-encoded tartrate-utilization was first demonstrated in an unusual *A. tumefaciens* strain isolated from grapevine (Gallie *et al*. 1984). Further studies have shown that conjugative tartrate utilization (pTr) plasmids are common in *A. vitis* and can be mobilized by *in planta* mating into recipient *A. tumefaciens* (Szegedi *et al*. 1992, Otten *et al*. 1995). The metabolism of tartrate by agrobacteria contributes to the colonization of host plant by the pathogen (Kado 1998, Salomone *et al*. 1998). Furthermore genes for tartrate utilization have also been found on some octopine/cucumopine Ti plasmids of *A. vitis* (Otten *et al*. 1995, Ridé *et al.* 2000) but their occurrence on other Ti plasmids has not yet been reported.

Previous studies have clearly shown that some grapevine isolates of *A. tumefaciens* can grow on tartrate. These strains contain an octopine/cucumopine pTi that is common in *A. vitis* (Otten *et al*. 1995, Salomone *et al*. 1996, Nascimento *et al*. 1999, Ridé *et al*. 2000). To get a more general information on the Ti plasmids of the grapevine isolates of *A. tumefaciens* and on their relation to *A. vitis* we have characterized 18 strains. Our results show that they contain 4 different Ti plasmids and that tartrate utilization is associated exclusively with octopine/cucumopine-, but not with other types of Ti plasmids in grapevine isolates of *A. tumefaciens*.

Material and Methods

B a c t e r i a l s t r a i n s : Strains used for experiments and their origins are listed in Tab. 1. They were grown on glucose/yeast-extract medium containing 1 % glucose (w/v), 0.5 % (w/v) yeast extract supplemented with AB minimal salts (Lichtenstein and Draper 1986) and 1.2 % (w/v) agar. Cultures were incubated at 27 °C. *A. tumefaciens* A281 (leucinopine pTi), A348 (octopine pTi) and C58 (nopaline pTi), the non-virulent UBAPF2, furthermore *A. vitis* Tm4 and AB3 (octopine pTi), AT1 (nopaline pTi) and S4 (vitopine pTi) were used as controls.

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List of bacterial strains used for experiments

A)

Strain	Geographical origin	Grapevine host	Isolated by	Source	Reference
Grapevine isolates of *Agrobacterium tumefaciens*					
Ag12	Portugal	Touriga Francesa/99R grafting	H. OLIVEIRA (1991)	H. OLIVEIRA	OLIVEIRA *et al*. 1998 NASCIMENTO *et al*. 1999
2654	France	Cabernet Sauvignon	M. RIDÉ (1987)	L. OTTEN	RIDÉ *et al*. 2000
2655	France	Cabernet Sauvignon	M. RIDÉ (1987)	L. OTTEN	RIDÉ *et al*. 2000
82.143	France	Danam	M. MICHEL (-)	M. MICHEL	SALOMONE *et al*. 1996
II-2/2	France	5C	E. SZEGEDI (2002)	This work	-
II-5/1	France	5C	E. SZEGEDI (2002)	This work	-
II-5/2	France	5C	E. SZEGEDI (2002)	This work	-
CG628	USA, New York	Riesling	T. J. BURR (1983)	T. J. Burr	BURR *et al*. 1999
CG632	USA, New York	Riesling	T. J. BURR (1983)	T. J. Burr	ARGUN *et al*. 2002
2946	Bulgaria	unknown	-	M. RIDÉ	SALOMONE *et al*. 1996
2947	Bulgaria	unknown	-	M. RIDÉ	SALOMONE *et al*. 1996
0	Hungary	Ezerjó	S. SULE (1976)	-	SULE and KADO 1980
1/21	Bulgaria	unknown	I. POPOVA (1978)	I. POPOVA	-
15/6	Hungary	Ezerjó	S. SULE (1976)	This work	-
15/8	Hungary	Ezerjó	S. SULE (1976)	This work	-
16/6	Hungary	Olaszrizling	S. SULE (1976)	This work	-
17/3	Hungary	Ezerjó	S. SULE (1976)	This work	-
18/6	Hungary	Kadarka	S. SULE (1976)	This work	-

B)

Strain	Ti plasmid type	Reference
Agrobacterium tumefaciens control strains		
A348	octopine	GARFINKEL *et al*. 1981
C58	nopaline	HOOYKAAS *et al*. 1980
A281	leucinopine	CHILTON *et al*. 1985
UBAPF2	-	HYNES *et al*. 1985
Agrobacterium vitis control strains		
Tm4	octopine/cucumopine	SZEGEDI *et al*. 1988
AB3	octopine/cucumopine	SZEGEDI *et al*. 1988
AT1	nopaline	SZEGEDI *et al*. 1988
S4	vitopine	SZEGEDI *et al*. 1988

Biochemical tests: 3-ketolactose production was tested as described by KERR and PANAGOPOULOS (1977). Tartrate utilization was tested on solid AB minimal medium supplemented with 0.5 % (w/v) sodium-potassium-tartrate, 25 $mg \cdot l^{-1}$ bromothymolblue and 1.2 % (w/v) agar. Results were scored 3 d after inoculations.

Agrocin 84 sensitivity: Sensitivity to *Agrobacterium radiobacter* strain K84 was tested on AB minimal medium containing 0.5 % (w/v) glucose and 1.2 % agar (w/v) as previously described (SZEGEDI *et al*. 1999). Results were scored 2 d after inoculations.

Virulence tests: Grapevines (*Vitis vinifera* cvs Cabernet franc and Chasselas) were grown in a greenhouse in a perlite:peat (4:1) mixture, pea (*Pisum sativum*) and kalanchoe (*Kalanchoe tubiflora*) plants were grown in garden soil. The stems of plants were inoculated through woundings made with a sterile needle dipped into young (2-3 day-old) bacterial cultures grown on glucose/yeast exctract/AB medium (see above) to test the tumorigenicity of strains and to obtain tumors for opine assays. Tumor formation was scored 6 weeks after inoculation. Control plants were inoculated with the plasmidless strain UBAPF2 or with a sterile needle.

Opine detection: Approximately 100 mg pieces of grapevine and/or *K. tubiflora* tumor samples were homogenized with 200 µl distilled water and centrifuged. Nine µl of the supernatants were spotted onto Whatmann 3MM paper in 3 µl aliquots. Safranin or anionic picrate were used as colour markers to follow the electrophoretic separation. Electrophoresis was carried out in formic acid: acetic acid: water (3:6:91, v/v/v, pH 1.8), or in 0.1 M formic acid titrated to pH 2.8 with 10 % (w/v) KOH. After electrophoresis papers were dried and stained. Octopine and nopaline were detected with phenanthrenequinone, cucumopine with Pauly-reagent, agropine and mannopine with alkaline silver nitrate, and silver chelating opines (leucinopine, vitopine) with reversed silver nitrate staining as described (DESSAUX *et al*. 1992). Opines were identified by comparing their relative electrophoretic mobilities to the used colour markers, to pure opine samples or tumor extracts induced by *A. tumefaciens* A281 (for leucinopine and agropine), A348 (for octopine and agropine), C58 (for nopaline), *A. vitis* Tm4 and AB3 (for octopine and cucumopine), AT1 (nopaline), or S4 (for vitopine).

PCR analysis: To prepare template DNA bacteria were lysed in Triton X-100/sodium-azide buffer (ABOLMAATY *et al*. 2000) by heating the samples at 95 °C for 10 min. Lysates were centrifuged and used directly for PCR or stored at -20 °C. Primers used for PCR reactions are listed in Tab. 2. The VCF/VCR primers are specific for the *A. tumefaciens virC* gene, VisF/VisR for the *A. vitis* vitopine synthase gene, TF/TR for the *6b* gene of *A. vitis* octopine pTis, NF/NR for the *6b* gene of *A. vitis* nopaline pTis and ttuCfw/ttuCrev for the tartrate dehydrogenase gene of the *A. vitis tar*-operon. The reactions were carried out in 25 µl volumes containing 1X *Taq* polymerase buffer, 5 % (v/v) DMSO, 1.5 mM $MgCl_2$, 0.5 µM of each primer, 200 µM of each dNTP, 1.25 unit of *Taq* polymerase and 1 µl of template DNA. The DNA amplification reaction was started at 94 °C for 1 min, then followed by 32 cycles at 92 °C for 1 min, 54-60 °C (see Tab. 2) for 1 min and 72 °C for 1.5 min. The reaction was completed with a final elongation step at 72 °C for 3 min. Samples were analysed after electrophoretic separation in ethidium-bromide stained agarose gels.

Results

We collected 18 grapevine isolates of *A. tumefaciens* from different geographical locations and compared them to

Table 2

Primers used for molecular characterization of *Agrobacterium tumefaciens* strains

Name	Sequence	Length of the amplified fragment	Annealing temperature	Reference
VCF/ VCR	5'-ATCATTTGTAGCGACT-3' and 5'-AGCTCAAACCTGCTTC-3'	730 bp	54 °C	SAWADA *et al*. 1995
VisF/ VisR	5'-CCGGCCACTTCTGCTATCTGA-3' and 5'- CCATTCACCCGTTGCTGTTATT-3'	561 bp	54 °C	SZEGEDI and BOTTKA 2002
TF/TR	5'-TGGCCGAAATTGTTTACTTCCACCC-3' and 5'-CTATGCCGAAAGACGGCTTGACCCT-3'	520 bp	60 °C	This work
NF/NR	5'-TTAACCCAAATGAGTACGATGACGA-3' and 5'-TTATTTCGGTACTGGATGATATTAG-3'	570 bp	58 °C	This work
ttuCfw/ ttuCrev	5'-GACTGGGGYTCGGACTATTACAAGAA-3' and 5'-ATCTCGTCCCACATCACCATGC-3'	479 bp	58 °C	This work

known *A. tumefaciens* and *A. vitis* strains. Three of these strains, Ag12, 2654 and 2655, have already been partially characterized (SALOMONE *et al*. 1996, NASCIMENTO *et al*. 1999, RIDÉ *et al*. 2000). As expected, all of them were 3-ketolactose positive, which is a key marker of *A. tumefaciens* (KERR and PANAGOPOULOS 1977, SULE 1978). Seven strains, namely Ag12, 2654, 2655, 82.143, II-2/2, II-5/1 and II-5/2 utilized tartrate. This property is specific for *A. vitis*, but atypical for *A. tumefaciens* (KERR and PANAGOPOULOS 1977, SULE 1978). These results confirm and extend previous observations concerning the occurrence of tartrate utilization in grapevine-derived isolates of *A. tumefaciens* (SALOMONE *et al*. 1996, NASCIMENTO *et al*. 1999, RIDÉ *et al*. 2000).

To test the presence of Ti and Tr plasmids we assayed these isolates in PCR reactions using various plasmid-specific oligonucleotides (Tab. 2.). First we used the *virC*-specific VCF and VCR primers that detect *A. tumefaciens* (SAWADA *et al*. 1995) but not *A. vitis* Ti plasmids (SZEGEDI and BOTTKA 2002). The 7 tartrate utilizing strains did not react in this test, but the remaining 11 tartrate-negative *A. tumefaciens* isolates generated specific bands with these primers (Fig. 1). The 7 tartrate utilizing strains were positive when they were probed with TF and TR primers specific for the *6b* gene of *A. vitis* octopine Ti plasmids (Fig. 2), and also with the *tar*-operon specific ttuCfw and ttuCrev primers (data not shown) confirming the presence of tartrate utilization genes in these strains. Neither *A. vitis* nopaline-type nor vitopine-type Ti plasmids were found in the tested isolates by PCR using the *6b*-specific NF/NR or the vitopine synthase-specific visF/visR primers.

All strains were pathogenic on *Vitis vinifera* cvs Cabernet franc and Chasselas, although to various degrees. None of the tartrate utilizing strains that lacked *virC*-specific sequences according to the PCR results induced tumors on pea (*Pisum sativum*) as observed earlier for *A. vitis*. The 11 tartrate-negative, but *virC*-positive strains induced tumors on pea like *A. tumefaciens* (SZEGEDI 1985).

Grapevine and kalanchoe crown galls were analysed for the presence of opines. Seven strains, Ag12, 2654, 2655, 82.143, II-2/2, II-5/1 and II-5/2 produced large amounts of octopine in tumors like *A. vitis* octopine strains (Fig. 3). These tumors also contained cucumopine, but not agropine or mannopine. Two isolates, 2946 and 2947 as well as the control strain *A. tumefaciens* A348 induced weak octopine synthesis that was detectable only in Kalanchoe tumors (data not shown) and they produced agropine and mannopine showing that these strains carry an octopine-type *A. tumefaciens* Ti plasmid. Two isolates, CG628 and CG632, produced only agropine and mannopine, but no other phenanthrenequinone-, or silver nitrate-positive, or silver chelating opines were detected. The remaining 7 strains, 0, 1/21, 15/6, 15/8, 16/6, 17/3 and 18/6, induced nopaline synthesis in tumors. These strains were sensitive to agrocin 84 produced by *A. radiobacter* K84 by which *A. tumefaciens* nopaline strains can be distinguished from *A. vitis* nopaline strains. Silver chelating opines (vitopine, succinamopine, leucinopine) were not detected in the tumors. Results of these studies are summarized in Tab. 3.

Discussion

Grapevine crown gall is caused predominantly by *A. vitis* (BURR *et al*. 1998, BURR and OTTEN 1999), although irregularly *A. tumefaciens* may also infect this host plant (KNAUF *et al*. 1983, SALOMONE *et al*. 1996, NASCIMENTO *et al*. 1999, RIDÉ *et al*. 2000, ARGUN *et al*. 2002). Recent studies have shown that many of the *A. tumefaciens* strains are able to utilize tartrate as sole carbon source and contain an *A. vitis*-derived octopine/cucumopine type Ti plasmid. To test the generality of these observations we have collected and identified by biochemical and molecular markers 18 pathogenic grapevine isolates of *A. tumefaciens* derived from different varieties and geographical regions.

Our studies have shown that these isolates can be divided into two main groups. Group I includes 7 of the 18 strains investigated that have an *A. vitis*-type octopine/cucumopine Ti plasmid which are able to grow on tartrate. Strains belonging to this group did not react with the *A. tumefaciens virC*-specific VCF/VCR primers, but yielded a specific band with the *A. vitis 6b* specific primers in PCR and were avirulent on pea like most *A. vitis* (SZEGEDI 1985). They induced a large amount of octopine in tumors which is a characteristic feature of *A. vitis*, but not of *A. tumefaciens* octopine strains (SZEGEDI *et al*. 1988). These tumors also produced cucumopine, but not agropine confirming that strains belonging to this group contain an *A. vitis*-type Ti plasmid. Thus they are most probably natural *in planta* transconjugants of *A. tumefaciens* and *A. vitis*.

The 11 strains belonging to group II contain *A. tumefaciens*-type Ti plasmid as shown by their positive reaction to the *virC*-specific primers, their virulence on pea, opine induction in tumors and agrocin 84 sensitivity. Tartrate utilizing strains were not found in this group suggest-

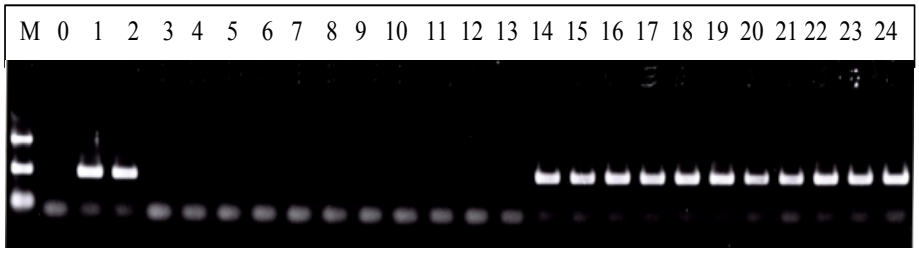

Fig. 1: Amplification of a *virC* specific fragment (730 bp) by PCR from DNA samples of *Agrobacterium tumefaciens* strains. M: size marker containing 1794, 753 and 191 bp long fragments, 0: reaction mix without DNA, lane 1: A348, lane 2: C58, lanes 3-6: *Agrobacterium vitis* Tm4, AB3, AT1 and S4 strains, respectively, used as controls. Lanes 7-13 are Ag12, 82.143, 2654, 2655, II-2/2, II-5/1 and II-5/2, lanes 14-24 are CG628, CG632, 2946, 2947, 0, 1/21, 15/6, 15/8, 16/6, 17/3 and 18/6 in the same order.

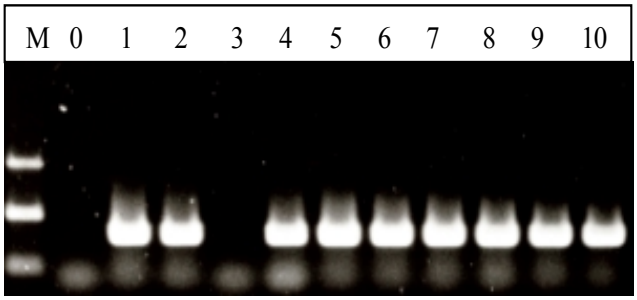

Fig. 2: Detection of a *6b*-gene fragment (520 bp) from *Agrobacterium tumefaciens* strains amplified by TF and TR primers specific for the *6b* gene of *Agrobacterium vitis* octopine Ti plasmids. M: size marker containing 1794, 753 and 191 base pair long fragments, 0: reaction mix without DNA, lanes 1 and 2 are *A. vitis* Tm4 and AB3, respectively, used as positive controls. Lane 3: *A. tumefaciens* A348 and lanes 4-10 are Ag12, 82.143, 2654, 2655, II-2/2, II-5/1 and II-5/2 in the same order.

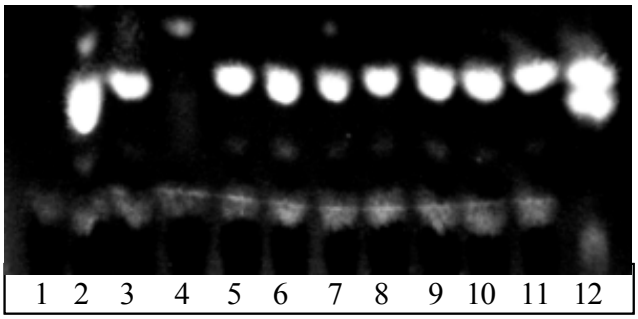

Fig. 3: Detection of octopine from grapevine (cv. Chasselas) tumors induced by tartrate utilizing *Agrobacterium tumefaciens* strains. Tumors were induced by *A. tumefaciens* A348 (lane 1), by *Agrobacterium vitis* Tm4 (lane 2) and AB3 (lane 3) used as controls, and by the tartrate utilizing *A. tumefaciens* strains Ag12 (lane 5), 2654 (lane 6), 2655 (lane 7), 82.143 (lane 8), II-2/2 (lane 9), II-5/1 (lane 10) and II-5/2 (lane 11). Lane 4 contains wound callus extracts from a non-inoculated plant, lane 12 contains 1 µg of octopine (upper spot) and 1 µg of nopaline (lower spot).

ing that this property, although contributing to the grapevine/*Agrobacterium* interaction (Kado 1998, Salomone *et al*. 1998), is not essential for the presence of *Agrobacterium* in grapes. On the basis of opines induced in the tumors they can be divided into three subgroups. Two strains belong to the *A. tumefaciens* octopine/agropine subgroup, two strains induced only agropine and the remaining 7 produced nopaline.

A. vitis strains may contain octopine/cucumopine-, nopaline-, or vitopine type Ti plasmids (Burr *et al*. 1998, Burr and Otten 1999). Of these we have detected only octopine/cucumopine type Ti plasmids in the grapevine isolates of *A. tumefaciens* that strongly correlated with tartrate utilization. On the other hand, *A. vitis* nopaline-, or vitopine type Ti plasmids so far have not been found in natural grapevine isolates of *A. tumefaciens*. We do not yet know if these Ti plasmids can be transferred to and maintained in *A. tumefaciens* under natural conditions.

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Table 3

Main characteristics of *Agrobacterium tumefaciens* strains isolated from grapevines

Group	Opines in tumors (strains)	Tartrate utilization	Virulence on grapevine	Virulence on pea	Reaction in PCR to VCF/VCR
Group I (with *A. vitis* type pTis)	octopine/cucumopine (Ag12, 2654, 2655, 82.143, II-2/2, II-5/1 and II-5/2)	+	+	-	-
Group II (with *A. tumefaciens* type pTis)	agropine/mannopine (2946, 2947)	-	(+)	+	+
	octopine and agropine/mannopine (CG628, CG632)	-	(+)	+	+
	nopaline (0, 1/21, 15/6, 15/8, 16/6, 17/3 and 18/6)	-	+	+	+

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