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Partial characterization of *Agrobacterium vitis* strains

by

S. MATSUMOTO[1]), K. OPHEL[2]); K. G. M. SKENE[3]) and N. S. SCOTT[3])

[2]) Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, S.A., Australia

[3]) CSIRO Division of Horticulture, Adelaide, S.A., Australia

Summary: Seventeen strains of *Agrobacterium vitis* (formerly classified *A. tumefaciens* biovar 3) were characterized using part of the T-DNA and *virA* regions of the Ti plasmid as probes. All strains except one were of the wide host range (WHR) strains and were classified into two groups depending on their ability to utilize octopine or nopaline. These WHR type oncogenic strains had homology with the limited host range type (LHR) *virA* gen of *A. vitis* but not with the WHR *virA* gene of *A. tumefaciens*.

The frequency of T-DNA excision in some *Agrobacterium* strains was estimated with the plasmid pTMA which mimics T-DNA excision from Ti plasmid DNA. In an *A. vitis* strain isolated from grapevine, T-DNA excision occurred after co-cultivation with grapevine tissues, but not with acetosyringone. In contrast, in *A. tumefaciens*, T-DNA excision occurred after co-cultivation with acetosyringone, but not with grapevine tissue.

Key words: Agrobacterium vitis, Agrobacterium tumefaciens, Vitis vinifera, vir genes.

Introduction

Agrobacterium species induce crown gall tumors on a wide variety of dicotyledonous plants. During infection, transferred DNA (T-DNA), a discrete segment of the tumor inducing (Ti) plasmid in oncogenic *Agrobacterium*, is transferred to plant cells and integrated into the plant genome (CHILTON *et al.* 1977). This mechanism is now the basis of transformation systems for many dicotyledonous plants. However, genetic transformation of *Vitis* species has been only partially successful when mediated by *A. tumefaciens*. Transgenic shoots (BARIBAULT *et al.* 1990) of the economically important *V. vinifera* were of mixed cell origin containing both transformed and untransformed cells. Solid transformed buds of *V. vinifera* obtained by MULLINS *et al.* (1990) did not develop further but the same authors successfully produced transformed plants of the rootstock *V. rupestris* cv. St. George (MULLINS *et al.* 1990).

Until recently *A. tumefaciens* strains have been classified into 3 biovars on the basis of chromosomally determined characteristics (KERSTERS and DE LEY 1984). Biovar 3 strains are associated with grapevines and are the strains most frequently isolated from crown gall tumours on these plants (KERR and PANAGOPOLOUS 1977). *A. tumefaciens* biovar 3 has now been classified as a separate species, *A. vitis* (OPHEL and KERR 1990) and the Ti plasmid of *A. tumefaciens* and of *A. vitis* strains examined are different in the T region and *Vir* region (YANOFSKY *et al.* 1985 a, b). While *A. tumefaciens* has undergone extensive development as a transformation vector for plants, it is possible that *A. vitis* could be the basis of a more suitable transformation vector for *Vitis* species.

We report here the partial molecular characterization of a number of *A. vitis* strains isolated in South Australia, and provide some evidence of the suitability of *A. vitis* as a transformation vector for *Vitis* species.

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[1]) On leave from Biochemical Research Institute, Nippon Menard Cosmetic Co. Ltd., Ogaki, Gifu-ken, 503, Japan

Material and methods

Bacterial strains and plasmids

Seventeen strains of *A. vitis* isolated in South Australia were provided by Prof. A. KERR together with strain K1069 which was originally isolated by Dr. T. C. BURR as strain CG-484. The following *Agrobacterium* strains were also used: LBA4404 (pAL4404) (HOEKAMA *et al.* 1983), A208 (pTiT37) (SCIAKY *et al.* 1978), C58C1Cm ($pTiB_6S_3tra^c$) (PETIT *et al.* 1978) and A856 (pTiAg162) (KNAUF *et al.* 1983). All strains were grown at 28 °C on LB plates or in MG liquid medium (MANIATIS *et al.* 1982).

The following plasmids were used: pTMA (MACHIDA *et al.* 1986); pPM1016-TE15 (HUSS *et al.* 1989) containing the TB *iaa* genes; the *Bam*HI-8 fragment of $pTiB_6806$ containing the TL *iaa* genes (GARFINKEL *et al.* 1981) cloned into pBR322 (supplied by Prof. Y. MACHIDA); the *Kpn*I fragment 10 of pTi15955 (nucleotide positions 2-4663, MELCHERS *et al.* 1987) containing the *virA* gene of WHR *A. tumefaciens* cloned into pTZ18R (supplied by Prof. Y. MACHIDA); and pBL-50 (LEROUX *et al.* 1987) containing the *virA* gene of LHR *A. vitis* strain, A856.

Opine utilization

The minimal medium of PETIT *et al.* (1978) was used to test opine catabolism of *A. vitis* strains. Octopine or nopaline were added to a final concentration of 0.2 % (v/v) and were used as the sole carbon and nitrogen source in the medium. Fresh cultures of the test isolates were streaked on opine-containing medium and growth was recorded after 5 to 7 d incubation at 28 °C. Where results were not clear, they were confirmed by the method of LIPPINCOTT *et al.* (1973).

Plant infections

Green soft stem fragments from grapevine (*Vitis vinifera* cv. Cabernet Sauvignon) grown in a glasshouse were surface sterilized for 10 min with 1.5 % (w/v) sodium hypochlorite solution and washed with 3 changes of distilled water. Fragments of ca. 1.5 cm were cut, and freshly cultured *Agrobacterium* from an LB agar plate was inoculated at 2 or 3 sites on each of 6 fragments using a sterile toothpick. The fragments were placed on MURASHIGE and SKOOG (MS) agar medium, and were transferred after 2 d to MS medium containing 500 mg/l cefotaxime. The stem fragments were maintained at 27 °C. *Kalanchoë daigremontiana* plants grown in a glasshouse were infected with *Agrobacterium* on the leaves of stems using a sterile toothpick. Carrots (*Daucus carota*, local variety) were purchased from the local market and discs were infected on the apical side with *Agrobacterium*. Gall formation was scored 3—6 weeks after infection, following culture at 27 °C.

Partial molecular characterization of Ti plasmid DNA from *A. vitis*

Total DNA of *A. vitis* isolates was digested with restriction enzymes, electrophoresed on 0.7 % or 2 % agarose gels and hybridized by the Southern blot method as previously described (MATSUMOTO *et al.* 1990).

Analysis of T-DNA excision

The frequency of the intramolecular recombination between 25 bp T-DNA border repeats following excision of T-DNA in the plasmid pTMA was measured (MACHIDA *et al.* 1986) and the recombination confirmed by measuring the size of the recombinant

plasmid DNA as described (MACHIDA *et al.* 1986), either by direct staining with ethidium bromide or by Southern blot hybridization using the plasmid pTMA as a probe.

Results and discussion

Opine utilization

Fifteen of the 18 *A. vitis* strains catabolized octopine as a sole carbon and nitrogen source but did not catabolize nopaline (Tab. 1). Two strains catabolized nopaline, and K1069, which is non-tumorigenic and does not contain a Ti-plasmid, did not catabolize either opine.

Table 1

Virulence of *A. vitis* and *A. tumefaciens* strains on selected host plants

Strains	Virulence			
	Carrot disc	Kalanchoë		Grapevine stem
		Leaf	Stem	
A. vitis				
octopine strains				
K1052, K1053, K1054 K1055, K1056, K1057 K1058, K1059, K1061 K1070, K1071, K1072 K306, K309	+	+	+[a]	+
K1076	+	+	±	±
nopaline strains				
K375, K377	+	+[b]	+	+
K1069[c]	−	−	−	−
A856[d]	−	−	−	+
A. tumefaciens				
A208	+	+[b]	+	±
C58C1Cm ($pTiB_6S_3tra^c$)	+	+	+	+
LBA4404[e]	−	−	−	−

\+ Tumour induced on every tissue sample
± Tumour induced on some tissue samples
a) Strain K306 induced shoots together with a tumor at the infection site
b) These strains induced teratomas together with tumors at the infection site
c) *A. vitis* strain without a Ti plasmid
d) Limited host range strain of *A. vitis*
e) *A. tumefaciens* strain without *onc* genes on the Ti plasmid

Host range of *A. vitis* isolates from South Australia

We examined the infectivity of the 17 different *A. vitis* isolates as described (GILLINGS and OPHEL 1992) from grapevines in South Australia. While the American LHR *A. vitis* strain, A856 showed no virulence on *Kalanchoë* and carrot, all the Australian isolates induced tumors on those plants and on grapevine stems (Tab. 1), sug-

gesting that they are all of the WHR type. There were minor differences in virulence between the strains which were difficult to score, but on *Kalanchoë* leaves, only the *A. vitis* nopaline strains, K375 and K377, induced teratomas together with tumors at the infection site. K1069 did not induce tumours on any plants.

Partial molecular analysis of *A. vitis*

Total DNA from each strain was digested with *Hin*dIII or *Eco*RI, and hybridized with parts of the T-DNA region of the *virA* region of *A. tumefaciens* and *A. vitis*. The non-oncogenic strain K1069 contains no Ti plasmid DNA and showed no homology with any of the probes. All other strains showed homologies to the T-DNA region and *virA* region probes in their Ti plasmid DNA. These homologies fell into a small number of groups and an example of each characteristic hybridization pattern is shown in Fig. 1 and discussed below. These hybridization analyses, together with their other properties allowed grouping of the *Agrobacterium* strains as shown in Tab. 2.

The octopine-utilizing WHR *A. vitis* strain Tm4, contains 2 T-DNA regions, TA and TB on the pTiTm4 plasmid (Fig. 1) (PAULUS *et al.* 1989). The TA region contains an *iaa*

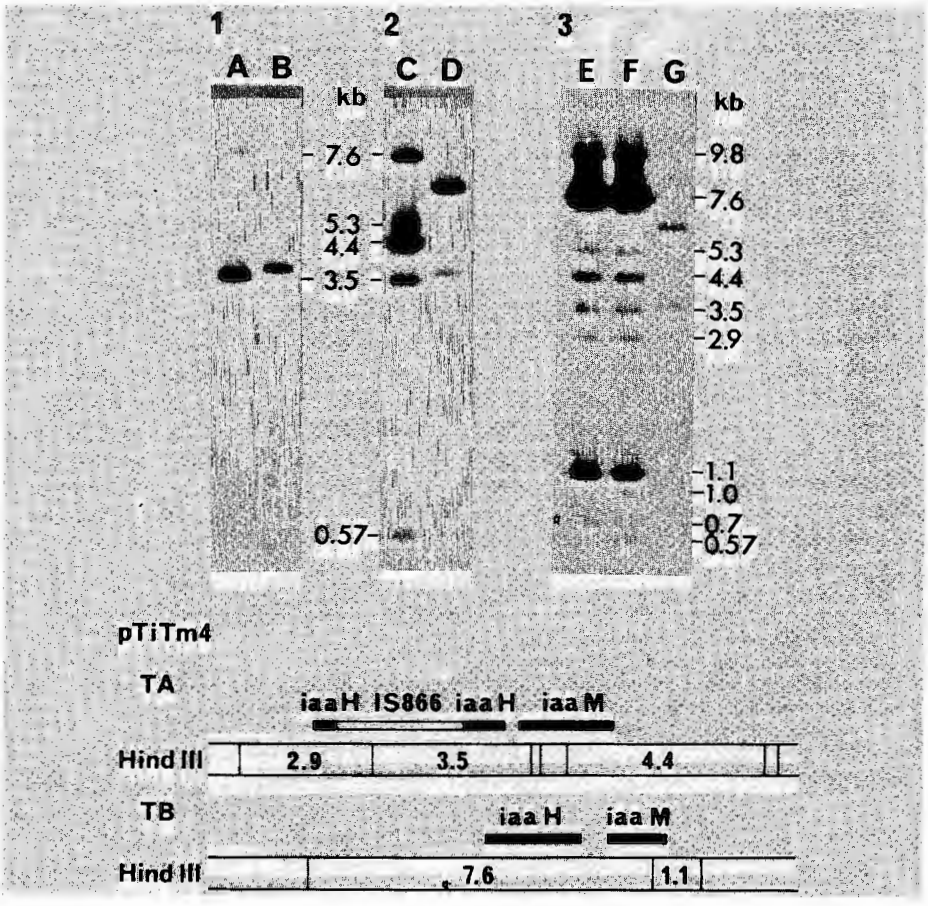

Fig. 1: Hybridization of Ti plasmid DNA in *Hin*dIII digests of total *A. vitis* DNA with TL iaa H (1), TL iaa M (2) and TB *iaa H* and *iaa M* (3). Lane A, C, E: K1052 (octopine strain), F: K306 (octopine strain), B, D, G: K375 (nopaline strain).

gene set (TA *iaa H* and TA *iaa M)* which resembles the *iaa* genes in the TL region of *A. tumefaciens* octopine strains such as C58C1Cm(pTiB$_6$S$_3$tra^c) apart from the addition of IS866 (Fig. 1). The TB region of *A. vitis* Tm4 also contains an *iaa* gene set (TB *iaa H* and TB *iaa M*) (Fig. 1). The TB *iaa* genes have only weak homology to the TA *iaa* genes and to the *A. tumefaciens* C58C1Cm (pTiB$_6$S$_3$tra^c) TL *iaa* genes. All the *iaa* genes in *A. vitis* except for TA *iaa H* are functional and essential for tumor induction on grapevine (HUSS *et al.* 1990). The Australian isolates of *A. vitis* were probed with the TL *iaa* of *A. tumefaciens* and TB *iaa* of *A. vitis* described above to examine the distribution of TA *iaa* and TB *iaa* in *A. vitis*. The octopine and nopaline strains of *A. vitis* fell into separate groups when classified following hybridization to the TL *iaa H*, Tl *iaa M* and TB *iaa* probes (Tab. 2, Fig. 1), and within the octopine strains, there was a further small difference in homology to the TB *iaa* probe.

Table 2

Hybridization patterns obtained with 5 different probes on total DNA of various *Agrobacterium* strains

Strains	Opine	Homology pattern[a]				
		TL iaa H	TL iaa M	TB iaa	LHR *virA*	WHR *virA*
K1052, K1053, K1054 K1055, K1056, K1057 K1058, K1059, K1061 K1070, K1071, K1072 K1076	Oct.	A	C	E	H	–
K306, K309	Oct.	A	C	F	H	–
K375, K377	Nop.	B	D	G	H	–
K1069[b]		–	–	–	–	–
A856[c]	Oct.				H	–
LBA4404[d]	Oct.				–	I

[a] The hybridization patterns produced with each probe were classified patterns A to I corresponded to lane A to I in Figs. 1 and 2, and bacteria containing similar Ti plasmid by this criterion are grouped together
[b] *A. vitis* strain without Ti plasmid
[c] Limited host range strain of *A. vitis*
[d] *A. tumefaciens* strain lacking T-DNA

The restriction fragments corresponding to those reported from pTiTm4 (PAULUS *et al.* 1989) were also found in the octopine strains of *A. vitis* (Fig. 1) isolated in Australia. In addition there were some fragments not accounted for by the map of pTiTm4. For example, a *Hin*dIII 5.3 kb fragment in the Australian strains was homologous to fragments containing both the TL *iaa M* and the TB *iaa* genes (Fig. 1), although hybridization with subfragments of the TB *iaa* region showed that the latter homology was only to TB *iaa M* (results not shown). The WHR *A. vitis* strain Tm4 belongs to the group of octopine-cucumopine strains with a large TA region (PAULUS *et al.* 1989) and it appears that the South Australian strains also belong to this group. Both LHR and WHR *A. vitis* octopine strains were isolated from the same locations in France (PAULUS *et al.* 1989) but all of the 15 octopine strains isolated from South Australia were classified as WHR and were similar to the French WHR isolates.

The two nopaline strains isolated in South Australia showed similar homologies (Fig. 1, Tab. 2) to those detected in the nopaline strains examined by PAULUS *et al.* (1989), suggesting that these French and Australian isolates are also related.

VirA protein acts as a sensor of plant derived inducer molecules such as acetosyringone (AS) and plays a critical role in the transcriptional induction of the *vir* regulon. The sequence of the *VirA* gene in the LHR *A. vitis* strain, A856, differs from that of WHR *A. tumefaciens*, and does not cross-hybridize in Southern hybridization (LEROUX *et al.* 1987). We examined the distribution of the *virA* gene of *A. vitis* using the *Pst*I/*Hin*dIII 2.0 kb fragment and the *Kpn*I 4.7 kb fragment containing the LHR *A. vitis* and WHR *A. tumefaciens virA* region, respectively, as probes. All of the oncogenic strains of *A. vitis* examined had homology to the LHR *virA*, but not to the WHR *virA* (Tab. 2, Fig. 2), and the restriction fragments corresponded to those of the LHR *virA* (Fig. 2).

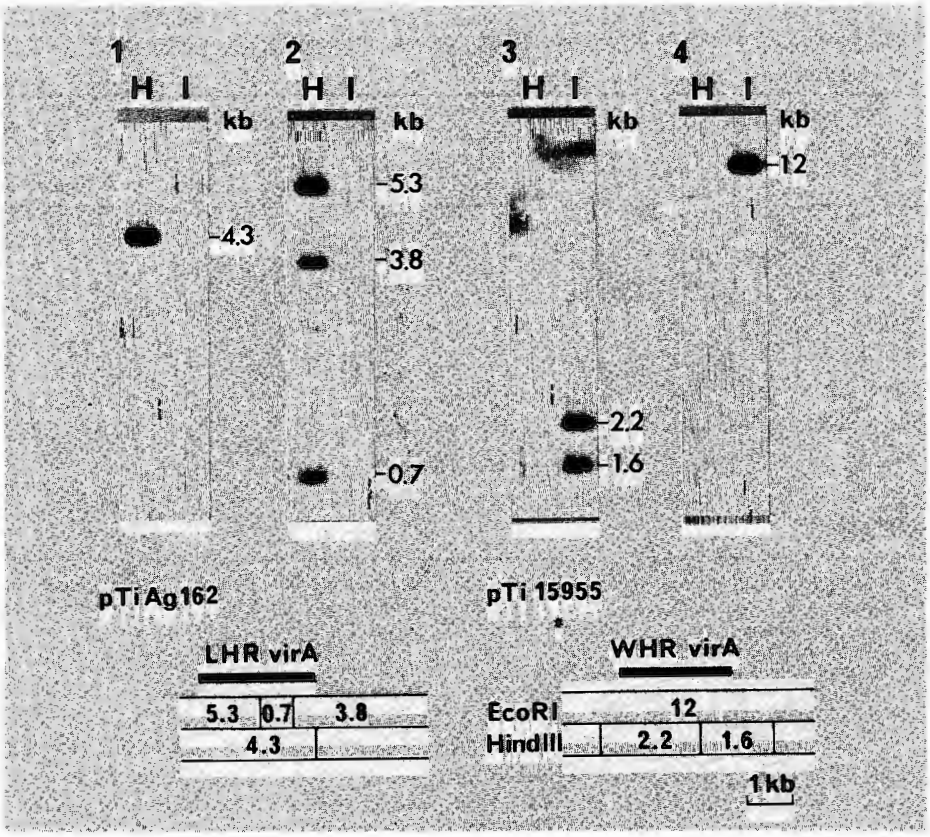

Fig. 2: Hybridization of Ti plasmid DNA in *Hin*dIII (1,3) or *Eco*RI (2,4) digests of total *A. vitis* and *A. tumefaciens* DNA with LHR *virA* (1,2) or WHR *virA* (3, 4). Lanes H: K1052, I: LBA4404.

A number of isolates of *A. tumefaciens* from Chinese grapevines showed similar homologies to the LHR *virA* sequence, although in contrast to the Australian isolates many of these did not cause galling on *Kalanchoë* (MA *et al.* 1987). The results suggest that the *virA* region described may be specific for both LHR and WHR *A. vitis* strains. This result may be of particular utility in the diagnosis of *A. tumefaciens* and *A. vitis* infections.

The frequency of T-DNA excision

The plasmid pTMA is a model system for detecting and assaying the circular forms of T-DNA generated in *Agrobacterium* by intramolecular recombination between 25 bp T-DNA border repeats following T-DNA excision (MACHIDA *et al.* 1986). The plasmid carries carbenicillin (Cb) resistance and two halves of a kanamycin (Km) resistance gene flanking the T-DNA. Thus recombination events are measured by the reconstitution of kanamycin resistance. This plasmid was introduced into *Agrobacterium* strains, and the frequency of circularization following excision of T-DNA was measured after incubation with AS or grapevine tissue. The excision of double stranded T-DNA had been reported as a first step in the transfer of T-DNA to host plants (KOUKOLIKOVA-NICOLA *et al.* 1985), but it seems probable that transfer of T-DNA to higher plants during the infection process involves the excision of only one strand of T-DNA (STACHEL *et al.* 1986). The mechanism proposed for the excision of single stranded T-DNA would also explain the generation of a small number of double stranded T-DNA excisions (STACHEL *et al.* 1986). Thus the excision of double stranded T-DNA is also likely to be a reflection of stimulation of the virulence genes of *Agrobacterium*. The results in Tab. 3 show that AS stimulated excision between the T-DNA border and circularization of pTMA in *A. tumefaciens* and grapevine tissues did not. The reverse was the case in *A. vitis* where AS had no effect but grapevine tissue stimulated circularization. This result is consistent with the observation that when the LHR *A. vitis*, A856, was incubated with AS, the *vir* gene was induced weakly, but when the *virA* gene of the WHR *A. tumefaciens* was introduced into the LHR *A. vitis*, there was strong induction of the *vir* gene (LEROUX *et al.* 1987).

Table 3

Frequency of recombination in pTMA due to T-DNA excision; expressed as number of Km resistant and Cb sensitive bacteria per 10^7 bacteria after co-cultivation for 48 hours with AS, grapevine, no inducer

Bacterial strain	Plasmid	Frequency		
		AS	Grapevine	No inducer
LBA4404[b]	pAL4404	2.0	< 0.08	< 0.15
A208[b]	pTiT37	8.3	< 0.07	< 0.03
A856[c]	pTiAg162	< 0.19[a]	0.43	< 0.20

a) The data with < indicate that no Km resistant and Cb sensitive colonies were found
b) *A. tumefaciens*
c) *A. vitis* (LHR)

As shown above, the homology to the LHR *virA* gene of *A. vitis* was also detected in WHR *A. vitis* (Tab. 2). All these strains were isolated from grapevine and are known to cause tumors in grapevine (Tab. 1). The isolation of methyl syringone from grapevine and the demonstration of its preferential induction of *virB* activity (SPENCER *et al.* 1990) also provides evidence that grapevines synthesize or secrete a plant signal other than AS which can be recognized by the VirA protein of *A. vitis*. It seems likely that there are similar inducers in *Kalanchoë* and carrot, since WHR *A. vitis* induces tumors on those plants.

These results indicate that there are significant differences between the structure and content of the T-DNA and *vir* regions of the Ti plasmid DNA in *A. vitis* and *A. tumefaciens*. They also suggest that an *Agrobacterium* based transformation vector for *Vitis* species may be more efficient if constructed from *A. vitis* rather than from *A. tumefaciens*.

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Correspondence to:

Dr. N. STEELE SCOTT
CSIRO Division of Horticulture
GPO Box 350
Adelaide, S.A. 5001
Australia