Transformation of tobacco plants with *virE1* gene derived from *Agrobacterium tumefaciens* pTiA6 and its effect on crown gall tumor formation

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Summary: The VirE1 protein plays a key role in the transport of VirE2 protein from the bacterium to the plant cell during crown gall tumor induction by *Agrobacterium*. The *virE1* gene of *A. tumefaciens* pTiA6 was cloned into the plant transformation vector pTd33 yielding pTd93virE1 that was introduced into *A. tumefaciens* EHA101 and used for tobacco transformation. The presence of the foreign DNA in the putative transgenic plants was confirmed by PCR analysis. Nine of the 41 transformed plants formed only small tumors following infection with the wild-type *A. vitis* octopine strain AB3. This property was inherited into the T1 generation. The expression of *virE1* gene in T1 plants was demonstrated by Northern blot analysis.

Introduction

Crown gall disease induced by *Agrobacterium tumefaciens (Smith & Townsend)* Conn or *Agrobacterium vitis* Ophel & Kerr causes serious damage worldwide on several plant species, e. g. on grapevine, fruit trees and raspberry (*De Cleene*, 1979). Although the loss can be reduced by using *Agrobacterium*-free planting material and resistant rootstock varieties, there is no efficient method yet that can be routinely used by grapegrowers to prevent this disease (*Burr & Otten*, 1999, *Burr* et al., 1998). Therefore genetic manipulation for crown gall resistance would have a great economic impact.

In the process of crown gall tumorigenesis pathogenic agrobacteria transfer a specific segment of their Ti (tumor inducing) plasmid, termed T-DNA into the plant chromosomal DNA resulting in tumorous growth and opine production (*Dessaux* et al., 1993, *Stafford*, 2000, *Weising & Kahl*, 1996, *Zupan & Zambryski* 1995). The T-DNA transfer process is determined by a set of virulence proteins (VirA-F). Genes coding for their synthesis are also located on the Ti plasmid (*Gelvin*, 2000, *Hooykaas & Beijersbergen*, 1994). Two of these virulence proteins, VirE2 and VirD2 are closely associated with the T-DNA transfer and integration process. VirD2 covalently binds to the 5'-end of the single stranded T-DNA which is then coated by several hundred molecules of VirE2. VirD2 plays a specific role in directing T-DNA into the plant nucleus, while VirE2 protects it from plant nucleases (*Citovsky* et al., 1989, *Sen* et al., 1989). Recent results suggest that the T-strand and VirE2 molecules are transported separately from the bacterium into the plant cell. Further experiments revealed that VirE2 protein export is mediated by VirE1 (*Chen* et al., 2000, *Deng* et al., 1999, *Gelvin*, 1998, *Lee* et al., 1999, *Stahl* et al., 1998, *Sundberg & Ream*, 1999, *Sundberg* et al., 1996). VirE1 strongly interacts with VirE2 and has been proposed to prevent the self-aggregation and binding of VirE2 molecules to the T-strand in the bacterium (*Deng* et al., 1999, *Sundberg & Ream*, 1999, *Zhou & Christie*, 1999).

Our starting assumption was that VirE1, if it is already present in the plant before *Agrobacterium* infection will bind VirE2 transferred with the infection. If this interaction is strong enough, it may prevent tumor formation and cause resistance to crown gall disease. To test this hypothesis we introduced the *virE1* gene of the *A.tumefaciens* octopine Ti plasmid pTiA6 into tobacco and the susceptibility of resulting plants to crown gall was tested with *A. vitis* AB3 and *A. tumefaciens* A348.

Material and method

Agrobacterium and *Escherichia coli* (Migula) Castellani & Chalmers strains used for the experiments are listed in *Table 1*. The *virE1* gene from pTiA6 was cloned into pTd33 (*Tinland* et al., 1995) by replacing the *gus*A gene within the p35S-tnos casette, thus yielding pTd93*virE1*. This plasmid was transformed into *E. coli* LE392 cells and mobilized with pGJ28 into *A. tumefaciens* EHA101 for plant transformation.

Table 1 – Bacterial strains used

Strains (plasmids)	Relevant characteristics*	Reference
Escherichia coli		
LE392 (pTd33)	plant transformation vector containing *nptII/gusA* genes as plant selectable markers, Gm^R	*Tinland* et al. 1995
LE392 (pTd93virE1)	*gusA* gene replaced by *virE1* gene of pTiA6 in pTd33	This work
JC2926 (pGJ28, pR64drd11)	mobilizing plasmid for pBR322 derivatives, Km^R, Gm^R, Tc^R	*van Haute* et al. 1983
Agrobacterium tumefaciens		
EHA101	disarmed pTi from pTiBo542, Km^R	*Hood* et al. 1986
EHA101 (pTd33)	Km^R, Gm^R	This work
EHA101 (pTd93*virE1*)	Km^R, Gm^R	This work
A348	pTiA6 in A136	*Garfinkel* et al. 1981
Agrobacterium vitis		
AB3	wild-type, octopine pTi	*Szegedi* et al. 1988

* Km^R: kanamycin resistance, Gm^R: gentamycin resistance, Tc^R: tetracycline resistance

Leaf discs of *Nicotiana tabacum* L. cv. Petit Havana SR1 plants were transformed according to *Horsch* et al. (1985). Selection was carried out on Murashige and Skoog medium containing 100 mgl^{-1} kanamycin to select transformed cells and 200 mgl^{-1} carbenicillin to eliminate bacteria. The regenerated plants were subcloned on the same medium and the rooted plants were transferred to the greenhouse. Stems of young tobacco plants were inoculated at 2–3 points with 10 μl suspension (OD_{600} = 0.5 – 1.0) of overnight cultures of *A. tumefaciens* A348 and *A. vitis* AB3 strains. Results were scored after four weeks of incubation in the greenhouse. Seeds of self-pollinated plants were collected from T0 plants. They were germinated and grown in sterile soil in the greenhouse and inoculated as described above.

Total nucleic acids were prepared from young apical leaves using the hot-phenol/SDS method as published by *Verwoerdt* et al. (1989). RNA was isolated by LiCl and DNA by ethanol precipitation, respectively from the phenol/chloroform extracted supernatant solution. DNA preparations were further purified by ribonuclease treatment. The presence of the *nptII* gene in putative transgenic plants was tested by PCR analysis carried out with a PTC-150-HB cycler (MJ Research, Inc., Watertown, USA). The reaction was carried out in 1x *Taq* polymerase buffer containing 1.5 mM l^{-1} $MgCl_2$, 200 μM l^{-1} of each dNTP, 0.2 μM l^{-1} of primers and 1.0 U *Taq* polymerase in 20 μl volume. After the initial denaturation step at 94 °C for 1 min, 30 cycles of 92 °C for 40 sec, 60 °C for 40 sec, 75 °C for 1 min 30 sec were performed followed by an elongation step at 75 °C for 5 min. The *nptII* specific primer pair resulting in amplification of a 700 bp region of *nptII* was originally described by *Hoffmann* et al. (1997). Northern blot hybridizations were carried out according to standard protocols (*Sambrook* et al. 1989) using a *virE1* specific probe. The probe DNA was prepared by PCR using the 5'–CCATCATCAAGCCGCA–3' (forward: 392–407 bp) and 5'–CTCCTTCTGACCAGCAAGA–3' (reverse: 582–564 bp) primers designed on the basis of the *A. tumefaciens* octopine Ti plasmid, pTiA6 sequence data (*Winans* et al., 1987).

Results and discussion

Leaf discs of *N. tabacum* SR1 plants were efficiently transformed both with EHA101(pTd33) and with EHA101(pTd93*virE1*) strains. Altogether 22 plants were obtained after transformation with pTd33 and 41 plants with pTd93virE1 in three independent experiments. All of the regenerated plants showed normal appearance. They could not be distinguished morphologically from the non-transformed SR1 plants. Plants obtained with pTd33 transformation were as sensitive to *A. vitis* AB3 as the non-transformed control tobacco plants. On the other hand, nine of the 41 pTd93*virE1*-transformed plants were moderately resistant since they formed only small swellings after four weeks of incubation as compared to the control plants (*Figure 1*). Similar reactions were not observed when *A. tumefaciens* A348 strain harboring the pTiA6 tumor inducing plasmid was used for inoculation, since all of the tested clones formed tumors. The presence of introduced DNA was confirmed by PCR analysis that showed the presence of the *nptII* gene in the tested putative transformants (*Figure 2*).

Three of the moderately resistant lines, designated I/12, II/6 and II/9 were self-pollinated and T1 seedlings were tested for susceptibility to AB3. This property was inherited into each of the three T1 families, but the segregation ratio was lower than the expected 3:1 Mendelian ratio for a single

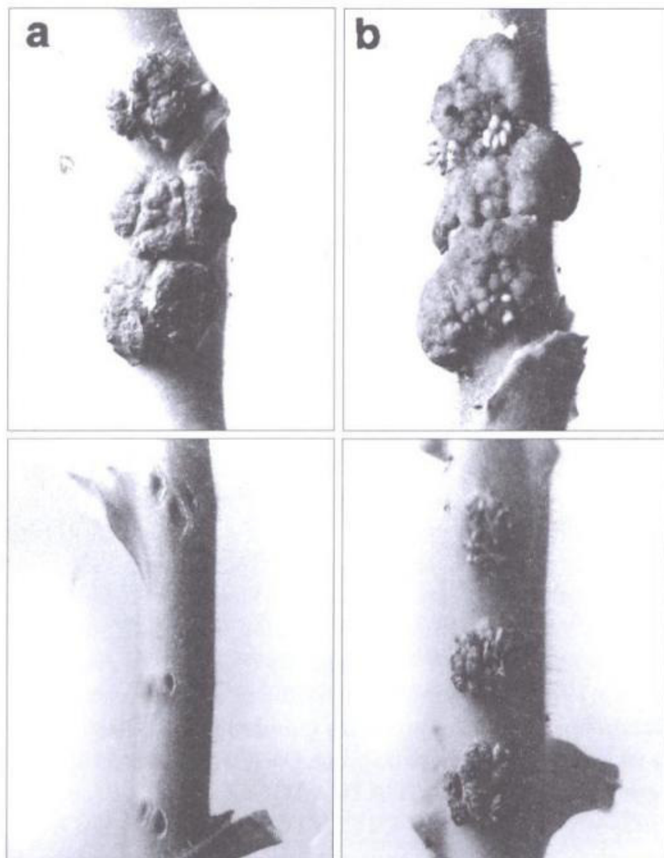

Figure 1 Susceptibility of virE1 transformed tobacco to Agrobacterium vitis AB3 (a) Non-transformed, (b) pTd33-transformed and (c) non-inoculated tobacco plants used as controls, (d) pTd93*virE1*-transformed plant (II/9)

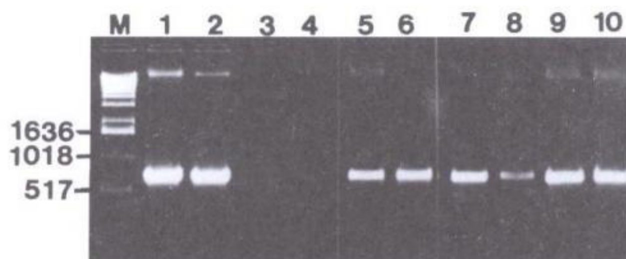

Figure 2 PCR detection of the transferred DNA in tobacco plants using nptII specific primers Lane 1: A. *tumefaciens* EHA101 (pTd33), lane 2: *A. tumefaciens* EHA101 (pTd93*virE1)*, lane 3: 0 control (without template DNA), lane 4: non-transformed *N. tabacum* SR1, lanes 5 and 6: two independent pTd33-transformed plants, lane 7 to 10 are four pTd93*virE1*-transformed moderately resistant plants I/12 (lane 7), II/1 (lane 8), II/6 (lane 9) and II/9 (lane 10)

dominant locus. Resistance to A348 harboring the *virE1* gene that was transferred to tobacco was not observed as in the case of T0 plants *(Table 2)*. Northern blot analysis of eight resistant T1 plants showed the expression of the *virE1* gene in these lines *(Figure 3)*.

It was suggested that in the process of crown gall tumor induction the T-strand and the VirE2 protein are transported separately from *Agrobacterium* into the plant cell and the export of VirE2 is mediated by VirE1 (*Gelvin*, 1998, *Lee* et al., 1999, *Stahl* et al., 1998, *Sundberg* et al., 1996).

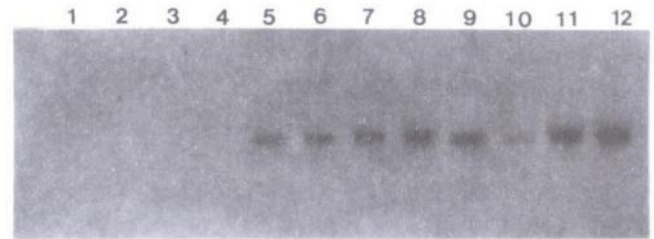

Figure 3 Northern blot analysis of resistant T1 plants using a virE1 specific probe Lanes 1 and 2 are two independent non-transformed *N. tabacum* SR1 RNA samples, lanes 3 and 4 are two pTd33-transformed samples used as sensitive (negative) controls. Lanes 5–7, 8–10 and 11–12 are independent moderately resistant T1 progenies of I/12 , II/6 and II/9 families.

Table 2 – Inheritance of resistance in T1

Family	T0 phenotype (susceptibility to *A. vitis* AB3)	*Agrobacterium* strain used for inoculation	Results (resistant: sensitive plants)
pTd33 II/2	sensitive (control)	A348	0:36
pTd93*virE1* I/12	resistant	A348	0:36
pTd33 II/2	sensitive (control)	AB3	0:36
pTd93*virE1* I/12	resistant	AB3	8:28
pTd93*virE1* II/6	resistant	AB3	9:21
pTd93*virE1* II/9	resistant	AB3	6:30

More recently protein interaction assays carried out in yeast-two-hybrid system confirmed that VirE1 protein interacts with VirE2 (*Deng* et al., 1999, *Sundberg & Ream*, 1999, *Zhou & Christie*, 1999). The potential biological role of this interaction may be to prevent self-aggregation and binding of VirE2 to T-strands in the bacterium. The *virE1* gene expressed in the plant cell reduces the crown gall tumor formation induced by the *A. vitis* octopine strain AB3. However no resistance was found to A348 of which the *virE1* gene used in our studies. Further studies are needed to test if VirE1 protein encoded by pTiA6 can interact with the heterologous VirE2 in the plant cell or it can interfere with another step of the *A. vitis*-induced transformation process.

Although we did not obtain complete resistance and a regular Mendelian genetic segregation we have shown that the *virE1* gene derived from the *A. tumefaciens* octopine Ti plasmid pTiA6 is able to reduce the size of tumors induced by octopine-type *A. vitis* strains AB3. From a practical point of view, the *virE1* gene may be a useful tool to reduce crown gall sensitivity of grapevine plants.

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