**Agrobacterium vitis** strains lack tumorigenic ability on *in vitro* grown grapevine stem segments

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**Summary**

Grapevine stem segments were cocultivated with three different *Agrobacterium tumefaciens* and three different *A. vitis* strains. *A. tumefaciens* strains induced tumors at variable frequencies, while *A. vitis*-infected stem segments never formed crown galls. The tumorous nature of tissues grown on hormone free medium was confirmed by opine assays. Bioinformatic and PCR analysis of the virulence regions of various *A. tumefaciens* and *A. vitis* Ti plasmids showed that *virH2* and *virK* genes are common in *A. tumefaciens* but they are lacking from *A. vitis*. Thus *virH2* and *virK* genes may be essential for grapevine stem segment transformation, but expression of certain T-DNA genes of *A. vitis* may also prevent the growth of transformed cells. Our data indicate that the tumorigenic ability of *A. vitis* is different on intact plant and on their explants, and that the specific host association of *A. vitis* on grapevine is probably determined by physiological and biochemical factors (e. g., better colonizing ability) rather than by its increased tumorigenic ability. Therefore it is not reasonable to develop „helper” plasmids for grapevine transformation from *A. vitis* pTis, unless their avirulence on *in vitro* explants is determined by T-DNA gene(s). Due to the inability of *A. vitis* to induce tumors on grapevine stem segments, the use of *in vitro* explant assays cannot be reliably used to select *A. vitis* resistant grapevine genotypes or transgenic lines.

**Key words:** crown gall, opines, Ti plasmids, *vir*-region, *Vitis*

**Introduction**

Tumorigenic agrobacteria (*Agrobacterium tumefaciens*, *A. rubi* and *A. vitis*) cause crown gall or cane gall disease on several, mainly annual crops. Rhizogenic strains (*A. rhizogenes*) cause intensive root formation called hairy root disease. Both diseases are based on the genetic transformation of the host plant leading to elevated hormone level or sensitivity, and opine production. The tumor-inducing (pTi) or root-inducing (pRi) plasmids contain two separate regions coding for this ability of agrobacteria. The *vir*-region carries genes for the DNA transfer from the procaryote bacterium into the eucaryote host plant through a highly sophisticated type IV transport system and directs its integration into the plant chromosome. The second region, called T-DNA, harbours genes that are transferred to the plant cells and are directly responsible for tumor formation. The length of T-DNA transported into the plant cell is determined only by its border sequences. This specific property of agrobacteria led to the development of so called „disarmed” or „helper” pTi plasmid derivatives lacking T-DNA. Such pTi derivatives have been widely used for decades to introduce useful traits into plants (Tzfira and Citovky 2008).

In the nature crown gall symptoms on grapevines are predominantly caused by *A. vitis* (Burr et al. 1998, Palacio-Belsa et al. 2009, Filo et al. 2013) but the occurrence of *A. tumefaciens* has also been reported (Szegedi et al. 2005, Palacio-Belsa et al. 2009, Rahimian and Abellati 2012, Abdellati et al. 2013). In contrast to the predominance of *A. vitis* on grapevine, exclusively *A. tumefaciens* (or sometimes *A. rhizogenes*) derivatives are used for grapevine transformation (Perl and Eshdat 1998, Martinelli and Mandolina 2001, Càrimi et al. 2012). The potential use of „disarmed” *A. vitis* to introduce foreign genes into grapevine has already been raised (Vivier and Pretorius 2000), but construction of such a plasmid has not been published yet. In a previous study an *A. vitis* strain showed extremely low transformation efficiency compared to *A. tumefaciens* and *A. rhizogenes* strains on grapevine embryogenic calli, thus it was found inappropriate for such purposes (Torregrosa et al. 2002). Although it has been shown that the host range pattern (profile) of various agrobacteria differs on various grapevine genotypes (Szegedi et al. 1984, Sule et al. 1994), transformation experiments are rarely preceded by such studies.

To test if *A. vitis* can be considered as an efficient gene vector for grapevine transformation we compared the tumor-inducing (transforming ability) of various *A. tumefaciens* and *A. vitis* strains on *in vitro* grapevine stem segments. Such *in vitro* explant assay may also be useful for early selection of resistant *Vitis* genotypes and transgenic lines. Our results showed that *A. vitis* strains are not tumorigenic on *in vitro* stem segments thus their use in grapevine transformation might provide invalid data when assaying genotype susceptibility.

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Material and Methods

Plant material: The following five grapevine genotypes were used during this work: *Vitis berlandieri* x *Vitis rupestris* 'Richter 110', the Seyve Villard 12375 x *V. vinifera* interspecific variety 'Fanny' and the *V. vinifera* cvs. 'Kadarka', 'Sauvignon blanc' and 'Ezerjó'. Plants were propagated *in vitro* in 380 ml glass bottles on ½ MS medium (MURASHIGE and SKOOG 1962) supplemented with 1.0 % saccharose and 0.25 % phytagel at 14 h photoperiod and at the light intensity of 50 μm² s⁻¹.

Strains: Bacterial strains used for the experiments and their relevant characteristics are listed in Tab. 1. Cultures for transformation of grapevine explants were grown on glucose/yeast extract medium as previously described (SZEGEDI et al. 2005).

Cocultivation and selection: Stems of *in vitro* grown plants were cut into 5-6 mm pieces in liquid B5 medium containing 1 % (w/v) saccharose (GAMBORG et al. 1968) to prevent drying and rinsed with bacterial suspensions (approx. 10⁷ cfu mL⁻¹) prepared also in liquid B5 medium. Then stem segments were transferred to solid hormone-free B5 medium containing 1% (w/v) saccharose and 0.6 % (w/v) agar. Explants were incubated at 25-27 °C for three weeks at 14 h photoperiod and at the light intensity of 50 μm² s⁻¹.

Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Disarmed helper strain/plasmid</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Agrobacterium tumefaciens</strong> strains</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A348</td>
<td>pTi A6 in C58 chromosomal background, agropine, mannopine/ octopine pTi</td>
<td>Not available*</td>
<td>GAREINKEL et al. 1981</td>
</tr>
<tr>
<td>C58</td>
<td>Wild type strain, nopaline/ agrocinopine A+B pTi</td>
<td>pMP90, MOG301</td>
<td>KONZ and SCHELL 1986, HOOD et al. 1993</td>
</tr>
<tr>
<td>A281</td>
<td>pTiBo542 in C58 chromosomal background, agropine, mannopine/ L,L-succinamopine pTi</td>
<td>EHA101, EHA105</td>
<td>HOOD et al. 1986, HOOD et al. 1993</td>
</tr>
<tr>
<td>GV3101(pTiTm4)</td>
<td>pTiTm4 in C58 chromosomal background</td>
<td>-</td>
<td>HUSS et al. 1989</td>
</tr>
<tr>
<td>II/5-1</td>
<td>Wild type isolate carrying an <em>A. vitis</em> octopine/cucumopine type pTi</td>
<td>-</td>
<td>SZEGEDI et al. 2005</td>
</tr>
<tr>
<td><strong>Agrobacterium vitis</strong> strains</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tm4</td>
<td>Wild type, octopine/cucumopine pTi</td>
<td>-</td>
<td>SZEGEDI et al. 1988</td>
</tr>
<tr>
<td>AT1</td>
<td>Wild type, nopaline pTi</td>
<td>-</td>
<td>SZEGEDI et al. 1988</td>
</tr>
<tr>
<td>S4**</td>
<td>Wild type, vitopine pTi</td>
<td>-</td>
<td>SZEGEDI et al. 1988</td>
</tr>
</tbody>
</table>

*Disarmed pTi have been developed from the very similar pTiB6 (MOG1, HOOD et al. 1993 and GV2260, DEBLAERE et al. 1985) and pTiAch5 (LBA4404, HOKIMA et al. 1983)

**Identical with the sequenced *A. vitis* S4 (SLATER et al. 2009).
was followed by 35 cycles of denaturation (94 °C, 40 sec), annealing (50 °C, 40 sec) and synthesis (72 °C, 1 min), and finally terminated (72 °C, 3 mins). Amplification products were analysed after electrophoresis in 1.5 % (w/v) agarose gel and ethidium-bromide staining.

**Statistical analysis:** Differences between tumor induction ability of different *A. tumefaciens* strains on various grapevine varieties were tested using the chi-square test. Observed frequencies of tumor formation were compared to expected frequencies. Expected frequencies were defined as the average tumor induction ability of all strains on all varieties.

**Results**

To test the potential suitability of *A. vitis* for grapevine genetic transformation and crown gall resistance assay we have tested the octopine/cucumopine strain Tm4, the nopaline strain AT1 and the vitopine strain S4 on *in vitro* grapevine stem segment explants. The tested strains were tumorigenic on these and/or on several other grapevine cultivars when intact plants were inoculated *in vitro* or in the greenhouse (data not shown). For comparison *A. tumefaciens* A348 (agropine/octopine pTi), C58 (nopaline/agropine agropine A+B pTi) and A281 (agropine/L,L-succinamopine pTi) were used. Disarmed derivatives of these or similar *A. tumefaciens* strains (Tab. 1.) have already been widely used to introduce foreign genes into grapevines. Embryogenic calli are most widely used for genetic transformation of grapevine (PERL and ESHID 1998, MARTINELLI and MANDOLINO 2001, BOUQUET et al. 2008, CARIMI et al. 2012), but stem sections or leaf discs are also considered as starting material (DAS et al. 2002, MAILLOT et al. 2006, NICHOLSON et al. 2012, NOOKARAJU and AGRAWAL 2013) thus we included them as well.

Grapevine stem segments collected from *in vitro* grown plants formed tumorous calli on hormone-free B5 medium at various degrees depending on the grapevine genotype after inoculation with *A. tumefaciens* strains (Fig. 1). In contrast to these observations, none of the three *A. vitis* strains, which are the natural agrobacterial pathogens of grapevines, induced tumors on stem segments of any of the tested five grapevine cultivars (Fig. 1). On the rootstock variety ‘Richter 110’ the A281 strain, on ‘Fanny’ and *V. vinifera* (European) grapes the C58 strain were the most efficient. C58-induced tissues growing on hormone free medium contained nopaline, while AT1 inoculated stem segments that did not show growth were nopaline negative (Fig. 1.) Similar results were obtained, although tumors were formed at lower frequencies, when ‘Richter 110’ and ‘Ezerjó’ leaf discs were cocultivated with *A. tumefaciens* or *A. vitis*. *A. vitis* strains never induced growth (tumor formation) on leaf discs on hormone-free medium (data not shown).

To further confirm the tumorous nature of the calli selected on hormone-free medium 12 independent tumor lines were analysed of each of the ‘Richter 110’/*A. tumefaciens* C58, ‘Richter 110’/*A. tumefaciens* A281, ‘Ezerjó’/*A. tumefaciens* C58 and ‘Ezerjó’/*A. tumefaciens* A281 combinations (altogether 48 tumor lines) for the presence of nopaline (C58-induced lines) or agropine and mannopine (A281-induced lines). Each line contained the appropriate opine (nopaline for C58-, and agropine/mannopine for A281-induced tumors) confirming that the selected tissues were true crown galls (Fig. 2).

Surprisingly, neither *A. tumefaciens* nor *A. vitis* strains transformed embryogenic calli of ‘Richter 110’. We could not select any lines growing on hormone-free MS or B5 media. Altogether 72 callus lines, 12 for each of the six strains, were assayed for the presence of the appropriate opines. All lines were opine negative confirming that transformation did not take place.

Next, stem segments of ‘Kadarka’, ‘Ezerjó’ and ‘Sauvignon blanc’ were coinoculated with 1:1 mixtures of *A. tumefaciens* C58 and *A. vitis* Tm4 cells. Thirty-six tumors,
To test if this avirulence of *A. vitis* on *in vitro* grapevine stem segments is due to chromosomal or Ti plasmid differences between *A. tumefaciens* and *A. vitis*, we tested also *A. tumefaciens GV3101 (pTiTm4)* and *A. tumefaciens II/5-1* strains, both carrying *A. vitis* type pTis (Tab. 1.), on 'Kadarka' explants. They showed the same negative results as the wild type *A. vitis* strains.

The results described above suggested that lack of certain virulence genes located on the pTis may be responsible for the different tumorigenic ability of *A. tumefaciens* and *A. vitis* on grapevine stem segments. Alignment of the virulence regions of *A. tumefaciens* 15955, C58 and Bo542, and *A. vitis* S4 showed that most of the well characterized *vir* genes are shared among the virulence regions of different *Agrobacterium* spp. strains (Fig. 3). Two virulence genes, *virH2* and *virK* commonly occurred in all *A. tumefaciens* strains, but they were lacking from *A. vitis* S4. *A. tumefaciens* C58 harbours two copies of *virE3*, which explains the scattered alignment of *virE3* sequences in different *Agrobacterium* spp. strains. While the majority of virulence genes shows a relatively high sequence homology in different strains, *virD3* shows peculiar distribution of gap-free alignments, where only the N and C terminal sequences seem to be conserved among *Agrobacterium* spp. strains. *VirF* genes from different *A. tumefaciens* show a low sequence similarity, the applied local alignment algorithm was unable to detect significant gap-free alignments for *virF* from strains C58 and 15955 (Fig. 3), although the latter also carries *virF*.

### Table 2

Transformation efficiency of various *Agrobacterium tumefaciens* and *Agrobacterium vitis* strains on grapevine stem segment assays.

Asterisks denote significant difference in tumor formation ability of different *A. tumefaciens* strains on the given variety (*p* = 0.05, ** = 0.01), while + denotes significant difference between the response of different varieties to a given *A. tumefaciens* strain (**p** = 0.01)

<table>
<thead>
<tr>
<th>Strain</th>
<th>'Richter 110'*</th>
<th>'Fanny'**</th>
<th>'Sauvignon blanc'**</th>
<th>'Kadarka'**</th>
<th>'Ezerjó'*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No of tumorous/No of total stem segments</td>
<td>% of tumorous stem segments</td>
<td>No of tumorous/No of total stem segments</td>
<td>% of tumorous stem segments</td>
<td>No of tumorous/No of total stem segments</td>
</tr>
<tr>
<td>Control</td>
<td>0/37</td>
<td>0</td>
<td>0/33</td>
<td>0</td>
<td>0/28</td>
</tr>
<tr>
<td>A348++</td>
<td>14/32</td>
<td>43.7</td>
<td>0/31</td>
<td>0</td>
<td>3/31</td>
</tr>
<tr>
<td>C58++</td>
<td>18/32</td>
<td>56.2</td>
<td>30/32</td>
<td>93.7</td>
<td>21/31</td>
</tr>
<tr>
<td>A281++</td>
<td>26/33</td>
<td>78.8</td>
<td>2/35</td>
<td>5.7</td>
<td>20/30</td>
</tr>
<tr>
<td>Tm4</td>
<td>0/37</td>
<td>0</td>
<td>0/32</td>
<td>0</td>
<td>0/28</td>
</tr>
<tr>
<td>AT1</td>
<td>0/38</td>
<td>0</td>
<td>0/32</td>
<td>0</td>
<td>0/29</td>
</tr>
<tr>
<td>S4</td>
<td>0/38</td>
<td>0</td>
<td>0/32</td>
<td>0</td>
<td>0/27</td>
</tr>
</tbody>
</table>
Agrobacterium vitis strains lack tumorigenic ability

We also searched for homologous sequences of *A. tumefaciens* Bo542 (NC_010929) and *A. vitis* S4 (NC_011982) have been aligned to the reference sequence of the virulence region of *A. tumefaciens* C58 (NC_003065). Each line shows gap-free local alignments between the query sequence and the C58 reference. Vertical position of the lines inside each box indicate percentage nucleotide similarity of the alignment.

Fig. 3: Local alignments of available *Agrobacterium* spp. virulence regions. The sequences of *A. tumefaciens* 15955 (NC_002377), *A. tumefaciens* Bo542 (NC_010929) and *A. vitis* S4 (NC_011982) have been aligned to the reference sequence of the virulence region of *A. tumefaciens* C58 (NC_003065). Each line shows gap-free local alignments between the query sequence and the C58 reference. Vertical position of the lines inside each box indicate percentage nucleotide similarity of the alignment.

We also searched for homologous sequences of *A. tumefaciens* virK and virH2 proteins in the complete pTi sequence of *A. vitis* S4 using tblastn. Significant similarity to VirK was not found at e-value threshold of 0.1, while *A. tumefaciens* queries showed significant similarity to *A. vitis* S4 VirH1. Phylogenetic analysis of *A. tumefaciens* and *A. vitis* VirH1 and VirH2 proteins orders *A. vitis* S4 VirH1 to the VirH1 protein sequences of different *A. tumefaciens* strains and not to VirH2 sequences (data not shown). Based on these results, virK and virH2 genes are indeed missing from *A. vitis* S4.

PCR analysis of the tested wild type agrobacteria, as expected, detected virH2- and virK-specific sequences in all *A. tumefaciens* strains. In contrast to these results we could not amplify any virH2- and virK-specific fragments with the primers used from *A. vitis* octopine (Tm4), nopaline (AT1) or vitopine (S4) strains (Fig. 4).

Discussion

An *in vitro* stem segment assay was expected to provide a simple method to test the tumorigenicity of various agrobacteria or susceptibility of various grapevine genotypes. We have shown that the natural grapevine pathogen *A. vitis* does not induce tumors on grapevine stem segments, while *A. tumefaciens* strains, although at variable frequencies depending on the grapevine cultivar, were tumorigenic in this assay. The reason of negative transformation results of embryogenic cell line with wild type agrobacteria is unknown. It may be due to the inappropriate media we used or due to the sensitivity of embryogenic cells to the hormone overproduction caused by *Agrobacterium*-transformation. Our data are not in agreement with some previous observations. Huss and coworkers (1990) successfully induced tumors on *V. vinifera* 'Chardonnay'...
A. vitis

Fig. 4: PCR analysis of marker (Fermentas SM0328), while C58 was more efficient on certain European grape rootstock 'Richter 110' more efficiently than A348 or C58, CG450 induced tumors is also possible that the T-DNA genes of A. vitis

 Torresgrosa et al. (2002) found that the frequency of transformation is determined both by the grapevine genotype and Agrobacterium strains. Our results confirm these observations. A. tumefaciens strain A281 transformed the rootstock 'Richter 110' more efficiently than A348 or C58, while C58 was more efficient on certain European grapevine cultivars than A281. These data may be considered for the selection of the appropriate disarmed ("helper") strain for introducing foreign genes into grapevine. Host range differences among various agrobacteria within Vitis spp. have also been observed earlier (Szegedi et al. 1984, Sule et al. 1994).

To get an insight into the possible role of the genetic background we tested two A. tumefaciens strains carrying A. vitis type pTis. Since neither GV3101 (pTiTm4) nor II/5-1 were tumorigenic, this property of A. vitis is probably determined by ptTi-encoded virulence or T-DNA genes. Until now data are available only for pTiS4 (Slater et al. 2009) that does not allow us a comprehensive comparison of these regions of A. tumefaciens and A. vitis.

The bioinformatic and PCR analysis of virulence regions suggested us that the presence (in A. tumefaciens) or absence (in A. vitis) of virH2 and virK genes are common and basic differences between A. tumefaciens and A. vitis pTis. The virH2 protein detoxifies the phenolic compounds formed after wounding plant tissues (Brencic et al. 2004). It looks unlikely that a small piece of stem segments produces sufficient amounts of phenolics to prevent transformation. The second gene, virK, also does not seem to be a basic virulence factor (Kalogeraki and Winans 1998). It is also possible that the T-DNA genes of A. vitis are transferred but, under the used circumstances, their expression prevents the growth of transformed cells. Thus further studies should be carried out to find which genes contribute to, or prevent tumor formation on in vitro grapevine stem segments.

Besides the bacterial virulence factors, genetic transformation of plants by Agrobacterium involves several host genes (proteins) as well (Gelvin 2010, Magori and Citovsky 2012, Tzera and Citovsky 2008). We should also consider that these contributing plant proteins are not produced in grapevine explants like stem segments or leaf discs. Manipulating such host factors may help us to understand grapevine-A. vitis interaction as well as to design strategies for crown gall resistance.

Another possibility is that competent cells of the stem segments and leaf discs (embryogenic calli) do not survive cocultivation with A. vitis. A. vitis produces polygalacturonase (pehA), a cell wall degrading enzyme encoded by a chromosomal gene. The pehA minus mutant strain CG50 derived from the A. vitis nopaline strain CG49 (Rodriguez-Palenzuela et al. 1991) showed the same negative reaction on grapevine stem segments as its wild type parent (Szegedi and Burr, unpublished observations). Besides polygalacturonase production A. vitis also induces tissue necrosis by a quorum-sensing regulated manner (Zhang et al. 2003). The necrosis-minus (aviR-) mutant of A. vitis S4 was also non-tumorigenic on grapevine stem segments like its wild type S4 (Szegedi and Burr, unpublished observations). Thus the chromosomally encoded tissue necrosis induced by A. vitis probably is not the key factor in the determination of non-tumorigenic response of grapevine explants to A. vitis.

Taken together, the susceptibility of intact grapevines and explants to A. vitis differs. A similar phenomenon was described for Kalanchoe daigremontiana stem segments inoculated with the A. tumefaciens octopine strain B6S3 and nopaline strain C58. Both strains induce tumors on intact Kalanchoe plants, but only B6S3 transformed its stem segments as shown by LpDH activity (octopine production). Additionally, B6S3 complemented the lacking avirulence of C58 (Otten 1982). This difference between the transforming ability of B6S3 and C58 was shown due to their different virF functions (Otten et al. 1985).

Our results suggest that specific adaptation of A. vitis to grapevine is primarily determined by physiological and metabolic factors, e.g. the ability of tartrate utilization from the bacterial side (Kado 1998, Salomone et al. 1998).
and the production of tartrate from the host side (Ruffner 1982) rather than by the host-specific virulence properties of the pathogen. Additionally, we confirm the previous observations (Torregrrosa et al. 2002) showing that A. vitis cannot be efficiently used as a tool for introduction foreign genes into grapevines, unless the avirulence of A. vitis on in vitro explants is determined by T-DNA genes. The method described previously (Huss et al. 2010, Krastanova et al. 2010) and here may provide an easy assay to test various helper plasmids for their utility for gene introduction into a given grapevine genotype. On the other hand, we show that stem segment assays cannot be routinely used to select A. vitis-resistant genotypes from natural or transgenic populations.

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References


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