Transgenic Res (2010) 19:949–958
DOI 10.1007/s11248-010-9373-x

ORIGINAL PAPER

Resistance to crown gall disease in transgenic grapevine rootstocks containing truncated *virE2* of *Agrobacterium*

Stoyanka V. Krastanova · Vasudevan Balaji · Michele R. Holden · Mary Sekiya · Baodi Xue · Esengul A. Momol · Thomas J. Burr

Received: 3 April 2009 / Accepted: 29 January 2010 / Published online: 25 February 2010

Abstract A truncated form of the Ti-plasmid *virE2* gene from *Agrobacterium tumefaciens* strains C58 and A6, and *A. vitis* strain CG450 was transferred and expressed in somatic embryos of grapevine rootstocks 110 Richter (*Vitis rupestris* × *V. berlandieri)*, 3309 Couderc (*V. rupestris* × *V. riparia*) and Teleki 5C (*V. berlandieri* × *V. riparia*) via *Agrobacterium*-mediated transformation to confer resistance to crown gall disease. Transformation was confirmed in 98% of the 322 lines by enzyme-linked immunosorbent assay for the neomycin phosphotransferase II protein and 97% of 295 lines by polymerase chain reaction for the truncated *virE2* transgene. Southern blot analysis revealed the insertion of truncated *virE2* at one to three loci in a subset of seven transgenic 110 Richter lines. In vitro resistance screening assays based on inoculations of shoot internode sections showed reduced tumorigenicity and very small galls in 23 of 154 transgenic lines. Non-transformed controls had a 100% tumorigenicity rate with very large galls. Disease resistance assay at the whole plant level in the greenhouse revealed seven transgenic lines (3 lines of 110 Richter, 2 lines of 3309 Couderc and 2 lines of Teleki 5C) were resistant to *A. tumefaciens* strain C58 and *A. vitis* strains TM4 and CG450 with a substantially reduced percentage of inoculation sites showing gall as compared to controls. No association was found between the level of resistance to crown gall disease and the source *Agrobacterium* strain of *virE2*. Taken together, our data showed that resistance to crown gall disease can be achieved by expressing a truncated form of *virE2* in grapevines.

Keywords Truncated *virE2* · Crown gall disease · Grapevine · *Agrobacterium tumefaciens* · *Agrobacterium vitis*

Electronic supplementary material The online version of this article (doi:10.1007/s11248-010-9373-x) contains supplementary material, which is available to authorized users.

S. V. Krastanova · V. Balaji · M. R. Holden · M. Sekiya · B. Xue · E. A. Momol · T. J. Burr (✉)
Department of Plant Pathology and Plant-Microbe Biology, Cornell University, New York State Agricultural Experiment Station, Geneva, NY 14456, USA
e-mail: tjb1@cornell.edu

Introduction

Crown gall is a serious bacterial disease of grapevines worldwide. It is caused by *A. vitis* and to a lesser extent by *A. tumefaciens* (Pearson and Goheen 1988; Burr et al. 1990). Crown gall can cause significant losses by reducing vigor and yield (30–50%) (Schroth et al. 1988). The infection process has been studied extensively (Burr et al. 1998; Burr and Otten 1999). Tumors form on canes, usually on the lower region of the trunk, near grafting sites. They are initiated following injuries caused by freezing temperatures (Burr and Katz 1984). On young vines crown gall is

particularly damaging and may cause death. The natural host range of *A. vitis* is restricted to grape. *A. vitis* survives systemically in vines and can be disseminated in symptomless propagation material. Several important grapevine rootstocks are susceptible to crown gall, among which 110 Richter (*V. rupestris* × *V.* berlandieri) and Teleki 5C (*V. berlandieri* × *V. riparia)*, are highly susceptible (Stover et al. 1997; Sule and Burr 1998).

Genetic engineering is an attractive approach to develop disease-resistant grapevine rootstocks. Several transgenic rootstock genotypes have been developed for resistance to viruses including *V. rupestris* cv. St. George, 110 Richter (*Vitis rupestris* × *V. berlandieri)*, 41B (*V. berlandieri* × *V. rupestris*, 3309 Couderc (*V. rupestris* × *V. riparia*), Teleki 5C, *V. riparia* cv. Gloire de Montpellier (*V. riparia*) and 101-14 Millardet et de Grasset (*V. riparia* × *V. rupestris*) (Colova Tsolova et al. 2001). In the case of crown gall, transgenic *V. riparia* cv. Gloire de Montpellier lines expressing a truncated *virE2* gene from *A. tumefaciens* strain C58 gene were generated (Xue et al. 1999). A truncated *virE2* construct lacking the 215 C-terminal amino acids was selected to confer resistance to crown gall disease in grapevine rootstocks based on a previous report showing reduced tumorgenicity in transgenic tobacco plants (Citovsky et al. 1994). To expand on our early attempts to engineer resistance to crown gall disease (Holden et al. 2003; Xue et al. 1999), we developed new grapevine rootstocks containing truncated *virE2* constructs with the aim of achieving practical resistance to *A. vitis*. Here, we report the transformation of the grapevine rootstocks 110 Richter, 3309 Couderc and Teleki 5C with truncated *virE2* constructs from *A. tumefaciens* strains C58 and A6, and from *A. vitis* strain CG450, and on the reaction of transgenic plants to infection by homologous and heterologous *Agrobacterium* strains.

Materials and methods

Construction of binary plasmid containing *virE2* expression cassette

An *Agrobacterium virE2* gene lacking the region coding for a single-stranded DNA binding domain, as previously described (Citovsky et al. 1992), was engineered from *A. tumefaciens* strains C58 and A6 and from *A. vitis* CG450. The *virE2* coding sequence from *A. tumefaciens* strain C58 (Hiroka et al. 1987) was amplified from its nopaline catabolic Ti-plasmid by polymerase chain reaction (PCR) with primers NOP-forward (5′ TACTTACCATGGATCCGAAGG CCGAAGGC 3′) corresponding to the 5′ coding region of the gene and NOP-reverse (5′ CTTGACCATGG**C TA**TCGATTCTCGC CGGCGAACTC 3′) hybridizing to nucleotide positions 1,000–1,020 of the gene to produce *virE2*-C58. Likewise, *virE2*-A6 was amplified by PCR from octopine catabolic Ti-plasmid from *A. tumefaciens* strain A6 (Winans et al. 1987) using the primer pair OCT-forward (5′ TACTTACCATGG ATCTTTCTGGCAATGAG 3′) corresponding to the 5′ coding region of octopine *virE2* and OCT-reverse (5′ AGATTCCCATGG**TCA**TC TATTTTCGCCAA CAAATTCCGCG 3′) hybridizing to nucleotide positions 927-951 of the gene. The *virE2* construct of *A. vitis* strain CG450 (Momol et al. 1998) was amplified by using primers NOP-forward and NOP-reverse. All the primers contained a *Nco*I site (underlined) to facilitate subsequent cloning. In addition, a translation stop codon (in bold) was incorporated into primers NOP-reverse and OCT-reverse to ensure translational termination of the truncated *virE2* constructs. Thus, expression of chimeric *virE2* yielded proteins lacking the single strand DNA binding region.

The three truncated *virE2* PCR products were digested with *Nco*I and directly cloned into the *Nco*I-restricted plant expression vector pEPT8 (Ling et al. 1997). The resulting plasmids were designated as pEPT8-virE2-C58, pEPT8-virE2-A6 and pEPT8-virE2-CG450. The *virE2* gene expression was regulated by the *Cauliflower mosaic virus* (CaMV) 35S promoter and CaMV 35S 3′ poly (A) signal. An *Alfalfa mosaic virus* (AIMV) leader sequence was placed upstream of the truncated *virE2* gene for enhanced expression (Ling et al. 1997). The expression cassette was excised from these plasmids with *Hin*dIII and ligated to the plant transformation vector pBIN19 (Bevan 1984) that was cut with the same restriction enzyme. The resulting vectors pBIN19-virE2-C58; pBIN19-virE2-A6 and pBIN19-virE2-CG450 (Fig. S1) were transferred to *A. tumefaciens vir* helper strain C58sZ707 by electroporation.

Plant material and anther cultures

Immature inflorescences of 110 Richter (*V. rupestris* × *V. berlandieri*), 3309 Couderc (*V. riparia* × *V. rupestris*) and Teleki 5C (*V. berlandieri* × *V. riparia*) were collected about 2 weeks before flowering. Anthers were dissected and cultured as described previously (Bouquet et al. 1982). Anthers were isolated from sterilized flower buds when pollen mother cells were at the uninucleate (GP1) or binucleate (GP2) developmental stages and plated on full strength basal Murashige and Skoog (1962) (MS) medium at a density of 40 floral buds per Petri dish (diameter 9 cm) and incubated at 28°C in the dark.

Induction of somatic embryogenesis and plant development

Induction of somatic embryogenesis and subculturing of embryogenic calli was carried out on solid, full strength MS medium containing 20 g/l sucrose, 0.2 mg/l 6-benzyl-aminopurine (BAP) and 1.1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) (induction medium) in the dark at 28°C (Krastanova et al. 1993, 1995, 2000). The pH was adjusted to 5.7 and Noble agar was added to a final concentration of 8 g/l. Cultures were maintained by transferring clumps of embryos to induction medium at 20-day intervals for calli multiplication. After 30–45 days, only secondary calli were transferred to half-strength MS medium supplemented with 3.6 ml/l glycerol for embryo differentiation. After 3 weeks, cultures were transferred to solid MS medium containing 1 g/l casein hydrolysate and 3.6 ml/l glycerol for embryo elongation. After 1–2 months elongated hypocotyls were transferred to the woody plant medium (WPM) (McCown and Lloyd 1981) supplemented with 0.1 mg/l BAP, 3 g/l activated charcoal, and 15 g/l sucrose, and grown at 25°C with a 16 h photoperiod under cool fluorescent light at 54 $\mu Em^{-2}\ s^{-1}$ to induce shoot and root formation. The pH of WPM was adjusted to 5.7 and 6 g/l Bacto agar (Sigma A-1296) was added for solidification. After 3–4 weeks, well-rooted and elongated plantlets were transplanted to Cornell potting mix (Boodley and Sheldrake 1982) and acclimatized to green house conditions following standard practices. After the first-year evaluation, plants were prepared for dormancy and then moved into storage at 4°C for 3 months. After dormancy, potted plants were pruned, fertilized and grown in the greenhouse for a second year evaluation.

Agrobacterium-mediated transformation and regeneration of putative transgenic grapevine rootstocks

Embryogenic calli of 110 Richter, Teleki 5C and 3309 Couderc were infected with *A. tumefaciens* strain C58sZ707 carrying the binary construct pBin19-EPT8-VirE2-C58 or pBin19-EPT8-VirE2-A6 or pBin19-EPT8-VirE2-CG450. A single recombinant *Agrobacterium* colony was grown overnight in 30 ml LB medium with 50 mg/l kanamycin and 100 mg/l acetosyringone at 28°C. The culture was centrifuged at 3,000×*g* for 10 min and the pellet suspended in half-strength MS with 100 mg/l acetosyringone to an OD_{600nm} of 0.5-0.7. Homogenous early globular embryos were immersed in the *Agrobacterium* suspension for 20 min, briefly blotted on sterile Whatman filter paper and transferred onto MS (induction medium) in the dark at 25°C. After 2 days of co-cultivation, embryogenic calli were fragmented and transferred to selective MS medium supplemented with 25 mg/l kanamycin, 300 mg/l cefotaxime, 200 mg/l carbenicillin and 100 mg/l acetosyringone in 90 mm plates. Calli were cultured at 28°C in the dark with subculturing every 20 days. After the first 3 weeks of culture, acetosyringone was not used anymore. After 6–8 weeks, newly formed secondary calli were transferred to half-strength MS containing 10 g/l sucrose, 3.6 ml/l glycerol, 8 g/l Noble agar amended with 25 mg/l kanamycin, 300 mg/l cefotaxime and 200 mg/l carbenicilin. After embryo differentiation, clumbs of globular and torpedo structures were transferred on MS with 20 g/l sucrose, 1 g/l casein hydrolysate and 3.6 ml/l glycerol supplemented with 25 mg/l kanamycin, 300 mg/l cefotaxime and 200 mg/l carbenicilin for hypocotyl elongation. Four months after transformation, elongated hypocotyls were transferred to WPM (McCown and Lloyd 1981) (Sigma-M6774, St. Louis, MO) containing 15 g/l sucrose, 3 g/l activated charcoal, 0.1 mg/l BAP and 6 g/l bactoagar (Sigma-A1296, St. Louis, MO) for plant regeneration (Krastanova et al. 2000). Single embryo-derived plantlets were micropropagated on the same medium by subculturing apical cuttings to produce individual plants.

Molecular characterization of putative transgenic plants

Putative transgenic plants were identified based on their ability to grow on kanamycin-containing selection medium and by detecting the expression of the neomycin phosphotransferase II (NPTII) protein in double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (5′-3′). Presence of transgenes was characterized by polymerase chain reaction and Southern blot hybridization, respectively with total plant DNA isolated from leaves of greenhouse-grown plants as described previously (Lodhi et al. 1994). For Southern blot hybridization, 20 μg of total DNA was digested with *Sac*I (Promega, Madison, WI), resolved by electrophoresis on 1% (w/v) agarose gels in 1× Tris–borate buffer and blotted onto Nytran N filter (Schleicher and Schuell, Keene, NH) according to the manufacturer's instructions. Blotted DNA was fixed by baking at 80°C for 1 h. Prehybridization was performed for 2 h. Transgene insertion was detected by hybridization with a [α-^{32}P(dCTP)]-labeled *virE2* gene probe, obtained using the RadPrime DNA labeling system (Gibco BRL, Gaithersburg, MD), as described (Sambrook et al. 1989).

Disease resistance assays

Plants of transgenic and non-transgenic lines were assayed for crown gall resistance using *A. tumefaciens* strains C58 (nopaline) and A6 (octopine), and *A. vitis* strain CG450 (vitopine) as inoculum. Bacterial inocula were grown at 28°C for 2 days on solid potato dextrose agar (PDA), suspended in sterile distilled H_2O to an optical density of 0.1 at A_{600nm} which is approximately 1.0×10^8 cfu/ml, and diluted to a concentration of about 1.0×10^6 cfu/ml. Resistance assays were performed first in vitro with internode sections from greenhouse-grown plants over a period of four consecutive years. Internode sections, 7–10 mm in length, were submerged for 1 h in *A. tumefaciens* suspensions or sterile distilled water, and transferred on half-strength B5 medium (Gamborg et al. 1968) for 2 days in the dark. Then internode segments were transferred to half-strength B5 medium containing 200 mg/ml cefotaxime and the fungicide Bravo (chlorothalonil—weather stik, 720 mg/l) at 25°C with a 16 h photoperiod under cool fluorescent light at 54 $\mu Em^{-2}\ s^{-1}$. An average of 20 internode segments was tested for each transgenic line each year. Susceptibility to *Agrobacterium* was evaluated by visual observation of tumor formation at 14 and 21 days post-infection (dpi). Tumor size was determined based on the following disease index: no galls (0 mm), small galls (1–2 mm), medium galls (2–4 mm), large galls (4–6 mm) and very large galls (>10 mm). Controls included internode segments from non-transgenic plants that were inoculated with *Agrobacterium* strains or with sterile distilled water. Transgenic plants were scored as resistant if no galls developed or half or less of the total explants developed smaller galls that were 1–2 mm diameter.

Resistance assays were also carried out on plants grown in a greenhouse by inoculation with *A. tumefaciens* strains C58 and *A. vitis* strains TM4 and CG450 (Otten et al. 1996) for two consecutive years. Greenhouse tests focused on plants from transgenic lines that showed resistance in vitro. Plants were screened for resistance after acclimatization from tissue culture during the first year and after dormancy and growth until a 20-node development stage during the second year. Inoculations were made closely below nodes by stab wounding tissue with a pin (0.8 mm in diameter) dipped into bacterial growth on PDA (Pu and Goodman 1992) beginning five nodes below the tip of growth on actively growing plants. Susceptibility, based on gall development, was evaluated 2 months after inoculation. Gall size was measured by following the same disease index used in in vitro assays. Controls included non-transgenic plants inoculated with *Agrobacterium* strains or with sterile distilled water. Transgenic plants were scored as resistant if no galls developed after 2 months or if half or less of the inoculated nodes developed galls that were small in size (1–2 mm).

Results

Generation and characterization of transgenic plants

Embryogenic calli derived from anthers of grapevine rootstocks 110 Richter, 3309 Couderc and Teleki 5C were transformed with truncated *virE2* sourced from *A. tumefaciens* strains C58 and A6, and *A. vitis* strain

CG450. The *virE2* expression cassette was contained in the pBIN19 binary vector backbone (Fig. S1). Putative transgenic plants were selected for further evaluation within 6–7 months following *Agrobacterium*-mediated transformation. About 322 putative transgenic lines were produced (33, 42 and 49 lines of 110 Richter; 20, 27 and 56 lines of 3309 Couderc; and 13, 39 and 43 lines of Teleki 5C with truncated *virE2* constructs from *A. tumefaciens* strains C58, and A6, and *A. vitis* strain CG450, respectively) on the basis of their ability to regenerate on selection medium and detectable expression of the neomycin phosphotransferase II (NPTII) protein by DAS-ELISA. Only a very limited number of 110 Richter (4%, 5 of 124) and 3309 Couderc (3%, 3 of 103) lines were negative for NPTII in DAS-ELISA, indicating that the selection conditions to recover transformed plants were stringent. To confirm the transgenic status of test plants, the insertion of the *virE2* constructs from *Agrobacterium* strains C58, A6 and CG450 was characterized by PCR and Southern blot.

Confirmation of T-DNA transfer and integration by PCR and Southern blot

About 295 lines of the three grapevine rootstock genotypes were analyzed by PCR with total plant DNA template and appropriate primers to detect the presence of truncated *virE2* transgene. Overall, expected *virE2* amplicons were obtained for 97% (285 of 295) of the transgenic lines tested by PCR, including all Teleki 5C lines. Only six transgenic lines of 3309 Couderc and four transgenic lines of 110 Richter that reacted positively for the NPTII protein in DAS-ELISA did not yield a *virE2* amplicon. As illustrated for eight 110 Richter transgenic lines, a DNA amplicon of the expected size (1,020 bp) was obtained (Fig. S2). No amplicon was obtained from DNA of non-transgenic plants (Fig. S2). Selected PCR-positive putative transgenic plants were further analyzed to determine transgene insertion loci and copies of T-DNA/plant junction fragments by Southern blot hybridization of a *virE2* probe from *Agrobacterium* strain C58 on *Sac*I-digested plant DNA. As shown in Fig. S1, *Sac*I is a unique site in the T-DNA of all the three binary constructs and the distance between the *Sac*I enzyme and right border (RB) is approximately 4 kb. Hence, the size of the right border junction fragment(s) is expected to be ≥4 kb. Plants of the seven transgenic 110 Richter lines tested showed insertion of the *virE2* construct at one to three loci (Fig. 1). As expected, the size of the *virE2* hybridizing fragments were at least 4.4 kb for all the lines tested, except line 828 for which a shorter truncated right border junction fragment was observed in addition to the major insertion fragment (Fig. 1).

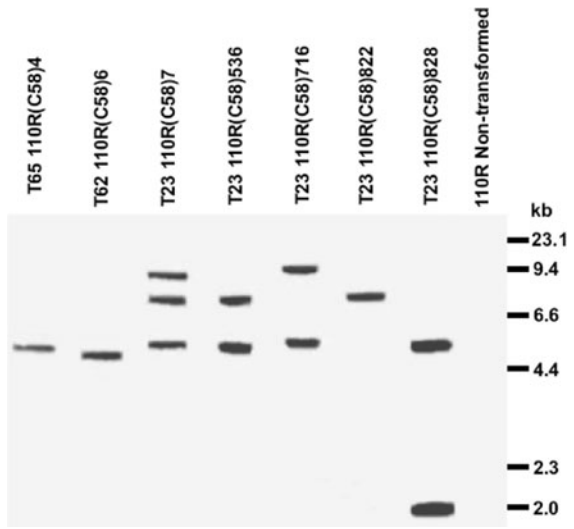

Fig. 1 Southern hybridization analysis of seven putative transgenic grapevine rootstock 110 Richter lines transformed with a pBIN19 binary plasmid encompassing truncated *virE2* derived from *A. tumefaciens* strain C58. Genomic plant DNA was restricted with *Sac*I, blotted onto a nylon membrane and hybridized with radio-labeled *virE2* as the probe. Total DNA from a non-transformed plant was used as a control. Signals obtained indicated the right border T-DNA/plant junction fragment(s). The hybridization patterns showed an insertion of the *virE2* construct at one to three loci. Lambda DNA digested with *Hin*dIII was used as the size marker

Reaction of transgenic rootstocks to inoculation with three *Agrobacterium* strains

The susceptibility to crown gall of a subset of 154 transgenic lines was evaluated by in vitro inoculation of shoot internode explants with *A. tumefaciens* strains C58 and A6 and with *A. vitis* strain CG450. This subpopulation of transgenic plants represented half of the transgenic population produced. Non-transgenic controls consistently developed very large (>10 mm) size galls between 14 and 21 days post-inoculation (dpi). Some transgenic lines exhibited resistance with half or less of the inoculated explants

developing galls. Of the 80 transgenic 3309 Couderc lines tested, ten were resistant to *Agrobacterium* infection (Table S1). Although the level of resistance varied among lines, resistance was observed for infection with both homologous and heterologous strains. For instance, transgenic line 46 expressing *virE2*-C58 showed an infection rate of 0, 15 and 20% when challenged with strains A6, C58 and CG450, respectively. Similarly, transgenic line 45 containing *virE2*-CG450 showed an infection rate of 2, 10 and 20% upon inoculation with strains C58, CG450 and A6, respectively. Interestingly, a higher level of resistance was achieved against the homologous *Agrobacterium* strain in majority of transgenic lines containing *virE2*-C58 and *virE2*-A6 (Table S1). In contrast, a higher level of resistance was obtained against the heterologous *Agrobacterium* strain C58 in transgenic lines containing *virE2*-CG450 (Table S1).

Of the 48 transgenic 110 Richter lines expressing *virE2*-C58 that were tested, seven lines exhibited resistance (Table 1). These seven lines were confirmed by Southern analysis to contain one to three copies of the *virE2* transgene (Fig. 1). Resistance to crown gall disease was higher in transgenic lines challenged with the cognate *Agrobacterium* strain than with heterologous strains. For instance, transgenic line 7 expressing *virE2*-C58 showed a 7, 12 and 22% infection rate following inoculation with *Agrobacterium* strains C58, CG450 and A6, respectively. A similar pattern was observed for the six other independent transgenic lines tested (Table 1). Of the 26 transgenic Teleki 5C lines screened for resistance to crown gall disease by in vitro inoculation assay, six were found to be resistant (Table 2). Interestingly, no association was found between the level of resistance and the source strain of *virE2* for this resistant genotype.

Table 1 Reaction of transgenic 110 Richter expressing a truncated *virE2* gene from *Agrobacterium* strain C58 to in vitro inoculation with *A. tumefaciens* strains C58 and A6, and *A. vitis* strain CG450

Transgenic line	Challenge *Agrobacterium* strain		
	C58	A6	CG450
828	9/82[a] (11)[b]	17/41 (41)	15/40 (37)
822	23/83 (27)	14/38 (36)	16/40 (40)
716	24/114 (21)	15/40 (37)	10/38 (26)
536	16/78 (20)	12/38 (31)	16/40 (40)
7	6/82 (7)	9/40 (22)	5/40 (12)
6	18/80 (22)	5/40 (12)	9/38 (23)
4	14/79 (17)	7/38 (18)	12/58 (20)
Control	23/23 (100)	17/17 (100)	19/19 (100)

[a] Number of shoot internode segments showing galls over the total number of shoot internode segments inoculated

[b] Percentage of shoot internode segments with galls

Transgenic grapevine rootstock lines with the highest level of resistance to *Agrobacterium* infection as revealed in the in vitro screening tests were further evaluated in the greenhouse for resistance by challenge inoculation with *A. tumefaciens* strain C58 and *A. vitis* strains TM4 and CG450 (Table 3). The objective of these experiments was to test whether resistance to *Agrobacterium* infection is manifested at the plant level. Two *A. vitis* strains were selected as inocula because our primary goal was to engineer practical resistance to the main causal agent of crown gall disease. All the VirE2-C58 expressing transgenic lines tested displayed significantly higher level of resistance to infection with the cognate strain C58 (Table 3) which was the source of *virE2* transgene. However, the transgenic lines exhibited a relatively lower level of resistance upon infection with heterologous *A. vitis* strains TM4 and CG450 as shown by an increase in the percentage of explants forming galls (Table 3). Transgenic 110 Richter lines 4, 6 and 828 were resistant to infection by the three *Agrobacterium* strains as well as transgenic 3309 Couderc lines 46 and 69 and Teleki 5C lines 6 and 11 (Table 3). In addition, plants of these resistant lines showed reduced gall size (1–6 mm) as shown for the 110 Richter line 828 (Fig. 2b). These results confirm the observations documented from in vitro resistance screening assays with internode explants. Notwithstanding, transgenic 110 Richter lines 7, 536, 716 and 822 were resistant to *A. tumefaciens* but not to one or the two *A. vitis* strains (Table 3). Overall, the level of resistance varied among transgenic lines and the percentage of transgenic plants that developed galls was significantly lower than those of non-transformed control (Table 3). Moreover, the gall size was very large (>10 mm) in non-transformed control plants (Fig. 2c) which developed galls after 20 dpi. In contrast, transgenic plants showed signs of gall formation only after 30–35 dpi and their gall size was reduced (1–6 mm) (Fig. 2b). Some of the transgenic lines such as 716 exhibited galls at a much later stage 40 days after inoculation with

Table 2 Reaction of transgenic Teleki 5C expressing a truncated *virE2* gene from *Agrobacterium* strains C58 and A6, and *A. vitis* strain CG450 to in vitro inoculation with homologous and heterologous strains

Agrobacterium source of the *virE2* transgene	Line	Challenge *Agrobacterium* strain		
		C58	A6	CG450
C58	6	2/40[a] (5)[b]	4/20 (20)	5/40 (12)
	11	6/40 (15)	4/40 (10)	7/40 (17)
A6	2	5/40 (12)	8/40 (20)	6/38 (15)
	16	10/38 (26)	8/30 (26)	10/40 (25)
CG450	1	4/20 (20)	4/40 (10)	2/20 (10)
	15	10/40 (25)	15/40 (38)	6/40 (15)
Control	NA	38/40 (95)	36/38 (95)	40/40 (100)

[a] Number of shoot internode segments showing galls over the total number of shoot internode segments inoculated

[b] Percentage of shoot internode segments with galls

Table 3 Reaction of transgenic 110 Richter, 3309 Couderc and Teleki 5C expressing a truncated *virE2* gene from *Agrobacterium* strain C58 to inoculation with homologous and heterologous strains in the greenhouse

Genotype	Line	Challenge *Agrobacterium* strains		
		C58	TM4	CG450
110 Richter				
	828	5/40[a] (12)[b]	1/38 (3)	2/55 (3)
	822	8/40 (20)	11/40 (27)	32/35 (92)
	716	1/65 (1)	26/38 (68)	2/30 (7)
	536	3/40 (8)	22/40 (55)	9/41 (21)
	7	1/60 (2)	24/42 (57)	8/44 (18)
	6	10/45 (22)	10/43 (23)	11/40 (27)
	4	8/40 (20)	14/40 (35)	10/40 (25)
	Control	40/40 (100)	38/38 (100)	40/40 (100)
3309 Couderc				
	46	11/60 (18)	10/45 (22)	11/46 (24)
	69	6/40 (15)	10/40 (25)	8/40 (20)
	Control	49/50 (98)	40/40 (100)	45/45 (100)
Teleki 5C				
	6	4/40 (10)	9/40 (23)	8/45 (18)
	11	8/40 (20)	11/45 (24)	9/44 (20)
	Control	40/40 (100)	38/38 (100)	45/45 (100)

[a] Number of internode showing galls over the total number of internode inoculated

[b] Percentage of internode with galls

A. vitis strain TM4. Most plants of transgenic line 716 inoculated with *A. tumefaciens* strain C58 (98.5%, 64 out of 65 plants) had no galls; only one plant had small galls (1–2 mm). Plants of transgenic lines 828 had small size galls after inoculation with *A. vitis* strains TM4 and CG450, but developed medium size galls after inoculation with *A. tumefaciens* strain C58. These results reveal different level of resistance conferred by *virE2* gene expression in transgenic grapevine plants. Consistently, gall formation in transgenic plants was delayed by at least 15–20 days when compared to the non-transformed controls. Taken together, our data clearly showed that resistance to crown gall disease can be achieved by expressing truncated *virE2* constructs in grapevine rootstocks.

Discussion

Different strategies have been deployed to engineer resistance to crown gall disease in grapevines and other plants. Some examples include engineered resistance based on the expression of antimicrobial peptides in *Vitis vinifera* 'Chardonnay' (Vidal et al. 2006), by expression of *virE2* lacking the C-terminus single-stranded binding domain in tobacco (Citovsky et al. 1994), antisense-mediated suppression of host factors associated with T-DNA trafficking, integration and cell transformation in *Arabidopsis thaliana* (Ward et al. 2002; Zhu et al. 2003), silencing of oncogenes present in the T-DNA (Escobar et al. 2001, 2002) and exogenous application of the phytohormone salicylic acid or its analogs before the onset of crown gall disease presents a possible means for achieving durable disease control (Anand et al. 2008).

The initial attempt to develop resistance to crown gall was by over-expressing a truncated *virE2*

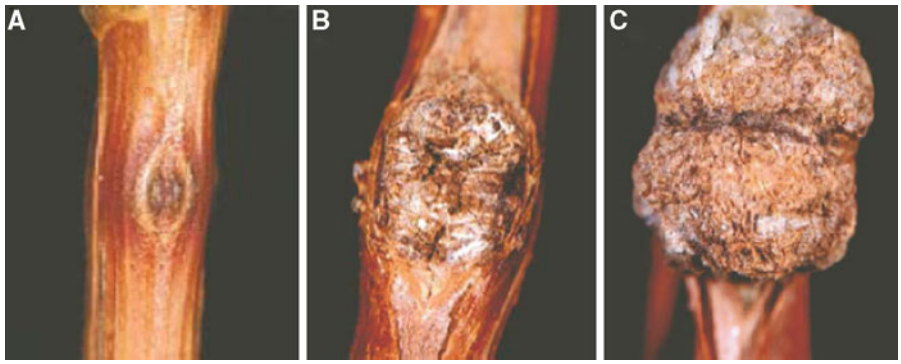

Fig. 2 Reaction of *virE2*-C58 containing transgenic grapevine rootstock 110 Richter to inoculation with *A. vitis* strains TM4 and CG450 in the greenhouse. Inoculated plants were scored for gall formation over a period of two consecutive years. Non-transformed plants were challenge-inoculated with *A. vitis* and included as control. **a** Transgenic line with no gall formation (0 mm). **b** Transgenic line with reduced gall (6 mm). **c** Non-transformed control plant with very large gall (>10 mm)

construct from *A. tumefaciens* strain A6 in transgenic tobacco plants. Transgenic plants were resistant to crown gall disease by developing fewer tumors than wild type controls (Citovsky et al. 1994). Though the molecular mechanism underlying resistance mediated by a chimeric *virE2* is unclear, it is hypothesized that the truncated VirE2 protein, devoid of a single stranded DNA-binding domain, competes with the wild type VirE2 of *A. tumefaciens* following infection for VirE2-interacting host factors essential for T-DNA transformation, such as VIP1 (Tzfira et al. 2001; Ward et al. 2002) and VIP2 (Anand et al. 2007) and possibly titrates them.

Here, we reported a practical application of the *virE2*-mediated resistance in an economically important commercial crop such as grapevine. We generated transgenic grapevine rootstocks expressing a *virE2* C-terminus deletion derivative from *A. tumefaciens* strains A6 and C58, and *A. vitis* strain CG450 and showed high level of resistance to crown gall disease in some of these transgenic lines. None of the transgenic grapevine rootstocks was immune to *Agrobacterium* infection except line 716 under the conditions used (Table 3). Resistant transgenic lines showed various levels of gall development and their size was generally much reduced than those of non-transformed controls. Furthermore, gall formation was delayed by at least 15–20 days in transgenic lines when compared to non-transformed control plants. Resistance was expressed as reduction in both the number of plants showing tumors and gall size, confirming previous observations (Citovsky et al. 1994).

Molecular characterization of seven 110 Richter transgenic lines by Southern analysis revealed integration of *virE2*-C58 at one to three loci. These plants along with those harboring either *virE2*-A6 or *virE2*-CG450 displayed tumors of significantly reduced size than non-transformed controls in the in vitro resistance screening assays and inoculation tests in the greenhouse. In addition, tumors developed much earlier and at a faster rate in non-transformed control plants compared with transgenic plants. The level of resistance exhibited by transgenic 110 Richter lines harboring *virE2*-C58 was higher upon challenge inoculation with *A. tumefaciens* strain C58 than with heterologous strains A6, TM4 and CG450 (Tables 1, 3). However, transgenic line 828 displayed relatively higher level of resistance to inoculation with all the three strains (C58, CG450 and TM4) tested (Table 3). Molecular analysis of transgenic line 828 showed a truncated right border junction fragment in addition to the expected full-length T-DNA copy. The presence of the truncated T-DNA copy raises the possibility that it might be linked to the broad-spectrum resistance observed in transgenic line 828. Similarly, two transgenic lines of Teleki 5C and 3309 Couderc were resistant to the three *Agrobacterium* strains used as inoculum, including the two *A. vitis* strains. Those lines displayed resistance to gall development under in vitro inoculation assays and greenhouse conditions. Resistance at the whole plant level was maintained for a period of two consecutive years. Interestingly, the gall size lowered over time. This observation is in contrast to the report by Vidal et al. (2006) where in

larger gall size was observed in the second year compared with the first year.

The *virE2* of *A. tumefaciens* strains exhibits significant sequence homology at the nucleotide and amino acid levels. In silico analysis of the *virE2* nucleotide and amino acid sequence of strains *A. tumefaciens* strains C58 and A6 showed an identity of 70.6 and 67.8%, respectively (data not shown). Based on the similarity at the molecular level, we anticipated that *virE2* derived from a given strain could confer resistance to gall formation incited by not only the cognate strain but also by heterologous strains. Our hypothesis was well supported by our results obtained with transgenic 110 Richter lines expressing VirE2-C58, which displayed resistance not only to challenge inoculation with cognate *Agrobacterium* strain C58, but also to A6 and *A. vitis* strain CG450. Our observation is in contrast to a previous study where transgenic tobacco expressing VirE2-C58 displayed resistance when challenged with the cognate *A. tumefaciens* strain C58 but developed tumors upon inoculation with *A. tumefaciens* strain A6 (Citovsky et al. 1994). Taken together, it is evident from our results that truncated *virE2* can be used to develop resistance to crown gall disease in grapevine where it is still a serious and persisting problem.

Lately, RNAi-mediated resistance is being employed to develop durable resistance in tree, nut and fruit crops, including grapevines. It is suggested to provide a broad-spectrum resistance to a wide-range of *Agrobacterium* biovars because of the highly conserved tumor-inducing hormone synthesis genes across species (Escobar et al. 2003; Viss et al. 2003). With the current developments in the elucidation of *Agrobacterium*-plant interactions, new insights are gained into the infection mechanism of the bacterium, enabling the development of novel and effective strategies to control crown gall disease. The use of *virE2* against the bacteria also has clear potential as a powerful and useful technology to engineer resistance to crown gall. Field tests of transgenic lines showing resistance need to be validated before commercialization of this technology can be realized.

Acknowledgments We are grateful to Dr. Marc Fuchs for critically reading the manuscript.

References

Anand A, Krichevsky A, Schornack S, Lahaye T, Tzfira T, Tang Y, Citovsky V, Mysore KS (2007) *Arabidopsis* VirE2 interacting protein2 is required for *Agrobacterium* T-DNA integration in plants. Plant Cell 19:1695–1708

Anand A, Uppalapati SR, Ryu CM, Allen SN, Kang L, Tang Y, Mysore KS (2008) Salicylic acid and systemic acquired resistance play a role in attenuating crown gall disease caused by *Agrobacterium tumefaciens*. Plant Physiol 146:703–715

Bevan M (1984) Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res 12:8711–8721

Boodley JW, Sheldrake R (1982) Cornell peat lite mixes for commercial plant growing. Cornell Univ Coop Ext Ithaca NY. Info Bul 43

Bouquet A, Piganeau AM, Lamaison AM (1982) Influence du genotype sur la production de cals d'embryoides et de plants entières. CR Acad Sci Paris 295:574–596

Burr TJ, Katz BH (1984) Grapevine cuttings as potential sites of survival and means of dissemination of *Agrobacterium tumefaciens* and *A. radiobacter* biovar 3. Phytopathology 77:1424–1427

Burr TJ, Otten L (1999) Crown gall of grape: biology and disease management. Annu Rev Phytopathol 37:53–80

Burr TJ, Norelli J, Rats B, Bishop A (1990) Use of Ti plasmid DNA probes for determining tumorgenicity of *Agrobacterium* strains. Appl Environ Microbiol 6:1782–1785

Burr TJ, Bazzi C, Sule S, Otten L (1998) Biology of *Agrobacterium vitis* and the development of disease control strategies. Plant Dis 82:1288–1297

Citovsky V, Zupan J, Warnick D, Zambryski P (1992) Nuclear localization of *Agrobacterium* VirE2 protein in plant cells. Science 256:1802–1805

Citovsky V, Warnick D, Zambryski P (1994) Nuclear import of *Agrobacterium* VirD2 and VirE2 proteins in maize and tobacco. Proc Natl Acad Sci USA 91:3210–3214

Colova Tsolova V, Perl A, Krastanova S, Tsvetkov I, Atanassov A (2001) Molecular biology and biotechnology of the grapevine. Roubelakis-Angelakis KA (ed) Kluwer, pp 411–443

Escobar MA, Civerolo EL, Summerfelt KR, Dandekar AM (2001) RNAi-mediated oncogene silencing confers resistance to crown gall tumorigenesis. Proc Natl Acad Sci USA 98:13437–13442

Escobar MA, Lesli CA, McGranahan GH, Dandekar AM (2002) Silencing crown gall disease in walnut (*Juglans regia* L.). Plant Sci 163:591–597

Escobar MA, Civerolo EL, Polito VS, Pinney KA, Dandekar AM (2003) Characterization of oncogene-silenced transgenic plants: implication for *Agrobacterium* biology and post-transcriptional gene silencing. Mol Plant Pathol 4:57–65

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:1515–1518

Hiroka T, Rogowsky PM, Kado CI (1987) Characterization of the *virE* locus of *Agrobacterium tumefaciens* plasmid pTiC58. J Bacteriol 169:1529–1536

Holden M, Krastanova S, Xue B, Pang S, Sekiya M, Momol EA, Gonsalves D, Burr TJ (2003) Genetic engineering of grape for resistance to crown gall. Acta Hortic 603: 481–484

Krastanova S, Walter B, Perrin M, Bardonnet N, Pinck L, Otten L (1993) Transfer and expression of the coat protein gene of grapevine fanleaf virus in grapevine. Extended abstracts of the 11th meeting ICVG, Montreux, Switzerland, pp 79–80

Krastanova S, Perrin M, Barbier P, Demangeat G, Cornuet P, Bardonnet N, Otten L, Pink L, Walter B (1995) Transformation of grapevine rootstock with the coat protein gene of grapevine fanleaf nepovirus. Plant Cell Rep 14:550–554

Krastanova S, Ling K, Zhu H, Xue B, Burr T, Gonsalves D (2000) Development of transgenic grapevine rootstocks with genes from grapevine fanleaf virus and grapevine leafroll associated closteroviruses 2 and 3. Acta Hortic 528:367–372

Ling KS, Zhu HY, Alvizo H, Hu JS, Drong RF, Gonsalves D (1997) The coat protein gene of grapevine leafroll associated closterovirus-3: cloning, nucleotide sequencing and expression in transgenic plants. Arch Virol 142:1101–1116

Lodhi MA, Ye GN, Weeden NF, Reisch BI (1994) A simple and efficient method for DNA extraction from grapevine cultivars and *Vitis* species. Plant Mol Biol Rep 12:6–13

McCown BH, Lloyd G (1981) Woody plant medium a mineral nutrient formulation for microculture of woody plant species. HortScience 3:453

Momol EA, Burr TJ, Reid CL, Momol SH, Otten L (1998) Genetic diversity of *Agrobacterium vitis* as determined by DNA fingerprinting of the 5′-end of the 23S rRNA gene and random amplified polymorphic DNA. J Appl Microbiol 85:685–692

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497

Otten L, Ruffray R, Momol EA, Momol MT, Burr TJ (1996) Phylogenetic relationships between *Agrobacterium vitis* isolates and their Ti plasmids. Mol Plant Microbe Interact 9:782–786

Pearson R, Goheen AC (1988) Compendium of grape diseases. APS Press, St. Paul

Pu X, Goodman RN (1992) Induction of necrogenesis by *Agrobacterium tumefaciens* on grape explants. Physiol Mol Plant Pathol 1:241–254

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning. A laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Schroth MN, McCain AH, Foottt JH, Huisman OC (1988) Reduction in yield and vigor of grapevine caused by crown gall disease. Plant Dis 72:241–246

Stover EW, Swartz HJ, Burr TJ (1997) Crown gall formation in a diverse collection of *Vitis* genotypes inoculated with *Agrobacterium vitis*. Am J Enol Vitic 48:26–32

Sule S, Burr TJ (1998) The effect of resistance of rootstocks to crown gall (*Agrobacterium* ssp.) on the susceptibility of scions in grapevine cultivars. Plant Pathol 47:84–88

Tzfira T, Vaidya M, Citovsky V (2001) VIP1, an *Arabidopsis* protein that interacts with *Agrobacterium* VirE2, is involved in VirE2 nuclear import and *Agrobacterium* infectivity. EMBO J 20:3596–3607

Vidal J, Kikkert J, Malnoy M, Wallace P, Barnard J, Reisch B (2006) Evaluation of transgenic "Chardonnay" (*Vitis vinifera*) containing magainin genes for resistance to crown gall and powdery mildew. Transgenic Res 15:69–82

Viss WJ, Pitrak J, Humann MC, Driver J, Ream W (2003) Crown gall resistant transgenic apple trees that silence *Agrobacterium tumefaciens* oncogenes. Mol Breed 4:283–295

Ward DV, Zupan JK, Zambryski PC (2002) *Agrobacterium* VirE2 gets the VIP1 treatment in plant nuclear import. Trends Plant Sci 7:1–3

Winans SC, Allenza P, Stachel SE, McBride KE, Nester EW (1987) Characterization of the virE operon of the Agrobacterium Ti plasmid pTiA6. Nucleic Acids Res 5:825–837

Xue B, Ling KS, Reid CL, Krastanova S, Sekiya M, Momol EA, Sule S, Mozsar J, Gonsalves D, Burr TJ (1999) Transformation of five rootstocks with plant virus genes and a *vir*E2 gene from *Agrobacterium tumefaciens*. In Vitro Cell Dev Biol Plant 35:226–231

Zhu Y, Nam J, Humara JM, Mysore KS (2003) Identification of *Arabidopsis* rat mutants. Plant Physiol 132:494–505