VirE1-Mediated Resistance to Crown Gall in Transgenic *Arabidopsis thaliana*

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ABSTRACT

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Crown gall disease, caused by *Agrobacterium tumefaciens*, remains a serious agricultural problem despite current biocontrol methods. *Agrobacterium tumefaciens* transfers single-stranded DNA (T-strands) into plant cells along with several virulence proteins, including a single-stranded DNA-binding protein (VirE2). In plant cells, T-strands are protected from nucleases and targeted to the nucleus by VirE2, which is essential for efficient transmission (transfer and integration) of T-strands.

VirE1 is the secretory chaperone for VirE2; it prevents VirE2 from forming aggregates and from binding the T-strands in bacterial cells. Therefore, we hypothesized that sufficient quantities of VirE1 expressed in plant cells might block T-DNA transmission by preventing VirE2 from binding T-strands. Here we show that root explants from *Arabidopsis thaliana* plants that expressed *virE1* formed 3.5-fold fewer tumors than roots from plants without *virE1*. Also, this resistance was specific for VirE2-mediated *Agrobacterium* transformation. Plants that have been genetically altered to resist crown gall may prove more effective than biological control.

Agrobacterium tumefaciens transfers the T-DNA portion of its tumor-inducing (Ti) plasmid into plant cells where it integrates into the plant genome. The T-DNA contains three oncogenes that encode synthesis of auxin (*iaaM*, *iaaH*) and cytokinin (*ipt*), which causes undifferentiated proliferation of plant cells (13). *Agrobacterium tumefaciens* secretes both the single-stranded DNA (ssDNA)-binding protein VirE2 and T-strands into plant cells via a type IV secretion system consisting of VirB1-VirB11 and VirD4 (3). Although VirE2 and T-strands are both exported through the VirB/D4 pore, VirE2 is exported into plant cells separately from T-strands (3). VirE2 produced directly in transgenic plant cells can replace bacterially encoded VirE2, indicating that VirE2 is only needed in plant cells (12).

VirE1 is a chaperone required for VirE2 stability and secretion (39). In bacterial cells, VirE1 binds VirE2 domains involved in ssDNA binding and self association (35). This prevents VirE2 from aggregating and prematurely binding T-strands, maintaining VirE2 in a transport competent state. The interaction between VirE1 and VirE2 is stronger than VirE2 self-interaction (8). Because VirE1 can prevent VirE2 from binding T-strand DNA (8), we predicted that expression of large amounts of VirE1 in plant cells might reduce or block T-DNA transmission. To test this hypothesis, we fused the *virE1* coding sequence to the *Figwort mosaic virus* (FMV) promoter and introduced this transgene into *Arabidopsis thaliana*. Among 19 *virE1*-transgenic lines tested, one was significantly less susceptible to tumorigenesis than control plants.

While this study was in progress, Szegedi et al. (36) observed reduced susceptibility to *Agrobacterium vitis* AB3 in transgenic tobacco that expressed *virE1*. However, these lines remained fully susceptible to *Agrobacterium tumefaciens* A348, a strongly pathogenic strain from which the *virE1* transgene originated (36).

MATERIALS AND METHODS

Plasmid construction. We constructed a plant transformation vector containing *virE1* in two steps. First, we fused the *virE1* coding sequence to an FMV promoter and a pea *rbcS* polyadenylation signal, which are contained in a ColE1-based plasmid derived from pCGN8063 (26). Next, we inserted this gene fusion into a plant transformation vector (pCGN5927) adjacent to a gene fusion (*Pnos-nptII-nos3'*) that confers kanamycin resistance to plant cells (26).

The *virE1* gene was amplified by polymerase chain reaction (PCR) from a pUC18 derivative (pGR1) that contains the *virE* operon from *Agrobacterium tumefaciens* octopine-type Ti plasmid pTiA6 (34). pGR1 DNA (200 ng) was linearized by cleavage with *Bam*HI and used as template for PCR. The 50- μ l PCR reaction contained 10 mM Tris, pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, deoxynucleoside triphosphates (0.2 mM each), primer oligonucleotides (0.3 μ M each), and 2.5 units of *Taq* DNA polymerase (Perkin Elmer, Boston, MA). The mixture was heated to 94°C for 1 min and then subjected to 30 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min. At the conclusion of cycling, the reaction was held at 68°C for 5 min. The 5' primer (5'-CGCGGATCCGC-CGCCACCATGGCCATCATCAAGCCGCATGCG-3') added a *Bam*HI site (bold italic) and Kozac sequence to the start of *virE1* (bold underline) and the 3' primer (5'-CCGGGTACCTCACTC-CTTCTGACCAGCAAGATTG-3') added a *Kpn*I site (bold italic) to the end. This 225-bp amplicon was cut with *Bam*HI and *Kpn*I and ligated to *Bgl*II and *Kpn*I sites downstream of the FMV promoter in plasmid pHA1, which we derived from pCGN8063 (26). The resulting plasmid was designated pHA1E1, and the sequence of the *virE1* transgene was confirmed. pHA1 was created by inserting a 20-bp self-complementary oligonucleotide (5'-AGCTG-GCGCGCCGGCGCGCC-3') with *Hind*III-compatible ends and two internal *Asc*I sites into the *Hind*III site of pCGN8063. The *virE1* gene fusion was excised from pHA1E1 as a 2,025-bp *Asc*I-*Not*I fragment and ligated into the binary vector pCGN5927 (26), which was also cut with *Asc*I and *Not*I. The resulting plasmid (pBVE1) contains the *virE1* gene fusion along with *nptII* between

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T-DNA borders (Fig. 1). The pBVE1 and pCGN5927 plasmids were each transformed into *Agrobacterium tumefaciens* EHA101 (16,17).

Plant transformation and selection. *Arabidopsis thaliana* plants (ecotype Columbia) were transformed by the floral dip method described by Clough and Bent (5). Plants were transformed with *Agrobacterium tumefaciens* EHA101 containing either pBVE1 or pCGN5927. The seeds were harvested and plated on Murashige Skoog (MS) (27) agar supplemented with kanamycin (50 µg/ml), vernalized at 4°C for 2 days, and then incubated at 24°C with a daily 16-h light cycle. Kanamycin-resistant plants (T0 generation) were transferred to soil and allowed to self-pollinate. T1 seeds were germinated on MS agar containing kanamycin, and these plants were allowed to self-pollinate to produce T2 seed. T3 and T4 generations were produced by additional self-pollinations; seeds were germinated on MS agar containing kanamycin.

Susceptibility to crown gall. Root explants from the T3 and T4 generations of 5927A (vector only) and BVE1/H (*virE1* transgenic) and untransformed plants were tested for susceptibility to crown gall tumorigenesis as described (28). Roots were excised from aseptically grown plants and infected with *Agrobacterium tumefaciens* strains: A348 (wild-type) (11), MX358(pVK100) (*virE2::Tn3-lacZ358* with cosmid pVK100) (15), or MX358(pLH338) (*virE2::Tn3-lacZ358* with pVK100 containing the *GALLS* gene from *Agrobacterium rhizogenes* 1724) (15) for 2 days on hormone-free MS agar. Root explants were then rinsed in timentin solution (100 µg/ml) and transferred to hormone-free MS agar containing timentin (100 µg/ml). Root explants were incubated under the same conditions as the plants. After 3 weeks, root pieces that had at least one tumor were counted as positive. Root explants with tumors larger than 3 mm were counted as large tumors, and tumors smaller than 3 mm were counted as small tumors.

Northern and Southern blot analysis. Total RNA was extracted as described (31) from whole plants (T2 and T3 generation) cultured on MS agar after initial selection on MS agar containing kanamycin. Samples containing 5 µg of total RNA were subjected to electrophoresis through a 2% formaldehyde-agarose gel and transferred to GeneScreen Plus nylon membrane (New England Nuclear, Boston, MA). Blots were hybridized with a *virE1* probe first, and then re-probed with a ubiquitin probe. Probes were labeled with [α -³²P] dCTP (3,000 Ci/mmol, MP Biomedicals, Irvine, CA) using a Random Primers DNA Labeling System (Invitrogen, Carlsbad, CA). Templates for probe synthesis were the 225-bp *virE1* amplicon and a plasmid with five copies of the *Arabidopsis thaliana* ubiquitin gene (pTB112) (2), which was linearized with *Aat*II.

DNA was extracted with urea (4) from 1 g of 8-day-old *Arabidopsis thaliana* plantlets (T4 generation) cultured in liquid MS (25 ml) with kanamycin (50 µg/ml). DNA (5 to 10 µg) was incubated with *Bcl*II, *Kpn*I, or *Xba*I and then precipitated with ethanol. The samples were separated by electrophoresis through a 0.8% agarose gel and transferred to GeneScreen Plus nylon membrane. The blots were probed with a 794-bp amplicon containing *nptII* sequences or a 1,355-bp amplicon corresponding to a *Kpn*I frag-

ment from pBVE1 (Fig. 1). The *nptII* probe was amplified from pCGN5927 (26) using primers 5'-ATGATTGAACAAGATGGA-TTGCACG-3' and 5'-TCAGAAGAACTCGTCAAGAAGGCGA-TAGAAGGC-3'. The *Kpn*I probe was amplified from pBVE1 using primers 5'-CTCACTCCTTCTGACCAGCAAGATTGTCTC-3' and 5'-CGTACCTAGGCGGCCAGCTT-3'. The probes were labeled by random prime synthesis.

Western blot analysis. Tissue (100 mg) from whole *Arabidopsis thaliana* plants was frozen in liquid nitrogen, ground to a fine powder, and proteins were extracted with 0.5 ml of extraction buffer (20 mM sodium phosphate, pH 7, 100 mM NaCl, 5 mM EDTA, 0.05% Tween 20, 1 mM phenylmethylsulfonyl fluoride, and 25 mM sodium ascorbate) on ice for 30 to 60 min. Insoluble material was removed by centrifugation in a microcentrifuge for 15 min at 4°C. Protein concentrations in the supernatant solutions were determined by Bradford assays (1), and samples (460 to 960 µg) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). His-tagged VirE1 was isolated from *Escherichia coli* BL21 (Invitrogen) containing *virE1* fused to a histidine tag in vector pPROEX-1 (Invitrogen) using Ni-NTA resin (Qiagen, Santa Clarita, CA) according to the manufacturer's instructions.

SDS-PAGE and blotting onto Hybond-P nylon membrane were done as outlined in Ream and Field (31). Protein extracts and His-tagged VirE1 (10 ng to 100 µg) were subjected to electrophoresis through a 15% polyacrylamide gel. A WesternBreeze kit (Invitrogen) was used with VirE1 antibodies (39) for chemiluminescent detection of VirE1. The antibodies were used at a 1:1,000 dilution.

RESULTS

***Arabidopsis* lines transformed with *virE1*.** *Arabidopsis thaliana* plants (ecotype Columbia) were transformed with a T-DNA containing *virE1* fused to the FMV promoter (Fig. 1). The T1 seeds were plated on MS plates containing kanamycin to obtain segregation ratios. Transgene mRNA levels in the T2 plants were assessed, and 19 lines producing *virE1* mRNA were screened for susceptibility to tumor formation. All plant lines exhibited normal growth and development. One line (BVE1/H), which showed significant resistance to crown gall, was chosen for further study along with one line (5927A) containing only vector sequences. The T1 segregation ratios for these lines were 60 Kan^r:26 Kan^s (2.3:1) for 5927A and 73 Kan^r:29 Kan^s (2.5:1) for BVE1/H. The lack of 3:1 segregation for line 5927A indicates that this line contained T-DNA at more than one locus, but the segregation ratio for line BVE1/H indicates that the transgenes may occupy one locus. These conclusions were confirmed by Southern blot analysis of T-DNA copy number and structure.

Transgene structure and copy number. Plant DNA was digested with *Kpn*I to assess T-DNA structure and to determine the number of T-DNA loci. Plant DNA was cut with *Kpn*I and probed with a radiolabeled *Kpn*I fragment containing *virE1* from the pBVE1 T-DNA (Fig. 1); an intact copy of the T-DNA should yield a 1,355-bp fragment. DNA from line BVE1/H produced a single band at 7,700 bp indicating a rearranged T-DNA structure (Fig. 2A, lane 2). No bands were observed for DNA from line

Fig. 1. Structure of the *virE1* T-DNA. The T-DNA is 4,337 bp long. RB = right border, FMVpro = *Figwort mosaic virus* promoter, HSP70L = *Petunia* HSP70 leader sequence, *virE1* = *virE1* gene from *Agrobacterium tumefaciens* A348, E9 3' = 3' end of pea *rbcS* E9 gene, *nos 3'-nptII-nos 5'* = kanamycin resistance, LB = left border. Numbers in parentheses refer to base pair coordinates. The first base of the right border is coordinate 1, and the last base of the left border is coordinate 4337.

5927A because the pCGN5927 T-DNA lacks homology with this probe (Fig. 2A, lane 1). *KpnI*-digested plant DNA was probed with an *nptII*-specific probe to assess transgene copy number. *KpnI* cuts the T-DNA upstream of the *nptII* gene and in plant DNA flanking the T-DNA. *KpnI*-digested DNA from line BVE1/H produced bands of 17 and 5.9 kb when probed with *nptII* sequences. The signal present in the 5.9-kb band was twofold greater than the signal in the 17-kb band, suggesting that the 5.9-kb band may contain two copies of *nptII* on a single *KpnI* fragment. Alternatively, the 5.9-kb band may represent two different *KpnI* fragments that co-migrate. Thus, BVE1/H has three copies of *virE1* (Fig. 2B, lane 2). *KpnI*-digested DNA from line 5927A produced two bands, indicating that this line contains two copies of the T-DNA (Fig. 2B, lane 1). The top band appears to contain a large (>20 kb) *KpnI* fragment and some partially cut DNA.

DNA from lines BVE1/H and 5927A was also digested with *XbaI* or *BclI* and analyzed by Southern hybridization. *XbaI* does not cut within the T-DNA, so each *XbaI* fragment that contains T-DNA sequences represents a separate locus. Line 5927A contained two loci (Fig. 2C, lane 1) and BVE1/H had three loci (Fig. 2C, lane 2), confirming the copy numbers observed with *KpnI*-digested DNA. *BclI* cuts 4,146 bp downstream of the T-DNA right border (Fig. 1). Plant DNA cut with *BclI* should yield fragments of varying length with one cut site within the T-DNA and another in the flanking plant DNA. DNA from line 5927A probed with *nptII* sequences produced two bands of 5,400 and 4,500 bp (data not shown); DNA from line BVE1/H yielded one intense band at 2,900 bp (Fig. 2C, lane 3).

***virE1* expression.** Northern blots were performed with three individual RNA preparations from whole plants to measure the *virE1* mRNA levels. The blots were probed with *virE1*- and ubiquitin-specific probes. The *virE1* mRNA is about 450 nt and ubiquitin mRNA is about 950 nt. BVE1/H plants accumulated high levels of *virE1* mRNA, and the vector-only line (5927A) produced no signal with this probe, as expected (Fig. 3). Both lines accumulated similar levels of ubiquitin mRNA (Fig. 3). The high levels of *virE1* mRNA in BVE1/H plants suggested that it may produce sufficient VirE1 protein to interfere with T-DNA

transmission by binding VirE2 transferred from *Agrobacterium tumefaciens*. We extracted proteins from plant lines BVE1/H and 5927A and subjected soluble proteins to SDS-PAGE. To monitor detection sensitivity, we loaded purified VirE1 (10 ng to 100 µg) into separate lanes. Proteins were transferred to a nylon filter by electroblotting, and we probed the blot with antibodies raised against VirE1 (39). We did not detect VirE1 among the proteins extracted from any plant line; however, the detection limit for purified VirE1 was 10 µg (data not shown).

Susceptibility to crown gall tumorigenesis. Roots of transgenic lines 5927A and BVE1/H were tested for susceptibility to

Fig. 3. Accumulation of *virE1* mRNA in transgenic plants. Each lane contains 5 µg of RNA. The blot was probed with *virE1* (top) or ubiquitin (bottom) sequences.

Fig. 2. T-DNA copy number in transgenic *Arabidopsis*. DNA (5 to 10 µg) from the plant lines 5927A (lanes A1, B1, C1) and BVE1/H (lanes A2, B2, C2 and 3) was cut with *KpnI* (A and B), *XbaI* (lanes C1 and 2), or *BclI* (lane C3). The blot was probed with a *virE1*-specific *KpnI* fragment (A) or *nptII* sequences (B and C).

crown gall tumorigenesis. *Agrobacterium tumefaciens* A348 (11) was used as the wild-type strain. Since the Columbia ecotype is less susceptible to crown gall than some other ecotypes of *Arabidopsis* (28), the nontransformed plants and control line 5927A (Figs. 4 and 5) did not have 100% tumorigenesis with A348. BVE1/H (Figs. 4 and 5) exhibited significant resistance to A348

compared with 5927A (Student's *t* test = 0.005). The majority of the tumors (90%) on BVE1/H were small (≤ 3 mm). In contrast, 44% of the tumors on line 5927A were large (>3 mm). When challenged with nonpathogenic *Agrobacterium tumefaciens* WR3095 (30), no tumors were observed on any plant line (data not shown).

Fig. 4. Tumor formation on *Arabidopsis thaliana* root explants of *virE1*-transgenic and vector-only plants with *VirE2*- and *GALLS*-dependent crown gall tumorigenesis. **A**, Vector-only (5927A) inoculated with wild-type *Agrobacterium tumefaciens* A348. **B**, Line BVE1/H inoculated with A348. **C**, 5927A inoculated with *GALLS*⁺ *virE2*⁻ *Agrobacterium tumefaciens* MX358 (pLH338). **D**, BVE1/H inoculated with MX358 (pLH338).

Fig. 5. Percentage of root explants with tumors. A348 = wild-type *Agrobacterium tumefaciens* A348. MX358(pLH338) = *GALLS*⁺ *virE2*⁻ *Agrobacterium tumefaciens*. MX358(pVK100) = *virE2*⁻ *Agrobacterium tumefaciens*. Black bars indicate tumors ≤ 3 mm and lined bars represent tumors >3 mm.

Because tumors on BVE1/H were significantly smaller than on 5927A, tumors resulting from infection with A348 were cultured on hormone-free MS agar for 11 months. Tumors from 5927A root segments were divided at least once, and each subtumor formed an unorganized callus 4 to 6 cm in diameter. Tumors from line H did not grow well enough to be divided (most did not survive) and were only about 2 cm in diameter (Fig. 6). In contrast to the unorganized tumors that formed on 5927A tissue, roots grew from the callus derived from tumors on line BVE1/H. This indicates that the T-DNA may be truncated in BVE1/H tumors (33).

Reduced tumor incidence on BVE1/H may result from production of sufficient VirE1 protein to interfere with normal T-DNA transmission. Alternatively, one of the T-DNAs may have disrupted a host gene required for tumorigenesis, creating a *rat* mutant, which is resistant to *Agrobacterium tumefaciens* transformation (29, 40). To distinguish between these possibilities, the plant lines were challenged with a strain that does not require VirE2 for pathogenesis. A *virE2* mutant (MX358) is nonpathogenic, but virulence is restored by a plasmid (pLH338) that encodes a protein called GALLS, which substitutes for VirE2 (15). GALLS is not homologous to VirE2, but like VirE2, it contains a putative nuclear localization signal and a C-terminal signal for secretion to plant cells (L. Hodges, A. Vergunst, P. Hooykaas, and W. Ream, *unpublished data*). Because GALLS and VirE2 do not resemble each other, we did not expect VirE1 to interfere with the function of GALLS. pVK100, the parent plasmid of pLH338, lacks GALLS and does not restore pathogenesis. If BVE1/H is a *rat* mutant, we expected it to be resistant to GALLS-dependent transformation. If the crown gall resistance of BVE1/H is indeed VirE1-mediated, GALLS-dependent tumorigenesis levels should be similar to those observed with A348. Both lines BVE1/H and 5927A had similar tumorigenesis levels (Student's *t* test = 0.652) when infected with MX358(pLH338) (Figs. 4 and 5). Therefore, the gall-resistant phenotype of line BVE1/H likely results from high levels of *virE1* expression. Alternatively, high levels of VirE1 may interfere with a host protein required for VirE2-mediated tumorigenesis, or one of the T-DNAs may prevent expression of such a host protein.

DISCUSSION

Arabidopsis lines that express VirE1 were constructed to test the model that VirE2 binds the T-strand inside plant cells. Our hypothesis was that the VirE1 expressed in the plant cells would bind incoming VirE2 and prevent it from binding T-strands. This would leave the T-strands susceptible to nuclease attack and ultimately prevent T-DNA integration and tumorigenesis.

Several experiments indicate that VirE2 binds the T-strands only after they are exported to plant cells. Plant cells that express VirE2 can complement *virE2*-mutant *Agrobacterium tumefaciens*, indicating that VirE2 is needed in plant cells but not in bacteria (12). VirE2 and T-strands can come from different bacterial cells in mixed infection experiments, in which two nonpathogenic strains (one lacking VirE2, another lacking T-DNA) can "complement" each other. VirE1 is needed for VirE2 secretion, but not for VirD2-T-strand export (34). Finally, T-strands do not associate with VirE2 in bacterial cells (3).

Reduced tumor incidence and size on line BVE1/H are consistent with our hypothesis. The poor growth and aberrant morphology of the tumors that formed on BVE1/H suggest that the T-DNA in these tumors is abnormal. Rossi et al. (33) observed truncated T-DNAs in rare tumors induced by *virE2*-mutant *Agrobacterium tumefaciens*. The unusual properties of tumors induced on BVE1/H indicate that they too may contain truncated T-DNAs, possibly due to inadequate protection by VirE2 caused by the presence of VirE1 in plant cells. Another possibility is that VirE1 expressed in plant cells interferes with the normal function of a plant protein needed in pathogenesis.

Currently, the only defense against crown gall disease is prevention. Commercially, plant roots or seed are inoculated with nonpathogenic biocontrol strains such as *Agrobacterium radiobacter* K84 (21,22) or K1026 (18,19,37) at planting. *Agrobacterium radiobacter* K84 and K1026 produce agrocin 84 (7,32), a bacteriocin that kills pathogenic *Agrobacterium tumefaciens* that contain agrocinopine catabolism (*acc*) genes (14). However, many pathogenic *Agrobacterium tumefaciens* are resistant to agrocin 84 because they lack the *acc*-encoded permease, which mediates agrocin 84 uptake. Spontaneous mutations in *Agrobacterium tumefaciens* can also confer resistance to agrocin 84 (6).

Recently, gene silencing has been used to prevent gall formation, but gall-resistant crops are not commercially available (9, 10,25,38). Gene silencing prevents T-DNA oncogene expression by degrading oncogene mRNAs. Double-stranded RNA elicits sequence specific destruction (silencing) of mRNA with sufficient sequence identity. Escobar et al. (9,10) observed resistance to tumor formation on roots and stems of tomato, *Arabidopsis thaliana*, and walnut by silencing both *iaaM* and *ipt*, and we produced transgenic apple rootstocks that are resistant to crown gall by silencing *iaaM* (25,38). Extensive testing of the effectiveness of these oncogene-silencing constructions on different *Agrobacterium tumefaciens* has not been done yet, but they are not effective on certain *Agrobacterium vitis* strains (E. Szegedi, J. Pitrak, and W. Ream, *unpublished data*). This may be due to lack of sufficient identity between the silencing construction and *iaaM* in these *Agrobacterium vitis* strains, or these strains may lack *iaaM* altogether. A transgene that blocks transmission of T-DNA into the plant genome, such as VirE1, may enhance the resistance of plants to crown gall.

The VirE1-mediated reduction in susceptibility to crown gall demonstrated in line BVE1/H was promising but not complete. However, it may be possible to modify the transgene to increase its effectiveness. First, the *virE1* gene we used contains four codons that are rarely used in *Arabidopsis thaliana*. Translation of *virE1* mRNA in *Arabidopsis thaliana* might increase significantly if these codons were replaced with synonymous codons that are frequently used in *Arabidopsis thaliana*. Second, the "ideal" sequence context for optimum translation of mRNAs in plants (AAA A/CAA AUG GCU) (20) differs from the Kozak consensus sequence (GCC GCC A/GCC AUG G) (23,24) we used; it may be possible to improve translation of *virE1* mRNA by changing the sequence immediately upstream of the start codon (AUG). Third, it may be possible to increase transcription of *virE1* in the specific root cell types that are susceptible to crown gall. Although the enhanced viral promoter we used gave high levels of transcription in whole plants, the level of expression in specific root cell types remains unknown. Strong root-specific promoters may provide better expression of *virE1* mRNA in root tissues, where crown gall is a problem. VirE1-mediated control of crown gall in species such as grape and raspberry, where aerial tumors are common, may require strong cambium-specific promoters.

Separately, none of the control methods for crown gall are 100% effective, but by combining and refining these methods, more

Fig. 6. Tumors cultured from BVE1/H and 5927A. **A**, Subculture of tumor tissue from vector-only line (5927A). **B**, Primary tumor from line BVE1/H.

reliable control may result. For instance, if the VirE1-mediated and oncogene-silencing methods were combined in a single transgenic line, the protection may broaden. Similarly, if VirE1 transgenics were used in conjunction with biocontrol strains, VirE1-mediated resistance might reduce damage caused by agrocin 84-resistant *Agrobacterium tumefaciens* pathogens.

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