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1 # Grapevine Crown Gall Suppression Using Biological Control
2 # and Genetic Engineering: A Review of Recent Research

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19 **Abstract:** Crown gall is a devastating grapevine disease often encountered in vineyards prone to winter
20 cold injury. *Agrobacterium vitis,* the predominant causal agent of this disease, moves from the roots via
21 xylem sap flow to freeze injury sites where genetic transformations then occur. Crown gall disrupts the
22 grapevine trunk vascular system, which prevents nutrient flow and leads to plant decline and death.
23 Viticultural practices designed to fight this disease are only partially effective, thereby requiring
24 alternatives. Genetic engineering and biological control could be more desirable approaches for disease
25 prevention. Biological control typically involves antagonistic organisms, which are applied to grapevine
26 roots to reduce the concentration of pathogenic *Agrobacterium* strains. Genetic engineering may prevent
27 infection and tumor formation by modifying grapevines and antagonistic organisms. In the grapevine, this
28 may be achieved by enhancing molecular mechanisms for producing bacterium-specific antimicrobial
29 peptides or preventing transferred deoxyribonucleic acid export, integration, and oncogene expression.
30 Alteration of antagonistic organisms enhances the production of bacteriocins effective against
31 agrobacteria. This paper reviews the potential use of biological control options and genetic engineering
32 tools for grapevine crown gall suppression and makes recommendations regarding directions for the
33 future.

34 **Key words:** *Agrobacterium vitis*, crown gall, control, biological technology, molecular biology

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36 **Crown Gall on Grape and *Agrobacterium* Nomenclature**

37 The unchecked growth of tumors on grape, commonly known as crown gall (CG), is one of the most
38 economically destructive diseases in temperate zone viticulture. The chief disease agent is the bacterium
39 *Agrobacterium vitis* although other *Agrobacterium* species may also cause CG on grapevine (Szegedi et
40 al. 2005, Kawaguchi and Inoue 2009). Crown gall results in yield reductions, grapevine decline, and vine
41 death (Süle and Burr 1998). It occurs worldwide and varies by viticultural regions with the most severe
42 impact in temperate climates. The world map of the Köppen-Geiger on climate classification (Kottek et
43 al. 2006) indicates that grapevine CG disease occurs in climate regions coded with Dfb (Tmin ≤ -3 °C).
44 Severe CG incidences are common in the Great Lakes region of the U.S. (Figure 1), the Canadian
45 province of Ontario, and Germany, Hungary, Slovakia, and the Czech Republic. The economic impact of
46 CG varies significantly between regions. For example, in the state of Pennsylvania, losses were estimated
47 over a six-year period at US$46,500 per 0.4 hectare vineyard (Steward and Wenner 2004).

48 Numerous taxonomic schemes have been proposed for the nomenclature of the *Agrobacterium*
49 species (Sawada et al. 1993, Willems and Collins 1993, de Lajudie et al. 1998). Nomenclature in this
50 review refers to the system proposed by Kerr and Panagopoulos (1977) and Ophel and Kerr (1990). Kerr
51 and Panagopoulos (1977) demonstrated that grapevine *Agrobacterium* isolates form a distinct group and
52 differ from strains isolated from other plants. They classified *Agrobacterium* spp. into three groups:
53 biotype-1, biotype-2, and biotype-3. Ophel and Kerr (1990) demonstrated, through biochemical tests and
54 differential levels of deoxyribonucleic acid (DNA) binding, phenotypic differences between the grapevine
55 *Agrobacterium* isolates and the previously described *A. tumefaciens, A. radiobacter, A. rhizogenes,* and *A.*
56 *rubi* spp. They proposed that grapevine biotype-3 isolates form a new separate *Agrobacterium* sp. named
57 *A. vitis*. Based on comparative 16S rDNA sequencing, renaming of agrobacteria to *Rhizobium*
58 *radiobacter, R. rhizogenes, R. rubi, R. undicola,* and *R. vitis* was proposed (Young et al. 2001, Willems
59 2006). Currently, the most commonly used nomenclature refers to biotype-1, biotype-2, and biotype-3
60 strains of *Agrobacterium* as *A. tumefaciens*, *A. rhizogenes,* and *A. vitis,* respectively. Tumorigenic
61 *Agrobacterium* spp. are also classified according to the type of their CG tumor-inducing or root-inducing
62 (Ti/Ri) plasmid, which determines the type of opine(s) synthesized and metabolized by each strain
63 (Paulus et al. 1989, Otten et al. 1996). Classification of *Agrobacterium* strains by opine production is
64 problematic because of unknown or non-existing opine types of several Ti/Ri plasmid-induced tumors.
65 Moreover, a single plasmid does not always induce only one specific opine type. In fact, inductions of
66 several opine types in various combinations are most often associated with a single Ti/Ri plasmid (Paulus
67 et al. 1989, Otten et al. 1996). *Agrobacterium* grapevine isolates predominantly metabolize nopaline,

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68 vitopine, or octopine/cucumopine and rarely other opine types (Otten et al. 1996). Currently 11 opine
69 types (octopine, nopaline, succinamopine, agropine, agropine/mannopine, mannopine,
70 chrysopine/succinamopine, chrysopine/nopaline, cucumopine/mikimopine, octopine/cucumopine, and
71 vitopine) are being classified (Petit et al. 1983, Szegedi et al. 1988, Dessaux et al. 1998, Pionnat et al.
72 1999, Moriguchi et al. 2001).
73

74 ## *Agrobacterium* Ecology and Etiology

75 ***Etiology.*** Agrobacteria commonly inhabit soils and roots of susceptible host plants (Figure 2).
76 *Agrobacterium vitis* survives in grapevine plant material including roots, trunks, cordons, canes, cuttings,
77 and debris. It also survives in vineyard soil. Agrobacteria are generally harmless to plants. However, they
78 can cause a disease if they possess a large (from approx. 200 to 800 kilobase pairs in size; Gelvin 2003)
79 Ti or Ri plasmid. Crown gall is triggered by the integration of transferred-DNA (T-DNA) into the plant
80 nuclear genome and the expression T-DNA genes encoding enzymes of plant hormone biosynthesis. Only
81 the T-DNA, that is only a segment of the Ti plasmid, is transferred into the plant nuclear genome. It is this
82 Ti plasmid that contains the T-DNA and the genes required for its export, integration, and oncogene
83 expression (van Larebeke et al. 1974, De Cleene and De Ley 1976, Portier et al. 2006).
84 ***The infection process.*** Crown gall infection is a three-step process. In step one the pathogen enters
85 the apoplastic space of the plant. *Agrobacterium vitis* is particularly concentrated in the rhizosphere of
86 grapevines and most commonly infects through roots and underground wound sites (Süle et al. 1995, Burr
87 et al. 1998). Step two centers on the colonization of the xylem by the bacteria. *Agrobacterium vitis*
88 systemically colonize grapevines and disseminate to shoots via xylem sap flow (Szegedi and Bottka 2002,
89 Szegedi and Dula 2006). The pathogen survives and persists in apparently healthy cuttings for extended
90 periods (Lehoczky 1968, Lehoczky 1971, Lehoczky 1978, Burr and Katz 1984, Burr et al. 1987, Burr et
91 al. 1988, Lehoczky 1989). Finally, step three involves the evasion of plant reactions and suppression of
92 plant defense mechanisms. A well-known example of such mechanism of evasion is the degradation of
93 hydrogen peroxide by the bacterium's catalase enzyme encoded by the *katA* gene (Xu and Pan 2000).
94 ***T-DNA export to plant cell.*** Wound healing processes, such as callus formation, development of graft
95 union vascular connection, and tissue repair and remodeling in response to grafting and freeze injury, as
96 well as physical damages caused by vineyard equipments, involve cell division and render grapevine cells
97 competent to *Agrobacterium* transformation. Consequently, these are the primary sites for CG
98 development (Creasap et al. 2005, Otten et al. 2008). The tumorous growth typically occurs around the
99 graft union (Figure 3 A, B), on the lower trunk (Figure 4), and at the base and disbudded points of

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100 cuttings as those are prevalent sites of wound healing and highly susceptible to T-DNA export. Many
101 proteins encoded by virulence (*vir*) genes play essential roles in the T-DNA transfer process. Nearly all
102 proteins encoded by the *vir* region of the Ti plasmid are required for T-DNA transfer to occur (Burr and
103 Otten 1999, Gelvin 2003, Matthysse 2006, Otten et al. 2008). VirA and VirG form a two-component
104 regulatory system. VirA works as sensor, while VirG works as transcriptional regulator. VirA senses the
105 presence of specific plant phenolic compounds induced at wound healing, while VirG increases the level
106 of *vir* gene transcription. VirD4 and VirB proteins are necessary for the transfer of the T-DNA and other
107 Vir proteins. VirD2 serves as a pilot protein that guides the T-DNA through the transfer process, while
108 VirE2 is required for efficient tumor formation. A detailed account of the T-DNA transfer process and the
109 role of individual Vir proteins are provided in recent reviews by Lacroix et al. (2008).

110 ***T-DNA integration into the plant nuclear genome.*** Induction of CG formation is unique because it
111 involves the inter-kingdom transfer of T-DNA from the bacterial pathogen into the plant nuclear genome
112 (Zupan and Zambryski 1995). The bacterial T-DNA is integrated into the plant chromosome where the
113 genetic transformation of the plant cell then begins. Plant cell transformation leads to elevated levels of
114 indole-3-acetic acid (auxin or IAA) and cytokinin production (Morris 1986). Expression of two
115 oncogenes (*iaaM* and *iaaH*) was required for auxin synthesis and tumor induction in grapevines
116 (Thomashow et al. 1986, Huss et al. 1990). Auxin is synthesized in a two-step biochemical pathway. Step
117 one includes the expression of the *iaaM* gene, which induces tryptophan mono-oxygenase production.
118 Tryptophan mono-oxygenase then converts tryptophan to indoleacetamide. Step two involves
119 indoleacetamide hydrolase production by the *iaaH* gene. Indoleacetamide hydrolase then converts
120 indoleacetamide to auxin (Thomashow et al. 1986, Huss et al. 1990). Interestingly, most *A. vitis* strains
121 only encode auxin synthesis in their T-DNA (Fournier et al. 1994). The expression of T-DNA genes
122 within the plant cell and the elevated level of hormone production break the regulation of the cell cycle
123 because the plant cell cannot regulate the expression of the T-DNA genes. These trigger the
124 transformation and abnormal proliferation of plant cells resulting in CG tumor or hairy root formation
125 (Petersen et al. 1989, Gaudin et al. 1994, Costacurta and Vanderleyden 1995).

126

127 Management of Grapevine Crown Gall

128 ***Traditional viticultural practices.*** Currently, there are no completely effective methods of controlling CG
129 disease in nurseries and vineyards. The stage is often set for CG during in-nursery callusing and rooting
130 or after the first winter in the vineyard generating problems during vineyard establishment (Figure 1).
131 Prevention of freeze injury (selecting vineyard sites that avoid low temperatures), graft union protection

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132 (through soil burial/mulching techniques), frequent trunk renewal (Figure 4), and removal of infected vine
133 parts are major viticulture strategies to suppress CG; however, they are only partially effective (Zabadal et
134 al. 2007). Prevention of infection is a critical disease management strategy. This involves producing
135 pathogen-free planting material by *in vitro* apical meristem or shoot tip tissue culture and inhibiting
136 bacterial infection, i.e., soil fumigation and soil solarization, (Burr et al. 1988, Pu and Goodman 1993a,
137 Burr and Otten 1999, Szegedi and Süle 2005, Otten et al. 2008,). Other strategies to reduce CG incidence
138 in both vineyard and nursery are heat and chemical treatments (e.g. hot water submersion and
139 oxyquinoline sulfate treatments) of dormant cuttings prior to grafting (Szegedi 1995, Burr et al. 1996,
140 Burr et al. 1998, Burr and Otten 1999, Otten et al. 2008). Breeding for grapevine CG resistance represents
141 another approach. However, resistant varieties that produce high quality fruit have not yet been developed
142 (Burr et al. 2003). Cultural practices to reduce CG incidence also include: use of own-rooted vines, cold
143 hardy cultivars, (e.g. Frontenac and La Crescent), multiple-trunk training systems (Figure 4), irrigation
144 management to avoid freeze injury (Matthysse 2006), and fumigation and solarization to reduce
145 *Agrobacterium* and nematode population density in soil (Süle et al. 1995, Pinkerton et al. 2000).
146 Nematode (*Meloidogyne hapla*) feeding sites on grapevine roots may serve as an entry point for *A. vitis*
147 (Süle et al. 1995).

148 Despite the multiple strategies, none of the currently available cultural practices provide acceptable
149 levels of CG control in temperate viticultural regions.

150 ***Novel approaches.*** The increasing global demand for disease-resistant and stress-tolerant grapevines
151 has prompted significant interest in research into genetic engineering-assisted grapevine breeding.
152 Genetic engineering, also called genetic modification, uses recombinant nucleic acid techniques involving
153 the formation of new combinations of genetic material. This material is produced by inserting externally-
154 produced nucleic acid molecules into a virus, bacterial plasmid, or other vector system and then
155 incorporating that vector into a host organism in which they do not naturally occur but in which they are
156 capable of continued propagation (Sands and Galizzi 2006). Organisms derived through genetic
157 engineering are referred to as: genetically engineered (GE), genetically modified (GM), and/or transgenic
158 (TG) organisms. For sake of clarity, this review adheres to the most commonly used acronym and refers
159 to such organisms as GM.

160 This approach to genetic engineering-assisted grapevine breeding taps the natural capability of
161 numerous *A. tumefaciens and A. rhizogenes* spp. to engage in inter-kingdom DNA transfer and has
162 enormously impacted plant science, led to rapid progress in plant molecular biology, and has now become
163 the essential embodiment of today's crop biotechnology.

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164 This same characteristic of *Agrobacterium* is utilized in efforts to suppress CG disease in viticulture.
165 Research addressing the development of CG-resistant grapevines through genetic engineering,
166 predominantly for less cold-hardy varieties (Vitis vinifera L.), emerged in the 21st century. Grapevine CG
167 resistance has been hypothesized to be related to a single gene (Szegedi et al. 1984, Szegedi and Kozma
168 1984). Research in this era targeted the introduction and expression of foreign genes, the efficacious
169 regeneration of transformed grapevines, and the efficiency of the transformation process (Nakano et al.
170 1994, Scorza et al. 1996, Mozsár et al. 1998, Torregrosa et al. 2002, Oláh et al. 2003). Deoxyribonucleic
171 acid fingerprint comparisons conducted on ancestral forms of *A. vitis* aimed to provide information on the
172 evolution of the pathogen (Argun et al. 2002). After overcoming the technological hurdles of grapevine
173 transformation, more recent studies addressed specific problems, such as stress tolerance and disease
174 resistance in grapevines (Burr and Otten 1999, Otten et al. 2008).

175 To sum these up to date, efforts to engineer CG resistance into grapevines have focused on three main
176 approaches: 1. blocking infection by expressing antimicrobial peptides in GM plants inhibitory to *A.*
177 *vitis*; 2. blocking T-DNA export and/or integration; and 3. blocking T-DNA oncogene expression
178 following its export and integration. Genetic engineering may create GM grapevines with resistance to
179 CG and GM biological control agents (BCAs) with higher CG control efficacy.

180 A biological control approach for CG disease caused by *A. tumefaciens* depends on the use of a non-
181 pathogenic biological control strain *A. radiobacter* K84. This strain produces the bacteriocin agrocin 84
182 which is toxic to certain strains of *A. tumefaciens* and *A. rhizogenes* (Kerr and Htay 1974, Kim et al.
183 2006). The mode of action of agrocin 84 is based on the agrocinopine (an analog of agrocin 84)
184 biosynthesis by the plant and its catabolism by pathogenic *A. vitis* strains (Kim et al. 2006). Application
185 of strain K84 results in agrocin 84 production by the K84 strain and agrocinopine biosynthesis by the
186 plant. The Ti plasmid genes of agrocin 84-sensitive tumorigenic strains encode for agrocinopine uptake
187 and catabolism. This agrocinopine-utilization system enables those strains to take up agrocin 84. Once
188 taken up, agrocin 84 is toxic to the bacterial cell (Kim et al. 2006). Unfortunately, strain K84 will not
189 control grapevine CG caused by *A. vitis* (Staphorst et al. 1985, Chen and Xiang 1986, Webster et al.
190 1986). However, the effectiveness of this BCA in controlling *A. tumefaciens*-mediated CG suggests that
191 biological control is a viable tool if an effective strain is found.

192 ***Biological control strategies to prevent infection.*** One of the most promising grape CG control
193 technologies is biological control and it has been defined in many different ways. This review adheres to
194 the definition of plant disease biological control as the control of a plant disease with a natural biological
195 process or the product of a natural biological process, including biological chemicals extracted from and

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196 delivered by living organisms and constitutive and elicited host resistances (Wilson 1997). This definition
197 allows clear dissociation from other means of plant disease control, such as physical, cultural, synthetic
198 chemical, or genetic engineering-assisted control. Research efforts to discover or develop living
199 organisms, or products created by those organisms, for grapevine CG suppression were initiated in 1972
200 (Kerr 1972).

201 The remarkable success of *A. radiobacter* strain K84 represents one of the most exhaustively
202 researched and commercially widely used antagonistic microorganisms (Cooksey and Moore 1982, du
203 Plessis et al. 1985, Farrand et al. 1985, Donner et al. 1993, Vicedo et al. 1993, Peñalver et al. 2000).
204 Effective CG control by the *A. radiobacter* strain was demonstrated on several plants, including *Prunus,*
205 *Rubus, Malus, Salix, Libocedrus, Chrysanthemum, Crategus, Carya, Rosa, Pyrus,* and *Humulus* spp.
206 (Moore and Warren 1979). Unfortunately, strain K84 failed to control grapevine CG caused by *A. vitis*
207 (Staphorst et al. 1985, Chen and Xiang 1986, Webster et al. 1986). Consequently, many alternative
208 bacterial strains have now been studied for their ability to inhibit grapevine CG infection and formation
209 with mixed results. A summary of the most important strains and their relevant characteristics is presented
210 in Tables 1 and 2.

211 ***A. radiobacter strains MI15 and HLB-2.*** One of the BCAs proved to be effective against *A. vitis*
212 strains was isolated from grapevines (Xuemei et al. 1993). The agrocin-producing *A. radiobacter* strain
213 MI15 (isolated from CG on Muscat Hamburg) inhibited tumor formation caused by *A. vitis* strains *in vitro*
214 and on inoculated grapevine shoots. Colonization of wound sites on grapevine stems by *A. radiobacter*
215 strain MI15 was also reported (Xuemei et al. 1993).

216 In another experiment, the *A. radiobacter* strain HLB-2 inhibited CG formation on inoculated young
217 grapevine shoots (Chen and Xiang 1986). *In vitro* growth of *A. vitis* strains harboring the nopaline or
218 octopine plasmid was inhibited by co-inoculations (Chen and Xiang 1986).

219 Further research on *A. radiobacter* strain HLB-2 demonstrated suppression of grapevine CG caused
220 by *A. vitis* strains Ag57, Ag63R, and G-1 (Pu and Goodman 1993b). Seven additional strains of
221 *Agrobacterium* spp. also showed sensitivity to *A. radiobacter* strain HLB-2. Greatest suppression was
222 achieved when *A. radiobacter* strain HLB-2 was applied at 10,000 times higher concentration than the
223 tumorigenic *A. vitis* strain Ag63R. In this case, 87% of inoculated grapevine stems did not exhibit tumor
224 symptoms. When the same *Agrobacterium* strains were co-inoculated with equal or lower concentrations
225 of *A. radiobacter* strain HLB-2, 35 and 0% of co-inoculated grapevine stems remained symptomless,
226 respectively (Pu and Goodman 1993b).

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227 Dipping of grapevine roots with *A. radiobacter* strain HLB-2 just prior to planting into
228 *Agrobacterium*-infested soil prevented CG formation (Pu and Goodman 1993a). Thirty-four months after
229 planting, 100% of the Catawba, Seyval blanc, and Vidal blanc and 78% of the Chancellor root-treated
230 grapevines had no CG symptoms. Twenty percent of the untreated Catawba and Vidal blanc and 100% of
231 the untreated Chancellor had CGs (Pu and Goodman 1993a).

232 Bazzi et al. (1999) demonstrated that strain HLB-2 reduces internal populations of *A. vitis,* thus
233 promoting grapevine vitality and increasing graft take. Internal populations of *A. vitis* strain CG49 were
234 reduced from approx. 1.0 x 10^9 to 7.35 x 10^6 colony-forming units per mL (CFU/mL) following HLB-2
235 treatment. Less than half the HLB-2 inoculated grapevines were dead or unmarketable compared to the
236 untreated and controls (Bazzi et al. 1999). Although strain HLB-2 reduced internal *A. vitis*, the magnitude
237 of the reduction cannot ensure prevention of CG incidence in the vineyard.

238 Results of these experiments (Chen and Xiang 1986, Pu and Goodman 1993a, Pu and Goodman
239 1993b, and Bazzi et al. 1999) cannot be compared because different treatment methods were used, like
240 grapevine root versus stem, cutting, or shoot inoculations. Each treatment mode aims to assess the
241 efficacy of select BCAs against pathogenic *Agrobacterium* strains. However, deployment of BCAs can be
242 accomplished through different plant tissue systems and by various ways. This suggests that efficacy
243 (including colonization and translocation) of BCAs may significantly vary by plant tissue systems and
244 ways used for deployment.

245 ***A. tumefaciens strain J73.*** One of the first demonstrations of the biological control potential of
246 grapevine CG, using *Agrobacterium* strains other than *A. radiobacter* and *A. vitis*, involved *A.*
247 *tumefaciens* strain J73 (Thomson 1986, Webster et al. 1986). This strain exhibited a broad spectrum of
248 activity against *A. tumefaciens* and *A. vitis* strains *in vitro*. J73 was classified as an agrocin-producing
249 biotype-2 strain with a nopaline-type Ti plasmid. A previous experiment (Staphorst et al. 1985) verified
250 sensitivity of four biotype-3 strains to *A. radiobacter* strain K84. However, *A. radiobacter* strain K84 was
251 effective only against strains harboring nopaline-type Ti plasmids. J73 inhibited all nopaline-, octopine-,
252 and agropine-type Ti plasmid strains (Thomson 1986, Webster et al. 1986).

253 Strain J73 was pathogenic on a number of plants other than grapevines. This restricted its *in vivo* use
254 as a BCA for grapevine CG. To prevent potential CG elicitation, it had to be cured of its nopaline-type Ti
255 plasmid to disable the inter-kingdom transfer of strain J73 T-DNA. This was accomplished by the
256 introduction of selectable plasmids carrying the origins of replication of either the nopaline Ti plasmid,
257 pTiC58, or the octopine Ti plasmid, pTi15955 (Webster and Thomson 1988).

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258 ***A. vitis* strain E26.** A non-pathogenic, agrocin-producing, *Agrobacterium* biotype-3 strain was
259 isolated in 1989 (Liang et al. 1990a). It was originally designated as *A. radiobacter* strain E26. Today it is
260 identified as *A. vitis* strain E26. It was effective *in vitro* and *in vivo* against 20 biotype-3 and one biotype-
261 1 *Agrobacterium* isolates (Liang et al. 1990a, Liang et al. 1990b). Although inhibition levels were not
262 specified, strain E26 exhibited both *in vitro* and *in vivo* inhibition of C58 (biotype-1), K27 (biotype-2),
263 pt12 (biotype-1), A6 (biotype-1), and Bo542 (biotype-2) strains. *In vivo* inhibition was obtained on 18
264 octopine, 1 nopaline, and 1 arginin plasmid harboring *Agrobacterium* biotype-3 strains when grapevine
265 stems were co-inoculated with equal concentrations of strain E26. Isolation of strain E26 from wounded
266 sites 1 month after inoculation verified its ability to colonize grapevine tissues. It was also effective when
267 tested against the Ti-plasmid cured avirulent *A. tumefaciens* strain C58. Liang et al. (1990a, 1990b)
268 concluded that resistance to strain E26 is chromosomally encoded.

269 When grapevines were inoculated with tumorigenic *A. vitis* strains, CG formation was inhibited in all
270 12 cases by strain E26 (Liang et al. 2001). Eleven of 12 *A. vitis* strains showed *in vitro* sensitivity to strain
271 E26. An agrocin compound designated as Ar26 was isolated and purified from E26. It was effective
272 against *A. vitis* strain MI3-2 (Liang et al. 2001, Wang et al. 2003).

273 E26 was able to achieve high efficacy rhizosphere and rhizoplane colonization (Li et al. 2005). It was
274 also able to block attachment of the tumorigenic *A. vitis* K308 to grape stem and seedling root cells. The
275 average 10^4 CFU E26 population per g of dry soil and fresh roots at 5 months post-planting (Li et al.
276 2005) also suggests that E26 is suitable for grapevine CG suppression.

277 Numerous molecular biology studies were done on E26. Recently, the role of a LuxR-LuxI type
278 quorum-sensing system involved in the induction of the hyper-sensitive response (HR) in tobacco by
279 strain E26 was shown (Wang et al. 2008). $AvsI_{E26}$ and $AvsR_{E26}$ components of the wild type strain E26
280 are believed to be responsible for long and short chain acyl-homoserine lactones signal induction required
281 for HR in tobacco. The $avsI_{E26}$ mutant was unable to elicit the HR. This proved that the AvsI quorum-
282 sensing system was necessary for induction of the HR. Biological control efficacy of the wild type E26
283 and the $avsI_{E26}$ mutant strains was not significantly different, as demonstrated in pot trials. Developed
284 galls were very small and their weights, 40 days following inoculation, for grapevines co-inoculated with
285 the pathogenic *A. vitis* strain K308 and strain E26 or $avsI_{E26}$ mutant strain, were greatly reduced as
286 compared to when only K308 was used as inoculum-negative control (Wang et al. 2008).

287 A genetic study was undertaken on the *vir* regions of strain E26 for risk determination of its
288 commercial use as a BCA in field applications (Wei et al. 2009). None of the five (*virA, virG, iaaH,*
289 *iaaM,* and *ipt*) genes required for tumorigenicity were detected by PCR and Southern blot analyses. Lack

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290 of *virA* and *virG* pathogenic determinants in strain E26 suggests its inability for CG symptom elicitation
291 in both host and non-host plants (Wei et al. 2009).

292 ***A. vitis* strain F2.** Another *A. vitis* strain, first isolated in South Africa and designated as strain F2,
293 redesignated as *A. vitis* strain F2/5 by Burr and Reid (1994), was proven to be non-tumorigenic on
294 grapevines and effective against grapevine CG (Staphorst et al. 1985, Burr et al. 1999). Using *in vitro*
295 testing, 17 of 25 serologically different non-agrocin-producing biotype-3 strains showed sensitivity to
296 strain F2. Crown gall formation by 12 biotype-3 strains was prevented in the greenhouse when co-
297 inoculation of 11 different non-agrocin-producing and four agrocin-producing tumorigenic *A. vitis* strains
298 was made with strain F2 (Staphorst et al. 1985, Burr et al. 1999).

299 Burr and Reid (1994) tested *A. vitis* strain F2/5 against 25 *A. vitis* and 19 *A. tumefaciens* strains *in*
300 *vitro* and 10 *A. vitis* and three *A. tumefaciens* on inoculated grapevine trunks. *In vitro*, 21 *A. vitis* and two
301 *A. tumefaciens* (biotype-1) strains exhibited sensitivity to strain F2/5. Greatest efficacy of CG suppression
302 was observed on grapevine trunks when suspensions containing equal concentrations of strain F2/5 and
303 the pathogen were co-inoculated. Reduction of gall size for seven *A. vitis* and for two *A. tumefaciens*
304 strains was observed. Only in cases where *A. vitis* strain CG78 was used, no reduction was exhibited in
305 the number of inoculated sites in which galls developed (Burr and Reid 1994).

306 The protective action of antagonist *A. vitis* strains F2/5, CG1077 (the agrocin-minus mutant of F2/5),
307 CG523, and *A. radiobacter* strain HLB-2 was tested (Bazzi et al. 1999). Those antagonists were used
308 against the pathogenic *A. vitis* strain CG49. Influences on grapevine vitality, graft take, and wood and root
309 productions were assessed. Eight months post-treatment, callus tissues were collected close to the graft
310 union and subjected to quantitative isolation of *A. vitis* strain CG4. Maximum decrease in the internal
311 concentration of this strain was achieved in grapevines treated with the antagonist F2/5-mutant strain
312 CG1077. Excessive lignification and severe tissue necrosis at both the graft union and vascular
313 parenchyma, throughout the vines treated with the wild-type strain F2/5, were reported. The strain F2/5
314 treatment significantly reduced graft take (from 97.5 to 67.5%) while there was only a slight decrease
315 (from 67.1 to 63.2%) for the control. Grapevines treated with the BCAs (other than F2/5) also exhibited
316 fewer necrotic symptoms on woody parenchyma tissues. Bazzi et al. (1999) concluded that establishing
317 vineyards with grapevines containing a high concentration of *A. vitis* may be difficult.

318 Burr et al. (1997) questioned whether the biological control ability of strain F2/5 is based on agrocin
319 production. Other hypotheses, including competition for attachment sites on grapevine cells and possible
320 prevention of T-DNA transfer by strain F2/5, were also addressed. Chardonnay vines were inoculated
321 with a 1:1 ratio of mixed suspensions containing the *A. vitis* strain CG49 and strain F2/5 or its agrocin-

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322 minus mutants CG1076, CG1077, CG1078, or CG1079. The number of cells of strain CG49 that attached
323 to grapevine shoot tissues was significantly reduced (from approx. 10^5 to 10^4 CFU/mL) following co-
324 inoculation with either F2/5 or CG1077 strains. Results confirmed that grapevine CG control by strain
325 F2/5 is neither associated with agrocin production nor with competition for attachment sites (Burr et al.
326 1997). Moreover, it is not associated either with its tartrate or octopine utilization plasmids (Szegedi et al.
327 1999).

328 Strain F2/5 was GM for trifolitoxin (TFX) peptide antibiotic production (Herlache and Triplett 2002).
329 The strain (*A. vitis* strain F2/5(pT2TFXK)) exhibited an enhanced level of CG disease suppression. It
330 contains a plasmid that is encoded for TFX production. Strain F2/5(pT2TFXK) is more effective against
331 all tested *A. vitis* tumorigenic strains than the wild type F2/5, as well as the TFX non-producing negative
332 control, F2/5(pT2TX3K). One month after co-inoculation of *Nicotiana glauca*, with wild type F2/5 and *A.*
333 *vitis* strains CG49, CG78, or CG435, gall formation occurred in all plants. However, when co-inoculation
334 was performed in the same manner, but with the GM TFX producing F2/5(pT2TFXK), gall formation
335 was reduced. In those cases, 10 of 14 (71.4%), 1 of 8 (12.5%), and 0 of 14 (0.0%) of the inoculants
336 exhibited tumor symptoms, respectively (Herlache and Triplett 2002). The competition of TFX
337 compounds with *Agrobacterium* and *Rhizobium* spp. is likely an important factor of its biological control
338 mechanism (Robleto et al. 1998, Scupham and Triplett 2006). More work is needed to specify TFX mode
339 of action.

340 Potential negative effects of strain F2/5 on grapevine tissues were previously reported (Bazzi et al.
341 1999) that include wound healing and graft-take reductions, partial callus formation, excessive
342 lignification, and tissue necrosis in cuttings. Those negative effects were confirmed (Creasap et al. 2005).
343 Necrotic symptoms were observed on cells generated following cambium activation at wounded sites.
344 This necrosis was suggested as a cause for abnormal wound healing and failure of graft union. The
345 necrosis is regulated by expression of a *luxR* homolog, *aviR* gene (Zheng et al. 2003, Hao et al. 2005).
346 The exact mode of action by which strain F2/5 controls grape CG remains unknown.

347 ***A. vitis strain VAR03-1.*** The non-pathogenic *A. vitis* strain VAR03-1 was the most effective strain
348 tested against pathogenic *A. vitis* isolates in several experiments. In the first series of experiments
349 (Kawaguchi et al. 2005), all the 11 tumorigenic *A. vitis* strains showed *in vitro* sensitivity to strain
350 VAR03-1. One year after *in vivo* co-inoculation of Neo Muscat seedling stems, no withered symptoms
351 were observed. In this case, strain VAR03-1 was co-inoculated with the tumorigenic *A. vitis* strain G-Ag-
352 27. In a separate experiment, significant reduction was achieved (as compared to when only G-Ag-27 was
353 used as inoculum-positive control) in the number of sites at which galls developed. Ten grapevine

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354 seedling stems were used for each replication and seven inoculations were made on each stem. Co-
355 inoculations were made with suspensions containing a 1:1 ratio of the pathogenic G-Ag-27 and VAR03-1.
356 Tumor formation reductions (TFRs), measured 12 weeks after inoculation, were from 66 of 70 to 0 of 70
357 (94.3% TFR), from 70 of 70 to 23 of 70 (67.1% TFR), and from 68 of 70 to 10 of 70 (82.9% TFR)
358 (Kawaguchi et al. 2005).

359 In the second series of experiments, grape CG biological control ability of strain VAR03-1 was
360 assessed against *A. vitis* strains G-Ag-27 and seven other *A. vitis* strains isolated in Japan (Kawaguchi et
361 al. 2007). Inoculation of Neo Muscat cuttings was made with suspensions containing approx. 10^8
362 CFU/mL of pathogenic inoculant-positive control. Co-inoculation was made with a 1:1 ratio of the
363 pathogenic strain and the BCA VAR03-1. Ten grapevine seedling stems were used for each replication
364 and six inoculations were made on each stem. Twelve weeks after inoculations, CG incidence were
365 reduced from 93.3% (highest TFR) to 33.3% (lowest TFR) when the BCA and strain A5-6 or strain A5-1
366 were used in co-inoculations. Statistically significant reduction in CG incidence by *A. vitis* strain G-Ag-27
367 was also reported after grapevine root treatments (Kawaguchi et al. 2007).

368 Recently, in the third series of experiments, high efficacy of strain VAR03-1 to control grape CG
369 caused by *A. vitis* strains MAFF 211674, MAFF 211676, At-90-23, and G-Ag-27 was reported
370 (Kawaguchi et al. 2008).

371 ***Antagonists outside the genus Agrobacterium.*** Although the majority of BCAs tested against grape
372 CG belong to the genus *Agrobacterium*, significant efforts have been made to identify potential biological
373 control organisms outside this genus. In one study, 24 of 851 bacterial isolates exhibited activity against
374 *A. vitis* strains (Bell et al. 1995). Biolog identification tests revealed that the few effective isolates were
375 strains of *Enterobacter agglomerans*, *Rahnella aquatilis,* and *Pseudomonas* spp. The most rapid decline
376 of population of *A. vitis* strain AA25 in soil was achieved when soil co-inoculations were made using a
377 10:1 ratio of AA25 and *Pseudomonas corrugata* strain JC583. *In vitro* and *in planta*, three *P. corrugata*
378 strains exhibited various levels of biological control ability as measured by growth and tumor formation
379 inhibition of tumorigenic *A. vitis* strains, respectively (Bell et al. 1995).

380 ***Pseudomonas spp. strains.*** *Pseudomonas aureofaciens* strain B-4117 and *P. fluorescens* strain
381 CR330D were tested for biological control activity against *A. vitis* strains Tm4 and Sz1 (Khmel et al.
382 1998). The pattern of decline over time in numbers of antagonistic bacteria on grapevine roots was similar
383 for all strains used. Rapid decline was observed 120 days after inoculation then remained constant for up
384 to 1 year. When cuttings were treated with antagonists, a decrease in CG incidence and lowered

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385 pathogenicity, as shown by reduction in tumor sizes, were observed as compared to water-treated control
386 vines (Khmel et al. 1998).

387 ***Rahnella aquatilis strain HX2.*** Although inhibitory effects of *R. aquatilis* bacteria towards various
388 isolates of *A. vitis* were already reported (Bell et al. 1995), only recently have extensive studies on this
389 potential BCA been initiated. *Rahnella aquatilis* strain HX2 isolated in China from vineyard soil was
390 shown to be effective against a number of *A. vitis* isolates (Chen et al. 2007). Experiments confirmed the
391 remarkable efficacy of HX2 for short term CG suppression (Chen et al. 2007). Recently, further
392 biological control assays and characterizations of strain HX2 were also made (Chen et al. 2009). Analysis
393 of the antimicrobial compound produced by this strain revealed that the compound: is likely to be
394 thermostable; contains one or more kind of sugars; is most active against *Agrobacterium* spp.; and
395 inhibits *Clavibacter*, *Pectobacterium*, *Pseudomonas* and *Xanthomonas* spp. It was determined that its
396 mode of action does not include bacterial cell lysis nor causes cytoplasmic material leakage at minimal
397 inhibitory concentration. Analyses conducted on the impacts of HX2 antimicrobial compound(s)
398 suggested that inhibition of RNA and protein synthesis, required for T-DNA transfer and tumor formation
399 processes, is likely to be the main factor involved in its mode of action (Chen et al. 2009).

400 ***Genetic engineering strategies to prevent infection.*** Several experiments targeted the prevention of
401 grape CG infection by expressing antimicrobial peptides in GM plants inhibitory to *A. vitis*. Genetically
402 modified Chardonnay grapevines, which possessed either the natural (*Mag-2*) or the synthetic (*MSI99*)
403 maganin short peptide antimicrobial compound-producing genes, were assayed. Their ability to confer
404 resistance to grape CG (*A. vitis*) and powdery mildew (*Erysiphe necator*) was tested (Vidal et al. 2006).
405 The development of strong resistance was not achieved; nonetheless, regenerated lines expressing the
406 *Mag-2* or *MSI99* genes exhibited significantly less CG incidence than non-GM controls. Enhancement of
407 transgene expression and the spectrum of resistance were suggested to achieve grape CG and powdery
408 mildew resistance with GM grapevines carrying genes encoded for maganin antimicrobial compound
409 production (Vidal et al. 2006).

410 Recently, CG suppression via GM grapevines carrying the *Mag-2* or *MSI99* genes was demonstrated
411 (Kikkert et al. 2009). Decreased pathogenicity by reduction in gall sizes was observed on greenhouse-
412 grown maganin-expressing Chardonnay inoculated with *A. vitis* strain TM4 or CG450. *In vitro* inhibition
413 of those *A. vitis* strains by *Mag-2* and *MSI99* compounds was also reported (Kikkert et al. 2009).

414 These results suggest the high CG resistance potential of grapevines expressing *Mag-2* or *MSI99*
415 genes. Increased resistance to CG may be achieved by expressing the *Mag-2* or *MSI99* peptides in GM
416 grapevines containing truncated *vir* genes. This may result in antimicrobial activity and inhibition of T-

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417 DNA export and integration simultaneously. This multiple inhibition of pathogenic strains may prevent
418 CG formation by simultaneous blocking of the infection and genetic transformation. Therefore, further
419 research with the *Mag-2* and *MSI99* peptides in GM grapevines is warranted.
420 ***Strategies to prevent T-DNA export to plant cell and integration into the plant nuclear genome.***
421 Recently, high efficacy CG-resistant GM grapevine generations were confirmed by transferring and
422 expressing the truncated form of the Ti-plasmid *virE2* gene in grapevine somatic embryos via
423 *Agrobacterium*-mediated transformation (Krastanova et al. 2010). According to several previous VirE2-
424 involved T-DNA export and integration blocking studies, the increased resistance is believed to be
425 associated with competition of the mutated VirE2 proteins with functional VirE2 proteins. Within the
426 plant cell the competition appears to block the integration of the T-DNA into the plant cell nucleus (Burr
427 and Otten 1999, Gelvin 2003, Matthysse 2006, Otten et al. 2008). The truncated *virE2* genes from *A.*
428 *tumefaciens* strain C58 and A6 and *A. vitis* strain CG450 were expressed in Teleki 5C, Richter 110, and
429 Couderc 3309 grapevine rootstocks. The truncated and transferred *virE2* genes were present in 314 of 322
430 (97.5%) GM lines, confirmed by double antibody sandwich enzyme-linked immunosorbent assay (DAS-
431 ELISA), and in 285 of 295 (96.6%) GM lines, confirmed by polymerase chain reaction. *In vitro*
432 susceptibility to *Agrobacterium* was evaluated 14 and 21 days post-inoculation by visual observation of
433 tumor formation on inoculated shoot internodes. Genetically modified plants with no galls or with galls
434 smaller than 2 mm diameter on half or less of the total number of explants were scored as resistant. When
435 *in vitro*, strain C58-*virE2* gene transformed Richter 110 shoot internode segments were inoculated, with
436 tumorigenic *A. tumefaciens* strain C58 (nopaline), A6 (octopine), or *A. vitis* strain CG450 (vitopine),
437 tumor formations were reduced significantly as compared to the non-GM control Richter 110. The highest
438 TFR was achieved when tumorigenic *A. tumefaciens* strain C58 was used in the inoculations of strain
439 C58-*virE2* gene transformed Richter 110. All of the non-GM control Richter 110 exhibited visible
440 tumors. When *in vitro*, strain C58, A6, or CG450-*virE2* gene transformed Teleki 5C shoot internodes
441 were inoculated, with homologous or heterologous tumorigenic *A. tumefaciens* strain C58, A6, or *A. vitis*
442 strain CG450, tumor formations were also reduced significantly as compared to the non-GM control
443 Teleki 5C.
444 The highest TFRs (90.0%), as compared to the non-GM control Teleki 5C (5.0 and 0.0%), were
445 achieved when tumorigenic A. tumefaciens strain C58 or A. vitis strain CG450 were used in the
446 inoculations of strain C58-*virE2* or CG450-*virE2* gene transformed Teleki 5C, respectively. In cases of
447 the non-GM control Teleki 5C inoculations, CG developed on 95.0, 94.7, and on 100.0% strain C58, A6,
448 or CG450 inoculated shoot internodes, respectively (Krastanova et al. 2010).

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449 This research confirmed the feasibility of GM rootstock grapevine production using truncated *virE2*
450 genes of tumorigenic *A. tumefaciens* strain C58, A6, and *A. vitis* CG450. Due to the highest TFR
451 performances exhibited by C58-*virE2* gene transformed vines, the truncated C58-*virE2* can be considered
452 one of the most effective genetic resources available for CG-resistant GM grapevine generation.
453 Evaluation of long-term CG resistance of the generated GM vines in vineyard settings and further
454 investigation on the efficacy of truncated *virE2* genes of other previously not tested *Agrobacterium* strains
455 are suggested.

456 The development of CG-resistant Richter 110 rootstock grapevines was achieved by blocking T-DNA
457 integration into the host genome (Holden et al. 2003). Genetically modified grapevines containing the
458 truncated *virE2* genes of *A. tumefaciens* strain C58 or A6, or *A. vitis* strain CG450 were generated and
459 assessed for CG susceptibility. Seven lines of shoot explants, transformed with the truncated C58-*virE2*
460 gene and inoculated with strain C58, expressed resistance. Two of these seven also showed resistance to
461 the other two *Agrobacterium* strains (Holden et al. 2003).

462 These results suggest that transformation of grapevines with strain-specific truncated *Agrobacterium*
463 *virE2* genes can result in grapevines expressing resistance to different *Agrobacterium* biotypes and
464 strains. As reported by Krastanova et al. (2010), this experiment confirmed the importance of truncated
465 *virE2* gene of *A. tumefaciens* strain C58 in CG-resistant grapevine generation.

466 A total of 928 putative GM grapevine combinations were generated by Xue et al. (1999) and tested
467 for resistance to grape CG, grapevine fanleaf virus (GFLV), and grapevine leafroll-associated
468 closterovirus-3 (GLRaV-3). Transformed vines contained a sense-oriented translatable gene of GFLV, an
469 antisense coat protein gene of truncated HSP90-related (heat shock protein 90) gene of GLRaV-3, and a
470 *virE2* of B gene (a truncated *virE2* that lacks 215 carboxyl-terminal amino acids; Citovsky et al. 1994)
471 from *A. tumefaciens* strain C58. Transformation efficiency of the five grape rootstocks with seven gene
472 constructs varied. Rates of transformation ranged from 45 to 100%. Transgenes were present in each case
473 as determined by PCR or Southern Blot analysis. Disease and virus resistance evaluations of the GM
474 vines are in progress (Xue et al. 1999).

475 ***Strategies to prevent expression of T-DNA oncogenes.*** Transferred-DNA oncogene expression
476 prevention (often referred to as gene silencing), using RNAi technology, involves within-plant cell
477 expression of messenger ribonucleic acid (mRNA) complementary to the mRNA of a gene whose
478 expression inhibition is targeted. Expression inhibition of a gene is accomplished through 21 to 25
479 nucleotides long RNA species (RNAs) that are derived to become RNA-induced gene silencing (Agrawal
480 et al. 2003). Employment of RNAs leads to the degradation of targeted RNA to nucleotides resulting in T-

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481 DNA oncogene expression prevention. Efficacy of gene silencing for high level CG resistance was
482 confirmed by several research experiments (Ebinuma et al. 1997, Escobar et al. 2001, Kovács et al. 2003,
483 Lee et al. 2003).

484 Feasibility of silencing the expression of an *A. vitis* oncogene, thus preventing tumor formation, has
485 been investigated (Kovács et al. 2003). This study demonstrated oncogene silencing in GM *Petunia*
486 *hybrida* containing double-transformed 35S-*iaaM* gene. One line of those transformed plants exhibited
487 reduced *iaaM* RNA levels, however, failed to attenuate tumorigenesis when challenged with *A. vitis*
488 isolates. A possible explanation for this phenomenon is that the 35S-*iaaM* transgene may have produced a
489 chimeric RNA molecule that was unable to induce silencing of the *A. vitis iaaM* gene (Kovács et al. 2003,
490 Kovács, personal communication). It was demonstrated by Lee et al. (2003) that the *iaaM* silencing
491 construct must contain the translation start site of the gene's sense strand in order for silencing to be
492 effective.
493

494 **Conclusion**

495 Whether biological control using natural bacterial strains, biological control by genetically modified
496 antagonistic strains, or genetic engineering of grapevines will lead to control grape CG disease is still
497 under investigation. Despite current limitations, biological control appears to have the greatest probability
498 for success in grapevine CG control. Moreover, it shall likely prove to be the most palatable strategy for
499 consumers, which expectedly will drive the preference of the industry accordingly. The genetic diversity
500 of *Agrobacterium* grapevine isolates is considered the major limitation for successful biological control.
501 For example, diversity in the *A. vitis* population could affect susceptibility to bacteriocins or antibiotics
502 produced by antagonistic strains. In numerous research experiments, the effectiveness of grapevine CG
503 BCAs was restricted to certain opine-metabolizing tumorigenic *A. vitis* strains. This suggests that the
504 sensitivity of various opine-metabolizing strains to biological control strains varies. Assessments of
505 opine-metabolizing strain sensitivities to BCAs may foster understanding of action mechanisms of
506 biological control strains. Deoxyribonucleic acid fingerprint comparison may also foster understanding of
507 the genetic background of the diversity of *Agrobacterium* spp., which is essential for developing more
508 effective BCAs. These may lead to the development of a BCA effective against each opine-metabolizing
509 *A. vitis* strain type. Besides efficacy against pathogenic strains, additional limitation of biological control
510 strains includes their persistence following deployment, including persistence in various soil types *in vivo*
511 and in different rooting media. Endophytic colonization by BCAs and systemic movement throughout the
512 plant are some other major limitations to their effective deployment. Nevertheless, the commercial

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513 success of *A. radiobacter* strain K84 and derivatives in controlling CG caused by *A. tumefaciens* confirms
514 that this approach could be utilized in grape CG management as well. Future research, of both previously
515 tested and potential new isolates of BCAs, is necessary to: determine specific mode(s) of action, complete
516 molecular and genetic understanding of biological control mechanisms, discover and develop new BCAs,
517 field-test all effective biological control strains, and develop combinations of various BCAs capable of
518 controlling grapevine CG disease regardless of the diversity of *A. vitis* strains.

519 Genetic mechanisms that trigger malignant cell growth and underlie CG formation are still not
520 completely understood. Determination of currently unknown grapevine and *Agrobacterium* genes
521 involved in the grape CG infection and genetic transformation processes and their exact roles would
522 enhance understanding of the mechanisms of this disease. Furthermore, technological approaches in GM
523 grapevine production, including the production of GM CG-resistant grapevines, must ensure that GM
524 grapevines will have the same positive horticultural and quality characteristics as the non-GM plants.

525 Continued interest of the grape industry in CG-resistant grapevines is another factor critically
526 necessary to achieve CG-resistant grapevine production. Virus-resistant GM grapevine production was
527 accomplished with significant support from the grape and wine industry after unacceptable economic
528 losses attributed to grapevine decline due to viral infections. Produced vines have been tested under
529 experimental field conditions in different regions of the world; however, they are not available
530 commercially. Therefore, to achieve CG-resistant grapevine production, close collaboration between
531 scientific institutions and grape growers is pivotal. Improvements in the following areas may further
532 foster the development of CG-resistant GM grapevine production: development of grape variety-specific
533 regeneration methods to increase regeneration efficiency; reduction of the 5 to 10-year time requirement
534 to generate testable GM grapevines; development of more rapid, accurate, and sensitive indexing systems
535 to increase the reliability of presence determination of inserted genes and for the selective detection of
536 tumorigenic and non-tumorigenic *Agrobacterium* strains within grapevines; and development of a
537 standardized evaluation system for CG resistance. In addition, successful commercial production of GM
538 CG-resistant grapevines cannot be achieved without public acceptance.

539 Although biological control and genetic engineering were discussed separately, successful
540 suppression of grapevine CG is likely to be achieved by incorporating and applying strategies from both
541 those areas. This was mainly indicated in this review by describing GM BCAs and their applications. The
542 importance of further collaboration between researchers from those fields and grape industry
543 representatives would seem essential to further progress.
544
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Table 1. List of selected bacterial spp. strains examined for biological control against grapevine CG and their relevant characteristics.

Bacterial spp.	Strains	Relevant characteristics	References
A. radiobacter	HLB-2	Inhibits CG formation on inoculated grapevine shoots. Root treatments prevent CG formation.	Pu and Goodman 1993a, 1993b Bazzi et al. 1999
A. radiobacter	K84	Reported to be ineffective against *A. vitis* strains. One contradictory report indicated inhibition of a few *A. vitis* strains in *vitro*.	Kerr and Htay 1974 Moore and Warren 1979 Donner et al. 1993 Vicedo et al. 1993
A. radiobacter	M115	Inhibits *in vitro* growth and tumor formation caused by *Agrobacterium* biotype-3 strains. Effective colonizer of wound sites on grape stems.	Xuemei et al. 1993
A. tumefaciens	J73	Agrocin-producing strain with broad spectrum activity against *A. vitis* and *A. tumefaciens*.	Thomson 1986 Webster et al. 1986
A. vitis	E26	*In vitro* and *in vivo* activity against *A. vitis* strains.	Liang et al. 2001 Li et al. 2005 Wang et al. 2008 Wei et al. 2009
A. vitis	F2	Non-tumorigenic on grapevines. Effective against grape CG caused by agrocin and non-agrocin producing *A. vitis*.	Staphorst et al. 1985
A. vitis	F2/5	Non-tumorigenic on grapevines. Biological control is specific to grapevine CG. Inhibits growth of all *A. vitis* strains tested. Causes necrosis of treated grapevine tissues.	Burr and Reid 1994 Burr et al. 1997 Bazzi et al. 1999 Burr et al. 1999 Szegedi et al. 1999 Zheng et al. 2003 Creasap et al. 2005 Hao et al. 2005
A. vitis	VAR03-1	Highly effective inhibitor of *A. vitis* pathogenic strains *in vitro* and *in vivo*. Root colonizer.	Kawaguchi et al. 2005, 2007, 2008
P. aureofaciens	B-4117	Inhibits CG development if grape cuttings are submerged in bacterial solution.	Khmel et al. 1998
P. fluorescens	CR330D	Reduces CG incidence and inhibits tumor formation.	Khmel et al. 1998
P. fluorescens	1100-6	Reduces CG incidence and internal populations of *A. vitis*.	Eastwell et al. 2006
P. corrugata	JC583	Varying levels of tumor formation inhibition in inoculated grapevine.	Bell et al. 1995
R. aquatilis	HX2	Inhibits CG formation. Effective against a number of *A. vitis* strains. Produces an antimicrobial compound.	Bell et al. 1995 Chen et al. 2007, 2009

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Table 2. *In vitro* growth, CG formation inhibition, colonization, persistence, and other relevant characteristics of selected bacterial spp. strains effective in biological control against grapevine CG.

Bacterial spp. strains	*In vitro* growth and CG formation inhibition	Colonization, persistence, and other characteristics
A. radiobacter HLB-2	Crown gall did not develop on root treated vines. Seventy-eight percent of root treated Chancellor did not exhibit CG (Pu and Goodman 1993a). Eighty-seven percent of treated vines did not exhibit CG (Pu and Goodman 1993b).	Reduced internal *A. vitis* concentration from approx. 10^9 to 7.35 x 10^6 CFU/mL (Bazzi et al. 1999).
A. tumefaciens J73	Inhibited *in vitro* growth of 9 of 16 *Agrobacterium* biotype-1, 11 of 14 biotype-2, and 22 of 27 *A. vitis* strains (Webster et al. 1986). Inhibited *in vitro* growth of all nopaline, octopine, and agropine type Ti plasmid strains (Webster and Thomson 1988).	Development of Ti plasmid transfer deficient strain J73 was accomplished (Webster and Thomson 1988).
A. vitis E26	Inhibited *in vitro* growth of 20 biotype-3 and one biotype-1 *Agrobacterium* strains. Inhibited *in vivo* CG formation of 18 octopine, one nopaline, and one arginine plasmid harboring *Agrobacterium* biotype-3 strains (Liang et al. 1990a, Liang et al. 1990b). Inhibited *in vivo* CG formation of all *A. vitis* strains (Liang et al. 2001). Inhibited *in vitro* growth of 11 of 12 *A. vitis* strains (Wang et al. 2003).	Was isolated from wound sites 1 month post-inoculation (Liang et al. 1990a). Approximately 10^4 CFU per g of dry soil and fresh roots was measured at 5 months post-planting. Blocked the attachment of tumorigenic *A. vitis* strain K308 to grape stem and seedling root cells (Li et al. 2005). The $avsI_{E26}$ mutant strain is as effective in controlling grape CG as the wild type E26 (Wang et al. 2008).
A. vitis F2	Inhibited *in vitro* growth of 17 of 25 non-agrocin producing biotype-3 strains (Staphorst et al. 1985). Inhibited *in vivo* CG formation of 12 *A. vitis* strains (Burr et al. 1999).	
A. vitis F2/5	Inhibited *in vitro* growth of 21 of 25 *A. vitis* strains. Two of 19 *A. tumefaciens* strains showed sensitivity to F2/5. Reduced *A. vitis* and *A. tumefaciens* tumor sizes. Number of sites at which CG developed was not reduced only when *A. vitis* strain CG78 was used in inoculations (Burr and Reid 1994).	Caused necrosis of treated grape tissues. This inhibited callus and graft union formation and reduced graft take (Bazzi et al. 1999, Creasap et al. 2005). Biological control by F2/5 is neither associated with agrocin production and competition for attachment sites nor with its tartrate or octopine utilization plasmids. Inhibition or prevention of T-DNA transfer is likely the main factor of its CG control mechanism (Burr et al. 1997, Szegedi et al. 1999). Necrosis induction may be regulated by *aviR* gene (Zheng et al. 2003). Regulatory role determination of *clp* genes in strain F2/5 is in progress (Burr, personal communication).

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A. vitis CG1077 F2/5	CG1077 inhibited pathogenic *A. vitis* more than strain F2/5 (Burr et al. 1997). Maximum reduction in internal *A. vitis* concentration, from approx. 10^9 to 1.24 x 10^6 CFU/mL, was achieved by CG1077 (Bazzi et al. 1999).	
A. vitis F2/5 CG1076 CG1077 CG1078 CG1079	They reduced the percentage of sites at which CG developed by *A. vitis* strain K306 or CG49 up to 92 and 100%. Reduced gall areas ranged from 3 to 21 mm^2. Average tumor areas and sites at which galls developed for inoculations only with strain K306 or CG49 were 140 and 40 mm^2 and 95 and 80%, respectively (Burr et al. 1997). (Percentage and mm^2 values of this experiment are approximated from Fig. 2).	
A. vitis F2/5(pT2TFXK)	Exhibited increased efficacy against all tested *A. vitis* strains as compared to the wild type F2/5 (Herlache and Triplett 2002).	Produces trifolitoxin (TFX) peptide antibiotic (Herlache and Triplett 2002).
A. vitis VAR03-1	Inhibited *in vitro* growth of all tumorigenic *A. vitis* strains (Kawaguchi et al. 2005). Reduced the number of sites at which CG developed up to 94.3% (Kawaguchi et al. 2005, Kawaguchi et al. 2007). Inhibited *in vitro* growth of all tested pathogenic *Agrobacterium* strains. Inhibition zones ranged up to 24.5 mm. Root treatment reduced CG incidence up to 26.7% *in vivo* and 46.7% in the greenhouse (Kawaguchi et al. 2008).	Eighteen months after root submersion treatment, detectable VAR03-1 ranged from approx. $10^{6.10}$ to $10^{6.28}$ CFU/g of fresh roots (Kawaguchi et al. 2008).
P. aureofaciens B-4117 and *P. fluorescens* CR330D	*In vitro* growth inhibition zones ranged up to 14 and 20 mm for *A. vitis* strain Sz1 and Tm4. Reduced *in vivo* CG incidence up to 5 fold. Reduced *in vivo* CG incidence up to 3.5 fold 2 months post-planting the submerged and sprayed vines in the vineyard (Khmel et al. 1998).	One hundred twenty days post-inoculation, population density declined from approx. 10^9 to 10^4 CFU/g of dry root. The approx. 10^4 CFU/g of dry root population density remained constant for up to 1 year. Twenty-four days post-inoculation, the mean colonization level for sterile and non-sterile soil by the antagonists was approx. 10^4 CFU/g of soil (Khmel et al. 1998).
R. aquatilis HX2	*In vitro* inhibition zones of *A. vitis* strains ranged up to 42.7 mm. Eight months after root submersion treatment, *in vivo* CG incidences were reduced with 62.5, 63.8, and 61.3% (Chen et al. 2007). Prevented CG formation by *A. vitis* strain K308 for 40 days (Chen et al. 2009).	

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Figure 1 Severe CG incidence and consequently grapevine mortality in a three-year-old vineyard planted with Riesling (clone 239) vines grafted to Couderc 3309 or Millardet et de Grasset 101-14 rootstocks. The photo was taken at a Chateau Grand Traverse vineyard (Old Mission Peninsula, Michigan, U.S.) on July 11, 2010. In April 2010, 1776 of 3077 vines (57.7%) exhibited CG and 525 of those 1776 (29.6%) did not exhibit any growth during the summer.

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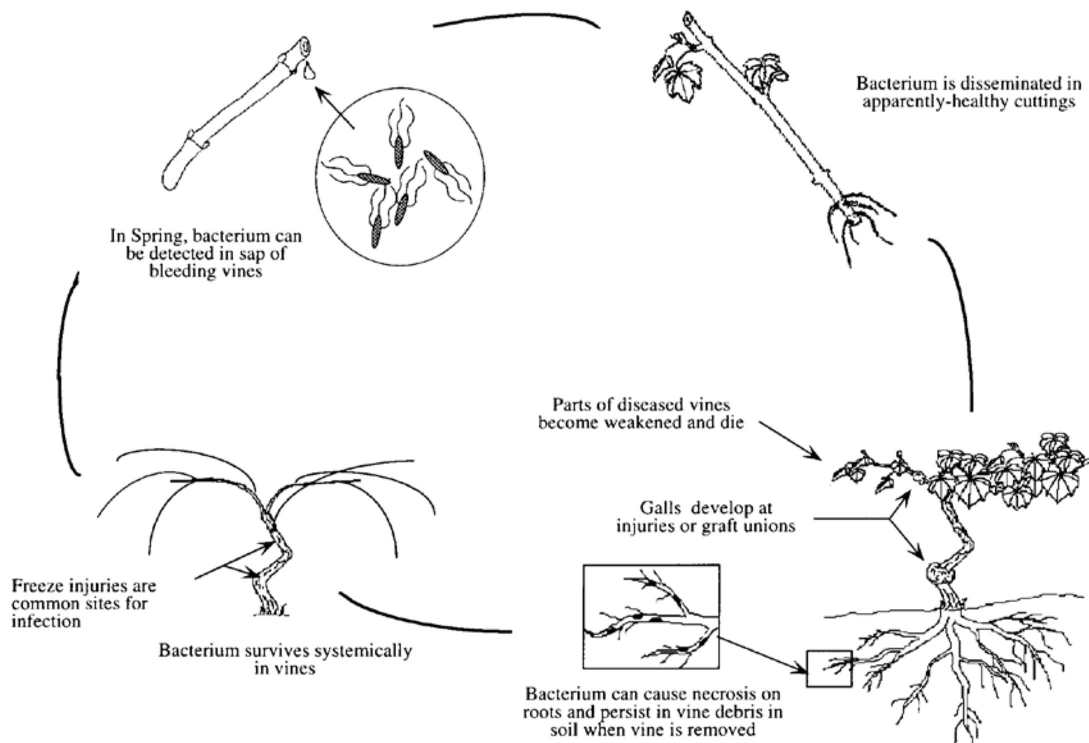

Figure 2 Disease cycle of grapevine CG (from Burr et al. 1998; reprinted by permission; © 1998 American Phytopathological Society).

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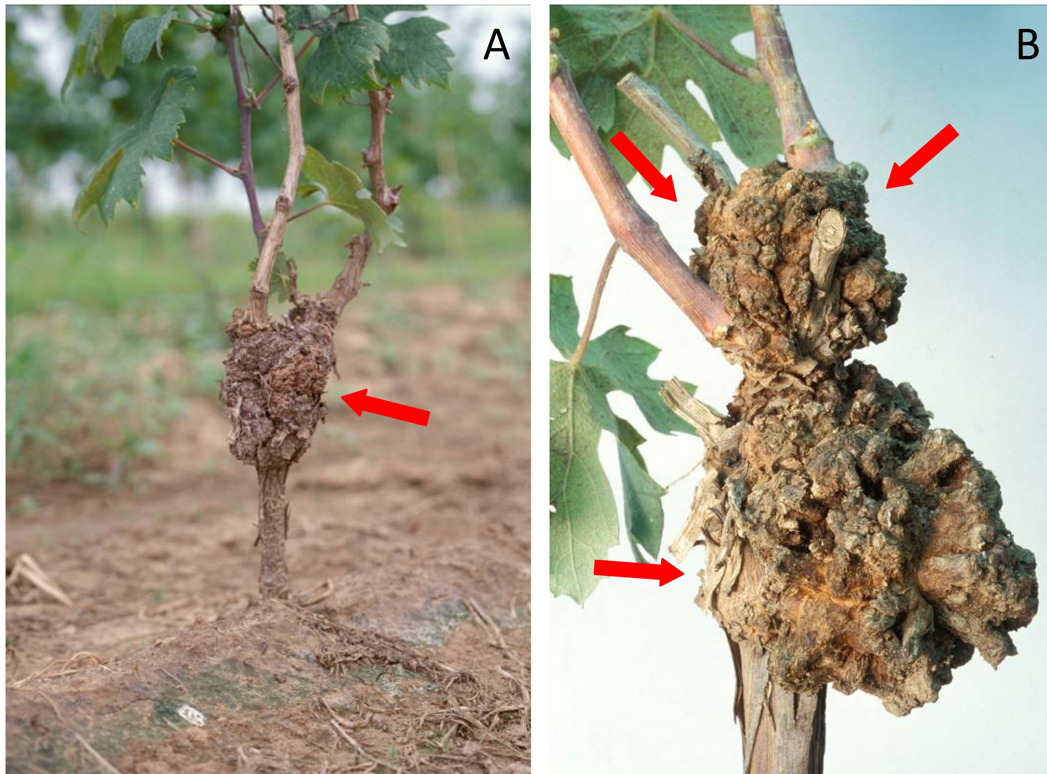

Figure 3 A, B Graft unions of Zinfandel grapevines exhibited CG shortly after vineyard establishment in the Canadian province of Ontario. Both vines will inevitably die. Crown gall developed at **A** the upper trunk, including the entire scion, and **B** wound sites around the graft union and the base of the shoots (red arrows).

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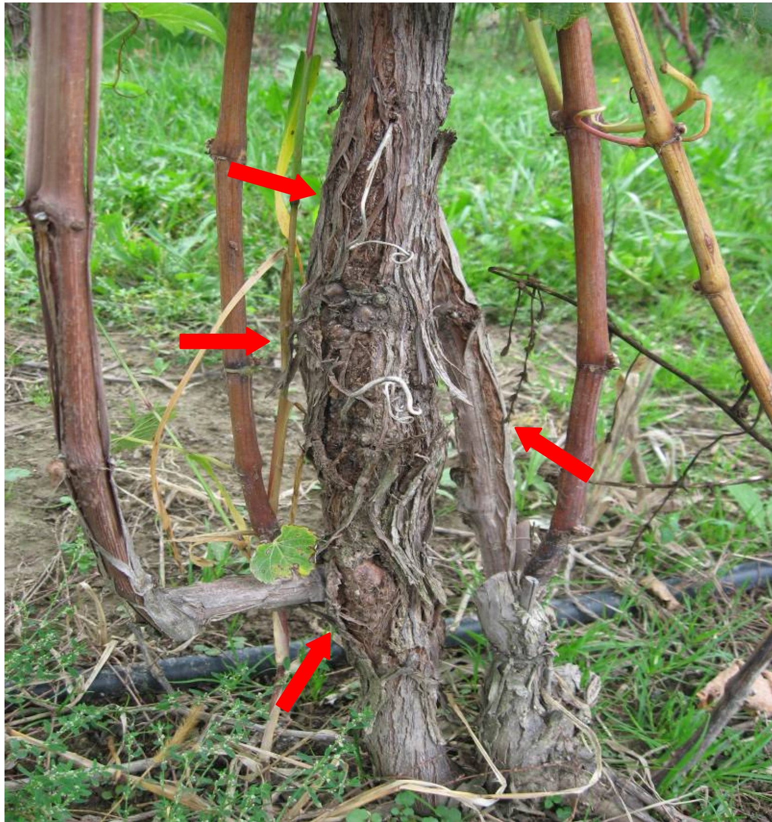

Figure 4 Own-rooted Cayuga White grapevine exhibits CG (red arrows). The photo was taken at the Horticulture Teaching and Research Center of Michigan State University (East Lansing, Michigan, U. S.). The use of multiple trunks helps to reduce the impact of the disease on the entire vine.