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Transformation of crown gall resistant and susceptible *Vitis* genotypes by *Agrobacterium vitis*

by

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S u m m a r y : Transformation of crown gall-susceptible and -resistant *Vitis* genotypes by *Agrobacterium vitis* strain CG49 was studied using *uid*A (GUS) in the p35SGUSINT construct. When greenhouse-grown material propagated through tissue culture was inoculated with CG49(p35SGUSINT) *in vitro*, the highly crown gall-susceptible *V. vinifera* Cabernet Sauvignon displayed GUS activity on 53 % of inoculated explants vs. 5 % for the resistant *V. amurensis* and 0 % for the resistant Couderc 3309. Response of Cabernet Sauvignon suggested a strong effect of shoot polarity on transformation. Inoculation of basal vs. apical explant surface in Cabernet Sauvignon indicated transformation in 88 % of basal inoculated explants with no transformation from apical inoculation. Basal inoculations indicated no transformation of *V. amurensis* and transformation in 10 % of Couderc 3309 explants. Inoculation of intact plants with CG49(p35SGUSINT) produced GUS-positive sites at 56 % of inoculated sites in Cabernet Sauvignon, 10 % of *V. amurensis* inoculated sites and 9 % of Couderc 3309 inoculated sites. Resistance to crown gall in these genotypes appears to be due to reduced susceptibility to transformation by *A. vitis* rather than post-transformation phenomena. These studies were complicated by production of GUS-positive spots from *in vitro* inoculations using wild-type CG49. Resident microorganisms producing b-glucuronidase may proliferate after tissue degradation by *A. vitis*-induced cell disruption. Use of *in vitro* internodal explants from tissue culture-propagated vines greatly reduced GUS expression from control CG49 inoculations and these were readily distinguished (by appearance and location) from GUS-positive spots resulting from transformation with *uid*A.

K e y w o r d s : Vitis, Agrobacterium, crown gall, disease resistance, transformation.

Introduction

Crown gall is a serious disease of grape in many regions of the world (BURR 1988). Although *Agrobacterium tumefaciens* biovar 1 is occasionally detected on grape, *A. vitis* is the predominant bacterial species causing grape crown gall (KERSTERS and DE LEY 1984, OPHEL and KERR 1990). It is known to exist systemically in grapevines including many that are asymptomatic (LEHOCZKY 1968 and 1971, BURR and KATZ 1983). As a result, *A. vitis* is often disseminated in grape nursery stock (BURR and KATZ 1984, GOODMAN *et al.* 1987). *A. vitis*-free material can be produced through shoot tip culture (BURR *et al.* 1988) but the pathogen survives in the grape rhizosphere (BURR *et al.* 1987 b) and *A. vitis*-free vines planted in *A. vitis*-infested soil have been shown to become infected (BISHOP *et al.* 1988). Since crown gall-susceptible vines apparently permit *A. vitis* entry through roots (BURR *et al.* 1987 b, BISHOP *et al.* 1988), the use of resistant rootstocks may reduce infection from soil inoculum and prevent subsequent infection of *A. vitis*-free but highly crown gall-susceptible scion cultivars. The work described here is part of a project directed toward identifying and characterizing crown gall-resistant grape rootstocks to test this hypothesis.

In the first part of this project, 43 *Vitis* genotypes were screened for susceptibility to crown gall by inoculation with a group of *A. vitis* strains from diverse geographic sources (STOVER 1993). Marked differences in genotype susceptibility were observed and differences in strain x genotype interactions suggest that there are at least two mechanisms of resistance to crown gall.

Infection of plants by crown gall pathogens and subsequent T-DNA transformation results in elevated levels of the plant growth regulators auxin and cytokinin which stimulate hyperplastic growths or galls (BINNS and THOMASHOW 1988). *Agrobacterium* strains with genes defective for auxin or cytokinin biosynthesis display different host ranges, suggesting that genotypic differences in plant hormone sensitivity can affect crown gall susceptibility (KAO *et al.* 1982, HOEKEMA *et al.* 1984). Reduced sensitivity of plants to plant growth regulators could result in no gall formation even though *Agrobacterium*-transformed cells are present and producing elevated levels of plant growth regulators. Alternatively, resistance to crown gall could result from suppression of transformation. In this study, the relative level of transformation induced by *A. vitis* in crown gall-resistant and susceptible *Vitis* genotypes was determined using *uid*A (GUS) as a marker gene. The gene product of *uid*A, β-glu-curonidase, can be easily assayed using the substrate 5-bromo-4-chloro-3-indolyl-D-glucuronic acid (x-gluc). When x-gluc is cleaved by β-glucuronidase, an insoluble blue dye is produced which identifies GUS-expressing tissues (JEFFERSON *et al.* 1987).

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Materials and methods

Bacterial strains: *E. coli* strain DH5 carrying p35S GUSINT (VANCANNEYT *et al.* 1990, gift of L. WILMITZER) containing *uid*A and *nptII* bracketed by T-DNA border regions was introduced into *A. vitis* strain CG49 through triparental mating (BOYER and ROULLAND-DUSSOIX 1969) using the helper plasmid RK2013. CG49(p35SGUSINT) was recovered on *Agrobacterium*-selective modified Roy and Sasser medium (BURR *et al.* 1987 b) containing 100 mg/l kanamycin, purified through single colony isolation, and maintained at -80 °C in cryogenic-storage medium (nutrient broth containing 22.5 % (w/v) glycerol). An intron in the GUSINT construct prevents expression outside of eukaryotic nuclei (VANCANNEYT *et al.* 1990), and no GUS activity was observed when bacterial pellets of CG49 or CG49(p35SGUSINT) were assayed. CG49 was isolated from a Riesling crown gall in Hammondsport, NY in October, 1979. It carries a nopaline type Ti plasmid and displays carbon source metabolism typical of *A. vitis* as outlined by SÜLE (1978).

Inoculum preparation: Prior to plant inoculation, CG49(p35SGUSINT) was grown at 28 °C on potato dextrose agar (Difco) containing 100 mg/l kanamycin for 48 h. Bacteria were suspended in sterile deionized water (SDW) and centrifuged at 7,800 *g* to remove kanamycin. The bacterial pellet was suspended in SDW and diluted to a final concentration of about 10^5 colony forming units per ml (cfu/ml) based on optical density (OD700=10^{-4}).

Plant material: Crown gall-susceptible *V. vinifera* cultivar Cabernet Sauvignon was compared with two crown gall-resistant genotypes, Couderc 3309 (*V. riparia* x *V. rupestris*) and *V. amurensis* 689 (USDA National Germplasm Repository for Apple and Grape, Geneva, NY accession number). The last two genotypes displayed very different *A. vitis* strain x *Vitis* genotype interactions in previous experiments (STOVER 1993).

In vitro experiments: Grape plants were maintained in the greenhouse for *in vitro* inoculation experiments. Young, actively-growing shoots were removed from plants and surface sterilized in 0.5% sodium hypochlorite (containing one drop of Tween 20/100 ml) for 20 min and then rinsed three times in SDW. Exposed ends of explants were removed and the remaining tissue was supported by inserting one end into culture medium in culture jars. 5 µl of the CG49(p35SGUSINT) suspension (10^5 cfu/ml) were spread over the exposed cut surface of each explant.

In the first set of *in vitro* experiments, the tissue used was collected from vines grown in the greenhouse by standard vegetative propagation. Nodal explants were placed in NITSCH and NITSCH (1969) medium without plant growth regulators. Control inoculations were made using SDW. Culture jars were kept in dim light and explants were sampled at 1, 2 and 4 weeks after inoculation in two trials and after 2 and 4 weeks in a third trial.

The second set of *in vitro* experiments was conducted with identical methods as the first except that control inoculations were made with a suspension of wild type *A. vitis* strain CG49. All plant material was assayed two weeks after inoculation.

In the third set of *in vitro* experiments, tissue was collected from *ex vitro* vines grown in the greenhouse from plants propagated through axillary shoot proliferation in tissue culture. Internodes were placed on NITSCH and NITSCH medium (1969) with 0.44 µM 6-benzylaminopurine, 0.049 µM indole butyric acid and 0.087 µM gibberellic acid. Control inoculations were made with CG49. Explants were kept under lights for 20 d before analysis.

GUS assays: The upper 5 mm of each explant were cut into 5 disks of 1 mm thickness, treated with fixative, and assayed for GUS using the indigogenic substrate x-gluc (JEFFERSON *et al.* 1987). Chlorophyll was removed from the tissue by sequential 24 h washes in 50, 75 and 95 % ethanol. Tissue pieces were examined with a microscope and the number of blue spots indicative of GUS expression and their position within the tissue were recorded.

Intact vine inoculations: In a single experiment, growth chamber-grown vines were inoculated with CG49(p35SGUSINT). Inoculum was prepared at about 10^9 cfu/ml in SDW (OD at 700 nm=1.0). 1 mm diameter holes were drilled through the nodes perpendicular to the shoot axis and 5 µl of bacterial suspension were applied to each wound. Inoculated sites were wrapped in parafilm and plants were placed in a growth chamber at 21 °C and 14 h day length. Tissues surrounding inoculated sites were excised and assayed for GUS activity at 6 and 10 weeks after inoculation as described above.

Statistical analysis: Data were examined using analysis of variance and means were separated using Fisher's protected LSD. All statements of significant difference indicate that means were found to be statistically different at the 0.05 level.

Results and discussion

In vitro experiments using source vines propagated from woody cuttings: In the first set of *in vitro* experiments, a significantly higher percentage of Cabernet Sauvignon explants (84.9 %) showed GUS expression than did either Couderc 3309 (32.1 %) or *V. amurensis* 689 (30.0 %) when inoculated with CG49(p35SGUSINT). When only the explants that showed some GUS expression where compared, Cabernet Sauvignon also had a significantly higher number of GUS positive sites per explant (46.8 for Cabernet Sauvignon, 23.1 for *V. amurensis* 689, and 21.9 for C 3309). GUS expressing sites were found in all of the tissue types on the inoculated surface of the explants and often extended below the inoculated surface especially in Cabernet Sauvignon. Results of later experiments strongly indicate

that a majority of these sites were not evidence of transformation but were caused by contaminating microorganisms with native β-glucuronidase activity.

Occasionally inoculation with CG49(p35SGUSINT) or SDW resulted in a blue smear on the explant surface following treatment with x-gluc that may have been due to the native β-glucuronidase activity of contaminating microorganisms. Only rarely were discrete spots observed in explants inoculated with SDW but the similarity between these spots and the more numerous spots from CG49(p35SGUSINT) inoculation was troubling. Wild type CG49, which has no GUS activity, was used for control inoculations in subsequent experiments because of concern that *A. vitis*-induced tissue disruption (Stover 1993) might enhance proliferation of β-glucuronidase expressing contaminants.

Nodal explants from source vines propagated from woody cuttings were inoculated with CG49(p35SGUSINT) or CG49 controls in one experiment. The CG49-inoculated explants displayed GUS expression in a high percentage of Cabernet Sauvignon (58.3 %) and *V. amurensis* (78.6 %) explants and in a lower proportion (7.7 %) of C3309 explants. The CG49(p35SGUSINT)-inoculated explants demonstrated GUS expression in a higher percentage of explants for all genotypes (92.3 % of Cabernet Sauvignon, 85.7 % of *V. amurensis,* and 15.4 % of C3309). In the controls, virtually all of the GUS expression found below the inoculated surface was in vascular elements. However, following inoculation with CG49(p35SGUSINT), 46 % of the Cabernet Sauvignon explants showed GUS expression in cambial tissue below the inoculated surface. This experiment demonstrates that grape shoots grown in the greenhouse often contain populations of microorganisms that produce β-glucuronidase. This is an important consideration when using the *uid*A system for comparing levels of transformation.

In vitro experiments using tissue culture propagated source vines: Since tissue culture of young shoots can effectively eliminate endophytes from plants (Podwyszynska and Hempel 1987, Burr *et al.* 1988), tissue culture (TC) propagated vines, reestablished into the greenhouse, were used in subsequent experiments to reduce spurious GUS activity from contaminating microorganisms. To further reduce the risk of microbial contamination, only the internodes were used since bracts surrounding lateral buds often harbor microorganisms. Low concentrations of plant growth regulators were included in the tissue culture medium to prevent senescence of internodes during the incubation period.

Even with TC source vines, some evidence of β-glucuronidase activity was observed in the explants inoculated with wild type CG49. This may have resulted from infection by endophytes after removal from TC or may represent low level infections that were maintained through the TC process. However, the number of GUS-positive sites in the control inoculations was very low (less than 0.2 spots/explant) and they were easily distinguished from transformed sites. β-glucuronidase activity in controls was seen in xylem vessels, pith cells and occasionally as a light blue smear on gall tissue after assaying using x-gluc. In contrast, GUS assays produced dark blue spots in the gall and cambial tissue of many explants inoculated with CG49(p35SGUSINT).

In the first experiment, where no effort was made to position the apical ends of explants in the same direction, 53 % of Cabernet Sauvignon explants inoculated with CG49(p35SGUSINT) were GUS positive as compared to 5 % of *V. amurensis*, and 0 % of C 3309 (Tab. 1). All GUS positive explants also had substantial cell proliferation (callus or gall tissue) on the inoculated surface where most of the GUS sites were observed. The extent of this proliferated tissue was larger and more frequent in Cabernet Sauvignon as compared to the other genotypes, but only a small portion (visually estimated as 2 %) of this tissue volume demonstrated GUS activity. It was previously reported that much of the tissue within crown galls is comprised of normal cells that have been stimulated into callus formation by diffusion of plant growth regulators from transformed tissue (Schröder 1987). In our experiments, the very low percentage of apparent gall tissue expressing GUS may indicate that the p35SGUSINT construct is transferred and expressed at a lower efficiency than the native T-DNA. 80 % of the p35SGUSINT-transformed Cabernet Sauvignon explants had GUS sites in the cambial region 1-2 mm below the inoculated surface. In Cabernet

Table 1

Comparison of GUS expression of *in vitro* internode explants from *ex vitro* vines two weeks after inoculation with CG49(p35SGUSINT) or wild type CG49. Orientation of internode explants was not determined so explants were randomly inoculated on either the apical or basal surface. Data indicate the percent of inoculated explants that were found to be GUS positive.

	percent of explants with GUS expression			number of
genotype	cell proliferation on inoc. surface	CG49 control inoculated	CG49(p35SGUSINT) inoculated	transformed sites per explant
'Cabernet Sauvignon'	48.7	0.0	53.0	1.47
V. amurensis 689	55.3[a]	0.0	5.0	0.05
Couderc 3309	36.6[a]	0.0	0.0	0.00

[a] callus or gall tissue was much smaller than in 'Cabernet Sauvignon'

Sauvignon the cambium below the inoculated surface often also had brown areas which may be associated with *A. vitis*-induced cell disruption. Frequently GUS-positive sites in the cambium were adjacent to these areas. In the first experiment using vines from TC, no GUS sites were observed below the inoculated surface in C 3309 or *V. amurensis*.

Swelling and callus typically develop on basal ends of grape shoots grown on TC medium containing plant growth regulators. Observations in this experiment suggested that GUS expression was only detected on explants where the apical surfaces were inserted into the TC medium and basal ends of the internodes were inoculated.

When this experiment was repeated, internode sections of all three genotypes were inverted into the TC medium so that only the basal surfaces were inoculated. The apical ends of a group of Cabernet Sauvignon internodes were also inoculated to test the effect of shoot orientation on transformation. No evidence of GUS expression from transformation was observed in Cabernet Sauvignon internodes where the apical surface was inoculated (Tab. 2). Only 13.4 % of these explants developed cell proliferation on the inoculated surface and the amount of this tissue was quite small compared to that which formed following basal inoculations. In contrast 100 % of the Cabernet Sauvignon internodes inoculated on the basal surface developed profuse cell proliferations on the inoculated surface and 88 % of these explants had sites indicating GUS expression from transformation. None of the *V. amurensis* internode explants displayed GUS activity, however, 76 % of these explants had slight callus tissue proliferation on the inoculated surface. In C 3309, 9.5 % of the internode explants displayed GUS activity and 58 % of inoculated explants had some callus proliferation.

Nodal explants of the *Vitis* hybrid Seyval blanc were reported by LOWE and KRUL (1991) to show enhanced gall formation when *A. vitis* or *A. tumefaciens* inoculations were applied to the basal surface rather than the apical end. Similar polarity responses have also been reported when carrot root discs were inoculated with *A. tumefaciens* (RYDER *et al.* 1985). In our work, and that of LOWE and KRUL (1991), explants containing buds did form some galls when the apical surfaces were inoculated. However, our studies using internodes without buds indicate no transformation or gall formation following inoculation of apical surfaces. In nodal explants, proximity of growing shoots to inoculated apical surfaces may enhance auxin concentration at these sites. To our knowledge, the work reported here provides the first evidence that transformation (as opposed to gall formation) is enhanced from inoculation of the basal explant surface.

Differential transformation of apical and basal surfaces in internode explants of the crown gall-susceptible Cabernet Sauvignon suggests that plant growth regulators influence the frequency of transformation. There is substantial evidence for basipetal movement of auxin in plants and numerous physiological processes appear to be dependent on basipetal but not acropetal auxin flow (TAMAS 1988). Basipetal movement of auxin may enhance the auxin concentration at the explant basal surface increasing the initial cell division that is critical for transformation (BINNS and THOMASHOW 1988). The differences observed in these experiments suggest the interesting possibility that variation in the levels of plant growth regulators, secreted by agrobacteria before transformation (SCHRÖDER 1987) or by plant tissues themselves, may influence the frequency of transformation.

Inoculation of intact vines with CG49(p35SGUSINT): Intact vines of the same *Vitis* genotypes were inoculated with CG49(p35SGUSINT). After 10 weeks the percentage of sites showing GUS expression was 56.2 % in Cabernet Sauvignon, 10.5 % in *V. amurensis* 689 and 9.1 % in C 3309. There were inoculated sites in each genotype where callus formed but there was no detectable GUS expression.

Potential causes of observed resistance to transformation: These results suggest that resistance to crown gall development by *A. vitis* in *V. amurensis* 689 and C 3309 is associated with a reduced level of transformation. Levels of transforma-

Table 2

The effect of shoot orientation on GUS expression of *in vitro* internode explants from *ex vitro* vines two weeks after inoculation with CG49(p35SGUSINT) or wild type CG49. Data indicate the percent of inoculated explants that were found to be GUS positive, the number of transformed sites per explant and the percent of explants displaying gall/callus proliferation.

		percent of explants with GUS expression			number of
genotype	orientation	callus on inoculated surface	G49 control inoculated	CG49(p35SGUSINT) inoculated	transformed sites per explant
'Cabernet Sauvignon'	B[a]	100[b]	50.0[c]	88.0	16.6
'Cabernet Sauvignon'	A	13.4	22.2[c]	10.5[c]	0.0
V. amurensis 689	B	76	0.0	0.0	0.0
Couderc 3309	B	58	0.0	9.5	0.1

[a] B=inverted shoots in medium with basal surface inoculated. A=upright shoots in medium with apical end inoculated.
[b] callus or gall tissue was much larger in inverted 'Cabernet Sauvignon' compared to all others.
[c] GUS expression in these explants was distinctly different from that scored as transformation.
Sites were located in xylem vessels, pith cells, or smeared on gall surface.

tion measured from *in vitro* assays using *uid*A (93 % for Cabernet Sauvignon, 5 % for *V. amurensis* 689, and 5 % for C 3309 averaged over two experiments) were consistent with the percent of inoculated sites forming galls in a study where intact plants were inoculated with a range of *A. vitis* strains (88.6 % for Cabernet Sauvignon, 29.1 % for *V. amurensis* 689, 4 % for C 3309) (STOVER 1993). The higher average percentage of inoculated sites forming galls in *V. amurensis* 689 was due to its susceptibility to a specific limited host range *A. vitis* strain (AG57) that was included in that study. When the response to AG57 is excluded, the incidence of gall formation in *V. amurensis* 689 drops to 16.3 %.

The reduced frequency of transformation in the crown gall-resistant C 3309 and *V. amurensis* could result from inhibition of any of the steps in the pathogenesis process leading up to transformation. When response of *Vitis* genotypes to *A. vitis*-induced cell disruption was measured, *V. amurensis* 689 and C 3309 showed significantly less cell disruption than Cabernet Sauvignon (STOVER 1993). The absence of detectable GUS expression below the inoculated surface and a reduced level of cambial browning in the crown gall-resistant genotypes may, in part, reflect more restricted movement of *A. vitis* due to minimal *A. vitis*-induced cell disruption. Significant *A. vitis*-induced cambial disruption (as indicated in Cabernet Sauvignon) may permit greater bacterial movement, which may increase the probability that *A. vitis* cells will encounter conditions ideal for gall formation.

The observed crown gall resistance in these grape genotypes could depend on reduced ability to induce pectolytic enzymes in *A. vitis*. This suggested the intriguing possibility that *A. vitis* pectolytic action may enhance the release of compounds that trigger induction of *A. vitis* virulence (*vir*) genes. However, when this was tested, there was no correlation between *vir* induction of *A. vitis* strains and crown gall susceptibility (STOVER 1993).

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