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Relative Virulence *of Agrobacterium* Strains on Strawberry

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Abstract. **Several strains *of Agrobacterium tumefaciens* and *A. rhizogenes* were shown to form tumors on runners of the diploid strawberry species *Fragaria vesca* L. Tumors, weighing from 0.1 to 8.3 mg, appeared from 2 to 4.5 weeks after infection. The majority of tumors tested for opine synthesis by high-voltage paper electrophoresis analysis showed positive results. These results demonstrate that diploid strawberry plants are susceptible to infection with *Agrobacterium* and that there are differences in the relative virulence of *Agrobacterium* strains.**

Agrobacterium is a genus of bacteria that resides in the soil and causes the common disease crown gall on a variety of dicotyledonous plants. Recently, this organism has gained widespread importance as a vector to transfer genes into plants. Currently, the infection of strawberry by *Agrobacterium is* not a problem in strawberry nurseries or production fields; therefore, it is not clear whether this plant can be transformed by *Agrobacterium. The* natural host range of *A. tumefaciens* and *A. rhizogenes* can be determined by demonstrating the ability of these bacteria to form crown gall or hairy root disease (De Cleene and De Ley, 1976, 1981).

Pathogenicity of *Agrobacterium* depends on the presence of a Ti or Ri plasmid. During infection, this pathogen transfers a portion of its Ti or Ri plasmid, the T-DNA segment, to the plant's genome (Chilton et al.,. 1977, 1982). Transcription and translation of genes present in the T-DNA causes crown gall tumors in *A. tumefaciens* or hairy root disease in *A. rhizogenes* (Chilton et al., 1977, 1982). Thus, the appearance of tumors or abnormally prolific roots, respectively, is a good indication that transformation has occurred. The T-DNA also contains genes that encode the synthesis of opines. The presence of these compounds in plant tissue extracts is unique to tissue infected with *Agrobacterium.* Different strains of *Agrobacterium* induce the synthesis of different opines; included among these are nopaline (NOP) and octopine (OCT) (Bomhoff et al., 1976), mannopine (MOP), mannopinic acid (MOA), agropine (AGR), and agropinic acid (AGA) (Petit et al., 1983).

We have used diploid strawberry *F. vesca* in these studies to determine its susceptibility to infection *by Agrobacterium.* It is the most extensively distributed species of this genus and can be found in the northern regions of America, Asia, and Europe. The cultivated strawberry is an octaploid and, therefore, genetically more complex than the diploid varieties.

Plant material. The plant source used in this investigation was a runner-producing derivative of the *F. vesca,* alpine clone (2n = 14, x = 7). Plants were grown in a growth chamber at 24C under a light intensity of 50 $\mu mol \cdot s^{-1} \cdot m^{-2}$. Runners used for infection were 4 to 6 weeks old.

Bacterial strains. Various tumor and root-inducing strains *of Agrobacterium* were used to infect the strawberry runners. Some characteristics of the *Agrobacterium* strains used in our experiments are shown in Table 1.

Culture medium and growth of bacteria. The *Agrobacterium* cultures were grown at 26 to 28C in 523 medium (Kado et al., 1972). Kanamycin (100 $\mu g \cdot ml^{-1}$) and spectinomycin (50 $\mu g \cdot m^{-1}$) were supplemented in the growth medium as required for bacteria that contained the corresponding resistance gene.

Tumor induction. Runners were wounded by cutting through the epidermal tissue with

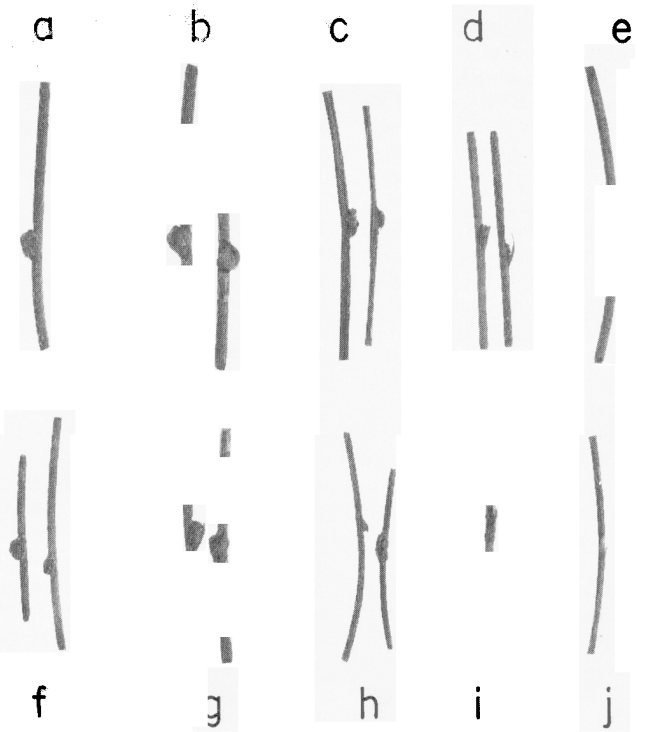

Fig. 1. Tumor formation on runners 4 weeks after inoculation with *Agrobacterium* strains. Note tumor size varies even within a given strain of *Agrobacterium.* Lanes a–h show runners inoculated with *A. tumefaciens* strains A208, C58, A722, A281, K12×562E, K12×167, B6S3×200, and 15955, respectively. Lane i shows runners inoculated with *A. rhizogenes* strain R1000. Lane j shows runners inoculated with 523 medium.

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Table 1. Opines produced by *Agrobacterium* strains used for the infection of strawberry.

Strain	Ti plasmid	Opine[z] OCT	NOP	AGR	AGA	MOP	MOA	References
A. tumefaciens								
A208	pTiT37	−	+	−	−	−	−	Sciaky et al., 1978
C58	pTiC58	−	+	−	−	−	−	Sciaky et al., 1978
A722	pTiA6	+	−	+	+	+	+	Garfinkel and Nester, 1980
K12	pTiA6	+	−	+	+	+	+	Dandekar et al., 1987
K12 × 562E	pTiA6:562E	+	−	+	+	+	+	Dandekar et al., 1987
K12 × 167	pTiA6:167	+	−	+	+	+	+	Dandekar et al., 1987
A281	pTiBO542	−	−	+	+	+	+	Petit et al., 1983
B6S3 × 200	pMON200II	+	+	+	+	+	+	Fraley et al., 1985
Ach5	pTiAch5	+	−	+	+	+	+	Petit et al., 1983
15955	pTi15955	+	−	+	+	+	+	Petit et al., 1983
A. rhizogenes								
A4T × 178	pRiA4T	−	−	+	+	+	+	R.C. Gardner, personal communication
A4	pHr(A4)	−	−	+	+	+	+	Moore et al., 1979
R1000	pRiA4b	−	−	+	+	+	+	White et al., 1985
232	ND[y]	ND	ND	ND	ND	ND	ND	Strobel and Nachmias, 1985

[z]Oct = octopine, NOP = nopaline, AGR = agropine, AGA = agropinic acid, MOP = mannopine, MOA = mannopinic acid
[y]ND = not determined.

Table 2. Analysis of tumor formation in strawberry.

Strain	Tumor formation[z]	Tumor wt[y] (mg)
A. tumefaciens		
A208	4/10	0.6–2.9 (1.9)
C58	2/10	4.6–5.3 (4.9)
A722	6/10	0.7–2.2 (1.3)
K12	3/10	0.6 (0.6)
K12 × 562E	7/10	0.4–2.9 (1.4)
K12 × 167	6/10	0.1–1.6 (0.8)
A281	5/10	0.4–0.9 (0.7)
B6S3 × 200	8/10	0.2–3.3 (1.4)
Ach5	1/3	8.3 (8.3)
15955	8/10	0.2–4.7 (1.6)
A. rhizogenes		
A4T × 178	6/10	0.6–1.5 (1.1)
A4	4/10	0.1–2.2 (1.0)
R1000	8/17	0.2–2.0 (0.9)
232	6/10	0.2–0.6 (0.4)
Control	0/10	---

[z]Number of tumors/number of infections.
[y]Range of tumor weights (average weight).

a sterile scalpel. One drop (2-5 µl) of a culture of *Agrobacterium* grown for *16* h and having a cell density of 5×10^8 cells/ml (as measured by absorbance at 420 nm) was placed on each wound. The wounded surfaces were then wrapped with parafilm to prevent drying. Tumors were harvested 4 to 8 weeks after inoculation. *Kalanchoe daigremontiana* plants were used as positive controls to check for oncogenicity of the various strains. The *Kalanchoe* plants were wounded by making incisions on the leaf surface and then were inoculated with the various *Agrobacterium* strains.

Opine analysis. Extracts for opine analysis were prepared by placing 1 to 4 mg of tumor tissue in an Eppendorf tube containing 200. µl of distilled water and boiling it for 10 min. The tumor tissue was removed by centrifugation (5000× *g*, 10 rein). The extract was then dried in a speed vac (Savant, New York) and resuspended in water to 0.5 $\mu l \cdot mg^{-1}$ of tissue. One to 2 µl of extract was spotted onto Whatman 3-mm paper along with 1 µl of standards of the NOP/OCT family (1 mg octopine, nopaline, and arginine/ml) or 1 µl of standards belonging to the mannityl opine family (1 mg MOP, MOA, AGP, and AGA/ml; authentic standards of these opines were provided by J. Tempé). Aqueous methyl green solution, which migrates just behind arginine, was used as a visual marker during electrophoresis. The paper was moistened with the appropriate electrophoresis buffer immediately before the current was applied. For analysis of the NOP/OCT family, the buffer used was 5% formic acid and 15% glacial acetic acid; for the mannityl opines, the buffer was 3% formic acid and 6% glacial acetic acid, Paper electrophoresis was performed at 10 $volts \cdot cm^{-1}$ for 40 min. The paper was then dried. Nopaline and octopine, which react with the fluorescent compound 9,10-phenanthrenequinone, were assayed as described by Otten and Schilperoort (1978). The detection of agropine, agropinic acid, mannopine, and mannopinic acid (silver nitrate-positive compounds) was performed as described by Petit et al. (1983).

Tumors appeared from 2 to 4.5 weeks after inoculation of strawberry runners with *Agrobacterium* (Fig. 1). Of 14 *Agrobacterium* strains (10 *A. tumefaciens* and four *A. rhizogenes)* used to infect strawberry runners, all 14 produced tumors of various sizes (Table 2). Strains containing pTi15955 (15955) and pMON 200 II (B6S3×200) were found to be the most infectious (Table 2), producing tumors in eight of the 10 sites inoculated: The average weight of the tumors incited by these strains was under 2 mg. Strains that contained pTiA6 (A722) or its recombinant derivatives K12×562E and K12×167 produced tumors in more than 60% of infected sites (Table 2); their average weights were also under 2 mg. The highly virulent strain A281 (Hood et al., 1986a, 1986b) produced average-sized tumors in five of 10 sites inoculated. Strains containing the Ti plasmids pTiAch5 and pTiC58 produced the largest tumors but were found to be the least infectious (one of three sites and two of 10 sites, Table 2). The tumor morphology in all cases was hard and compact with no visible differentiated structures (buds, leaves, or roots).

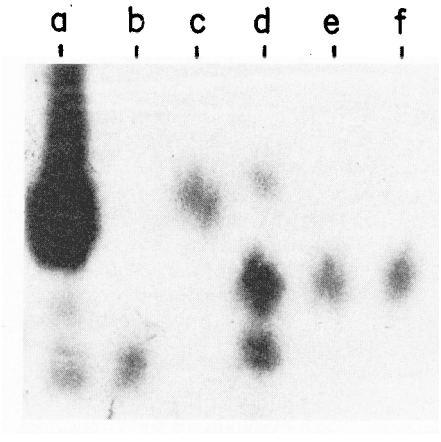
No root formation was observed when *A. rhizogenes* was used to infect strawberry; tumors were found, however, at the infection sites. The *A. rhizogenes* strains 232 and A4T×178 were the most infectious of the *Rhizogenes* strains tested (Table 2), and they produced tumors on six of 10 infected sites. All tumors weighed under 2 mg. Control runners inoculated with sterile medium failed to produce any tumors.

Opine expression in tumors was verified by analysis of tumors for the presence of opine compounds. The aim of the analysis was to determine the presence of the nopaline/octopine family and/or the mannityl opine family (AGR, AGA, MOP, MOA). Sixty-three tumors were analyzed for opines, 53 of which were analyzed for all six opines. Ten tumors that were too small to be analyzed for both opine families were analyzed only for the presence of the mannityl opine family. The analysis for the presence of both families of opine compounds was necessary because many of the strains were capable of synthesizing more than one class of opine (Table 1). Both agropine and mannopine could be clearly distinguished in our analysis (Fig. 2), but mannopinic acid comigrated with mannopine, and agropinic acid comigrated

Table 3. Characterization of opines present in strawberry tumors.

Strain	Opines[z]			
	AGR	MOP/MOA	NOP	OCT
A208	0/4	0/4	3/3	0/3
C58	0/2	0/2	2/2	0/2
A722	0/4	4/4	0/4	0/4
K12	1/1	1/1	ND[y]	ND
K12 × 562E	7/7	7/7	0/6	4/6
K12 × 167	6/6	6/6	0/4	4/4
A281	5/5	5/5	ND	ND
B6S3 × 200	8/8	8/8	7/7	0/7
Ach5	1/1	1/1	0/1	1/1
15955	4/4	4/4	0/3	3/3
A4T × 178	2/6	2/6	0/4	0/4
A4	2/3	2/3	ND	ND
R1000	0/8	0/8	0/7	0/7
232	0/4	0/4	0/2	0/2

[z]Number of positive/number tested.
[y]Not determined.



Pig. 2. Electrophoretic analysis of the opines AGR (lane b), AGA (lane c), MOA (lane e), and MOP (lane f) standards as compared to a tumor extract from B6S3×200 (lane a). Lane d contains a mixed standard of AGR, AGA, MOA, and MOP.

with neutral sugars that form the bulk of silver nitrate-positive cellular components. Therefore, we have scored for either AGR or MOP/MOA m our results (Table 3). All tumors formed after infection by strains containing pTiA6 (A722, K12) or its derivatives (K12×562E, K12×167) produced the mannityl opines; 50% to 60% of these tumors also produced octopine (Table 3). Similar results were found for the other octopine-producing strains Ach5 and 15955. All tumors from B6S3×200 contained nopaline and the mannityl opines but not octopine.

Two sets of tumors formed by *A. rhizogenes* R1000 and 232 failed to show any signs of opine synthesis, either in the OCT/NOP assay or the MOP/MOA/AGR assay. Control runners not inoculated or inoculated with sterile medium did not produce any opine compounds.

Fourteen tumor- and root-forming strains *of Agrobacterium* were used to determine the efficiency of infection of diploid strawberry *F. vesca.* Interestingly, infection of strawberry with *A. rhizogenes* resulted in the formation of tumors at the infection sites rather than the formation of roots. This observation is consistent with those made by De Cleene and De Ley (1981). No organized microstructure were seen by microscopic examination of tumors formed *by A. rhizogenes* or *A. tumefaciens.*

A majority of the octopine-producing tumors also were found to contain mannityl opines, suggesting the transfer of both T_L and T_R-DNA. In the case of octopine-producing *Agrobacterium,* it is known that the T-DNA is not contiguous but is comprised of two separate T-DNA structures, T_L and T_R (Thomashow et al., 1980). T_L-DNA contains genes for the synthesis of octopine (Barker et al., 1983; Garfinkel et al., 1981; Leemans et al., 1982), whereas T_R-DNA contains genes that encode synthesis of the mannityl group of opines (Barker et al., 1983; Salomon et al., 1984).

Fragaria vesca alpine clones are known for aroma and good flavor, but are not commercially significant because they are highly susceptible to viral infections (used as a viral indicator plant). The genome size is small, ≈ 22 times that of *E. coli* (Ahmadi et al., 1988). They are highly inbred (isogenic), with the availability of many isolated single-gene mutants. Therefore alpine clones are suitable for molecular studies and could be developed as an experimental system for perennial woody plants. They have a generation time of only 7 weeks (seed to seed), a rapid asexual mode of propagation, and can be readily regenerated from various vegetative tissues (P.L. Schuerman and A. M.D., unpublished data). *Fragarai vesca* can hybridize with octoploid species with ease (Ahmadi et al., 1990; Scott, 1951). Their pentaploid hybrids usually produce decaploid offspring (unreduced gamete) or may be doubled synthetically to obtain fertile decaploid derivatives. The identification of suitable strains, shown here, is the first step in the development of a functional *Agrobacterium*-mediated gene transfer system. Useful traits, such as herbicide, pest, and disease resistance, once introduced via transformation in *F. vesca,* could be introgressed into octoploid species, resulting in new cultivars with desirable genes.

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