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Genotypic variation in susceptibility of *Pisum sativum* to crown gall and characterization of one cultivar of pea with reduced susceptibility to crown gall

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GENOTYPIC VARIATION IN SUSCEPTIBILITY OF PISUM SATIVUM TO CROWN GALL AND CHARACTERIZATION OF ONE CULTIVAR OF PEA WITH REDUCED SUSCEPTIBILITY TO CROWN GALL

by

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ABSTRACT

Thirty-four cultivars of pea (Pisum sativum) were assayed for tumorigenesis after inoculation with Agrobacterium tumefaciens strain B6. The response of the 34 cultivars fell into 3 significantly different groups based on tumor weights: high, medium, and low susceptibility. The least susceptible cultivar, Sweet Snap, also formed the smallest tumors in comparison to 5 other cultivars when inoculated with 5 other strains of Agrobacterium. Preliminary experiments indicate that neither chemotaxis, binding, vir-gene induction, nor T-DNA expression are limiting factors in Sweet Snap's reduced susceptibility. In an inheritance study, the F1, F2, and F3 progeny from an initial cross between Sweet Snap and Wando (a more susceptible cultivar) formed tumors that were intermediate in weight between the two parents.

INTRODUCTION

Agrobacterium tumefaciens

A.tumefaciens was first isolated and found to be the cause of crown gall disease by Smith and Townsend (1907). The genus Agrobacterium is in the family Rhizobiaceae and has been classified by two systems: 1) a system in which the genus is broken down into four species and 2) a system in which the genus is broken down into 3 biovars. In the first system, used by Bergey's Manual of Determinative Bacteriology (Buchanen and Gibbons 1974), the species are differentiated according to their phytopathogenicity. The four species are: (1) A.tumefaciens (Smith and Townsend) Conn, which is pathogenic on most dicotyledonous plants, (2) A.radiobacter (Beijerinek and Van Delden) Conn, an avirulent species; (3) A.rhizogenes (Riker et al.) Conn, which causes hairy root disease; and (4) A.rubi (Hildebrand) Stan and Weis, the causal agent of cane gall on raspberries, blackberries and other related species in the genus Rubus. The other clasification system, developed by Keane et al. (1970) and Kerr and Panagopoulos (1977), divides Agrobacterium into 3 biovars based on biochemical tests, serotyping, and electrophoretic protein patterns. Biovars I and II have been isolated from a variety of hosts and biovar III is naturally restricted almost entirely to grapevine but can infect other hosts experimentally (Knauf et al 1982, Kerr and Panagopoulos 1977).

A.tumefaciens has been extensively studied at the molecular level, especially since the early eighties when it was discovered that it can be used as a vector for genetic engineering. A.tumefaciens contains a large plasmid (200 kb), the Ti (tumor inducing) plasmid. Within the plasmid is a region of DNA called T-DNA (transfer DNA) which is integrated into the genome of a susceptible plant. Expression of T-DNA genes results in tumorigenesis in the host (Chilton et al. 1977). In order to induce tumors, the bacteria must first bind to the plant cell. This process is controlled by chromosomal virulence genes whose products are constitutively produced in A.tumefaciens (Douglas et al 1985). The transfer of the T-DNA into the plant cell is controlled by another region of

the Ti plasmid, the virulence (vir) region which itself is not transferred into the host genome.

The vir region contains 7 different complementation groups : vir A,B,C,D,E,G, and pin F. The vir A and vir G genes are constitutively expressed (Stachel et al. 1985). It has been hypothesized that the vir A product is a protein permease that promotes the uptake of phenolic signal molecules such as acetosyringone that are exuded by susceptible plants (Stachel and Zambryski 1986). The vir A protein is also involved in host range determination as it has been found to be in part responsible for the limited host range of a biovar III grapevine isolate of A.tumefaciens (Yanofsky et al 1985). The vir G protein whose production is both constitutive and plant inducible, might interact directly with the plant signal molecule and undergo a conformational change. Then the vir G protein might interact with the vir promoter sequence to enhance recognition by RNA polymerase (Stachel and Zambryski 1986). Vir B,C,D, and E are plant inducible. Vir B and vir D might control the process of transferring T-DNA and Vir C has been implicated in specifying the host range of A.tumefaciens (Stachel and Nester 1986). The product of vir D generates nicks at the borders of the T-DNA which initiate its excision from the Ti plasmid (Wang et al. 1987). Then the single stranded T-DNA is transferred into the host plant cell and integrated into the host genome (Chilton et al.1977). The mechanism of action by which the T-DNA is integrated into the genome is not known (Jorgenson et al. 1987).

After integration into the host genome, the T-DNA directs the synthesis of auxins and cytokinins. Gene 1 of the T-DNA codes for tryptophan 2-monoxygenase which converts tryptophan to indole-3-acetamide (Van Onckelen et al. 1986). Gene 2 of the T-DNA encodes an amidohydrolase which converts indole-3-acetamide to indole-3-acetic acid, an auxin (Schroder et al. 1984). Gene 4 of the T-DNA encodes for dimethyl allyl transferase, an enzyme involved in cytokinin biosynthesis (Akiyoshi et al. 1984). Because the pathways for hormone production encoded by the T-DNA are different from the pathways in the plant, the plant cannot control the excess hormone production and tumors result (Shroder et al. 1984). The T-DNA also has genes for the production of opines which are unusual amino acid derivatives. Separate Ti plasmid genes whose products direct the

catabolism of opines so agrobacteria can proliferate near the transformed tumor cells (Petit et al. 1978).

Different strains of A.tumefaciens have varying host ranges with some capable of causing tumors on most dicotyledonous plants and some strains only capable of infecting plants within a few genera. These strains are designated as wide host range (WHR) and limited host range (LHR) respectively (Buchholz and Thomashow 1984, Unger et al. 1985). As mentioned above, the virulence region of the Ti plasmid also plays a role in determining the host range of A.tumefaciens. Yanofsky et al. (1985) has shown that a defective cytokinin gene limits the host range of a LHR isolate of A.tumefaciens. A LHR A.tumefaciens isolate from Lippia canescens, which is restricted in pathogenicity to Lippia canescens, members of the cucurbit family, and Nicotiana glauca, contains T-DNA which does not show homology to the T-DNA of WHR strains of A.tumefaciens (Unger et al. 1985).

Crown Gall

Crown gall, caused by A.tumefaciens is a common disease of most dicots and some gymnosperms (De Cleene and Deley, 1976). Crown gall disease occurs world wide and is of economic importance on a wide range of agricultural and ornamental plants (Tarbah and Goodman 1986). Crown gall causes a reduction in yield in grapevines (Schroth and McCain, 1988). In California crown gall was rated as one of the three most important pathogens on eight different crops (Anonymous 1965). It also causes problems in roses (Cramer 1981), fruit trees (Schroth et al. 1971), and ornamentals (Nesme et al. 1987). Disease inception can occur at any time during the life of the host plant as a result of wounds caused by frost damage or mechanical injuries. The wounds on the roots or the crown of the plant allow A.tumefaciens in the soil to infect the plants. The worst damage occurs when when young trees or grape vines are infected and the tumors progress as the tree matures. The tumor radiates from the center of the tree and restricts the flow of nutrients. Dead tumors also provide an entry for other pathogenic organisms like heart rot fungi

(Schroth et al. 1971). In grape vines, aerial tumors can form on the vines due to the presence of agrobacteria in the vascular tissue (Tarbah and Goodman 1986).

The disease has been controlled by various chemicals such as chloropicrin, methyl bromide (Shroth et al 1971), copper and mercury compounds (Grimm and Vogelsanger 1987) and streptomycin sulfate (Grimm and Sule 1981, Cramer 1981). Unfortunately, many of these compounds are phytotoxic and antibiotics only give a short term control. An oil-water emulsion containing 1,2,3,4 - tetrahydronaphthalene dimetylmethane, dimethyl naphthalene, 2,4, xylenol and m-cresol kills tumor tissue without affecting healthy tissue. This has been used on tumors of almond, peach, apricot, plum, cherry, and olive knot tumors incited by Pseudomonas savastanoi. This selective action of the chemical on tumorous but not healthy tissue may be due to differential permeability of tumorous cells compared to nontumorous cells as tumorous cells have an increased capability to take up solutes from dilute solutions (Scroth and Hildebrand 1968). Although the emulsion efficiently kills tumorous tissue it can only be applied to visible above-ground tumors. In Australia, biological control of crown gall has been successful by using the A.radiobacter strain 84. This strain produces agrocin 84, a toxin to many strains of Agrobacterium. Biological control has been 100% effective using this strain (Kerr 1980). However all biovar III isolates tested and three biovar II isolates from peach are not controllable with agrocin 84 (Kerr and Panagopoulos 1977). As with other plant diseases, the best way to prevent crown gall is through the use of resistant varieties. Although two species of grape are resistant to crown gall Vitis labrusca and Vitis amurensis (Szegedi et al. 1984) most commercial cultivars of grape and other economic hosts are susceptible (R.N. Goodman personal communication, University of Missouri). Theoretically, understanding the mechanism of resistance and identifying the genes responsible for this resistance can improve the chance that agronomically useful resistant plants can be selected or genetically engineered.

Host Determinants

Engineering a plant to be resistant to infection by A.tumefaciens would be facilitated by understanding the biochemical, physiological and genetic mechanism of resistance. Although there is extensive literature about the molecular genetics of A.tumefaciens, little is known about the host's contribution to infection. The plant's role is still undefined with respect to the interaction of WHR and LHR strains of A.tumefaciens and susceptible plants (Knauf et al. 1982, Yanofsky et al. 1985, Buchholz and Thomashow 1984). Some of the possible factors involved in the susceptibility of a plant to crown gall disease are given by Owens and Cress (1985): "Included might be traits that affect the initial survival of the agrobacteria in the wounds; the subsequent binding of bacteria to the plant cell wall; the transfer of DNA from the bacteria into the host cell; the integration of DNA into the plant chromosome; and the endogenous phytohormone levels in the stem that could enhance or inhibit tumor growth and possibly determine the age at which galls begin to senesce". Although their exact biochemical nature is not known, plant cell walls have been proposed to have attachment sites for A.tumefaciens (Krens et al. 1985). Specific vir-inducing compounds have been isolated from the exudates of susceptible plants to crown gall (Stachel et al. 1985) and chemotaxis has been observed to acetosyringone (Ashby et al. 1987), one of the vir-inducing molecules isolated from susceptible hosts to crown gall. How the plant cell integrates the DNA into its nuclear chromosome is not known, although Jorgensen et al. (1987) have shown that in transgenic tomato plants the T-DNA is organized in direct repeats in the plant chromosome and have suggested that host machinery for DNA repair and synthesis is involved.

Knowledge of the preliminary steps in pathogenesis of A.tumefaciens can aid in elucidating the mechanism of resistance to crown gall. Assays are available for chemotaxis (Ashby et al. 1987, Hawes et al. 1988); binding (Hawes and Pueppke 1987); influence of vir gene inducing molecules (Sheikholeslam and Weeks 1987); cellular transformation (Jefferson et al. 1987) and hormone production (Weiler and Spanier 1981). Many mutants of A.tumefaciens are available to study pathogenesis. However an analogous system is lacking in a susceptible host species in which there

is defined genotypic variation with respect to susceptibility to crown gall. Crown gall is an economically important disease of grapes and stone fruit but the lengthy reproductive time and growth habit of grapes and deciduous trees makes genetic and molecular studies impractical. Most monocotyledonous plants are resistant to crown gall (DeCleene and Deley 1976) however their anatomy and biochemistry are so different from dicots that it would be infeasible to study them for potential genetic transfer to dicots. The response to infection by A.tumefaciens has been studied amongst different cultivars of various species of dicots including chrysanthemum (Miller, et al. 1975), castor bean (El Khalifa and Nur 1970), Medicago sativa L. (Mariotti et al. 1984), moth bean (Eapen et al. 1987), and soybean (Owens and Cress 1985). Pea (Pisum sativum) has been chosen for the host plant to study as certain cultivars are highly susceptible to crown gall (Kurdjian et al. 1968), it has a short reproductive time, and genetic maps of pea are available.

Objectives

The objectives of this research were to: (1) assay commercially available cultivars of peas for susceptibility to crown gall and to select for genotypic variation in tumorigenesis, (2) determine what preliminary infection steps might be blocked in a resistant cultivar or one with a reduced susceptibility, and (3) study the inheritance of resistance to crown gall in peas.

MATERIALS AND METHODS

I. Selection for Genotypic Variation.

Seeds: The cultivars of pea used in the following experiments and their sources are as follows: Burpee Seed Company: Alaska, Burpeeana Early, Freezonian, Green Arrow, Grenadier, Knight, Maestro, Mammoth Melting Sugar, Oregon Sugar Pod II, Snappy, Snowbird, Sugar Bon, and Sugar Daddy. Park Seed Company: Blizzard, Giant Melting Sugar, Novella, Patriot, and Sugar Ann. Roger Brothers Seed Company: Alaska 423, Honey Pod, Oregon Sugar Pod, Progress #9, Salvo, Sparkle, and Target. Royal Seed Company: Dwarf Grey Sugar, Laxantonian, Laxton's Progress, Little Marvel, Sugar Snap, Sweet Snap, Thomas Laxton, and Wando. University of Saskatchewan Department of Horticulture: Century.

Bacteria Strains: The bacteria strains used in these experiments are descried in Table 1. A.tumefaciens cultures were maintained in 50% glycerol:yeast extract mannitol (YEM) at -80 C. Cultures used for inoculations were derived from single colony isolations and grown overnight on YEM at 28 C. Concentrations were determined turbidimetrically using a Beckman spectrophotometer and confirmed by dilution plating on YEM.

Assay for Genotypic Variation

All 34 cultivars listed above were inoculated by the following procedure: Pea seeds were surface sterilized prior to germination by soaking in 95% ethanol for 5 minutes and then soaking for 5 minutes in 50% commercial bleach. Seeds were then rinsed 3 times with sterile distilled water and placed on sterilized discs of germination paper on 0.5% water agar in 9 cm petri dishes. The petri dishes containing the seeds were placed in an incubator at 27 C for 2 to 3 days to germinate. For each trial, approximately 30 seedlings were used per cultivar. Three trials were performed for most of the cultivars inoculated with A.tumefaciens strain B6 (the actual numbers of seedlings inoculated for each cultivar are given in Table 2 of the Results section).

Table 1. Strains of A.tumefaciens and E.coli used in the experiments for this research and background strains of A.tumefaciens.

Bacteria Strain	Opine	Onc*	Biovar	Source or Description	Reference
A.tumefaciens					
B6	Octopine	+	I	Apple gall, Iowa	Sciaky et al. 1978
T37	Nopaline	+	I	Walnut gall, California	Sciaky et al. 1978
A281	L,L-Succinamopine	+	I	Conjugation of Bo542 with A136	Hood et al. 1986
Bo542	L,L-Succinamopine	+	I	Dahlia gall,Germany	Sciaky et al. 1978
A136	-	+	I	Plasmid cured C58	Watson et al. 1975
A723	Octopine	+	I	B6-806 in NT-1	Garfinkel and Nester 1980
B6-806	Octopine	+	I	Derived from B6	Sciaky et al. 1978
NT-1	-	-	I	Plasmid cured C58	Sciaky et al. 1978
C58	Nopaline	+	I	Cherry gall, New York	Sciaky et al. 1978
LBA4404	Octopine	-	I	Streptomycin resistant	Bevan 1984
LBA4404(pBI121)	Octopine	-	I	Contains the GUS gene	Jefferson 1987
A.rhizogenes					
R1000	-	+	-	Derived from A4T	White et al. 1985
A4T	-	+	-	Rose gall, California	Moore et al. 1979

E.coli strains used:

Bacteria strain	Marker	Characteristics	Source	Reference
HB101(RK2013)	Kn**	Conjugative plasmid	Clontech	Bevan 1984
HB101	Str ***		Clontech	Boyer et al. 1969

* Oncogenic on sunflower and kalanchoe (Sciaky et al 1974). Also oncogenic on pea (this research).

** Kanamycin sulfate resistant. *** Streptomycin sulfate resistant.

An incision 1mm deep and 1mm long was made in the hypocotyl of the seedlings with a flame-sterilized scalpel. To inoculate the wounded seedlings, they were immersed in a concentrated bacterial suspension (10^9 bacteria/ml) of A.tumefaciens strain B6 for 5 minutes and then placed in growth pouches (Northup King) with 3 to 6 seedlings per pouch (Fig. 1). Controls were wounded but not inoculated. The seedlings were grown in a growth chamber (Conviron) and grown at 20 C with 12 hours of light per day with fluorescent bulbs. Two weeks after inoculation, the plants were removed from the growth pouches, the efficiency of tumorigenesis was determined and the tumors which formed were excised (Fig. 2) and weighed. The efficiency of tumorigenesis was designated as the percentage of inoculated seedlings that formed tumors. If the wound site of an inoculated seedling could not be distinguished from a non-inoculated control section, then that seedling was classified as not forming a tumor. If there was any amorphous tissue growth at the inoculated site then that seedling was classified as having a tumor. The weights of the control wounded sections were subtracted from the weights of the inoculated sections to obtain the tumor weight for each cultivar. The tumor weights were analyzed using an analysis of variance on the software package of SPSS.

Strain Specificity

Inoculations were also performed with A.tumefaciens strains A281, T-37, A723, and A.rhizogenes R1000 described in Table 1 with the cultivars Sweet Snap, Alaska, Dwarf Grey Sugar, Freezonian, Sugar Snap, and Thomas Laxton. Inoculations were performed by the same procedure listed above. Tumors were excised and weighed for these as described above.

Figure 1. Two-week-old pea plants grown in a growth pouch.

Figure 2. Excision of a tumor from a pea plant with a scalpel.

II. Characterization of Variation of Susceptibility in Pea to Crown Gall.

Chemotaxis

Pea seeds were germinated as described above. For the cultivars Thomas Laxton, Sweet Snap, Dwarf Grey Sugar, and Wando, root cap cells were isolated and their concentrations were standardized as described above. Ten ul of the root cap cell suspension was placed at the edge of a petri dish containing 0.2% water agar and phosphate EDTA buffer as described by Hawes et al. (1988). A 10 ul droplet of a 10^9 cfu/ml suspension of A.tumefaciens was placed in the center of the water agar. After 24 hours the distance that the bacteria had moved towards and away from the root cap cells was measured for each cultivar. The chemotaxis ratio was calculated by dividing the distance that the bacteria had moved towards the root cap cells by the distance that the bacteria had moved away from the root cap cells. Five replications were performed for each cultivar. In addition to using isolated root cap cells, root tips were also used to determine their chemoattractiveness. Three root tips about 5mm in length were placed at the edge of the petri dishes containing 0.2% water agar and after 24 hours, and the chemotaxis ratio was calculated.

Binding of Agrobacteria to Root Cap Cells.

A binding assay utilizing isolated root cap cells and A.tumefaciens was used (Hawes and Peuppke 1987). Pea seeds were surface sterilized by soaking in 95% ethanol for 5 minutes followed by 5 minutes in a 50% solution of commercial bleach. The seeds were then rinsed 3 times with sterile distilled water and placed on sterile germination paper on 0.5% water agar in 9cm petri dishes to germinate. After 3 days the seeds germinated and the root cap cells were isolated from seedlings with roots approximately 2cm in length as described by Hawes and Peuppke (1987). The root tip of each seedling was immersed for 5 minutes in 100 ul of sterile distilled water. The water was agitated to dislodge the root cap cells. The number of root cap cells was counted with a hemacytometer and root cap cell suspensions were standardized to 2×10^4/ml

Root cap cells from the pea cultivars Dwarf Grey Sugar, Freezonian, Sugar Snap, and Sweet Snap were obtained as described above. The root cap cell concentrations were adjusted to a density of 2 X 10^4 by dilution with water or by concentration through a 10um mesh filter. The root cap cell samples (100ul) were placed into wells of a 96 well polystyrene microtiter plate. A.tumefaciens strain B6 was used in the binding assay. A 100ul sample of a suspension of bacteria (10^7cfu/ml) was added to each root cap cell sample, then the mixture was incubated for 1-2 hours at 20C. After incubation, the mixture was stirred and 20 ul samples were taken out and placed into a cone made from a 10 um mesh nylon filter. To rinse off the unbound bacteria 100 ul of sterile water was added and the liquid was removed by touching the end of the cone to a tissue. To determine the number of bacteria bound to the root cap cells, the root cap cells with the bacteria were examined microscopically. The number of bacteria bound to the periphery of each root cap cell was counted for about 20 root cap cells for each trial. For each cultivar, 3 trials were performed.

Vir-Gene Induction

A. Test for ability of acetosyringone to enhance tumorigenesis:

Acetosyringone was added to A.tumefaciens strain B6 (10^9cfu/ml) to a concentration of 70 uM. This was incubated for 24 hours prior to inoculation. Thirty Sweet Snap seedlings were inoculated with the acetosyringone induced bacteria by the inoculation procedure described above.

B. Test vir inducing ability of wound exudates:

Wando seedlings were aseptically germinated as described above. The roots from 20 of these seedlings were excised and added to 50ml of a suspension of A.tumefaciens strain B6 (10^9 cfu/ml). This was placed on a shaker for 24 hours after which Sweet Snap seedlings were inoculated with this suspension of induced bacteria. The inoculated Sweet Snap seedlings were placed in growth pouches and grown in the growth chamber as described earlier. After two weeks the inoculated sections were excised and weighed.

Nuclear Transformation

A. Plant DNA extraction:

DNA was extracted according to the method of Rob Martienssen (personal communication, Universtity of California Berkeley). DNA was extracted from the stems of 2-week-old Sweet Snap and Wando plants by the following method: Five grams each of Wando and Sweet Snap tissue was frozen in liquid N_2 and ground to a powder in a mortar and pestle. The frozen powder was added to 5 ml of extraction buffer (0.1 M Tris-HCL, pH 8.5, 0.1 M NaCl, 50 mM EDTA, 2% SDS and 100 ug/ml proteinase K) and incubated at room temperature for 2 hours. Five ml of phenol/chloroform solution (equilibrated with DNA extraction buffer not containing proteinase K) was added to the solution of plant tissue and extraction buffer. After 5 minutes the solution was centrifuged for 5 minutes at 4350 rpm. The aqueous layer was transferred with a cut off pasteur pipette to a clean tube. Five ml of extraction buffer without proteinase K was added to the tube from which the aqueous layer was extracted and this was allowed to sit for 5 minutes before centrifugation at 4350 rpm for 5 minutes. After centrifugation the aqueous layer was removed and added to the previously extracted aqueous layer. An equal volume of phenol/chloroform solution was added to the aqueous layers and then mixed, allowed to sit for 5 minutes and centrifuged at 4350 rpm for 5 minutes. The aqueous layer was then extracted and retained. An equal volume of chloroform/isoamyl alcohol solution was added to the aqueous solution, mixed, allowed to sit for 5 minutes, and centrifuged at 4350 rpm for 5 minutes. The aqueous top layer was extracted and retained. To precipitate the DNA enough 3 M sodium acetate was added to the aqueous solution to make the final concentration 0.3 M. Then, an equal volume of absolute ethanol was layered on top and gently rolled until the DNA precipitated out of solution. The DNA fibers were twirled onto a glass pipette and transferred to a microfuge tube to drain the excess EtOH, however the DNA fibrils were not completely dried out. The DNA pellet was resuspended in .5 ml of TE (10mM Tris HCL, pH 7.5 1 mM EDTA) buffer and gently mixed by hand. To remove the RNA form the solution, RNase was added to a final concentration of 100 ug/ml and incubated at 37 C for 3 hours. To extract proteins from the solution,

an equal volume of phenol/chloroform solution was added and the solution was mixed gently and allowed to sit for 5 minutes and then centrifuged at 4350 rpm. The aqueous layer was extracted and equal volume of chloroform/isoamyl alcohol was added and the solution sat for 5 minutes before centrifugation at 4350 rpm for 5 minutes. The aqueous layer was extracted and dialyzed in TE buffer overnight at 4 C. The DNA was stored at 4 C.

B. Micro-gel analysis of DNA:

Ten ul of the extracted DNA from Sweet Snap and Wando tissue and 1ul of L Hind III endonuclease (International Biotechnologies Incorporated) was loaded onto a 1% agarose minigel in tris-borate buffer. The gel ran for 5 hours at 40 volts and 11 mAmps with the whole apparatus submerged in an ice bath.

C. Tissue culture of Sweet Snap and Wando tumors:

Sweet Snap and Wando seeds were germinated and inoculated with A.tumefaciens strain B6 and grown in the growth chamber as described above. The tumors formed on Sweet Snap and Wando seedlings were excised and surface sterilized in 95% EtOH for 1 minute and 25% clorox for 2.5 minutes and then rinsed 2 times in sterile distilled water. These tumor pieces were placed on Murashige and Skoog medium (1962)without auxins and placed in the growth chamber at 20 C and a 12 hour photo period.

Cellular Transformation

A. Triparental mating procedure:

The plasmid pBI121 (Clontech) was used which contains the B-glucuronidase (GUS) gene with a cauliflower mosaic virus (CaMV) 35S promoter and a nopaline synthase (NOS) terminus. The pBI121 plasmid was delivered in the E.coli strain HB101. In order to mobilize the GUS gene in A.tumefaciens, a tri-parental mating was performed with E.coli HB101(pBI121), E.coli HB101(RK2013), and A.tumefaciens LBA4404 all described in Table 1. These were all streaked onto Luria Broth (LB) media and placed in the incubator at 29 C for 24 hours. The bacteria were then

washed off the plate with sterile distilled water and 0.1ml of the bacterial suspension was spread over LB media containing kanomycin (50ug/ml) and streptomycin (50ug/ml) and placed in the incubator at 29 C. After 48 hours single colonies were transferred to LB media with kanamycin (50ug/ml) and streptomycin (50ug/ml). Ketolactose production was tested by the method of Bernaerts and DeLey (1963). Single colonies were streaked onto lactose plates and Benedict's solution was poured over the colonies. Ketolactose production was monitored by development of yellow circles around the colonies.

B. Histochemical Assay:

X-Gluc solution stocks:	Volume used for 5 ml of solution:
0.2M $NaPO_4$ buffer pH 7.0	2.5 ml
(made from 62 ml of 0.2 M Na_2HPO^4 and 38 ml of 0.2 M NaH_2PO_4)	
Distilled water	2.4 ml
0.1 M $K_3[Fe(CN)_6]$ Potassium ferrocyanide	0.025 ml
0.1 M $K_4[Fe(CN)_6].3H_2O$	0.025 ml
0.5 M EDTA	0.10 ml
X-Gluc (5-bromo-4-chloro-3-indolyl-B-glucuronide)	5.0 mg

Twenty each of Sweet Snap and Wando seedlings were inoculated with A.tumefaciens strains LBA4404(pBI121) and LBA4404 described in Table 1. by the same procedure employed in the quantitative tumorigenesis assay. Controls were wounded, but not immersed in bacteria. The inoculated seedlings were then placed in growth pouches and grown in the growth chamber for 2 weeks. Thin sections of the inoculated and wounded regions were made by cutting the seedlings with a razor blade. The thin sections were then placed in microtiter wells. Enough X-Gluc solution (Maude Hinchee, Monsanto St. Louis Mo. personal communication) was used to cover each thin section. The microtiter plates containing the plant tissue were covered, wrapped in parafilm and

placed in an incubator at 37 C for 4 or 12 hours. After incubation the thin sections were rinsed in water and placed on microscope slides and observed under the light microscope.

Alternatively, thin sections were peeled off of the Sweet Snap and Wando pea seedlings with tweezers of then placed in a suspension of A.tumefaciens LBA4404(pBI121) for : 1) Two days, 2) Two hours and then placed in MS media for 2 days, 3) Two hours and then placed on water agar without rinsing for 2 days, 4) Two hours and then placed on water agar for 2 days and then placed on water agar containing ampicillin (100ug/ml) and rifampicin (10ug/ml) for 1,2,3, and 4 days to kill the bacteria, and 5) Two hours and then placed on water agar with carbenicillin (50ug/ml) and tetracycline (100ug/ml) for 7 days.

C. Test for root cap cell transformation:

Isolated root cap cells from Sweet Snap and Wando were also incubated with the agrobacteria containing the GUS gene. The root cap cells from each cultivar were quantified microscopically with the use of a hemacytometer and 100ul aliquots were placed in sterile microtiter wells. Ten ul of a suspension of A.tumefaciens LBA4404(pBI121) (10^9 cfu/ml) was added to each suspension of root cap cells and allowed to bind to the root cap cells for 2 hours. The unbound bacteria were rinsed off with sterile distilled water and the root cap cells were resuspended in MS media. After 2 days, 10 ul of X-gluc was added to the root cap cells.

Expression of T-DNA

Twenty aseptically germinated seedlings each of Sweet Snap and Wando were inoculated with A.tumefaciens strain B6, and 10 control seedlings of each cultivar were wounded but not inoculated. All plants were grown for two weeks in growth pouches in the growth chamber as described earlier. After two weeks the inoculated sections were excised and weighed. Octopine was extracted from 2 grams of the inoculated tissues of each cultivar by the following procedure (De Framond et al. 1983): The plant tumor tissue and the control non-inoculated tissue was macerated in an equal weight of 70% ethanol. One hundred ul aliquots of the extract were applied to Whatman

3MM chromatography paper. Five ul of a .02 mM octopine solution in water was spotted on the chromatography paper as a standard. The chromatography paper was placed in a solvent containing methyl ethyl ketone: 2-butanol: t-butanol: diethyl amine: water (40:20:20:1:25) which resolves octopine from interfering fluorescent substances in the tumor extract. After 7 hours the chromatography paper was dipped in a solution prepared by mixing equal volumes of 0.02% phenanthrenequinone in anhydrous ethanol and 10% NaOH in 60% EtOH. After the paper dried, it was examined under a UV lamp to detect guanidine groups.

III. Inheritance Study.

A. Determination of flowering times:

At the time the inheritance study was initiated, only 13 cultivars of pea were available. All of these cultivars were grown at the Campbell Avenue Farms greenhouses at the University of Arizona and in a growth chamber (Conviron) set at a 12 hour photoperiod and 20 C.

B. Crosses of Wando and Sweet Snap:

To initiate crosses, 150 Sweet Snap and 100 Wando seeds were planted at the Campbell Avenue Farms. One week after germination all seedlings were inoculated by dipping a sterile needle in a culture of A.tumefaciens strain B6 and then stabbing this into the plant stem. After 2 weeks, Sweet Snap seedlings with large tumors were discarded and Wando plants without tumors were discarded. When the plants bloomed, reciprical crosses were made of Wando and Sweet Snap. Four months after planting, the seeds of crosses as well as selves of Wando and Sweet Snap were harvested.

Of the F1 progeny, 23 WxS and 36 SxW were planted to collect the F2 seeds. Six WxS and 12 SxW seeds were germinated and inoculated with A.tumefaciens strain B6 in order to measure tumor weights of the F1 progeny. After the F1 plants had matured, 237 F2 seeds were collected and assayed for tumorigenesis. Twenty of the F2 plants were grown to maturity to collect the F3 progeny at the Campbell Avenue Farms. Sixty F3 seeds were collected from the F2 plants and assayed for tumor formation as described earlier. The tumor masses were analyzed using the statistics software package on SPSS.

RESULTS

I. Selection for Genotypic Variation.

The assay described in the Materials and Methods section proved to be suitable for selection of genotypic variation in susceptibility of pea to crown gall. The peas grew vigorously in the growth pouches in the growth chamber up to 17 days (Fig. 1). Beyond 18 days the plants began to deteriorate. Tumors developed on susceptible plants in 6 to 10 days, so 14 days allowed sufficient time for tumor development without the plants becoming overgrown or invaded by saprophytic fungi. One wound 1 mm deep was large enough to allow tumorigenesis, yet tumors that formed were not so large that they supressed the growth of the plant. When peas were stabbed all the way through the hypocotyl at 3 sites and then inoculated, plants were severely stunted (Hawes et al. 1989 in press). The genotypic variation in response of the 34 cultivars of pea to crown gall infection is represented in Table 2. Excised tumors from 3 cultivars are shown in Figure 3. Mean tumor mass fell into three statistically different groups: small, medium and large. Sweet Snap formed the smallest tumors (4mg +/- 1) and was in a class by itself. The group with the medium response was composed of 31 cultivars with tumor masses ranging from 12 +/- 1 to 30 +/- 3 mg. The large tumor-forming group consisted of two cultivars, Oregon Sugar Pod and Target which developed tumors whose mean weights were 43 +/- 4 mg and 47 +/- 6 mg, respectively.

The efficiency of tumorigenesis for Sweet Snap was 64%. Some of the inoculated Sweet Snap seedlings were very difficult to distinguish from uninoculated controls except for a small amount of amorphous tissue growth in the inoculation site. Only plants that did not have any amorphous tissue growth were scored as not forming tumors. This was significantly lower than the efficiency of tumorgenesis for all of the other cultivars, which had efficiencies of 89 to 100%.

Table 2. Genotypic variation in tumor weight of 34 cultivars of pea inoculated with A.tumefaciens strain B6. Tumor weights were determined by subtracting uninoculated controls.

Cultivar	n	Mean Tumor Mass (mg)	Efficiency
Sweet Snap	67	4 +/- 1	64%
Dwarf Grey Sugar	89	12 +/- 1	96%
Alaska	94	13 +/- 1	96%
Knight	63	13 +/- 2	89%
Laxantonian	57	14 +/- 2	97%
Maestro	71	16 +/- 2	96%
Alaska 423	55	16 +/- 3	96%
Green Arrow	72	17 +/- 3	94%
Snappy	44	17 +/- 2	94%
Wando	91	17 +/- 2	98%
Burpeeana Early	48	19 +/- 3	89%
Freezonian	90	19 +/- 3	98%
Century	95	20 +/- 3	91%
Sugar Bon	46	21 +/- 3	96%
Giant Melting Sugar	33	21 +/- 4	100%
Thomas Laxton	91	21 +/- 3	95%
Grenadier	60	21 +/- 3	93%
Honey Pod	23	21 +/- 5	96%
Patriot	44	22 +/- 3	93%
Novella	44	22 +/- 3	93%
Salvo	22	23 +/- 4	100%

Table 2. continued.

Cultivar	n	Mean Tumor Mass (mg)	Efficiency
Sugar Ann	46	24 +/- 4	100%
Sparkle	24	24 +/- 3	96%
Little Marvel	67	25 +/- 2	92%
Mammoth Melting Sugar	91	26 +/- 2	97%
Blizzard	46	26 +/- 3	94%
Snowbird	86	27 +/- 2	99%
Laxton's Progress	78	27 +/- 3	90%
Sugar Snap	74	28 +/- 3	91%
Sugar Snap	74	28 +/- 3	91%
Oregon Sugar Pod II	46	28 +/- 2	96%
Sugar Daddy	43	30 +/- 3	97%
Progress #9	29	30 +/- 3	89%
Oregon Sugar Pod	48	43 +/- 4	98%
Target	24	47 +/- 6	100%

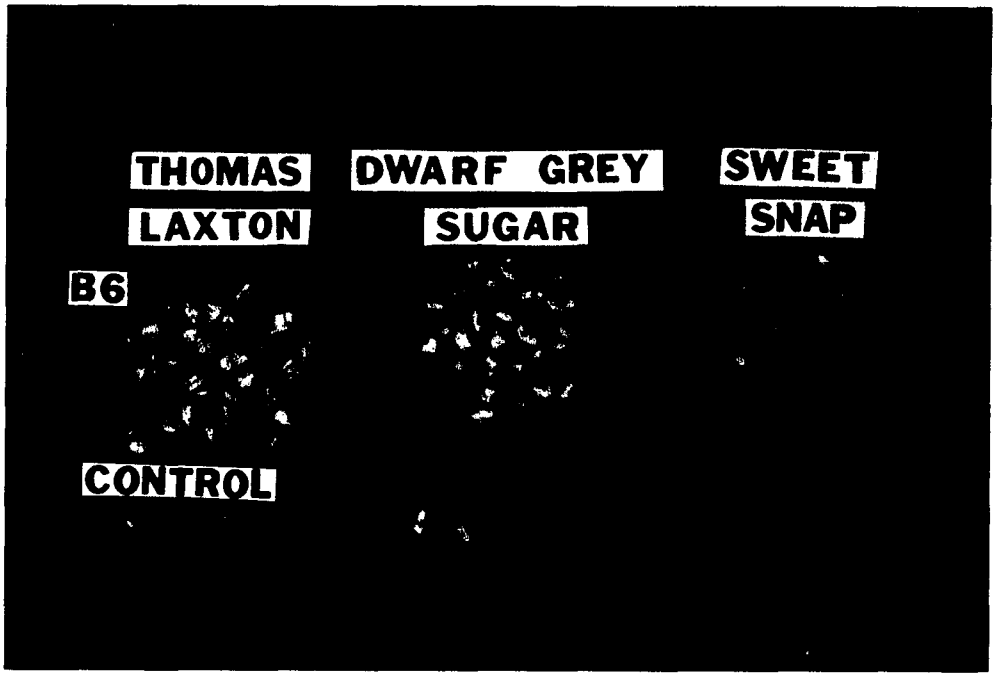

Figure 3. Excised tumors from cultivars Thomas Laxton, Dwarf Grey Sugar, and Sweet Snap after inoculation with A.tumefaciens strain B6.

Strain Specificity

To determine if the observed variation in response to strain B6 among different cultivars would also occur in response to inoculation with other strains of A.tumefaciens and one strain of A.rhizogenes, 6 cultivars that exhibited varying susceptibility to A.tumefaciens strain B6 were chosen. At the time these experiments were initiated the highly susceptible cultivars Target and Oregon Sugar Pod were not available. The tumor masses resulting from these inoculations are given in Table 3 and excised tumors after A.rhizogenes inoculations are shown in Figure 4. The efficiencies were the same as for the B6 inoculations for all strains and cultivars except for Sweet Snap inoculated with A.tumefaciens strain A281. The efficiency of tumor development in Sweet Snap inoculated with A281 was 84%. In general, B6 induced the smallest tumors followed by A723, T37, R1000 and A281 for all 6 cultivars with a few exceptions: Freezonian formed larger tumors when inoculated with A723 than when inoculated with T37 or R1000, Sugar Snap formed larger tumors when inoculated with B6 than when inoculated with A723 or T37 or R1000. The response of the cultivars with the different strains did not parallel the B6 inoculations as indicated in Table 4. While inoculating with a different strain altered the ranking of the most of the cultivars, Sweet Snap's response was unique in that it consistently formed the smallest tumors.

Table 3. Average masses (mg) of inoculated sections of pea cultivars inoculated with different strains of A.tumefaciens and one strain of A.tumefaciens (R1000).

	Agrobacterium strain				
Cultivar	B6	A723	T37	R1000	A281
Sweet Snap	4 +/- 1	5 +/- 1	8 +/- 1	6 +/- 1	28 +/-3
Alaska	13 +/- 1	21 +/- 4	25 +/- 3	32 +/- 5	41 +/-6
Dwarf Grey Sugar	12 +/- 1	16 +/- 2	16 +/- 2	37 +/- 4	43 +/-5
Freezonlan	19 +/- 3	39 +/- 3	27 +/- 2	26 +/- 3	50 +/-5
Sugar Snap	28 +/- 3	16 +/- 3	15 +/- 3	27 +/- 3	52 +/-7
Thomas Laxton	21 +/- 3	27 +/- 7	25 +/- 3	31 +/- 6	53 +/-6

Table 4. Rank of tumor masses for 6 different cultivars inoculated with 4 strains of A.tumefaciens and 1 strain of A.rhizogenes The cultivars and their respective abbreviations are as follows: Dwarf Grey Sugar (D.G.S.), Freezonian (Freez), Sugar Snap (Sug. Snp.), Sweet Snap (Swt. Snp.), Thomas Laxton (T.L.).

	Agrobacterium strain				
Rank	**B6**	**A723**	**T37**	**R1000**	**A281**
1 (small)	Swt. Snp.	Swt. Snp.	Swt. Snp.	Swt. Snp.	Swt. Snp.
2	D.G.S.	Alaska	Sug. Snp.	Freez.	Alaska
3	Alaska	D.G.S.	D.G.S.	Sug. Snp.	D.G.S.
4	Freez.	Sug. Snp.	Alaska	T.L.	Freez.
5	T.L.	T.L.	T.L.	Alaska	Sug. Snp.
6 (large)	Sug. Snp.	Freez.	Freez.	D.G.S.	T.L.

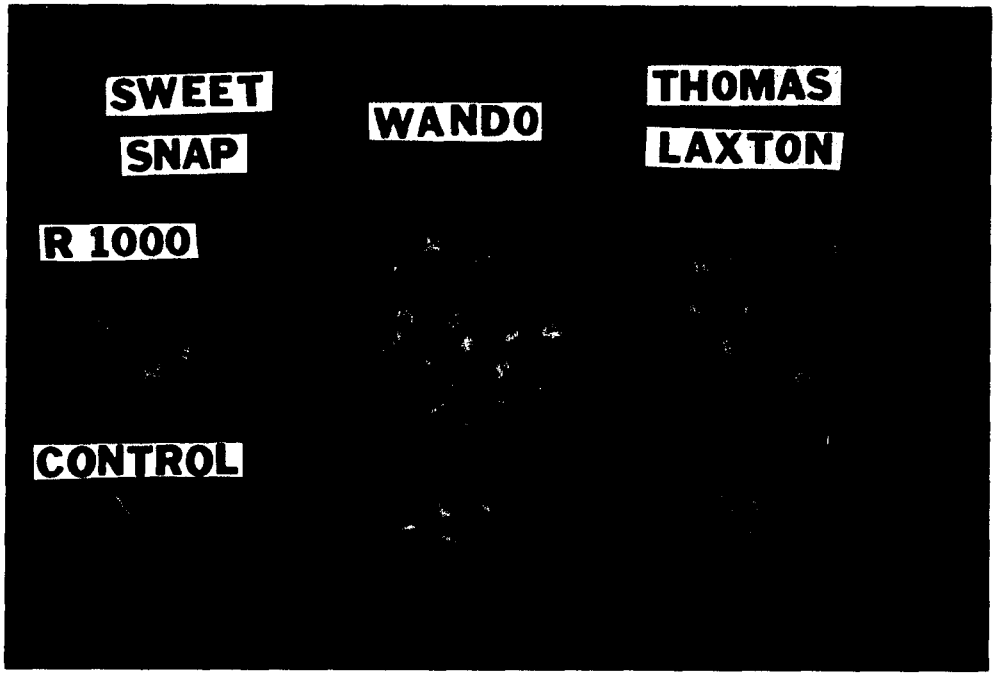

Figure 4. Excised tumors of cultivars Sweet Snap, Wando, and Thomas Laxton after inoculation with A.rhizogenes strain R1000.

II. Characterization of Variation of Susceptibility in Pea to Crown Gall.

Chemotaxis

As chemotaxis has been proposed to be important in the host recognition in crown gall disease (Ashby et al. 1987), a chemotaxis assay was employed to determine if the variation observed in susceptibility in pea to crown gall was correlated to chemotactic attraction. The chemotaxis ratios were obtained for 4 different cultivars varying in their susceptibility to crown gall. The chemotaxis ratios for Sweet Snap, Dwarf Grey Sugar, Thomas Laxton and Wando are given in Table 5. The halo formed by the bacteria on the water agar was difficult to see under the fluorescent lights in the lab so the plates were observed with the indirect light of a desk lamp (Figure 5). The chemotaxis ratios were more variable when root tips of the 4 cultivars were used than when the isolated root cap cells were used for the assay. Chemotaxis was not correlated with relative susceptibility to crown gall. The chemotaxis ratio for root tips of Sweet Snap was higher than the other cultivars and the chemotaxis ratio for isolated root cap cells was the same as the chemotaxis ratio for root cap cells of Dwarf Grey Sugar and Thomas Laxton.

Table 5. Chemotaxis ratios* of A.tumefaciens strain B6 to root tips and isolated root cap cells of 5 different cultivars of Pisum sativum.

	Chemotaxis Ratios	
Cultivar	Root tips	Isolated Root Cap Cells
Dwarf Grey Sugar	1.7 +/- 0.20	1.7 +/- 0.08
Sweet Snap	1.9 +/- 0.65	1.7 +/- 0.27
Thomas Laxton	1.46 +/- 0.07	1.7 +/- 0.30
Wando	1.35 +/- 0.30	1.59 +/- 0.24

*Chemotaxis ratio = (distance bacteria move towards chemo-attractant)/(distance bacteria move away from chemo-attractant)

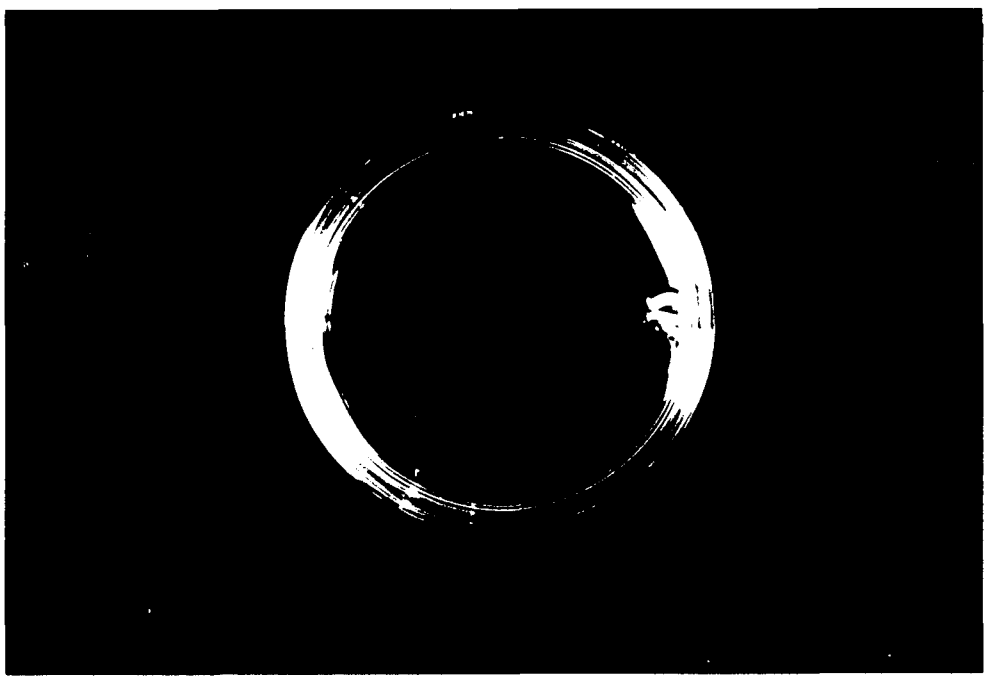

Figure 5. Chemotaxis assay showing swarm of bacteria moving towards pea root tips.

Binding of Agrobacteria to Root Cap Cells.

The next proposed step in crown gall infection, binding, was studied. A binding assay was used to determine if binding of agrobacteria to isolated root cap cells was correlated with the genotypic variation observed in the susceptibility of pea to crown gall.

When root cap cells were isolated for the assay, I observed that suspensions containing high concentrations of root cap cells from sugar peas (Sweet Snap, Dwarf Grey Sugar, and Sugar Snap) were more foamy than the root cap cell suspensions from other cultivars of pea like Freezonian, Wando, and Target. Genotypic variation was also observed in the number of root cap cells liberated from each cultivar. Quantifying the cell concentration in the suspensions with the hemacytometer helped to alleviate this problem. The binding of bacteria to the root cap cells was a time dependent process (Hawes, personal communication), thus it was essential that all assays were examined after the same amount of time. The mean number of bacteria bound to the periphery of the root cap cells for each of 4 cultivars is given in Table 6 and an example of a root cap cell with bacteria around the periphery is given in Figure 6. The mean number of bacteria bound to the isolated root cap cells of Sweet Snap was lower than the number of bacteria bound to the root cap cells of Freezonian and Sugar Snap and about the same as Dwarf Grey Sugar, however the values were not significantly different.

Table 6. Mean numbers of bacteria bound to the periphery of isolated root cap cells of 4 pea cultivars.

Cultivar	n	Mean # of bacteria bound to root cap cell
Dwarf grey sugar	70	13 +/- 7
Freezonian	40	26 +/- 11
Sugar Snap	75	21 +/- 10
Sweet Snap	65	13 +/- 8

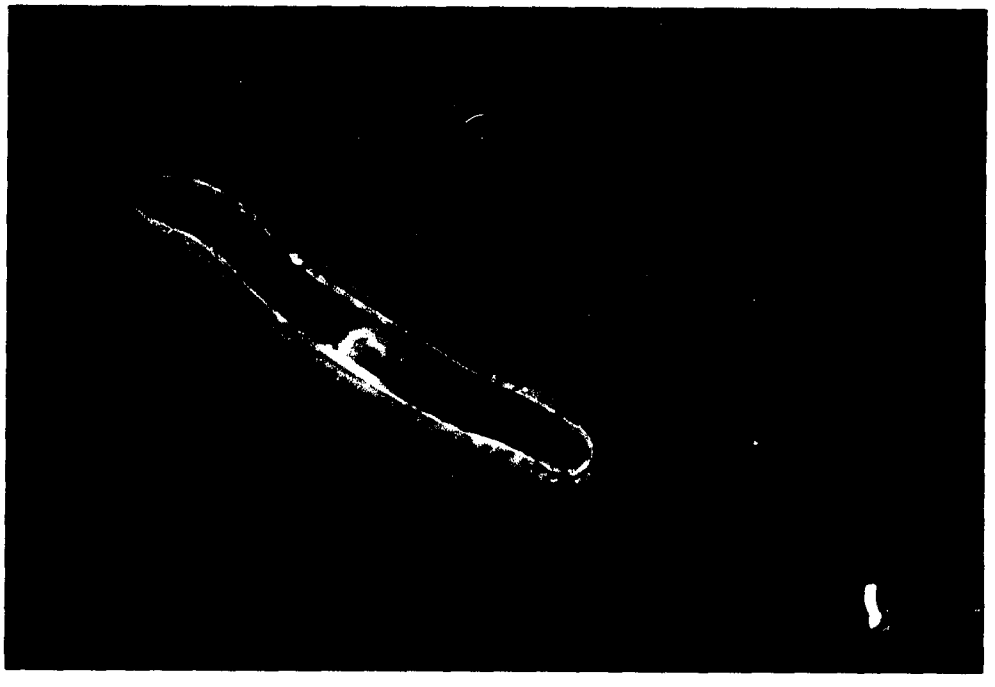

Figure 6. Isolated root cap cell of pea with bound agrobacteria around the periphery.

Vir-gene induction

Induction of vir genes is one of the primary steps in the infection of A.tumefaciens (Stachel et al. 1986). The genotypic variation in tumorigenesis we observed in pea could be due variable amounts of vir-inducing compounds produced by the various cultivars. To determine if incubating agrobacteria with As (a vir-gene inducer) would enhance tumor formation in Sweet Snap, seedlings of Sweet Snap were inoculated with As-induced bacteria and the resulting tumors were weighed. The appearance of the tumors formed on Sweet Snap plants inoculated with As-induced bactera did not differ from the tumors on plants inoculated with uninduced agrobacteria. The average tumor weights of Sweet Snap inoculated with induced bacteria was 3 $\pm$ 1 mg which were not significantly different from tumor weights resulting from inoculations with uninduced bacteria.

It is possible that a highly susceptible cultivar contains more vir- inducing compounds than a cultivar with low susceptibility. To test this assumption, agrobacteria were incubated with the exudates of Wando and used to inoculate Sweet Snap. The average of the ensuing tumor masses was 4 $\pm$ 1 mg which was not signficantly different from tumor masses resulting from uninduced bacteria.

Nuclear Transformation

It is possible that agrobacteria may be chemotactically attracted to the plant cell, bound to the plant cell, respond to vir gene inducing molecules, and have the T-DNA excised from the Ti plasmid but not integrated into the genome. If the host somehow inhibits integration of the T-DNA into the genome tumors would not develop, or could be reduced in size due to low levels of hormone producing T-DNA genes. The presence of T-DNA in the transformed plant cell can be validated by molecular hybridization techniques (Thomashow et al. 1980). A Southern blot analysis was attempted to confirm that the small tumors in Sweet Snap did in fact contain T-DNA.

Control DNA was extracted from non-transformed plant tissue of Wando and Sweet Snap. During the extraction process of DNA from Sweet Snap and Wando stem tissue, the viscous DNA

had precipitated, however very small amounts were obtained as indicated by the absorbance on the spectrophotometer.

Sweet Snap A280 = 0.877 A260 = 0.0

Wando A280 = 0.0557 A260 = 0.0

The absorbance at 280nm is due to proteins and the absorbance at 260 is due to the DNA. Although there was not enough DNA to be detected by spectrophotometry, faint bands of DNA could be seen on the agarose minigel after soaking in ethidium bromide and observation under UV light.

The very small size of Sweet Snap tumors precluded sufficient DNA for analysis. An attempt was made therefore to increase transformed callus tissue in culture. To obtain callus tissue for analysis of transformed plant tissue, tumors were surface sterilized and placed on tissue culture media to grow. These callus tissue survived for 2 weeks but they did not grow very well and died.

Cellular Transformation

Although capable of being transformed by A.tumefaciens, a plant could still exhibit reduced tumor size and efficiency because of a reduction in the number of cells transformed. A histochemical assay for foreign gene expression in transformed plant tissue using the B-glucuronidase (GUS) gene (Jefferson 1987) was used in an effort to determine if there was a quantitative difference in the number of cells transformed in Sweet Snap compared to other cultivars.

Thin sections from the inoculated regions of the Sweet Snap and Wando seedlings which had been inoculated with LBA4404(pBI121) 2 weeks prior to analysis were assayed for GUS activity. After 4 hours in the 37C incubator no blue product had formed, however after 12 hours a blue product had formed. Due to the nature of the wounded sections, it was difficult to get a good cross section of inoculated tissue for histochemical analysis of transformed cells. Another method of inoculating peeled-off tissues from the hypocotyl and assaying these tissues for GUS activity was employed.

The results of the different procedures used to analyze the thin sections for GUS activity are as follows:

1. When the thin sections were immersed in a suspension of LBA4404(pBI121) for 2 days and then rinsed in water and assayed for GUS activity, the sections were blue in both Wando and Sweet Snap, however large clumps of bacteria did not rinse off the sections so it was difficult to see individual transformed cells expressing the GUS gene. Sections that were inoculated with A.tumefaciens LBA4404 did not show any GUS activity nor did control sections without bacteria.

2. When Wando and Sweet Snap sections were inoculated for 2 hours with LBA4404(pBI121), placed on MS media for 2 days then rinsed and assayed for GUS activity, no blue stained plant or bacteria cells were observed.

3. When Wando and Sweet Snap sections were inoculated with LBA4404(pBI121) for 2 hours , placed on water agar for 2 days, rinsed in sterile water, and assayed for GUS activity, blue cells could be observed in these sections. However, the bound bacteria also apparently expressed the GUS gene as they were also blue. The blue product formed from the reaction of B-glucuronidase and X- glucuronide had stained adjacent cells so it was difficult to see individual transformed cells.

4. In an effort to reduce the background activity of GUS expressed by the bacteria, thin sections were inoculated with A.tumefaciens LBA4404(pBI121), placed on water agar for 2 days and then placed on water agar with ampicillin (100ug/ml) and rifampicin (10ug/ml) for 1-4 days. Blue stained bacterial cells could still be observed after the sections had been on the antibiotic media for four days.

5. The procedure of placing thin sections inoculated with A.tumefaciens LBA4404(pBI121), on water agar for 2 days, then placing the sections on water agar with carbenicillin (50ug/ml) and tetracycline (100ug/ml) for 7 days, and rinsing in water and assaying for GUS activity was the best method of removing the background expression of GUS by the bacteria. These sections did not have any blue transformed cells or blue bacteria. Controls of bacteria on water agar instead of antibiotic were still viable as they expressed GUS.

Isolated root cap cells incubated with LBA4404(pBI121) for 2 hours and rinsed of unbound bacteria were assayed for GUS activity. The background of the bacteria expressing the GUS gene made it difficult to tell whether or not the root cap cells were transformed or if the blue product was only produced by the bacteria.

Expression of T-DNA

The expression of T-DNA in transformed pant tissue is evident by tumor formation, octopine production, or expression of foreign genes cloned into the T-DNA. It would still be possible for a plant to not develop tumors even though the T-DNA is transcribed in the cell perhaps due to altered phytohormone biosynthesis or regulation. Because no definite results of GUS expression were obtained the expression of T-DNA in inoculated Sweet Snap and Wando plants was tested by an octopine assay.

The octopine from inoculated and control wounded sections of Wando and Sweet Snap was extracted with ethanol and applied to chromatography paper. After eluting in the solvent for 7 hours and staining with phenanthrenequinone reagent, purple spots were visible above the sections that were from the tumor tissue. These spots had migrated the same distance as the methylene green marker. No purple spots were observed over the non-inoculated control sections. Not a high enough concentration of standard was used as no spot was observed.

III. Inheritance Study.

A study of the inheritance of the variation in susceptibility was initiated in order to understand the genetic basis for the reduced susceptibility phenotype observed in Sweet Snap.

The phenotypic variation in flowering time and pea height are given in Table 7. The cultivar Wando was selected to cross with Sweet Snap because it bloomed at the same time as Sweet Snap, and because Wando has distinct genetic markers which are different from Sweet Snap. Wando forms medium size tumors, it is a dwarf and the leaves are speckled in contrast to Sweet Snap which forms small tumors, is tall and has unspeckled leaves. There was also a difference in the ability of tumors to continue to grow beyond 2 weeks. Sweet Snap tumors remained small (Figure 7) whereas Wando tumors continued to grow until the plants matured (Figure 8). The cultivar Target which forms the largest tumors was not available when the inheritance study was initiated, thus it was not used in the crossing experiments.

The phenotype of the F1 progeny was tall plants (Figure 9) with speckled leaves (Figure 10) which are two described dominant phenotypes expressed in the parents (Tedin 1925, Mendel 1865). The tumors formed on the F1 progeny were intermediate between the 2 parents (Figure 11).

The heights of the F2 and F3 plants varied from tall, medium and dwarf and most of them had speckled leaves. The mean tumor masses and ranges for the parents, F1, F2, and F3 plants are given in Table 8.

Table 7. Bloomming time for 13 different pea cultivars and their heights at maturity.

	Blooming time (days)		Height at maturity (cm)	
		Growth		Growth
Cultivar	Greenhouse*	Chamber**	Greenhouse	Chamber
Alaska	19	26	30	55
Century	46	-	80	-
Dwarf Grey Sugar	38	39	38	50
Freezonian	21	25	32	70
Grenadier	31	27	7	24
Laxantonian	23	31	8	25
Laxton's Progress	23	31	8	18
Little Marvel	25	33	13	20
Snowbird	25	24	18	19
Sugar Snap	46	-	80	-
Sweet Snap	38	41	75	70
Thomas Laxton	22	31	45	70
Wando	39	41	30	30

* Pea plants were grown at the Campbell Avenue Farms in steam pasteurized potting soil consisting of sand, soil from the farm and peat moss in the ratio of 1:1:1. The plants were fertilized weekly with 1 teaspoon of osmocote per pot and they were watered daily. Seeds were planted July 3, 1987.

** The Conviron growth chamber was set at a 12 hour photoperiod at 22C.

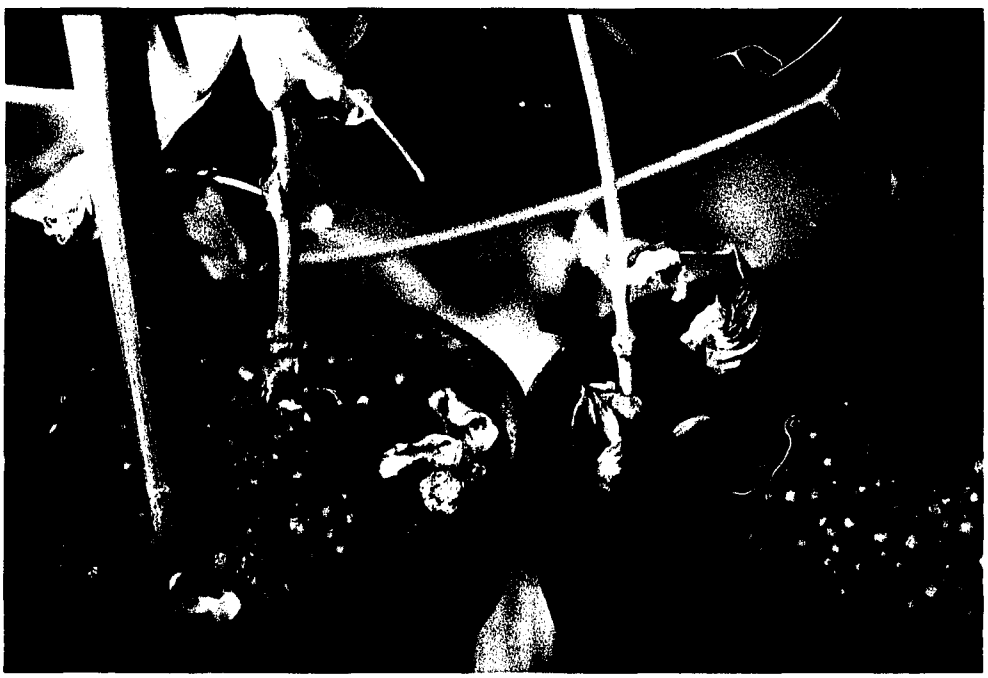

Figure 7. Tumors on Sweet Snap plants 8 weeks after inoculation with A.tumefaciens strain B6.

Figure 8. Tumors on Wando plants 8 weeks after inoculation with A.tumefaciens strain B6.

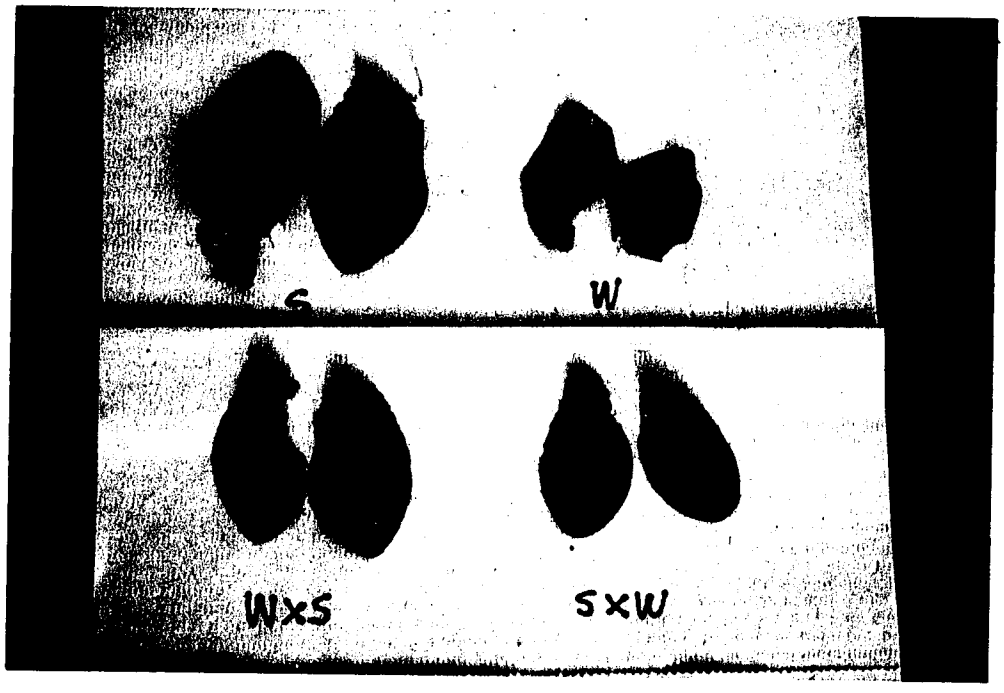

Figure 9. Leaves of Sweet Snap (top left corner), Wando (top right corner), and leaves from the F1 progeny resulting from reciprical crosses of Wando and Sweet Snap (bottom left and right corners.

Figure 10. Mature pea plants of: (from left to right) Sweet Snap, Wando, F1 resulting from a S X W cross, and F1 resulting from a W X S cross.

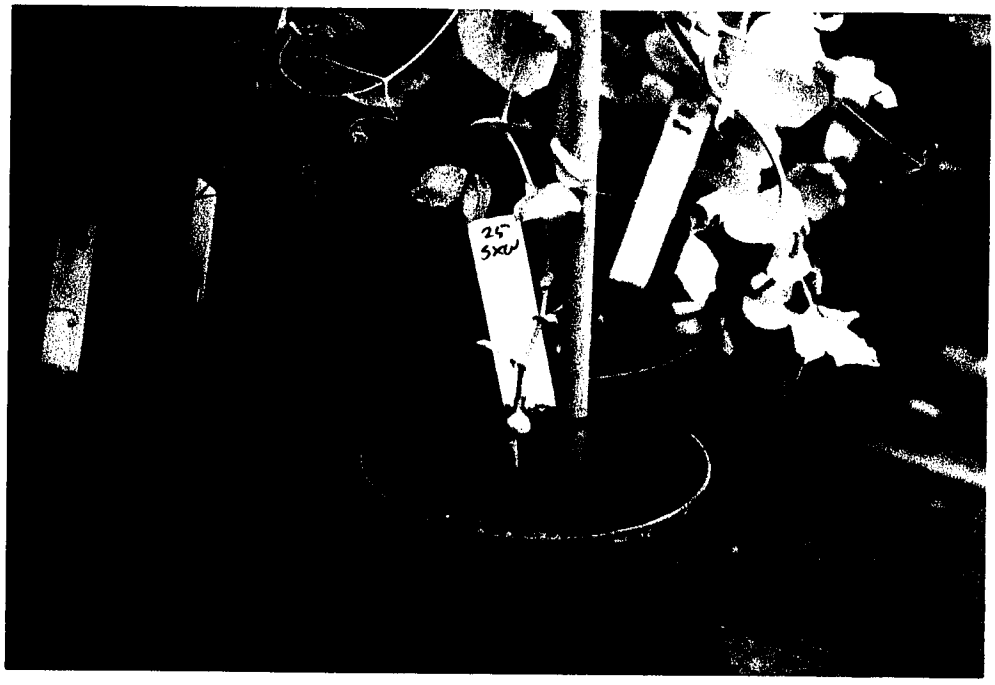

Figure 11. Tumor on a F1 plant resulting from a S x W cross.

Table 8. Statistics of F1, F2, and F3 tumors (the tumor masses in this table are the masses of the whole inoculated section rather than the net masses given in the other tables because there were no controls for the F1, F2, and F3 progeny)

Cultivar	n	Mean Tumor Mass (mg)	Range
Sweet Snap	67	22.5 +/- 7.5	7.7 - 40.6
Wando	91	37.0 +/- 14.5	7.4 - 92.7
F1	15	27.5 +/- 10.7	15.7 - 52.6
F2	236	26.3 +/- 11.0	6.3 - 64.7
F3	60	25.78 +/- 13.21	7.9 - 67.3

DISCUSSION

Pisum sativum has proven to be an excellent choice for a model system to study host factors involved in crown gall disease. Although studies with other hosts on the cultivar dependence on crown gall infection have been performed, the hosts employed would not be as practical as pea for this type of comprehensive study. Peas have a short growing time, tumors develop quickly, genotypic variation amongst cultivars with respect to tumorigenesis can be detected with a simple assay, and genetic markers of pea are available.

The genotypic variation to crown gall infection has been studied in soybeans, however a period of 8 weeks after infection is required to observe tumor development (Owens and Cress 1984). In chrysanthemum, 4 weeks is required to observe tumors (Miller et al. 1975). In this study we found that only 2 weeks was required to observe tumor development in peas.

Among only 34 cultivars of pea assayed, significant variation in tumorigenesis was observed after inoculating with A.tumefaciens strain B6. The response of the 34 cultivars to crown gall infection fell into three statistically different groups based on tumor masses: low, medium, and high susceptibility. Target, the most susceptible cultivar, formed tumors 10 times larger than the least susceptible cultivar, Sweet Snap. In addition, 89% to 100% of inoculated seedlings formed tumors for 33 of the cultivars and only 64% of the inoculated seedlings of the cultivar Sweet Snap, formed tumors. Different cultivars of alfalfa have been compared for their response to infection by A.tumefaciens, but the cultivars assayed were not very susceptible as in the most susceptible cultivar only 14% of the inoculated plants developed tumors (Mariotti et al. 1984).

The procedure employed in this research of uniformly inoculating approximately 60 seedlings per each pea cultivar in the hypocotyl has resulted in consistent results with respect to tumor weight and efficiency of tumorigenesis. Some assays have not been quantitative as only a numerical, positive or negative designation of tumor formation was given for tumor formation on chrysanthemum (Miller et al. 1985), alfalfa (Mariotti et al. 1984), and grapes (Knauf et al. 1982). A

quantitative assay for tumor formation on castor bean leaves has been described by El Khalifa and El Nur(1970). However, in their assay only the number of tumors formed was observed which might not be correlated with the size of the tumors formed. The inoculation procedure employed by Owens and Cress (1984) involved wounding the plants in the stem with 8 punctures and then spreading a suspension of A.tumefaciens over the wound. Utilizing this inoculation procedure, they observed that tumor size and the number of tumors formed per wound area were not correlated. They also observed genotypic variation in the number of tumors formed per wound area. Perhaps if the peas were wounded in more than 1 site, similar results might be obtained.

In Owens and Cress's (1984) study, genotypic variation was observed in the masses of tumors that developed on soybeans but only 6 plants of each cultivar were inoculated. With such a small number of plants assayed, reliable frequencies of the number of plants that formed tumors were not obtained. Owens and Cress (1984) also noted genotypic variation in the growth of galls beyond their 8 week experiment period. A difference in the ability of galls to grow beyond two weeks was noted when the pea cultivars Wando and Sweet Snap were grown in the greenhouse for the inheritance study. Galls on inoculated Wando plants continued to grow whereas the galls on Sweet Snap stayed the same size. Other cultivars were not studied for their ability to continue to grow tumors beyond the 2 week experiment period.

Although genotypic variation to crown gall infection has also been observed in castor bean (El Khalifa and El Nur 1970), chyrsanthemum (Miller et al. 1985), and grapes (Knauf et al. 1982), the genetics of peas have been studied more thoroughly and genetic maps and markers of pea are available. Even among only 34 cultivars of pea studied in this research, various phenotypes were expressed with respect to height, leaf and tendril morphology, flower color, seed color and texture, seeds per pod, and other qualities (ie. some were sugar peas, snap peas, canning peas, or freezing peas). The reduced susceptibility phenotype of Sweet Snap was not correlated with any other observable phenotype as other more susceptible cultivars expressed at least one of Sweet Snap's phenotypes (ie. white flowers and yellow smooth seeds) however it is interesting to note that Sweet

Snap is reported to be resistant to powdery mildew and legume yellows (Park Seed Catalogue 1987).

Specific plant cultivar-bacterial strain interactions have been observed in studies involving A.tumefaciens and soybean (Owens and Cress 1984), chrysanthemum (Miller et al. 1975), alfalfa (Mariotti et al. 1984), and grapevine (Knauf et al. 1982). The response to inoculation of 6 cultivars of pea with 4 different strains of Agrobacterium was interesting in that the ranking of cultivars according to their tumor masses varied depending on what bacteria strain was used (Table 4). Other groups have inoculated with a disarmed strain of A.tumefaciens as a control to determine the effects of hormones produced by the bacteria and not the transformed plant cells however no tumors have ever resulted from inoculations with these strains (Owens and Cress 1984, Yanofsky et al. 1985). Although different rankings were observed for the different strains, Sweet Snap consistently formed smaller tumors compared to the five other cultivars tested. The fact that Sweet Snap forms medium sized tumors when inoculated with the hypervirulent strain A281 shows that Sweet Snap is capable of forming substantial tumors. The hypervirulence of A281 is due to the high expression of vir G which might cause a more rapid transformation than non-hypervirulent strains (Jin et al. 1987). Besides forming larger tumors, a higher transformation efficiency was achieved by inoculating Sweet Snap with strain A281 (85%) compared to B6 (64%). The rapid transformation ability of A281 may allow it to overcome factors which attenuate the transformation with other strains. Some of these factors which may attenuate the transformation include: 1) a hypersensitive reaction, 2) phytoalexin production, or 3) dehydration of the wound site. A hypersensitive reaction has been observed after inoculating grapes with certain wide host range strains of A.tumefaciens (Yanofsky et al. 1985), however the response of Sweet Snap seedlings that did not form tumors looked more like uninoculated controls than a hypersensitive response. Phytoalexins in Pisum sativum have been discovered which are active against pathogenic fungi (Van Etten 1975) and bacteria (Wyman and Van Etten 1978). Sometimes phytoalexin production is induced by pathogenic bacteria (Gnanamanickam and Patil 1976,Cruickshank, and Perrin 1971), thus A281 might be able to

transform cells before the host is able to synthesize the phytoalexin. In soybeans, larger tumors resulting from A.tumefaciens inoculations have been obtained by enclosing the wound site in a plastic tube to prevent dehydration (Pederson et al. 1983). This procedure could be employed when inoculating Sweet Snap to determine if larger tumors will develop after inoculation with A.tumefaciens. However, because the inocuated site was protected by the moist growth pouch, it is doubtful that enclosure of the wound would alter the response.

This variation in susceptibility to crown gall in pea indicates that genotypic variation exists which can be exploited for analysis of host determinants in crown gall disease. Since Sweet Snap consistently forms extremely small tumors, has a low efficiency of tumorigenesis, and is a commercially available cultivar, it was compared with other more susceptible cultivars to determine if any of the following steps in crown gall tumorigenesis were blocked or attenuated: 1) chemotaxis, 2) binding, 3) vir-gene induction, 4) cellular transformation, and 5) expression of T-DNA. The assays utilized to characterize Sweet Snap's reduced susceptibility will be used in the future to assay other cultivars found to exhibit a reduced susceptibility to crown gall. Among 1200 pea lines, 15 have been found to be resistant to crown gall (Hawes, Marx, and Pueppke, unpublished).

Chemotaxis may give A.tumefaciens a competitive advantage in establishing itself in the rhizosphere. Ashby et al. (1988) have observed chemotaxis of A.tumefaciens to acetosyringone, a vir-gene inducer. They have proposed that this might enable A.tumefaciens to find susceptible plants. Other groups, however, have not observed chemotaxis of A.tumefaciens to acetosyringone (Hawes et al. 1988, Parke et al. 1987). No other studies have been performed with chemotaxis of A.tumefaciens to susceptible and resistant cultivars, however Chet et al. (1973) have shown that Pseudomonas lachrymans is chemotactically attracted to extracts of both susceptible and resistant plants. Chemotaxis did not appear to be a limiting factor in Sweet Snap's reduced susceptibility: the chemotaxis ratios of Sweet Snap were not significantly different from chemotaxis ratios of more susceptible cultivars. These results are not surprising as chemotaxis has been reported for A.tumefaciens wild type strains to a wide variety of sugars and amino acids (Hawes et al. 1988)

which are probably found in the root exudates of most peas (Rovira 1956) and to monocots (Hawes et al. 1988, Ashby et al. 1988) which are mostly resistant to infection by A.tumefaciens. Another reason why it is unlikely that chemotaxis limits tumorigenesis in Sweet Snap is that the seedlings were immersed in a high inoculum concentration. Under these conditions, the bacteria were in contact with the roots and the fact that the bacteria were chemotactic would not have given the bacteria an advantage. Attachment of A.tumefaciens to the plant cell wall has been proposed to be one of the initial steps in crown gall tumorigenesis (Glogowski and Galasky 1978, Lippincott and Lippincott 1969) The bacterial cell wall of A.tumefaciens contains 2-linked-B-D glucans which are thought to be involved in the binding process (Puvanesarajah et al. 1985). It has been suggested by Neff et al. (1987) that the plant site for attachment contains pectin. No studies have been done to determine if there are biochemical difference in the cell wall structure of resistant and susceptible plants to crown gall. Hawes and Pueppke (1987) have shown that root cap cells of resistant plants bind significantly fewer bacteria than do root cap cells from susceptible plants. The binding ability of Sweet Snap root cap cells to A.tumefaciens was compared to other susceptible cultivars. The observation that the number of agrobacteria bound to the periphery of Sweet Snap root cap cells was not significantly different from the number of bacteria bound to the periphery of root cap cells of more susceptible cultivars indicates that binding is not a factor in the reduced susceptibility observed in Sweet Snap. The root cap cells are not the site of infection for A.tumefaciens so perhaps another assay could be employed to observe the binding to root tissues as these might vary morphologically from the root cap cells.

As binding and chemotaxis did not appear to be limiting factors in the reduced susceptibility of Sweet Snap, vir-gene induction, another preliminary step in the infection process of crown gall disease was studied. By incubating agrobacteria with acetosyringone prior to inoculation Sheikholeslam and Weeks (1987) were able to increase the rate of transformation of alfalfa presumably because levels of vir-inducing compounds limit transformation in Arabidopsis. Owens and Smigocki (1988) were able to increase the incidence and size of galls formed on soybean by

inoculating with acetosyringone-induced agrobacteria. Only one of five cultivars of soybean showed a significant response to acetosyringone possibly because this cultivar secreted suboptimal amounts of acetosyringone. In this research, inoculation of pea seedlings with agrobacteria incubated with acetosyringone resulted in tumors that were not significantly different from the tumors formed with uninduced bacteria. There might be some similarities between a mononocot and a resistant cultivar. Schafer et al. (1987) have postulated that monocots are resistant to crown gall because they lack vir-inducing compounds. They were able to transform a monocot by incubating the agrobacteria in wound exudates of a susceptible dicot. When Sweet Snap seedlings were inoculated with agrobacteria incubated with root exudates of Wando, a susceptible cultivar, tumor size was unaffected therefor evidently Wando does not have more vir-gene inducing compounds than Sweet Snap, and that the reduced tumor formation in Sweet Snap is not due to the lack of vir-gene inducing molecules. In order to quantitatively test for the presence of vir-inducing molecules another assay utilizing vir-lac gene fusions can be employed (Stachel et al. 1985, Stachel and Zambriski 1986). In these fusions the expression of B-galactosidase is placed under control of the virulence genes and the expression of B-galactosidase can be measured spectrophotometrically and quantitatively. These constructs can be used to determine the if there is any variation in the ability to induce vir genes among cultivars with high and low susceptibility to crown gall.

Since Sweet Snap is apparently able to induce vir-genes, one of the next possibilities limiting transformation is the inability of T-DNA to be integrated into the plant genome. Gheysen et al. (1987) have proposed that the T-DNA is transferred as a DNA-protein complex from the bacterial cell to the plant cell. They also have suggested that plant-encoded recombination and repair activities are involved in integrating T-DNA into the plant genome. The integration of T-DNA into the plant genome could be prevented by proteinases in the plant cell that degrade the carrier protein of the T-DNA. There is also the possibility that the T-DNA is degraded by endonucleases in the plant cell. Virtis et al. (1987) have reported that T-DNA is degraded in the plant cell. The presence of T-DNA in the genome of transformed host tissue can been verified by a molecular hybridization

techniques (Thomashow et al. 1980). In the case of Sweet Snap, the development of small tumors indicates that T-DNA has been incorporated into the genome. Unfortunately, Sweet Snap formed such small tumors that not enough tissue could be obtained for the detection of T-DNA in these tissues for a routine analysis, even though DNA was extracted from control noninoculated plant tissue successfully. In future experiments, Sweet Snap could be inoculated with a hypervirulent strain like A281 since the tumors produced are at least 6 times as big as the tumors formed after inoculation with B6. It would be interesting to see if the "resistant" individual plants that do not form tumors have the T-DNA incorporated into them as well.

Although the T-DNA might be integrated into the genome of the host, production of hormones could be prevented in a resistant plant by a block in transcription or translation of the T-DNA. A northern blot analysis might reveal if mRNA is being transcribed from the T-DNA. Translation of the T-DNA usually results in the production of opines and hormones so opine assays are routinely used to verify that cells have been transformed (Ellis et al. 1979, de Frammond et al.1983). Untransformed plant tissues have been reported to contain opines (Johnson et al.1974, Christou et al. 1986), but in this study octopine was only detected in the inoculated sections and not in the control non-inoculated plants.

In the case of Sweet Snap it is obvious that the T-DNA is expressed as tumors are formed. One possible reason for the reduction in size of these tumors is that there are fewer cells initially transformed because tumor size is believed to be proportional to the number of cells transformed. A histochemical assay to detect individual cells transformed by agrobacteria containing the B-glucuronidase gene (GUS) was utilized to test this possibility. GUS has been used effectively to verify that plants have been transformed by A.tumefaciens (Jefferson 1987, Jefferson et al. 1987). One of the problems encountered when GUS was employed in this assay was that the agrobacteria also have GUS activity. This has been controlled with the use of antibiotics. I was not able to detect GUS activity in the epidermal sections. In future assays, cross sections through the stem can be inoculated and assayed for GUS as Jefferson et al. (1987) have reported that the highest GUS

activity was detected in the phloem tissue of transformed plants. Since it was difficult to observe individual cellular transformations a flourometric or spectrophotometric GUS assay can be employed (Jefferson 1987). If a fluorometric or spectrophotometric assay were used, individual transformed cells could not be observed, however by observing the same mass of inoculated tissues for each cultivar, quantitative differences in GUS expression should be observed if they exist.

There is a possibility that abnormal hormone regulation in the plant limits the amount of cell differentiation and growth in a cultivar that is resistant to crown gall. Hormonal regulation and synthesis can vary in different species of plants. A strain carrying the limited host range A.tumefaciens plasmid pTiAg63 is avirulent on some hosts due to a defective cytokinin gene, but is virulent on other hosts (Buchholz and Thomashow 1984). There might be a correlation between auxin resistance and resistance to Agrobacterium as auxin resistant mutant plants of tobacco have been regenerated from protoplasts which are also resistant to infection by A.rhizogenes but susceptible to A.tumefaciens. (Tourneur et al 1985). The auxin resistance of tobacco plants has been correlated with the lack of membrane-bound auxin binding proteins (Nakamura et al. 1988). Perhaps a similar situation exists in Sweet Snap.

An inheritance study was performed in order to aid in understanding the genetic basis of resistance to crown gall in pea. In grapevine, resistance to crown gall is controlled by a single dominant gene (Szegedi and Kozma 1984). In other studies on the genotypic variation in response to crown gall in other plants, inheritance studies were not performed (Owens and Cress 1984, Mariotti et al. 1984, Miller et al. 1975, El Khalfa and Nur 1970, Eapen et al. 1987). Since the response of the F1, F2, and F3 progeny to infection by crown gall was an intermediate response, it does not appear that the reduced susceptibility phenotype observed in Sweet Snap is due to a single dominant gene. At the time the inheritance study was initiated the highly susceptible cultivar Target was not available. Target will be used next in crosses as there is a greater distinction between the tumors formed on it in comparison to Sweet Snap. It will also be interesting to see the outcome of a completely resistant cultivar and a highly susceptible cultivar.

Although the reason for Sweet Snap's reduced susceptibility has not been elucidated, my results do not support the hypothesis that preliminary steps in pathogenesis (chemotaxis, binding and possibly vir gene induction) are involved. In characterizing the reduced susceptibility exhibited by this cultivar or another cultivar found to be resistant, it is essential to assay the primary steps before going on to further complicated assays. The assays utilized to characterize Sweet Snap's reduced susceptibility will be used in the future to assay other cultivars found to exhibit a reduced susceptibility to crown gall. Among 1200 pea lines screened, 15 have been found to be resistant to crown gall (Hawes, Marx, and Pueppke, unpublished).The development of these assays for the characterization of resistance to crown gall in pea may also be of beneficial use for studying other plant-pathogen interactions which involve some of the same processes as A.tumefaciens.

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