Plant Physiol. (1990) 92, 1226-1232
0032-0889/90/92/1226/07/$01.00/0

Received for publication August 17, 1989
and in revised form November 9, 1989

Extended Host Range of *Agrobacterium tumefaciens* in the Genus *Pinus*[1]

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ABSTRACT

Two-to 4-month-old seedlings of nine pine species (*Pinus eldarica* Medw., *Pinus elliottii* Engelm., *Pinus jeffreyi* Grev. & Balf., *Pinus lambertiana* Dougl., *Pinus ponderosa* Laws., *Pinus radiata* D. Don, *Pinus sylvestris* L., *Pinus taeda* L., *Pinus virginiana* Mill), Douglas fir (*Pseudotsuaa menziesii* (Mirb.) Franco) and incense cedar (*Libocedrus decurrens* Torr.) were inoculated with five strains of *Agrobacterium tumefaciens*. Transformation occurred in all conifer species tested as determined by gall formation and opine production. The frequency of gall formation varied by host species, by bacterial strain, and was related to the age of the stem when inoculated. Galls were visible 8 to 12 weeks after inoculation and were small (often less than 2.5 millimeters in diameter). Fewer than half (230 of 502) of the galls originally formed on the trees were present after 1 year, and 26 of these grew to diameters greater than 2 centimeters. The majority of these larger galls (18 of 26) were found in *P. radiata*. Bacterial strain-specific opines were found in 67 of the 81 gall tissues sampled.

Agrobacterium-mediated DNA transfer occurs in many dicotyledonous plants (8) and has considerable potential as a gene vector system (18). Realization of this potential is determined, in part, by the efficiency of DNA transfer (13). Transformation efficiency is often established by measuring the frequency of gall formation produced by a specific strain in the plant population, *i.e.* species, cultivar, or variety, to be transformed (5). Although the host range of *Agrobacterium* is extensive in dicots (8), many gymnosperms (8, 23) and most monocots (15) do not readily form galls in response to inoculation. The lack of gall formation has been interpreted as the inability of *Agrobacterium* to transfer DNA, even though this was shown not to be the case (15).

Gall formation is the result of transfer of genes whose expression in transformed cells results in the production of the phytohormones IAA (16) and isopentenyl adenosine (1). However, gall formation requires more than the transfer of genes responsible for auxin and cytokinin production (3). Transformed plant cells must interpret the resulting cytokinin and auxin synthesis as the signal for cellular proliferation which results in gall formation. Bacterial gene-directed hormone synthesis has been shown to be regulated by plant mechanisms (4, 12). Therefore, gall formation and growth is the result of gene transfer, expression, and the interaction of these genes and their products with the plant's phytohormone regulatory mechanisms.

The host range of *Agrobacterium* in gymnosperms has been recently reexamined by monitoring gall formation in response to inoculation with defined strains (7, 9, 10, 19, 21, 24). In our previous work (21, 24), we reported the successful inoculation of loblolly (*Pinus taeda* L.) and sugar pine (*Pinus lambertiana* Dougl.), extending the host range of *Agrobacterium* into the most economically important conifer genus. Here, we have continued our host range studies in conifers by screening five *A. tumefaciens* strains in nine economically important pine species, as well as Douglas fir and incense cedar. Our successful inoculation, transformation, and tumor induction in all pine species screened extends the host range of *Agrobacterium* within this genus and suggests that all pines are susceptible. However, the low frequency of continued gall growth and chronic gall establishment suggests that conifers may have phytohormone regulatory mechanisms different from readily infectable annual dicotyledenous host plants.

MATERIALS AND METHODS

Host range studies were done by inoculating young seedlings in 11 coniferous species (Table I) and scoring for gall formation and opine synthesis. To break dormancy, seeds were cold-stratified for various times before sowing. Seedlings were greenhouse-grown under mist with weekly fertilization in RL Super Cells (R Leach, Cone-tainer, Canby, OR[3]) in a peat-sand-vermiculite-perlite soil mix under natural light at the Institute of Forest Genetics in Placerville, CA.

Five *Agrobacterium tumefaciens* strains were tested for gall

[1] This work was supported in part by a grant from the USDA/CGRO in Forest Biology 85-FSTY-9-0132. Paper No 12302 of the Journal Series of the N. C. Agricultural Research Service, Raleigh, NC 27695-7643.

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[3] Use of tradenames in this publication does not imply endorsement by the North Carolina Agricultural Experiment Station of the products named, nor criticism of similar ones not mentioned.

Table I. *Conifer Species Screened for Susceptibility to Agrobacterium tumefaciens*

Species	Common Name	Seed Source
Pinus eldarica Medw.	Afghan pine	USDA-FS Nursery, Placerville, CA
Pinus elliotti Engelm.	Slash pine	Forestry Dept., Univ. of Florida, Gainesville, FL
Pinus jeffreyi Grev. & Balf.	Jeffrey pine	USDA-FS Institute of Forest Genetics, Placerville, CA (IFG)
Pinus lambertiana Dougl.	Sugar pine	IFG
Pinus ponderosa Laws.	Ponderosa pine	USDA-FS Nursery, Placerville, CA
Pinus radiata D. Don	Monterey pine	Univ. of California, Berkeley, CA
Pinus sylvestris L.	Scots pine	IFG
Pinus taeda L.	Loblolly pine	University-Industry Tree Improvement Co-op, N.C. State Univ., Raleigh, NC (UITIC)
Pinus virginiana Mill.	Virginia pine	UITIC
Libocedrus decurrens Torr.	Incense cedar	USDA-FS Nursery, Placerville, CA
Pseudotsuga menziesii (Mirb.) Franco	Douglas fir	USDA-FS Nursery, Placerville, CA

formation. Two strains, M2/73 and U3, were selected because inoculation resulted in gall formation and opine production in *Pinus taeda* (21). Strain C58 was chosen because of its broad host range (17). Two other strains, A281 and 542, were selected for their hypervirulence in dicots (14). Control inoculations with avirulent *Agrobacterium* strain A136 was used to test the effect of bacteria on wound response.

Seedlings were inoculated with individual *Agrobacterium* strains when their epicotyls had reached 3 to 5 cm high. The plants were heavily watered before inoculation. Bacterial cultures, streaked on potato dextrose agar (Difco), were used as the source of inoculum. Seedlings were inoculated by stabbing the epicotyl stem several times with a No. 11 scalpel blade that had been dipped in overnight bacterial cultures. After wounding, the seedlings were tented with plastic wrap and placed under greenhouse benches to reduce light intensity, helping to prevent the wounds from drying during the infection period. The plastic wrap was removed after 3 d and seedling growth was maintained as before. Control seedlings were similarly handled and wounded without inoculation or inoculated with avirulent *Agrobacterium* strain A136. The number of seedlings used in each screening test varied due to poor seed germination and/or limited numbers of available seeds.

Seedlings were first scored visually for gall formation 8 to 12 weeks after inoculation and were later examined at variable times to assess gall growth. Seedlings which exhibited continued gall growth were transplanted to larger "TreePots" (TreePot Enterprises, Garden Grove, CA) and were maintained at the Institute of Forest Genetics.

Opine analysis was performed on ethanol extracts of gall tissues. Galls with minimal, normal, stem tissue were excised from actively growing plants, weighed, and ground in 80% ethanol using 2 mL of ethanol per g (fresh weight) of tissue. For tissue quantities less than 0.5 g, the tissue was ground in 0.5 mL of 80% ethanol. All extracts were centrifuged at 16,000*g* for 10 min in a microcentrifuge. Supernatants of those ground in excess ethanol were dried in a Speedvac centrifuge (Savant), and the residue was dissolved in the appropriate volume (1:2, w/v) of 80% ethanol. All samples were spotted onto 3MM paper and subjected to high voltage electrophoresis (21). Strains M2/73 and C58 are known to produce nopaline and its lactam (23); strains 542, A281, and U3 are known to produce agropine and mannopine (11, 21). Opines were identified as described previously (21).

RESULTS

This study confirms our earlier observations of *Pinus taeda* and *Pinus lambertiana* susceptibility to *Agrobacterium* (21, 24) and extends the host range of *Agrobacterium* into seven additional pine species. At the end of one year, 230 galls remained of the total 502 scored in 2116 inoculated seedlings in 11 conifer species. Twenty-six galls had diameters greater than 2 cm at 1 year. In addition, 67 extracts from 81 gall tissues sampled produced the expected opines, markers for cell transformation. These data show that *Agrobacterium* can transfer DNA to cells in at least nine pine species.

Gall formation was observed in all 11 species tested 8 to 12 weeks postinoculation (Table II). Gall formation ranged from 0% in *P. ponderosa* inoculated with strain C58, to 86% in *P. radiata* inoculated with strain 542. A seedling was scored as having a gall if a tumor-like hypertrophy was clearly visible on the stem (Fig. 1a). At 8 to 12 weeks, these tumors were small, rarely having the diameter of the stem, but still clearly distinguishable from wound swelling or swelling resulting from inoculation with avirulent A136. In all cases, gall morphology was tumorous; no shooty teratomas or rooty galls were observed. *Agrobacterium* strains 542 and A281 induced gall formation in *P. menziesii*, confirming a previous report (19). Incense cedar proved to be an excellent host, confirming our earlier observations.

We observed upon subsequent scoring that many galls did not continue to enlarge but rather dried up and were sloughed off as the trees grew. Scoring trees 1 year postinoculation showed that only 200 of the 472 original galls remained and 30 new galls had appeared (Table II). In *P. lambertiana* inoculated with strains C58, M2/73 and U3, an increase in the number of galls was observed at 1 year. The number of U3-induced galls observed after 1 year in *P. virginiana* increased as well. Of those remaining galls, most had enlarged

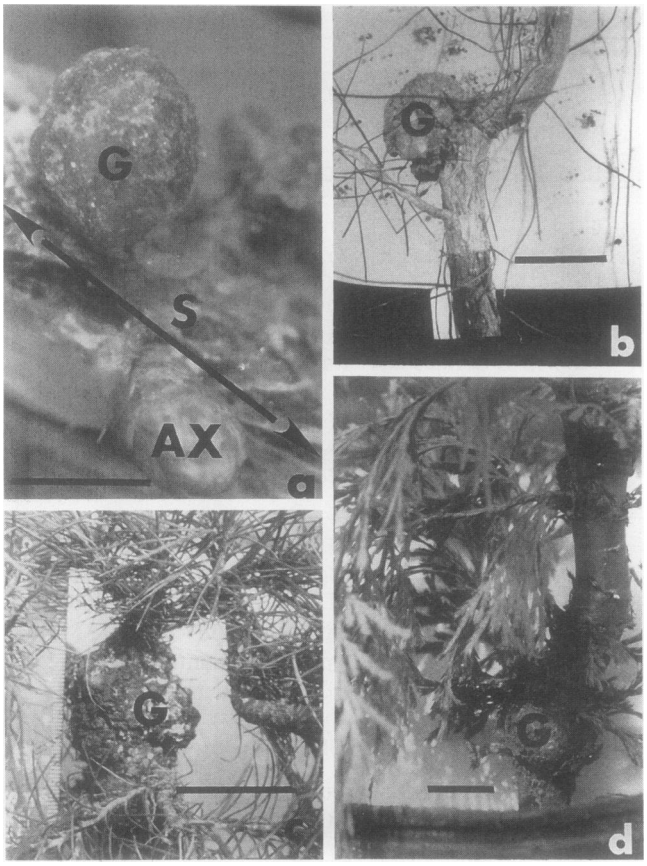

Figure 1. Gall formation in three conifer species. a, Gall caused by strain C58 inoculated into *P. taeda* seedling 8 to 12 weeks postinoculation. Scale marker represents 1 mm. b, Gall caused by strain 542 inoculated into *P. taeda* 18 months postinoculation. Scale marker represents 2 cm. c, Gall caused by strain U3 inoculated into *P. radiata* 3 years postinoculation. Scale marker represents 2 cm. d, Gall caused by strain 542 inoculated into *L. decurrens* 3 years postinoculation. Scale marker represents 2 cm. G, gall; Ax, axillary bud; S, seedling stem.

but were still less than 1 cm in diameter. However, 26 of the total 230 galls enlarged more rapidly. Figure 1, b to d, shows large gall (diameters greater than 1 cm) induced on *P. taeda, P. radiata,* and *L. decurrens.* Eighteen of these 26 galls were found on *P. radiata* which had been inoculated by A281, 542, M2/73, or U3. The largest galls at 1 year were also found among the *P. radiata*-inoculated plants.

Chi-square tests for homogeneity of both the first and 1-year scoring data gave high levels of significance for nonhomogeneity of variation in the ability of the different *Agrobacterium* strains to infect a given species (overall χ^2 Table III). Homogeneity statistical tests were also calculated on the variance partitioned among *Agrobacterium* strains and pine species (Table III). This analysis also gave significant evidence for nonhomogeneity within species and within strains. These results indicate that there is host-pathogen specificity among the *Agrobacterium* strains and the pine species tested.

Gall tissue extracts were tested for the presence of opines (Table IV). Opines were found in 67 of 81 extracts. The opines found were those known to be synthesized by the strains used for inoculation. Opines were not found when uninoculated stem tissue of each species was assayed. In four of the galls which did not show opine synthesis, the fresh weight of the gall tissue extracted was 10 mg or less, which makes opine assay quite difficult. In the other extracts which showed no opine synthesis, sufficient gall tissue was available to detect opines if they were present.

To obtain stronger evidence for DNA transfer, Southern hybridization analysis is necessary. Callus cultures are required for DNA isolation because the amount of living gall tissue does not yield sufficient amounts of DNA for analysis. Therefore, *in vitro* culture of gall tissue from *P. taeda, P. radiata,* and *P. lambertiana* was attempted. In all cases, no callus cultures could be established, despite the use of several different media, supplied with and without exogenous cytokinin and/or auxin. Although we have been unsuccessful in culturing callus from galls on greenhouse-grown seedlings, galls from tissue cultured shoots of *P. lambertiana* readily produce hormone-autotrophic callus (24). Southern hybridization analysis of this callus gave clear evidence for DNA transfer and integration. We have also cultured callus from galls on *P. taeda* seedlings grown *in vitro.* However, this callus grew poorly, was not hormone autotrophic, and yielded insufficient callus for DNA isolation (21). We have no explanation for these observed differences in callus growth.

DISCUSSION

Although it is clear that several *Agrobacterium* strains produced galls in the coniferous species tested, determination of quantitative effects, *e.g.* ranking of strains for infectivity or ranking species for resistance, was not straightforward for several reasons. Quantitative interpretation was confounded because the proportion of galled trees is a function of the time after inoculation, clearly seen by comparing the 8- to 12-week scoring data with the 1-year data in Table II. In 22 of the 30 inoculation combinations, the number of galls remaining after 1 year decreased; in 4 cases the number remained constant; and in 4 cases the number increased. These observations suggest that the relationship between the number of galls and postinoculation time in all combinations may not be identical. Therefore, to rank *Agrobacterium* strains as to their ability to induce galls, or pine species as to their relative susceptibility to gall formation, would require time-dependent scoring data on larger numbers of galls for each strain/species combination.

Infection quantitation is further complicated because the initial host-pathogen interaction appears to be dependent on the developmental state of the plant. Our experience in inoculating conifer seedlings in this screening study and in more extensive experiments with *Agrobacterium*-inoculated *P. taeda* seedlings (to be published elsewhere) indicates that there is an inverse relationship between the degree of stem woodiness and/or plant age and the frequency of gall formation. We have observed that the frequency of gall formation can vary from 0 to about 80% in *P. taeda* with the same *Agrobacterium* strain but with plants differing in age and morphology. Highest rates of gall formation have been consistently observed in seedlings inoculated at the earliest stage capable of surviving initial wounding. The epicotyl stems of these seedlings are green and succulent and have not yet become woody. However, the degree of woodiness alone does not seem to be

Table II. *Numbers of Galls Formed Following Inoculation by Specific Strains of* Agrobacterium[a]

Species	Acrobacterium Strains				
	A281	542	C58	M2/73	U3
			number		
P. taeda					
Plants inoc.	92	147	50	98	133
Galls 8–12 wks.	24	38	11	8	23
Galls 1 yr.	7	29/2[b]	0	0	6
P. elliotti					
Plants inoc.	48	97	NT[c]	49	48
Galls 8–12 wks.	14	8		6	5
Galls 1 yr.	10/1[b]	8		0	2/1[b]
P. ponderosa					
Plants inoc.	NT	33	49	49	44
Galls 8–12 wks.		3	0	21	5
Galls 1 yr.		0	0	5	4/1[b]
P. lambertiana					
Plants inoc.	97	97	35	112	82
Galls 8–12 wks.	13	12	2	9	10
Galls 1 yr.	8	8	8	25	14
P. radiata					
Plants inoc.	48	98	NT	98	40
Galls 8–12 wks.	36	85		50	34
Galls 1 yr.	11/3	10/2[b]		16/5[b]	12/8[b]
P. sylvestris					
Plants inoc.	NT	31	11	33	NT
Galls 8–12 wks.		3	6	21	
Galls 1 yr.		3	4	20/1[b]	
P. virginiana					
Plants inoc.	NT	82	NT	80	10
Galls 8–12 wks.		1		11	4
Galls 1 yr.		1		7	8
P. jeffreyi					
Plants inoc.	NT	NT	46	NT	NT
Galls 8–12 wks.			2		
Galls 1 yr.			1		
P. eldarica					
Plants inoc.	NT	NT	NT	NT	27
Galls 8–12 wks.					7
Galls 1 yr.					3
P. menziesii					
Plants inoc.	49	49	NT	NT	NT
Galls 8–12 wks.	4	23			
Galls 1 yr.	3	18			
L. decurrens					
Plants inoc.	NT	NT	15	39	NT
Galls 8–12 wks.			2	24	
Galls 1 yr.			2	10/2[b]	

[a] Seedlings were first scored for gall formation 8–12 weeks after inoculation. [b] The number of galls remaining after 1 year which were greater than 2 cm in diameter. [c] Not tested.

the limiting factor to gall formation. Inoculation of new, succulent growth induced by pruning 3- to 4-month-old loblolly pine seedlings gave no gall formation with *Agrobacterium* strains known to produce high rates of gall formation in younger seedlings (A-M Stomp, unpublished data). This sensitivity of gall formation to the age or state of the pine seedlings may explain why earlier workers failed to infect pine.

Early efforts by Smith (23) were unsuccessful in producing galls in *P. sylvestris* inoculated with 'pure cultures of the crown gall organism.' De Cleene and De Ley (8) also showed that pines were not susceptible to *Agrobacterium*. Although Smith does not describe the age or woodiness of the pine plants he used, these plants were in 5-gallon containers, suggesting that they were large and woody. De Cleene and De Ley (8) do not describe the pine plants they used except to say that the plants were less than 1 year old, healthy, and growing. Loblolly pine seedlings at the stage of development inferred from these previous reports would produce very low frequencies of gall formation in our experience.

The heterogeneity of plant genotypes within species also confounds quantitative interpretation of the data. An assumption of the statistical test for homogeneity is that all

Table III. *Chi-Square Test for Homogeneity*

	χ^2	df	Significance Level
Strain			
A281	11.27	3	0.005
542	26.54	6	0.005
C58	38.17	4	0.005
M2/73	153.98	6	0.005
U3	54.97	6	0.005
Species			
P. elliotti	15.48	3	0.005
P. lambertiana	76.01	4	0.005
P. ponderosa	8.42	3	0.05
P. radiata	9.10	3	0.05
P. sylvestris	18.00	2	0.005
P. taeda	37.72	4	0.005
P. virginiana	65.60	2	0.005
Overall	337.87	29	0.005

The variance of 8–12 week scoring data (Table II) was analyzed using the Chi-square test for homogeneity (26) as:

$$\chi^2 = \frac{(n_{i2}^2/n_{i\cdot}) - n_{\cdot 2}^2/n_{\cdot\cdot}}{n_{\cdot 1}n_{\cdot 2}/n_{\cdot\cdot}^2}$$

where:

n_{i2} = number of plants with galls
$n_{i\cdot}$ = number of inoculated plants in the treatment
$n_{\cdot 2}$ = total of plants with galls in all treatments
$n_{\cdot\cdot}$ = total number of inoculated plants in all treatments
$n_{\cdot 1}$ = total number of plants without galls in all treatments

individuals sampled are identical in their response to the particular test, in this case susceptibility to *Agrobacterium*. The seed sources used in these experiments were bulk seed lots, unselected for susceptibility to *Agrobacterium*. Therefore, it is reasonable to hypothesize that the sample is unbiased, but heterogeneous, in susceptibility. Although this lack of homogeneity violates one of the underlying assumptions of the statistical test, if samples of reasonable size, *e.g.* greater than 25 individuals, are taken their means should be distributed normally around the population mean. In our study, the sample size in all but two strain/species inoculations is greater than 25. Therefore, we expect that the frequency of gall formation obtained here would be a reasonable estimator of the mean infection frequency of each specific strain/species inoculation.

Detection of opine synthesis may not always be sufficient evidence of transformation by *Agrobacterium*. Christou *et al.* (6) reported that untransformed cultures of soybean, and to a lesser extent cotton, will synthesize nopaline when grown on medium containing high amounts of arginine. Although the authors state that arginine feeding was necessary for opine production, these data are cited as evidence against the use of opines to confirm transformation. The opine data presented here are from gall tissue, and the opines detected were those predicted for each of the five strains. In addition, opines could not be detected in control extracts from untransformed stem tissue. Therefore, we find it improbable that opine synthesis resulted from plant production of opines, but rather that transfer and expression of T-DNA has occurred.

For genetic engineering purposes, events leading to 'successful' transformation consist of recognition, transfer, insertion, and T-DNA gene expression. In contrast, when 'success' is defined as gall formation, the entire pathological process from recognition through sustained cellular proliferation leading to chronic gall formation is required. Appearance of small galls in all inoculated pine species indicates that transformation events through initial hormone imbalance and cell proliferation are quite similar between gymnosperms and dicotyledonous plants. However, the inhibition of chronic gall establishment suggests that pines are able to overcome infection, either through plant defense mechanisms or by regulation of the phytohormone imbalance produced in transformed tissue.

Screening a variety of pine species showed that chronic gall

Table IV. *Opine Synthesis in Gall Tissue of 11 Pine Species*

Three galls induced by the same strain were harvested from different trees of each species if possible. Galls were harvested from trees taking as little of the stem tissue as possible. High-voltage paper electrophoresis of 80% ethanol extracts of gall tissue was used to separate and identify opines.

Species	Strain				
	A281	542	U3	M2/73	C58
P. taeda	+++[a]	+++	+++	++–[b]	NT[c]
P. elliotti	+++	+++	+++	NT	NT
P. ponderosa	NT	+++	NT	+++	NT
P. radiata	+++	+++	++–	+– –	NT
P. lambertiana	+++	+++	++–	+++	– – –
P. sylvestris	NT	++b[d]	NT	+++	+bb
P. jeffreyi	NT	NT	NT	NT	–bb
P. virginiana	NT	+–b	++b	++b	NT
P. eldarica	NT	NT	++b	NT	NT
P. menziesii	++b	+++	NT	NT	NT
L. decurrens	NT	– –b	NT	+++	– –b

[a] Pluses indicate the presence of the predicted opine in gall extract. [b] Minuses indicate the absence of any opine in the gall extract. [c] Not tested as this species/strain inoculation was not done. [d] Fewer than three galls were available for opine testing.

growth was not equally distributed among species. Eighteen of the 26 galls which attained a diameter greater than 2 cm were from inoculations of *P. radiata.* Although we have produced large galls in several other species, including *P. taeda, P. radiata* is exceptional in our experience for both the frequency of continued gall growth and their size.

P. radiata shows another uncommon characteristic in that it is one of a small number of pines which readily stump sprout. Stump sprouting is the renewed growth of dormant, axillary buds, which is believed to be regulated by auxin and/or cytokinin (25). Exceptional responsiveness of *P. radiata* to the manipulation of these two hormones forms the basis of the highly efficient *in vitro* plant regeneration system in this species (2). These observations suggest to us that chronic gall establishment, stump sprouting, and the ability to manipulate a species *in vitro* with cytokinin and auxin may be related. This hypothesis makes some testable predictions.

One prediction is that a higher frequency of chronic gall growth should be found in pine species which stump sprout. *P. echinata, P. serotina, P. oocarpa,* and *P. eldarica* exhibit stump sprouting and therefore can be tested for *Agrobacterium* susceptibility and sustained gall growth. Although *P. eldarica* was included in our test, this species needs to be reexamined because a very small sample, 27 trees, was inoculated.

It is also possible to test if continued gall growth correlates with the degree of stump sprouting within species. This characteristic is known to vary from tree to tree. Such differences in morphology, a bushy *versus* an apically dominant habit, have given rise to inbred lines or cultivars in a variety of annual plants. Work with soybean (13, 20), squash and pumpkin (22) has shown that gall formation is a function of cultivar. However, the possibility of a correlation between plant morphological habit and gall growth has not been examined. Although inbred lines and cultivars are not available in pines, this correlation can be evaluated by producing clonal populations of bushy and strongly apically dominant pine plants from tissue culture or rooted cuttings. Sustained gall growth in these clonal populations can be compared to test the hypothesis. Such studies may also lead to the identification of individuals highly susceptible to *Agrobacterium.* Hinchee *et al.* (13) state that the successful production of transgenic plants in soybean is critically dependent on using soybean cultivars with high suceptibility to *Agrobacterium* infection. To test the predictions above, we are in the process of evaluating chronic gall growth in more pine species and have begun clonal studies to identify susceptible genotypes in several pine species. Identification of susceptible genotypes will be valuable for further development of transgenic pine plants.

ACKNOWLEDGMENTS

We thank Gayle Dupper and Annie Delfino-Mix, Institute of Forest Genetics, for excellent greenhouse work.

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