CELL BIOLOGY & MOLECULAR GENETICS

Inheritance of *Agrobacterium tumefaciens*-Induced Tumorigenesis of Soybean

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ABSTRACT

Soybean [*Glycine max* (L.) Merr.] genotypes differ widely in response to inoculation with wild-type strains of *Agrobacterium tumefaciens* (Smith and Townsend) Conn. The objective of this work was to study the inheritance of tumorigenesis in soybean so as to gain a better understanding of the *A. tumefaciens*-soybean interaction. A rapid, nondestructive assay for tumorigenesis was developed, which consisted of inoculating hypocotyls of partially etiolated seedlings at multiple wound sites. Four soybean genotypes were screened for tumorigenesis and each had a quantitatively distinct phenotype. 'Peking', a highly tumorigenic genotype, was crossed with two relatively non-tumorigenic genotypes, 'Century' and 'Thomas'. The extent of tumor formation of F_1 plants was intermediate to that of the parents, while the F_2 frequency distributions were continuous and no distinct phenotypic classes were observed. Variance component heritability on a plot basis was 0.30 for Peking × Century and 0.44 for Peking × Thomas, and on an entry-mean basis was 0.62 for Peking × Century and 0.76 for Peking × Thomas. The results indicate that tumorigenesis of soybean is a quantitative trait and heritability estimates were moderate to high. Thus, it should be possible to introgress tumor forming capacity into resistant genotypes. Such an approach may help alleviate genotype restrictions to genetic transformation.

MOST STUDIES of the *Agrobacterium tumefaciens*-plant interaction have focused primarily on bacterial molecular mechanisms which result in the transfer, integration, and expression of transfer DNA (T-DNA) in the host (for recent reviews see Charles et al., 1992, Zambryski, 1993). In contrast, study of host effects on infection of plants with *A. tumefaciens* has been essentially limited to documentation of variability among plants of different genera, species, and genotypes (e.g. De Cleene and De Ley, 1976; Knauf et al., 1982; Owens and Cress, 1985), and to investigations of inheritance in grapevine, *Vitis* spp., (Lowe and Krul, 1991; Szegedi and Kozma, 1984) and pea, *Pisum sativum* (L.) (Robbs et al., 1991). Soybean was initially considered not to be a host for *A. tumefaciens* (De Cleene and De Ley, 1976). Subsequently, it was shown that tumors form on soybean in response to infection with *A. tumefaciens*, but not to the extent observed in other dicotyledons such as tobacco (*Nicotiana tabacum* L.) (Pedersen et al., 1983; Wyndaele et al., 1985; Hawes and Pueppke, 1987). Owens and Cress (1985), Byrne et al. (1987), and Delzer et al. (1990) documented genotypic differences in tumorigenic response, and Peking was consistently the most responsive genotype tested. Byrne et al. (1987) and Delzer et al. (1990) documented a soybean genotype × *Agrobacterium* strain interaction.

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Variability for tumorigenesis among genotypes of soybean permits a genetic analysis. Our long-term objective is to examine the potential for reducing genotypic restrictions for *Agrobacterium*-mediated transformation of soybean through genetic modification of the host response. Towards this end, the objectives of this study were (i) to assess the response of diverse soybean genotypes to infection with wild-type strains of *A. tumefaciens* with an assay suitable for genetic studies, and (ii) to estimate the heritability of tumorigenesis.

MATERIALS AND METHODS

Screening of Soybean Genotypes and *Agrobacterium* Strains

Four soybean genotypes were selected for study: Peking for its highly tumorigenic response, Century for its adaptation to the northern USA (Maturity Group II), Thomas for its adaptation to the southern USA. (Maturity Group VII), and 'Masshokutou 502' (PI 417138) for its high regeneration capacity via somatic embryogenesis (Bailey et al., 1993; Komatsuda and Ohyama, 1988). Seeds of PI 417138, Peking, and Century were obtained from the germplasm collection at Urbana, IL. Seeds of Thomas were available from the second author.

Agrobacterium tumefaciens strains A281 (*L,L*-succinamopine), C58 (nopaline), and A6 (octopine) were chosen as commonly studied representatives of three major opine groups. These strains were provided by E.W. Nester, Univ. of Washington, Seattle, WA. Strain NTI (C58 cured of Ti-plasmid) was used as a negative control and was provided by G. Moore, Univ. of Florida, Gainesville, FL.

Bacterial inoculum was prepared by growing single colony isolates overnight at 28 °C in LB broth (Maniatis et al., 1989). A 100-µL aliquot of log-phase culture was spread on 30 mL of solidified LB medium and incubated for 2 d at 28 °C. Soybean seeds were planted at a density of four per 6.35-cm pot in a 1:1 mixture of sand and Hyponex potting mix (Hyponex Corp., Maryville, OH). Seedlings were maintained for 1 wk in flats at 25 ± 2 °C, with a photon flux density of 50 µmol m^{-2} s^{-1} and a 23-h photoperiod. These conditions resulted in seedlings with elongated hypocotyls that were suitable for multiple inoculation sites. Five vertical punctures of 0.3 cm in length and spaced at 0.5- to 1.0-cm intervals were made on each hypocotyl by inserting a no. 11 scalpel blade completely through the stem. A 2-d-old mucilage of bacteria was immediately rubbed into one side of the wound site with a spatula until bacteria were observed to completely fill the incision. Inoculated plants were transferred to a growth chamber (24 ± 2 °C, 75% relative humidity, photon flux density of 150 µmol m^{-2} s^{-1}, and a 23-h photoperiod). After 3 d, plants were thinned to two per pot. Plants were watered daily by subirrigation and pots were fertilized weekly with 20 mL of a 5 g L^{-1} nutrient solution (Peters 20-20-20 Soluble Plant Food, Grace-Sierra Horticultural Products Co., Milipitas, CA).

Abbreviations: ANOVA, analysis of variance; LSD, least significant difference; LB, Luria-Bertani medium; T-DNA, transfer DNA.

A total of 20 plants per bacterial strain/soybean genotype combination was evaluated. At least two plants of each genotype were wounded without inoculation or inoculated with strain NTI. Plants were evaluated for tumorigenesis after 21 d. The response evaluated was a composite measure of tumor size, the frequency of tumors per wound site, and the frequency of wound sites with tumors. This response is referred to as *tumor index* and was derived as follows. The diameter of each tumor or collection of confluent tumors at a wound site was measured to the nearest millimeter, and these values were summed to derive a total tumor diameter per wound site. Tumor index of a plant is the mean of the total tumor diameters of the five wound sites. Analysis of variance was conducted with SAS PROC ANOVA (SAS, 1988). Multiple comparisons among soybean genotypes and bacterial strains were made with Fisher's Protected LSD.

Inheritance of Tumorigenesis

Peking was crossed as the maternal parent with Century and Thomas. Five F_1 plants of Peking × Thomas and 10 of Peking × Century were inoculated with strain A281 and evaluated for tumor index. Seven plants each of Peking, Century, and Thomas were also inoculated with A281. As negative controls, three plants each of Peking, Century, and Thomas were inoculated with strain EHA101. EHA101 is a disarmed derivative of A281 and was supplied by E. Hood (Hood et al., 1987). Two F_2 populations consisting of 143 plants of Peking × Century and 127 plants of Peking × Thomas were inoculated with strain A281 and evaluated for tumor index. Fifty plants each of Peking, Century, and Thomas were inoculated with strain A281 and assayed concurrently with the F_2 generation. The frequency distribution of tumor index was continuous in the F_2 populations from both crosses, so heritability estimates were calculated. Forty-five $F_{2:3}$ families were randomly selected from each F_2 population. Sixteen plants each of Peking, Century, and Thomas and of each $F_{2:3}$ family were inoculated with strain A281 and evaluated in four replicates (plots) of four plants each. A plot consisted of two pots with two plants each. At least five plants each of Peking, Century, and Thomas were inoculated with strain EHA101 as negative controls. Plots were randomized in a growth chamber after inoculation. Heritability was computed on a plot basis and on an entry mean basis from variance components obtained using PROC VARCOMP of SAS (SAS, 1988). The formulas were as follows:

Heritability on a plot basis,

$$h^2 = \frac{\sigma_G^2}{\sigma_\delta^2/4 + \sigma_e^2 + \sigma_G^2}$$

Heritability on an entry mean basis,

$$h^2 = \frac{\sigma_G^2}{\sigma_\delta^2/16 + \sigma_e^2/4 + \sigma_G^2}$$

where h^2 = heritability, σ_δ^2 = plant to plant variance, σ_e^2 = replicate × genotype variance, σ_G^2 = genetic variance. Standard errors of heritability estimates were calculated as described by Hallauer and Miranda (1988).

RESULTS AND DISCUSSION

Screening of Soybean Genotypes and *Agrobacterium* Strains

Various wounding protocols have been used to evaluate the tumorigenic response of soybean to wild-type strains of *A. tumefaciens* and have ranged in severity from whole plants stabbed with needles (Owens and Cress, 1985) to decapitated seedlings (Byrne et al., 1987). The assay described here was designed for use in genetic studies to be reproducible, rapid, nondestructive, and amenable to quantitation. In preliminary experiments we determined that single wound sites on a plant would be inadequate to score unique individuals of an F_2 population since tumors were occasionally absent at single wound sites of Peking, the most responsive genotype. Therefore, replicate inoculation sites per plant were used to obtain an average score for each individual (Fig. 1). Use of week-old, partially etiolated seedlings and maintenance of plants under a 23-h photoperiod prevented variation

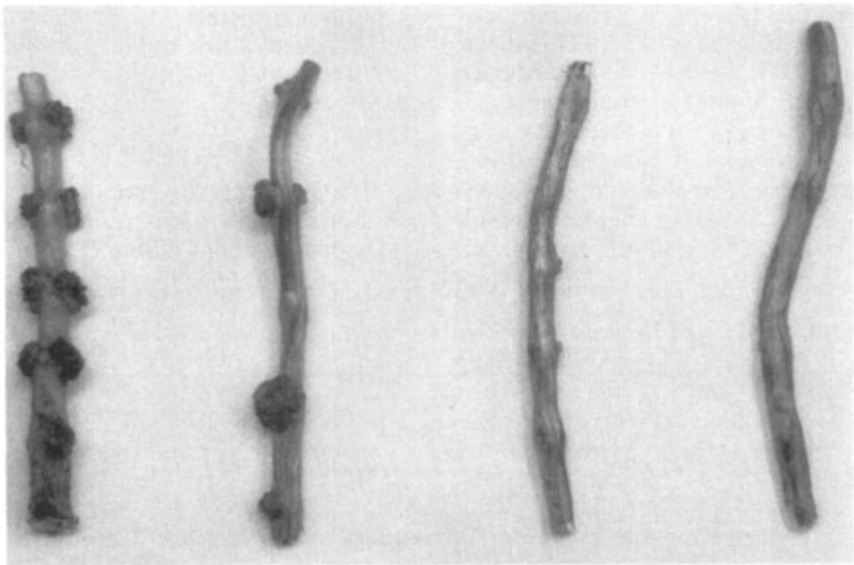

Fig 1. Tumors on hypocotyls of four soybean genotypes 21 d after inoculation with strain A281 of *A. tumefaciens*. Genotypes, from left to right, are Peking, PI 417138, Thomas, and Century.

Table 1. Mean tumor index of four soybean genotypes 21 d after inoculation with three strains of *A. tumefaciens*.

Soybean genotype	*Agrobacterium* strain A281	C58	A6	Mean†
	Tumor index‡			
Peking	5.67§	5.47	4.93	5.34
PI 417138	4.04	3.83	3.63	3.83
Thomas	0.79	0.82	1.23	0.95
Century	0.15	0.25	0.20	0.20
Mean†	2.66	2.59	2.50	

† Protected LSD (0.05) for genotype means = 0.48 and for strain means = 0.42.
‡ Tumor index = per plant mean of summed tumor diameters at 5 wound sites (see also text).
§ Values for each genotype/strain combination are means of 20 plants.

related to the timing of flowering and senescence among genotypes of different maturity groups.

At least some plants of each genotype formed tumors after inoculation with *A. tumefaciens* strains A281, C58, and A6. No tumors were observed on wounded, uninoculated plants or on plants inoculated with the disarmed strain NTI. Tumor index was different among the four genotypes studied ($p < 0.001$), and each genotype had a tumor index different from that of all other genotypes (Fig. 1, Table 1). As reported previously, Peking was the most tumorigenic genotype (Owens and Cress, 1985; Byrne et al., 1987; Delzer et al., 1990). A wide diversity of soybean germplasm has now been tested for response to several strains of wild-type *A. tumefaciens* by many investigators under a range of conditions (Byrne et al., 1987; Delzer et al., 1990; Hinchee et al., 1988; McKenzie and Cress, 1992; Owens and Cress, 1985; Pedersen et al., 1983; this study). Taken together, these studies reveal the general pattern that most, if not all, soybean genotypes form tumors in response to inoculation with *A. tumefaciens*, but the magnitude of response varies considerably.

Strains of *A. tumefaciens* were not different for tumor index ($p = 0.99$) and there was no significant strain × soybean genotype interaction ($p = 0.19$). These results differ from previous studies (Byrne et al., 1987; Delzer et al., 1990; Hood et al., 1987; Jin et al., 1987). Apparently, the relative magnitude of tumorigenesis conferred by different bacterial strains on a given soybean genotype is dependent upon the assay employed and/or the criteria used to estimate tumorigenesis. For example, we found no differences in tumor index of Peking after inoculation of intact plants with strains C58 and A281, whereas

Table 2. Mean (± SE) tumor index of three parents and two F_1 populations 21 d after inoculation with strain A281 of *A. tumefaciens*.

Parent or cross	*N*	Mean ± SE
		Tumor index†
Peking	7	2.49 ± 0.16
Century	7	0.00 ± 0.00
Thomas	7	0.26 ± 0.10
Peking × Century	10	0.72 ± 0.10
Peking × Thomas	5	1.68 ± 0.25

† Tumor index = per plant mean of summed tumor diameters at five wound sites (see also text).

Delzer et al., (1990) observed that C58 was more tumorigenic on cotyledon explants of Peking than was A281. One explanation for this disparity is that diverse bacterial strains require different conditions for optimal tumorigenesis and that such conditions vary among tissue types of the same genotype. Alternatively, differences in the criteria used to estimate tumorigenesis may account for the failure of strains to rank similarly in different studies. In this study, the score for tumorigenesis (i.e., tumor index) reflects tumor size, the number of tumors per wound site, and the number of wound sites with tumors. Delzer et al. (1990) used a visual scoring scale which was largely a measure of the number of tumors per wound site. It is possible that C58 produced more numerous, but smaller, tumors than A281 in our assay such that the total gall diameter at a wound site was equal between the strains. We did not count individual tumors within a wound site since they were often confluent.

Plants of Peking, Century, and Thomas, used as checks, were inoculated with EHA101 or A281 in three separate trials concurrent with screening of plants from the F_1, F_2, and F_3 generations of Peking × Century and Peking × Thomas. No tumors were observed after inoculation with EHA101. For a given parental genotype, the tumor index following inoculation with A281 varied widely among the separate trials, but the ranking of genotypes remained the same (compare Tables 1-3, Fig. 2).The considerable within-genotype variability among trials suggests that tumor index is strongly affected by environmental conditions.

Inheritance of Tumorigenesis

The F_1 means for tumor index following inoculation with strain A281 were intermediate to those of the parents in both populations (Table 2). The F_2 frequency distribu-

Table 3. Plot means (± SE) and plot ranges for tumor index of parents and $F_{2:3}$ lines, and broad-sense heritabilities for two $F_{2:3}$ soybean populations.

Parent or $F_{2:3}$ population	Mean ± SE	Range	h^2 ± SE Plot	h^2 ± SE Entry mean
	Tumor index†			
Peking	7.46 ± 0.33	5.67-8.90		
Century	0.23 ± 0.04	0.00-0.40		
Thomas	0.98 ± 0.17	0.35-1.65		
Peking × Century	2.55 ± 0.02	0.20-6.50	0.30 ± 0.09	0.62 ± 0.20
Peking × Thomas	3.54 ± 0.03	0.15-7.65	0.44 ± 0.11	0.76 ± 0.20

† Tumor index = per plant mean of summed tumor diameters at five wound sites (see also text).

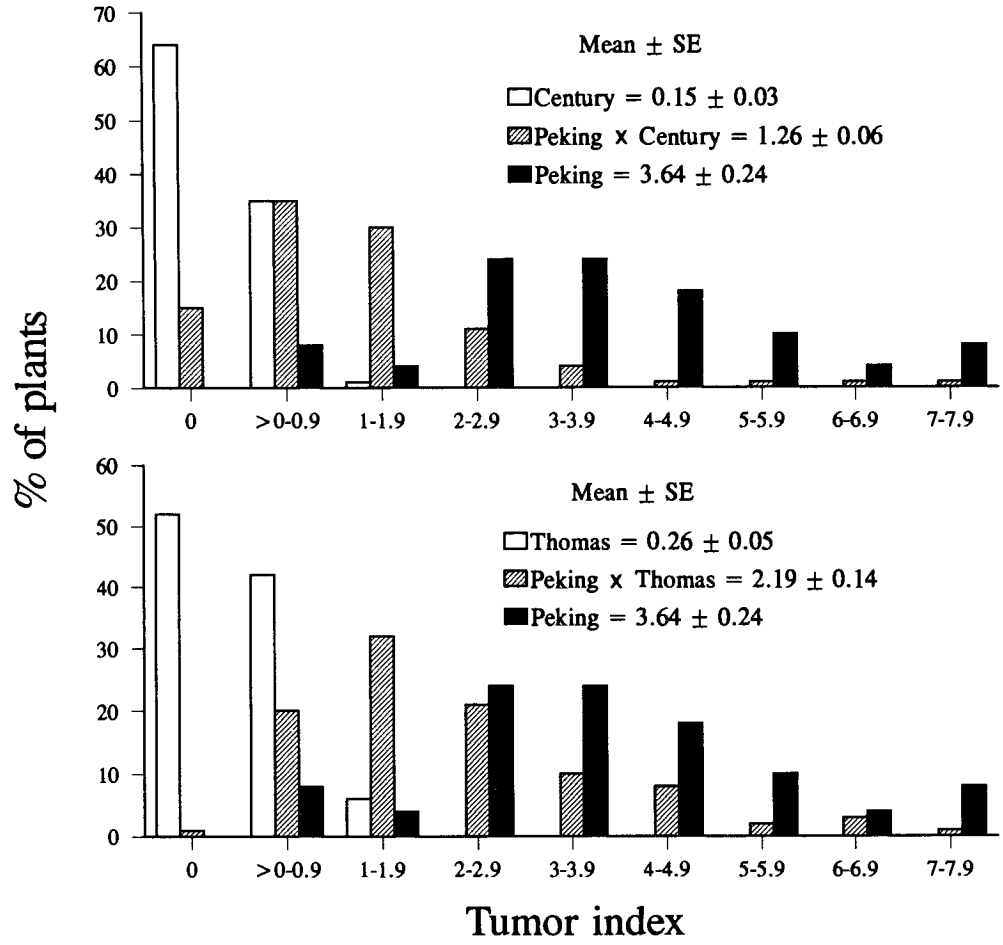

Fig 2. Means (±SE) and frequency distributions of tumor index for three parents and two F_2 populations 21 d after inoculation with strain A281 of *Agrobacterium tumefaciens*.

tion was continuous for both populations and distinct phenotypic classes were not apparent (Fig. 2). Tumor index for Peking was highly variable and a few plants of Peking were within the range of Century and Thomas plants. The mean tumor index of the Peking × Century F_2 population was lower than that of Peking × Thomas. Transgressive segregation for high tumor index did not occur, since no F_2 individuals from either population exceeded that of the highest Peking individual (Fig. 2).

The F_2 frequency distributions indicate that tumor index is a quantitatively inherited trait and that the genetic control of tumorigenesis is somewhat different for these two populations. Century may have genes for resistance not found in Thomas, since parent means for tumor index were consistently lower for Century (Tables 1-3, Fig. 2). Broad-sense heritability estimates on a plot basis (0.30 and 0.44) and on an entry-mean basis (0.62 and 0.76) were moderate to high for both $F_{2:3}$ populations (Table 3). Therefore, selection for tumor index in early segregating generations should be possible. The lower mean and heritability estimates of the Peking × Century $F_{2:3}$ population relative to that of the Peking × Thomas population support the hypothesis that Century has a resistance gene(s) not found in Thomas (Table 3).

The quantitative mode of inheritance for tumorigenesis in these soybean genotypes is similar to that of pea. Robbs et al. (1991) evaluated F_1 and F_2 generations from crosses of resistant × susceptible pea genotypes. The F_1 progeny had intermediate tumor weights to those of the parents, and tumor weights among individuals of an F_2 population were widely distributed with a lack of distinct phenotypic classes. It was concluded that tumorigenesis in the pea genotypes studied was conditioned by multiple host genes.

Implications for Soybean Transformation

Genotype differences for tumorigenesis are not necessarily a reflection of the frequency of integration or level of T-DNA expression in the host (Facciotti et al., 1985; Klee et al., 1987; Szegedi et al., 1989). Instead, genotype effects may be manifested at later stages of tumor development through modulation of oncogene expression, functional complementation of oncogenes by endogeneous plant hormones, and/or sensitivity to oncogene-directed phytohormone production (Lowe and Krul, 1991; Robbs et al., 1991; van Wordragen et al., 1992). If such oncogene-dependent mechanisms are the primary

determinants of genotype specificity in soybean, then disarmed strains containing scoreable markers such as β-glucuronidase are expected to more accurately predict the potential of genotypes to integrate and express genes of agronomic importance (van Wordragen, 1992; Higgins et al., 1992).

In this regard, no unequivocal relationship between tumorigenesis and the efficiency of T-DNA transfer and/or expression has been established for soybean. Facciotti et al. (1985) inoculated 'Forrest' soybean with a cointegrate vector containing oncogenes of pTiA6 and observed no tumor formation, but when wounded tissue was excised and placed on growth-regulator-free medium the explants proliferated callus that tested positive for octopine. Thus, transformation occurred in the absence of tumor formation. Similarly, Owens and Cress (1985) found that when cotyledons of 'Biloxi' were inoculated with strain A348 and incubated on water agar, no tumors were formed. However, culture of inoculated cotyledons on medium with (2,4-dichlorophenoxy) acetic acid promoted the growth of calli that later exhibited growth regulator-autonomous growth, and octopine production. Thus, the failure of tumor formation on water agar did not reflect a failure of T-DNA integration. Nonetheless, Hinchee et al. (1988) and Parrott et al. (1989) found that Peking tissue was more responsive to transformation with a neomycin phosphotransferase gene than tissue of most other genotypes, suggesting that the highly tumorigenic response of Peking is at least partially a result of increased T-DNA expression rather than a soybean genotype-specific response to oncogenes. It is noteworthy that the highly tumorigenic response of Peking that has been documented in whole plants is also observed in isolated tissues which are amenable to regeneration (Hinchee et al., 1988; Parrott et al., 1989; Delzer et al., 1990).

The distinct response phenotypes of the soybean parents identified here will be of use to dissect the ultimate causes of genotype effects on tumorigenesis. Hawes et al. (1989) proposed that the biochemical and molecular causes of genotypic variability for susceptibility in pea could be examined by analyzing resistant and susceptible genotypes with the quantitative assays that are available for several stages of the infection process. Conceivably, once the factor(s) which limit stable T-DNA expression in resistant genotypes are identified, appropriate mutants of *A. tumefaciens* (e.g., Ankenbauer et al., 1991; Mozo and Hooykaas, 1992) or alterations of infection conditions (Godwin et al., 1991; Turk et al., 1991; Chang and Chan, 1992) could be employed to overcome genetic or physiological host limitations. From the $F_{2:3}$ lines generated in this study it will also be possible to generate soybean lines homozygous for genes that confer various levels of tumorigenesis. Testing of these lines for T-DNA expression with scoreable markers will provide insight into the extent that tumorigenesis and T-DNA transfer are correlated. Since heritability estimates were moderate to high it should also be possible to introgress tumorigenic capacity into resistant genotypes of agronomic importance. Such an approach may help alleviate genotype restrictions to genetic transformation.

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