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Genetics

Inheritance and expression of foreign genes in transgenic soybean plants

(*Glycine max*/particle acceleration/genetics/transformation)

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ABSTRACT **DNA-coated gold particles were introduced into meristems of immature soybean seeds using electric discharge particle acceleration to produce transgenic fertile soybean plants. The lineages of integrated foreign DNA in two independently transformed plants were followed in the first (R_1) and second (R_2) generation of self-pollinated progeny. One plant (4615) was transformed with the *Escherichia coli* genes for β-glucuronidase and neomycin phosphotransferase II; the other (3993) was transformed only with the gene for β-glucuronidase. Segregation ratios for the introduced gene(s) were approximately 3:1 for plant 4615 and 1:1 for plant 3993 in the R_1 generation. DNA analysis showed 100% concordance between presence of the foreign gene sequences and enzyme activity. Moreover, all copies of the foreign genes are inherited as a unit in each plant. Plant 3993 segregated in a 1:1 ratio in the R_2 generation. R_1 plants derived from plant 4615, which expressed both genes, gave either 100% or 3:1 expression of both genes in the R_2 generation, demonstrating recovery of both homozygous and heterozygous R_1 plants. Our results show that foreign DNA introduced into soybean plants using electric discharge particle acceleration can be inherited in a Mendelian manner. Results also demonstrate cotransformation of tandem markers and show that both markers are inherited as closely linked genes in subsequent generations. These results indicate that whole plants can be derived from single transformed cells by a *de novo* organogenic pathway.**

Technologies are being developed to transform the more important agronomic crops since *Agrobacterium*- or protoplast-based transformation methods are currently unsuccessful or inefficient with these plants. We described (1) how electric discharge particle acceleration can stably transform a major commercial cultivar of soybean. This method bypasses traditional tissue culture operations involving protoplast and callus cultures and may thereby reduce varietal restrictions and problems of somaclonal variation frequently associated with regeneration of plants from tissue culture. To evaluate and characterize the stability of introduced genes in transgenic plants obtained by this method, the expression and organization of two foreign marker genes were analyzed in sexually derived progeny over two generations.

MATERIALS AND METHODS

Plasmids. pAcX1100P and pAcX1021P were constructed by inserting the chimeric β-glucuronidase (GUS) (2) and neomycin phosphotransferase II (NPT II) (3) genes from pCMC1100 (1) and pCMC1021 (4), respectively, into the unique *Xho* I site of pAc3 (5). The insertions in these plasmids are oriented such that the direction of transcription is the same as that of the *Ac* element (6). Plasmids were prepared as described (1).

Preparation of DNA and Particle Delivery. DNA-coated gold particles were prepared by mixing equal molar amounts of pAcX1100P, pAcX1021P, and pAc3 with 1.5-μm gold beads at a rate of 1 mg of beads to 0.01–0.1 μg of DNA as described (1, 7). Particle delivery into the target tissue was performed as described (7).

Explant Preparation and Plant Regeneration. Embryonic axes used as target tissue were isolated from immature seeds of *Glycine max* (cv. Williams) plants grown in the greenhouse. The axes were excised and arranged on water/agar plates (1). After particle delivery, plant regeneration was accomplished by organogenesis on a cytokinin-enriched medium (1).

Progeny Analysis. Primary regenerants (R_0) refer to transgenic plants recovered from the explant originally subjected to particle acceleration, R_1 and R_2 plants (first and second generation) are seed-derived plants obtained from self-pollination of R_0 and R_1 plants, respectively. R_1 and R_2 seeds were germinated in the greenhouse at 26°C day and 21°C night temperatures on a 16-hr photoperiod. Leaf punches were taken from 10-day-old seedlings and assayed for GUS (8) and NPT II activity (9). For determining GUS activity in pollen, flowers were harvested and anthers were removed. Pollen grains were separated from the anthers and incubated in the GUS assay buffer for 4 hr at 37°C. Stained pollen grains were observed microscopically and counted.

Pollen Viability Tests. Pollen isolated from flowers of transgenic plants was stained with acetocarmine (10) or germinated on the pollen germination medium of Pfahler containing calcium (0.03%) and boric acid (0.01%) (11).

Southern Blot Analyses. DNA was isolated from 1 g of fresh leaf tissue from greenhouse grown plants using the method of Dellaporta *et al.* (12). Conditions for restriction endonuclease digestion of plant DNA, transfer to nylon membranes, preparation of hybridization probes, membrane hybridization, washing, and autoradiography were described (1, 4, 7). Membranes were prepared for rehybridization with additional probes by three successive washes in 5 mM Tris·HCl, pH 7.5/2 mM EDTA/0.1% SDS at 80°C. Washed filters were then either immediately prehybridized to begin a second round of hybridization or stored in 0.18 M NaCl/10 mM sodium phosphate, pH 7.5/1 mM EDTA/1.0% SDS, at 5°C.

RESULTS AND DISCUSSION

We have shown that electric discharge particle acceleration can be used for the delivery of foreign DNA, its stable integration in plant cells (7), and the recovery of transgenic soybean plants (1). The technique allows the recovery of transformed plants from tissues and lines that cannot be

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Abbreviations: R_0, primary regenerant; R_1 and R_2, first and second generation of self-pollinated progeny; GUS, β-glucuronidase; NPT II, neomycin phosphotransferase II.

transformed using conventional methods, such as *Agrobacterium* or electroporation. In addition, it bypasses the need for time-consuming and labor-intensive tissue culture manipulations involving protoplast and callus cultures. The method involves introduction of DNA into soybean meristems and propagation of shoots from these meristems on high cytokinin-containing medium. The shoots were rooted on hormone-free medium or grafted on soybean seedlings if rooting difficulties were encountered.

Expression of the marker genes in R_0 plants was determined by assaying leaf discs for GUS and NPT II activity. The nature of the histochemical GUS assay facilitates such analysis by permitting assays of hundreds of plant samples in a short time. R_0 plants obtained by this method can be divided into two groups based on the pattern of foreign gene expression. Most R_0 plants gave both enzyme-positive and enzyme-negative samples when different nodes of the plant were analyzed, suggesting that they were sectored into transformed and nontransformed regions; however, other plants exhibited enzyme activity in all leaf samples.

To characterize integration of the transforming DNA into the soybean genome and its inheritance, we analyzed R_1 and R_2 plants derived from self-pollinated seed of primary regenerants (R_0). Two R_0 plants that expressed foreign genes in all of their leaves were further studied. Both plants were recovered from experiments in which the GUS and NPT II genes were introduced on separate plasmids. One of these plants (4615) expressed both GUS and NPT II activity, demonstrating cotransformation with two independent markers. The other plant (3993) expressed only GUS activity.

Fig. 1 indicates the distribution of seeds inheriting GUS activity versus those not inheriting the transformed phenotype for plant 4615. Transformed seeds were randomly distributed among the pods. Frequently, both transformed and nontransformed seeds were identified in the same pod, consistent with independent fertilization of ovules in a pod by individual germinating pollen grains. No correlation could be established between the position, or number of seeds per pod, and transformation events. Additionally, transformed seeds are distributed throughout the plant, consistent with the observation of GUS activity in all parts of the plant.

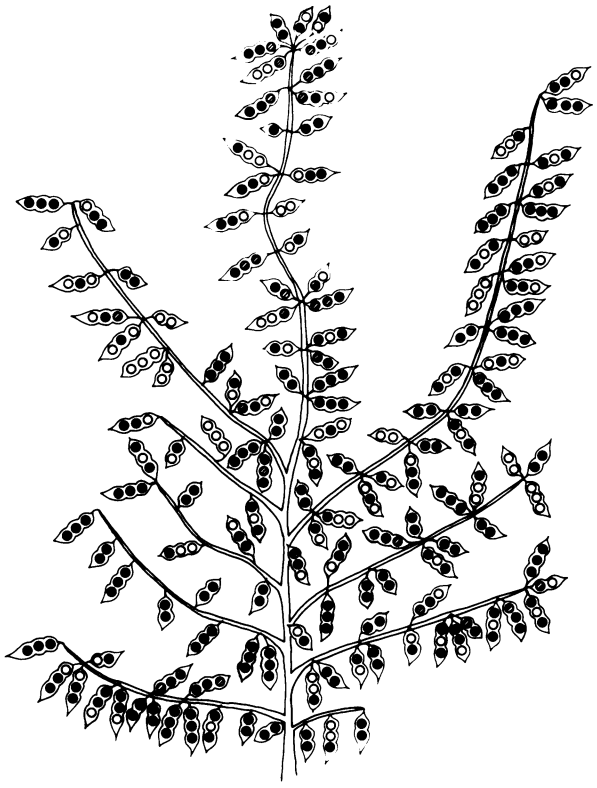

FIG. 1. Distribution of transformed R_1 seeds in primary transgenic soybean plant 4615. Seeds were collected and their location on the plant was recorded. The seeds were then germinated and 3-mm punches of the seedling leaf tissue were assayed for GUS as shown in Fig. 3. Solid circles, GUS^+ seedlings; open circles; GUS^- seedlings; and hatched circles, aborted seeds and seeds that did not germinate.

Table 1. Segregation and expression of the GUS gene in R_2 progeny of plant 4615

Plant	GUS^+ seeds, no.	GUS^- seeds, no.	χ^2
6-2*	22	8	0.040
11-2*	28	5	1.710
14-3†	32	0	—
32-1*	24	9	0.090
49-2†	33	0	—
65-3*	25	8	0.010
76-2†	33	0	—
121-2†	85	0	—

*Probable heterozygous plants.
†Probable homozygous plants.

A segregation of 2.3:1::GUS^+:GUS^- was observed in the R_1 generation of the 4615 lineage. χ^2 analysis indicated that this ratio is best approximated by a 3:1 segregation model. The R_2 generation comprised two groups of segregants: certain families expressed both genes in 100% of the progeny, whereas others showed a segregation of 3:1 (Table 1). These results are consistent with Mendelian segregation of a single dominant gene. Moreover, the observation of nearly normal segregation ratios in R_1 suggests that plant 4615 was uniformly heterozygous for the transformed trait. Segregation of NPT II activity was completely concordant with GUS activity in both R_1 and R_2 generations. Thus, both markers behave as typical dominant genes and are tightly linked (map distance, <1 centimorgan) in the 4615 lineage.

GUS staining of pollen from these two groups of R_1 plants confirms the homozygous and heterozygous genotypes indicated by the pattern of segregation in R_2 (data not shown). Pollen derived from flowers belonging to families 14-3, 49-2, and 121-2 (Table 1) showed expression of the GUS gene in 100% of the pollen grains. In families of plants segregating in a 3:1 ratio in R_2 (Fig. 2), pollen also segregated into expressing and nonexpressing populations.

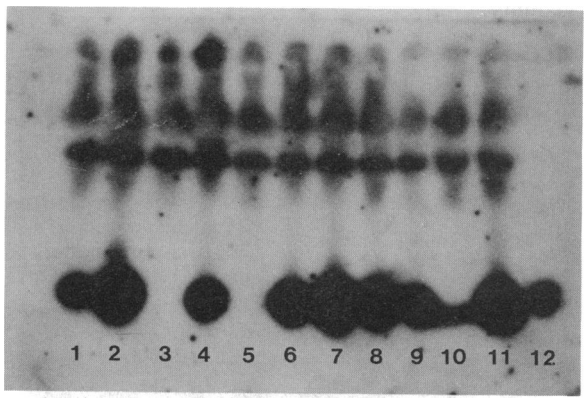

FIG. 2. NPT II activity in R_2 progeny of transgenic soybean plants. A 3:1 segregation of the inherited gene is shown. Lanes: 1, 2, 4, 6, 7, 8, 9, and 11, leaf tissue of NPT II-positive plants; 3, 5, and 10, leaf tissue from nonexpressing plants; 12, positive control (purified bacterial NPT II enzyme). Negative controls from soybean leaves of nontransformed plants did not show NPT II activity (data not shown).

Table 2. Segregation and expression of the GUS gene in R_2 progeny of plant 3993

Plant	GUS^+ seeds, no.	GUS^- seeds, no.	χ^2
10-1	22	27	0.735
14-1	26	21	0.765
59-2	21	23	0.090
59-3	20	25	0.350
119-2	20	24	0.360
184-1	19	17	0.120

R_1 segregation was 1:1.

In the R_1 of lineage 3993, GUS activity segregated at a 1:1 ratio (Table 2). This result can be interpreted as indicating that plant 3993 was chimeric, comprising both transformed and nontransformed cells, the latter accounting for a higher proportion of nontransformed progeny in the R_1 generation than expected. However, segregation of GUS activity in the R_2 generation continued to show a 1:1 pattern (Fig. 3 and Table 2). These results suggest that the aberrant segregation ratio is intrinsic to the 3993 genotype and that this R_0 plant is also heterozygous for the transformed trait. Pollen from GUS-positive plants of the 3993-R_1 generation that was stained for GUS activity showed very few (<1%) GUS-positive grains. Acetocarmine staining indicated that only 50% of all pollen grains were viable. Pollen germination tests confirmed that 50% of the pollen was nonviable. Moreover, *in situ* GUS staining of pollen after germination indicated that pollen grains expressing GUS were nonviable. Nonviable pollen grains that did not exhibit detectable GUS activity were also observed, but none of the viable pollen showed GUS activity staining. Thus the 1:1 segregation ratio observed for the 3993 lineage likely reflects a failure to pass the transformed phenotype through pollen. The recovery of plants that are homozygous for the transformed genotype in the 4615 lineage (Table 1) and the observation of GUS activity in pollen from these plants demonstrates that pollen infertility is not a necessary consequence of GUS activity, though effects of subtle differences in GUS expression in the two lineages cannot be ruled out. Alternatively, integration of foreign DNA into the genome in the 3993 lineage may have produced an insertion mutation in an essential gene. Plants in the 3993 lineage produce normal quantities of seed so it is unlikely that these plants carry a recessive embryo-lethal mutation. It is more probable that these plants carry a recessive mutation in an essential gene for pollen formation or viability.

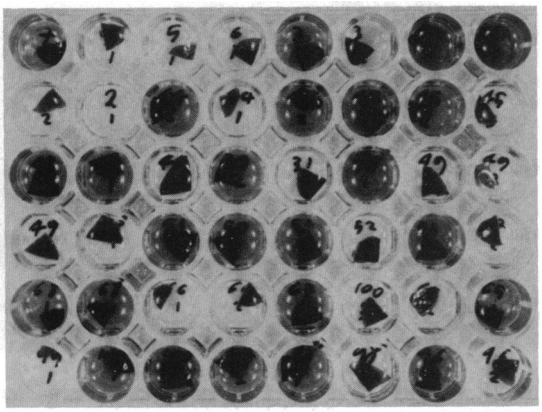

FIG. 3. Expression of GUS activity in R_2 progeny of transgenic soybean plants derived from plant 3993. A 1:1 segregation is shown. Segregations of 3:1 as well as homozygous situations were observed in transgenic plants derived from independent R_1 transformants of the 4615 lineage (Table 1).

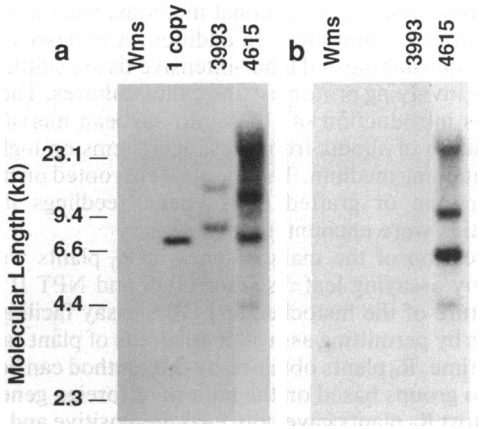

FIG. 4. Southern analysis of transgenic R_0 soybean plants. DNA samples (5 μg) were digested with *Pvu* I and the resulting fragments were resolved by electrophoresis and transferred to a nylon membrane. (*a*) Membrane was hybridized with a ^{32}P-labeled RNA probe corresponding to the minus strand of the *gus* coding region. (*b*) The GUS probe was removed and the membrane was rehybridized with a ^{32}P-labeled RNA probe corresponding to the minus strand of the NPT II coding region. Hybridizing fragments were detected by autoradiography for ≈17 hr in each case. Lanes Wms contain DNA from a nontransformed soybean (cv. Williams) plant. Lane 1 copy was constructed by adding GUS plasmid DNA to Williams control DNA in an amount approximately equal to one plasmid copy per soybean genome, based on a genome size of 6×10^9 nucleotide pairs (13). Lanes 3993 and 4615 contain DNA samples from the corresponding R_0 plants. The positions and lengths, in kilobase pairs, of the molecular size markers are indicated.

Southern blot analysis reveals that plant 3993 contains two copies of the chimeric GUS gene, and plant 4615 has several copies (Fig. 4*a*). Additional mapping (data not shown) indicates that plant 3993 contains one gene that is interrupted within its promoter by recombination with nonplasmid DNA. The recombination breakpoint is located in a region ≈150 base pairs upstream of the start of transcription. Deletion analyses indicate that disruption of the cauliflower mosaic virus 35S promoter in this region inactivates promoter function (14). Therefore, it is likely that plant 3993 contains a single active GUS gene. Mapping of the GUS genes in plant 4615 is complicated by the number of genes present but is consistent with the occurrence of one to three copies present as a direct tandem repeat and several additional copies or partial copies present as nontandem insertions.

Hybridization of the blots with an NPT II probe (Fig. 4*b*) indicates that plant 4615 also contains multiple copies of the NPT II gene whereas plant 3993 does not contain hybridizing fragments. These results are consistent with results from enzyme assays and further show that plant 3993 does not contain silent NPT II genes. Analysis of digests with enzymes that do not cleave the input plasmids (e.g., *Bgl* II, *Sac* I, and *Spe* I; data not shown) revealed two GUS fragments in plant 3993 and multiple GUS and NPT II fragments in plant 4615, indicating that separate copies of the marker genes are interspersed with nonplasmid DNA in these plants.

Our use of plasmids that include marked and unmodified *Ac* elements was designed to test the feasibility of using plant transposable elements as transformation vectors analogous to the *P*-element vectors widely used in *Drosophila* systems (15). At the current level of resolution, neither of the Southern patterns clearly indicates integration of the transforming DNA as an intact, marked *Ac* element. To the contrary, the Southern pattern observed for plant 3993 demonstrates that neither of the integration events in this lineage includes both of the *Ac* borders. The situation in plant 4615 is less clear cut, due to the complexity of the Southern pattern; but again the

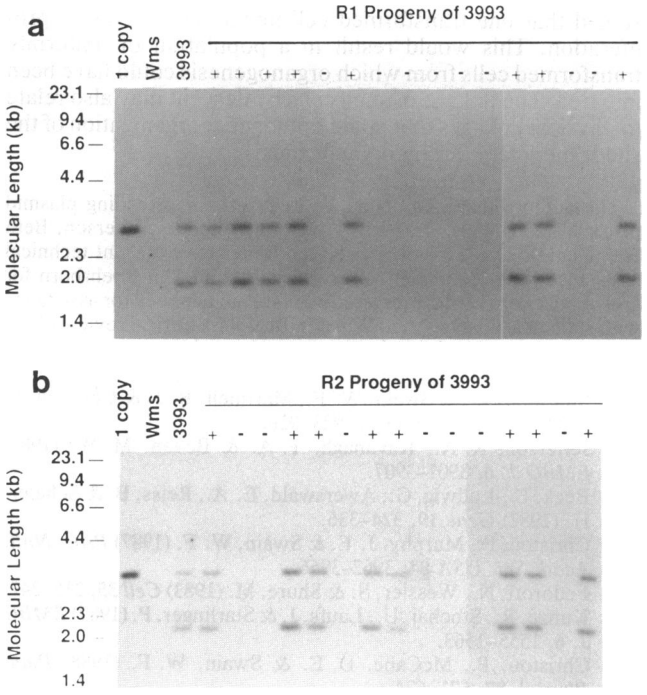

FIG. 5. Southern analysis of R_1 and R_2 progeny of plant 3993. Southern blots were made as described in Fig. 4, except that digestion was with *Eco*RI. Lanes 3993 contain DNA from the parental R_0 plant for comparison. Lanes + or − contain DNA samples from self-pollinated R_1 (*a*) or R_2 (*b*) progeny; the symbols indicate the presence (+) or absence (−), respectively, of GUS enzyme activity in leaf samples from the same plant. The positions and lengths, in kilobase pairs, of the molecular size markers are indicated.

pattern is inconsistent with predominant integration by transposition of the marked *Ac* elements. Further analysis (data not shown) indicates that plant 4615 also contains portions of an unmarked, but incomplete, *Ac* element. Thus, in these two plants the integration of introduced DNA into the soybean genome appears to have occurred nonspecifically as the integration of any other plasmid (1) and without regard for the *Ac* element in which the marker genes were imbedded.

Southern-blot analysis of GUS genes in self-pollinated progeny of lineages 3993 and 4615 indicates all copies of the GUS genes are inherited as a unit (Figs. 5*a* and 6*a*). Moreover, in plant 4615 all copies of the NPT II genes cosegregate with the GUS genes (Fig. 6*b*). Inheritance of the foreign genes in self-pollinated R_2 progeny of each lineage follows the same pattern (Figs. 5*b* and 6 *c* and *d*). Data from Southern analysis is thus completely concordant with enzyme assay data: plant material exhibiting enzyme activity also exhibits the complete DNA repertoire, whereas material from nonexpressing plants contains no detectable input DNA. These segregation and cosegregation data indicate that the transformed phenotypes in the 3993 and 4615 lineages each derived from a single transformation event.

Tissue from plants derived from *in vitro* regeneration of embryonic axes of immature R_1 seeds was also subjected to enzyme and DNA analyses to determine whether such manipulations would influence gene expression in regenerated plants. Embryonic axes were plated on standard regeneration medium and multiple shoots were recovered. Uniform GUS activity was observed in all plant tissues recovered in this fashion. Southern blot analyses on plant tissues grown *in vitro* demonstrated that they, too, were identical to their *in vivo* counterparts (data not shown).

Our results indicate that transgenic soybean plants produced by particle acceleration methods are genotypically and

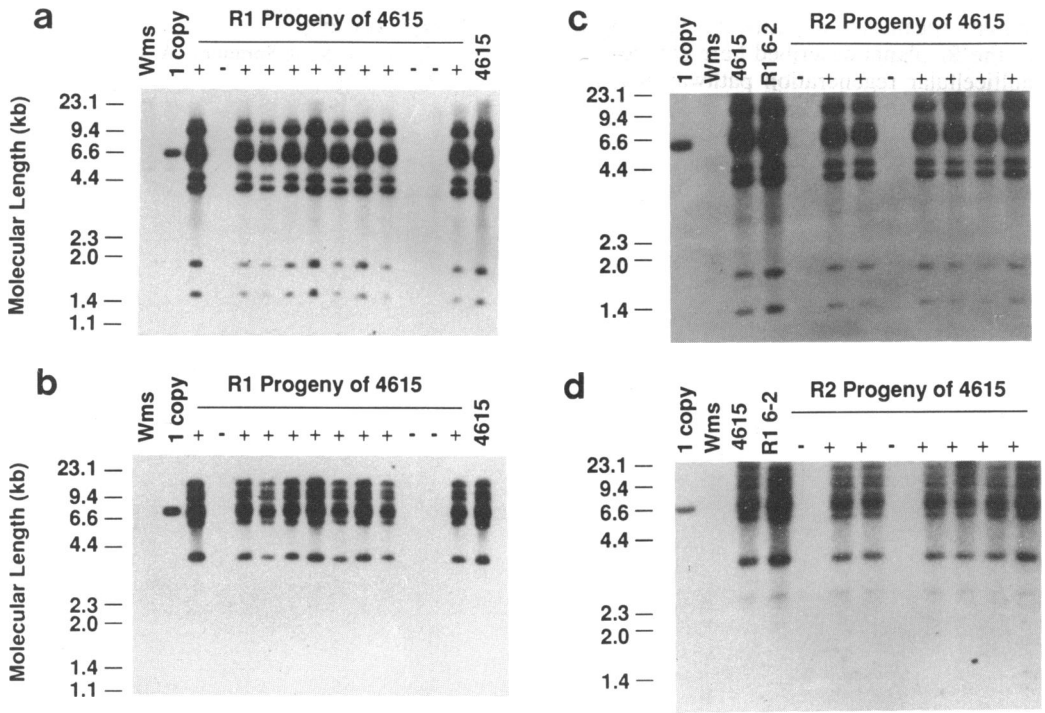

FIG. 6. Southern analysis of R_1 and R_2 progeny of plant 4615. Southern blots were made as described in Fig. 4 except that digestion was with *Bam*HI, and 1 copy reconstructions included both GUS and NPT II plasmids. (*a* and *c*) Samples from self-pollinated R_1 and R_2 plants, respectively. The membranes were hybridized with a ^{32}P-labeled RNA probe corresponding to the minus strand of the *gus* coding region. (*b* and *d*) The gus probe was removed and the membranes were rehybridized with a ^{32}P-labeled RNA probe corresponding to the minus strand of the NPT II coding region. Lanes Wms contain DNA from a nontransformed soybean (cv. Williams) plant. Lanes 4615 (*a* and *b*) or 6-2 (*c* and *d*) contain DNA samples from the parental R_0 or R_1 plants, respectively. Lanes + or − contain DNA samples from self-pollinated R_1 (*a* and *b*) or R_2 (*c* and *d*) progeny. The symbols indicate the presence (+) or absence (−) of GUS and NPT II enzyme activity in leaf samples from the same plant. The positions and lengths, in kilobase pairs, of the molecular size markers are indicated.

phenotypically stable. Transgenic plants containing a few copies of the transforming genes or many copies have been characterized. Enzyme assays and DNA analyses show complete concordance between the presence of enzyme activity and the corresponding chimeric genes. With the exception of their expression of bacterial enzyme activities the transgenic plants do not show any obvious phenotypic differences. The plants appear identical to untransformed greenhouse-grown Williams plants in terms of morphology, growth habit, age to maturity, flowering, seed yield, and germination. In most cases, the transforming genes behave as a single dominant locus exhibiting normal Mendelian segregation, as exemplified in this analysis by the 4615 lineage. Segregation patterns observed in the R_2 generation of this lineage demonstrate that both homozygous and heterozygous R_1 plants were obtained from the self-pollinated seed of plant 4615. The transforming genes in the 3993 lineage also behave as a single dominant locus, but in this case they are linked to an inviable-pollen phenotype. Consequently, aberrant, but consistent, segregation ratios are observed in R_1 and R_2 generations of this lineage, and plants homozygous for the transformed genotype are not produced.

Agreement between the segregation patterns observed in the R_1 and R_2 generations of each lineage suggests that the R_0 plants were uniformly transformed and were heterozygous for the transformed genotype. Although restriction mapping reveals interspersion of multiple gene copies with nonplasmid DNA, genetic analysis indicates that all copies of the foreign genes are tightly linked. Genetic linkage was also observed between independent markers cotransformed into a single plant. From these results, we conclude that plants 3993 and 4615 were ultimately derived from single cells. This is a surprising conclusion in relation to the widely held view that organogenesis, as opposed to somatic embryogenesis, follows a multicellular regeneration pathway. The apparent clonal nature of the R_0 plants described here can be reconciled with a multicellular regeneration pathway if it is assumed that one transformed cell underwent extensive proliferation. This would result in a population of uniformly transformed cells from which organogenesis could have been initiated. The linkage of such cells by descent may also relate to physical linkages that could influence reorganization of the shoot meristem during organogenesis.

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