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Cytological characterization of transgenic soybean

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Abstract Some of the transgenic soybean [*Glycine max* (L.) Merr.] plants produced by bombarding embryogenic suspension cultures with DNA-coated particles exhibit morphological aberrations, including stunted plant growth, leathery dark green leaves and partial-to-total seed sterility. In general, cultures from two Asgrow soybean lines (A2242, A2872) that were maintained for 8 months or longer produced primary transformants with reduced fertility. Cytological examination (mitotic pro-metaphase to metaphase chromosomes) of cells of suspension cultures, of roots from germinating somatic embryos, and of plants (R_0 and R_1) derived from A2242, revealed, besides diploidy ($2n = 40$), various chromosomal aberrations such as deletions, duplications, trisomics and tetraploidy. Diploid transgenic plants with a normal karyotype from A2242 generally exhibited good fertility. No chromosomal abnormalities were observed in A2872-derived plants. However, plants regenerated from relatively old cultures of A2872 (more than 1 year in culture) showed a range of phenotypic abnormalities although they all contained $2n = 40$ chromosomes. These results indicate that soybean genotypes differ in their susceptibility to chromosomal instability induced by tissue culture. Therefore, chromosome analysis of cell cultures and the plants derived from them can help eliminate chromosomally and genetically abnormal material from gene-transfer experiments.

Key words Soybean · *Glycine max* (L) Merr. · Transformation · Particle bombardment · Chromosomal aberrations

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Introduction

Recent advances have made feasible the genetic transformation of many important crop species. Embryogenic suspension cultures of soybean can be initiated from globular-stage somatic embryos arising on immature cotyledons (Finer and Nagasawa 1988) and genes can be delivered to the embryogenic cultures by particle bombardment (Parrott et al. 1989; Finer and McMullen 1991). However, the first transgenic plants produced by this procedure were sterile and displayed a range of phenotypic abnormalities (Finer et al. 1995). Subsequent work showed that fully fertile plants can be produced by introducing genes into relatively young cultures (Stewart et al. 1996). Plant cells propagated in culture can undergo various genetic and chromosomal abnormalities (Singh 1993). Apparently, embryogenic suspensions of soybean are particularly prone to abnormalities induced by tissue culture.

This study presents data on the fertility of transgenic soybean produced by the bombardment of embryogenic suspensions. Cytological analysis was performed in an attempt to determine the cause of morphological variation in the transgenic soybean.

Materials and methods

Embryogenic cultures from two Asgrow soybean genotypes, A2242 and A2872 in FN medium, were initiated from immature cotyledons as described by Finer and Nagasawa (1988). We maintained the cultures in 35 ml of FN media containing 10 μg/ml of 2,4-D. Flasks (250-ml) were placed on a rotary shaker (150 rpm) at 28°C with fluorescent light and a 16-h photoperiod. Sub-culturing was carried out about every 2 weeks by inoculating approximately 35 mg of tissue into 35 ml of fresh medium.

The hygromycin resistance gene (Gritz and Davies 1983; Kaster et al. 1983), consisting of the hpt coding region under the control of the 35*s* promoter (Odell et al. 1985) and the nos (nopaline synthase) 3′ end (Depicker et al. 1982) together with other genes designed to alter the amino-acid or lipid profile in mature seed, were co-introduced. These genes will be the subject of future publications. The protocols of Parrott et al. (1989) and Finer and McMullen (1991) were followed for bombarding the cells, selecting for hygromycin-resistant cell lines, and regenerating plants from these cell lines. Gold particles (1 μm) coated with DNA were accelerated with a PDS 1000/He gene-gun (Bio-Rad, Hercules Calif.) into about 500 mg of embryogenic tissue contained in 4.5-cm Petri dishes. An average of six plates were bombarded in each experiment. Following bombardment, about 250 mg of tissue was re-suspended in 35 ml of FN medium in a 250-ml flask and placed on a rotary shaker for 7–10 days. The tissue was then transferred to fresh FN medium containing hygromycin (50 μg/ml). Subsequent transfers to fresh hygromycin-containing medium were done every week for the first 3 weeks and then bi-weekly for another 4 weeks. Transgenic cell clusters were observed after 2–6 weeks in the hygromycin-containing medium. Each cell cluster was removed and placed into an individual flask containing liquid medium and allowed to grow for an additional 4–6 weeks before using the tissue for plant regeneration.

The chromosome counts (mitotic pro-metaphase and metaphase) in embryogenic suspension cultures (non-transgenic) and in roots from developing somatic embryos (either transgenic or non-transgenic) from R_0 and R_1 transgenic plants were determined according to Singh (1993).

Results

The chromosome counts in Asgrow soybean genotype A2242

Seeds from A2242 (control) contained 2n = 40 chromosomes with no apparent chromosomal abnormalities. The chromosome counts of somatic tissues from A2242 revealed that this genotype is not chromosomally stable in culture. The plants derived from these cultures also exhibited chromosomal abnormalities (Table 1). Chromosomes of nine R_1 populations from culture 22-1 were examined. Regenerated R_0 plants were in contact with 2,4-D for 6.43–9.00 months. Although R_0 plants from eight cultures expressed normal diploid morphological features, R_1 seedlings from four populations contained 2n = 80 chromosomes and four populations had 2n = 40 chromosomes (Fig. 1 A).

Characterization of transgenic plants based on morphological traits is not always reliable. For example, R_0 plants from a 22-1 culture were morphologically tetraploid. These plants showed dark-green leathery leaves and produced mostly one-seeded pods. Chromosome counts from five R_1 seedlings showed 2n = 39 + 1

Table 1 Chromosome analysis at somatic metaphase in the transgenic Asgrow soybean genotype A2242

Culture ID	R_0 phenotype	Origin of roots	Months on 2,4-D	No. samples	2n	Karyotype
A2242		Control		5	40	Normal
22-1	Diploid	R_1	6.43	7	40	Normal
22-1	Tetraploid	R_1	6.90	5	40; 41	39 + (1); 38 + (3)[a]
22-1	Diploid	R_1	6.90	2	40	Normal
22-1	Diploid	R_1	6.96	9	40	Normal
22-1	Diploid	R_1	6.96	2	80	Normal
22-1	Diploid	R_1	7.00	8	40	Normal
22-1	Diploid	R_1	7.30	9	80	Normal
22-1	Diploid	R_1	7.96	8	80	Normal
22-1	Diploid	R_1	9.00	1	80	Normal
22-1	Tetraploid	R_0	11.47	3	80	Normal
22-1	Tetraploid	R_0	15.36	1	80	Normal
22-1	–	Embryo	15.36	5	80	3, 80; 2, 79 + 1[b]
22-1	–	Embryo	16.73	4	80	3, 80; 1, 40 + 80[c]
817	–	Embryo	7.17	4	40	3, 40; 1, 39 + 1[d]
817	–	Embryo	7.33	5	40	4, 40; 1, 39 + 1[d]
817	–	Embryo	7.83	5	80	3, 80; 2, 79 + 1[e]
817	–	Embryo	8.70	1	40	Normal
817	–	R_0	9.13	1	40	Normal
817	–	Suspension	11.26	1	80	Normal
817	–	Embryo	12.43	2	80	Normal
825	–	Suspension	6.86	1	40	Normal
828	–	Embryo	2.86	1	40	Normal
826	–	Suspension	4.20	1	80	Normal

[a] Three small metacentric chromosomes
[b] One megachromosome
[c] Chimaera 40 + 80 chromosomes
[d] Long chromosome
[e] One sample with 79 + 1 dicentric chromosome and other sample with 79 + 1 fused centromeric chromosome

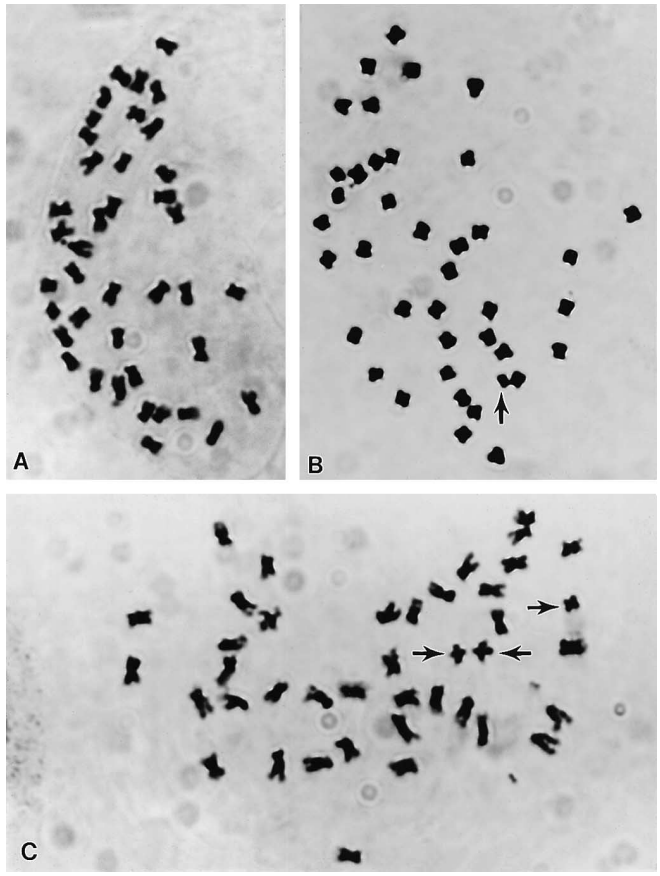

Fig. 1A–C Mitotic chromosomes in root tips of seedlings from Asgrow soybean line A2242 R_1 generation. **A** $2n = 40$ showing normal karyotype; **B** $2n = 39 + 1$ small metacentric chromosome (*arrow*); **C** $2n = 38 + 3$ small metacentric chromosomes (*arrows*)

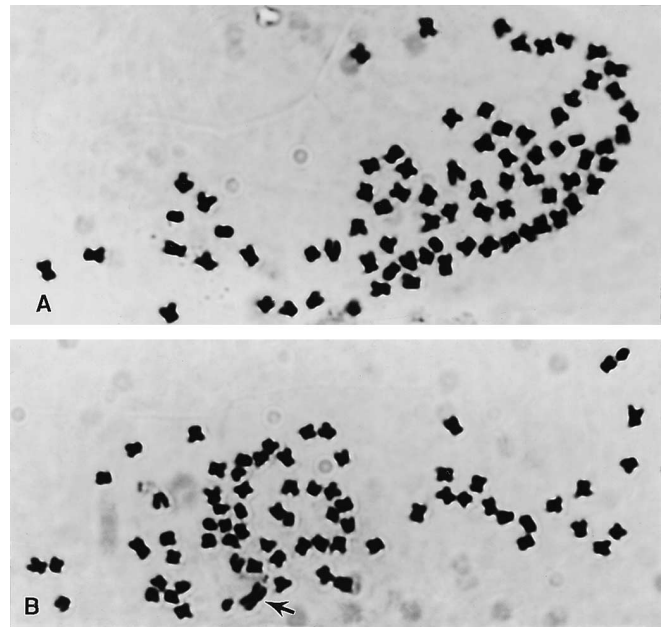

Fig. 2A, B Mitotic metaphase chromosomes in root tips of seedlings from Asgrow soybean line A2242 grown in agar. **A** $2n = 80$ showing normal karyotype; **B** $2n = 79 + 1$ mega (monocentric) chromosome (*arrow*)

small metacentric chromosome (Fig. 1 B) in three plants, and one plant each contained $2n = 38 + 3$ small metacentric chromosomes (Fig. 1 C) and $2n = 40$ chromosomes. The 40-chromosome plant may have had a small deletion, which however, could not be detected in the condensed metaphase chromosomes, or may have carried desynaptic or asynaptic genes.

Cytological abnormalities, especially tetraploidy, were frequently observed in A2242 cultures. The appearance of tetraploids also was somewhat dependent on the age of cultures. Embryo suspensions of culture 826, which was 4.20-months old, displayed cells with $2n = 80$ chromosomes. Four R_0-derived plants from the 22-1 culture exhibited tetraploid morphological traits, such as slow plant growth, thick dark-green leaves, and set mostly one seed per pod. Their tetraploid nature was confirmed cytologically as all plants carried $2n = 80$ chromosomes. Five germinating somatic embryos from culture 22-1 were 15.36-months old. Roots from all of these embryos carried $2n = 80$ chromosomes (Table 1). Three had 80 normal chromosomes (Fig. 2 A) and two had $79 + 1$ megachromosome each (Fig. 2 B).

The chromosome counts of suspension cultures and germinating somatic embryos from culture 817 are extremely informative. Cells in 11.26-month-old suspension cultures showed $2n = 80$ chromosomes. Transgenic plants recovered from this culture were tetraploid. This was shown by chromosome analysis of germinating somatic embryos from a 7.83-month-old culture. We examined roots from five samples of culture 817 cytologically. Three samples showed $2n = 80$ chromosomes with a normal karyotype. Two samples, in addition to tetraploidy, carried one aberrant chromosome; one sample had $2n = 79 + 1$ dicentric chromosome (Fig. 3A), while the other sample contained $2n = 79 + 1$ chromosome with centric fusion (Fig. 3B). Some germinating embryos contained $2n = 40$ normal chromosomes (Table 1). Occasionally, counting chromosomes at somatic metaphase may prove misinformative. Since soybeans possess 40 small and nearly metacentric chromosomes, an excellent chromosome spread and keen observation are pre-requisites for reaching a precise conclusion. For example, somatic embryos from 7.17- and 7.33-month-old cultures produced one seedling each with $2n = 39 + 1$ long chromosome, while other seedlings showed a normal 40-chromosome karyotype (Table 1). The unusual chromosome may be an acrocentric and could be easily confused with the nucleolus organizer chromosome.

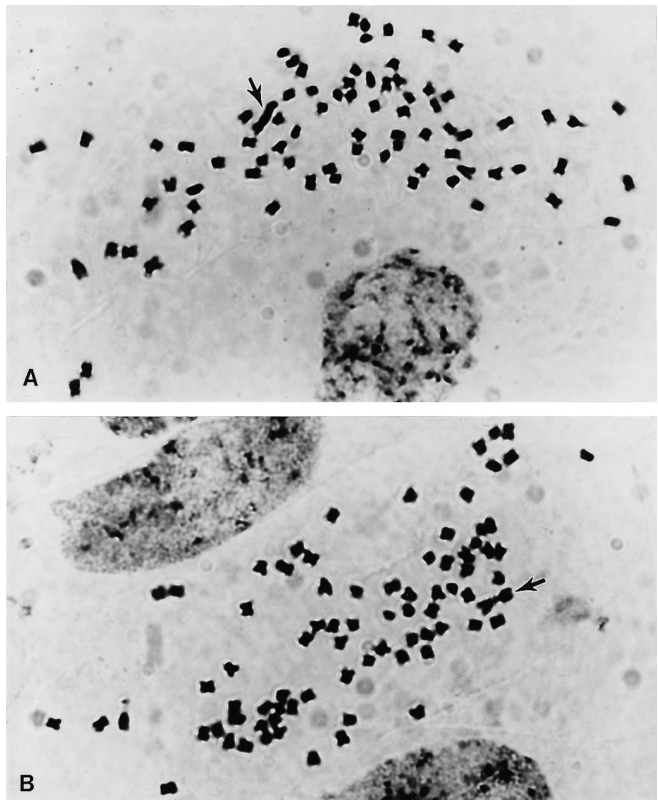

Fig. 3A, B Mitotic metaphase chromosomes in root tips of seedlings from Asgrow soybean line A2242 grown in agar. **A** 2n = 79 + 1 mega (dicentric) chromosome; **B** 2n = 79 + 1 mega (centric fusion) chromosome

The chromosome counts in Asgrow soybean genotype A2872

In contrast to material from A2242, tissues from A2872 did not display any chromosomal abnormalities. Nine selfed seeds from A2872 showed 2n = 40 chromosomes with a normal karyotype. R_0 and R_1 plants derived from suspension cultures contained 2n = 40 chromosomes (Table 2). Culture age ranged from 8.26 to 32.30 months. The R_0 plants expressed normal diploid-like phenotypes and, as expected, all plants carried 2n = 40 chromosomes. However, five R_1 plants from a 29.43-month-old culture of 5-2 had tetraploid morphological features with one-seeded pods, but showed 2n = 40 chromosomes (Table 2). These abnormalities may be genic, as desynaptic or asynaptic plants express tetraploid phenotypes with partial-to-complete sterility.

Discussion

During the past decade substantial progress has been made in plant transformation and this progress has been summarized in several reviews (Fisk and Dandekar 1993; Christou 1994; Klein and Zhang 1994; Vasil 1994; Casas et al. 1995; Songstad et al. 1995; Puddephat et al. 1996). However, an understanding of the cytogenetic changes induced by tissue culture in these transgenic crops is lacking. The present study provides extensive cytological information on transformed soybeans produced by the bombardment of embryogenic suspension cultures. The fertility of the primary transformants was variable. In general, transgenic seed can be recovered from cultures that are less than 8-months old. Morphological variants, particularly seed sterility, are often recorded in transgenic crops (Christey and Sinclair 1992; Conner et al. 1994; Ghosh Biswas et al. 1994; Austin et al. 1995; El-Kharbotly et al. 1995; Finer et al. 1995; Fütterer and Potrykus 1995; Lynch et al. 1995; Schulze et al. 1995; Shewry et al. 1995; Widholm 1995; Hadi et al. 1996; Liu et al. 1996). Lynch et al. (1995) observed that transgenic rice plants were shorter, took longer to flower and showed partial sterility. Schulze et al. (1995) recorded fruit development after selfing transgenic cucumber, but none of the harvested fruits contained seeds. Similarly, Ghosh Biswas et al. (1994) transferred 29 rice primary transformants to soil in a greenhouse. Only 11 plants flowered but they did not set seed, while two control plants were completely fertile. Liu et al. (1996) recorded morphologically abnormal flowers without seed in greenhouse-grown transgenic soybean plants. These investigators did not examine the sterile plants cytologically. However, the present observations indicate that sterility can have a chromosomal basis.

Our study demonstrates that chromosomal aberrations are induced during culture at an early stage and are probably genotype dependent. The occurrence of tetraploid (2n = 80) and aneuploid cells in 4.2-month-old suspension cultures and a 7.17–7.83-month-old embryo of soybean genotype A2242 suggests that chromosomal aberrations are induced in the culture. These cultures will generate aneuploid and tetraploid partial-to-total sterile plants. Primary transformants from A2872 showed 2n = 40 chromosomes even from 32.30-month-old cultures. This suggests that a genotype may be highly responsive to culture conditions but may be prone to chromosome aberrations (Hermsen 1994). Chromosomal aberrations are routinely recorded in cell and tissue culture and are transmitted to their regenerants (Singh 1993). Thus chromosome counts of embryo suspensions will help to maintain chromosomally normal cultures.

Unexpected segregation and low expression or disappearance of foreign genes have been observed in transformed crops (Chupeau et al. 1989; Fromm et al. 1990; Somers et al. 1994; Fütterer and Potrykus 1995; Meyer 1995). Somers et al. (1994) examined Gus activity in R_1 and some R_2 and R_3 generations in 15 transgenic families of oats. Six families showed an aberrant segregation ratio, seven families segregated in a 3:1 GUS + :GUS-ratio, and two families segregated 15:1

Table 2 Chromosome analysis at somatic metaphase in the transgenic Asgrow soybean genotype A2872

Culture ID	R_0 phenotype	Origin of roots	Months on 2,4-D	No. samples	2n	Karyotype
A2872		Control		9	40	Normal
821	–	Suspension	8.26	1	40	Normal
821	–	Embryo	9.23	5	40	Normal
2-1	Diploid	R_1	22.76	1	40	Normal
2-2	Diploid	R_1	11.20	1	40	Normal
2-2	Diploid	R_1	18.03	1	40	Normal
2-4	Diploid	R_1	13.16	1	40	Normal
2-4	Diploid	R_1	20.00	1	40	Normal
5-1	Diploid	R_1	20.50	2	40	Normal
5-1	Diploid	R_1	21.50	2	40	Normal
5-2	Diploid	R_1	19.06	4	40	Normal
5-2	Diploid	R_1	20.20	1	40	Normal
5-2	Diploid	R_1	20.73	1	40	Normal
5-2	Diploid	R_1	21.20	2	40	Normal
5-2	Diploid	R_1	29.00	3	40	Normal
5-2	Diploid	R_1	29.43	3	40	Normal
5-2	Tetraploid	R_1	29.43	5	40	Normal
90-2	Diploid	R_1	19.06	1	40	Normal
90-2	Diploid	R_1	32.30	4	40	Normal
801	–	Embryo	9.23	2	40	Normal

for GUS activity. An extensive review on transformation research in the Poaceae by Fütterer and Potrykus (1995) revealed that the expression of transgenes in the progeny of transgenic plants could be quite unpredictable. Genes may be physically present but gene activity may be poorly expressed or totally lost in subsequent generations. This is generally attributed to the poorly understood phenomenon of co-suppression, or gene silencing (Jorgensen 1990; Matzke and Matzke 1995; Stam et al. 1997). The present investigation furnishes a cytological clue that may help explain aberrant segregation ratios or the loss of transgene sequences which may be applicable in some cases. For example, the selfed population of a plant with $2n = 39 + 1$ metacentric chromosomes identified in soybean genotype A2242 is expected to segregate plants in a ratio of 1 ($2n = 40$):2 ($2n = 39 + 1$ metacentric):1($2n = 38 + 2$ metacentrics). Diploid plants will be normal and fertile and may not express the introgressed gene if this gene is in the deleted chromosome. Matzke et al. (1994) attributed an erratic inheritance in a transgenic tobacco line to aneuploidy ($2n = 49$ or 50).

In conclusion, we recommend an early chromosome count of embryo suspensions before conducting transformation experiments to ensure the isolation of the most-fertile plants. However, it should be pointed out that somatic metaphase chromosome analysis identifies only major chromosomal structural aberrations. Meiotic chromosome pairing, particularly at pachytene, and molecular methods, on the other hand, may detect small chromosome aberrations such as deletions, duplications, inversions and translocations.

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