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Growth characteristics and transformability of soybean embryogenic cultures

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Abstract Embryogenic cultures of soybean [*Glycine max* (L.) Merr. cv. Jack and Asgrow A2872] were established in liquid Finer and Nagasawa medium, maintained by transfer to fresh medium at biweekly intervals, and subjected to microprojectile bombardment over time. Cultures were not amenable to transformation until they were at least 6 months old. Over time, different cell lines of the same genotype acquired very different culture phenotypes. Histological analysis of cell lines differing in transformation ability showed that the most transformable cultures had cytoplasmic-rich cells in the outermost layers of the tissue. In contrast, the outer layers of less transformable cultures contained cells with prominent vacuoles. Although fresh weight accumulation of the cultures was curvilinear during the 2-week subculture period, a burst of mitotic activity was evident shortly after transfer to fresh medium. This activity usually lasted from the 2nd to the 6th day following subculture, and peaked on the 4th day. Tissues at or near this stage always produced more transient expression of a reporter gene than did bombardments at other times. In addition, the cell lines most amenable to transformation also exhibited the highest mitotic index. Thus any treatment to increase the mitotic index, especially when the cell lines are less than 6 months old, may facilitate the transformation of cell lines from which efficient recovery of transgenic plants is still possible.

Key words Microprojectile bombardment · Mitotic index · Somatic embryogenesis · Transformation

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Abbreviations *DAPI* 4,6-Diamino-2-phenylindole · *FN medium* Finer and Nagasawa medium · *GUS* β-Glucuronidase · *MI M*itotic index

Introduction

The use of repetitive embryo systems has been effective for the transformation of soybean [*Glycine max (*L.) Merr.] using microprojectile bombardment (Finer and McMullen 1991; Sato et al. 1993; Parrott et al. 1994). However, this method has not come into general use as the number of stable transformation events recovered can be highly variable and is generally low when cell lines under 6 months old are used (Stewart. et al. 1996). A second limitation to this system is the recovery of cell lines which will not convert, or will convert into plants which are either sterile or which have limited fertility (Cho et al. 1995; Finer et al. 1996), a problem that traditionally has been greater when older cell lines are used.

Currently, little information is available on the cell growth dynamics of cell cultures from any large-seeded legume. In addition, the biological factors that contribute to the variability of the biolistic process have not been characterized for any transformation system. Consequently, we undertook this study to characterize more precisely the growth dynamics of soybean embryogenic cultures within their normal 2-week subculture period, and to determine if differences in growth dynamics between cultures contribute to transformation ability. We also show that morphological differences between cultures may be related to transformation ability.

Materials and methods

Culture initiation and maintenance

Embryogenic cultures of soybean cultivars Jack and A2872 were established in liquid Finer and Nagasawa (1988) (FN) medium as pre-

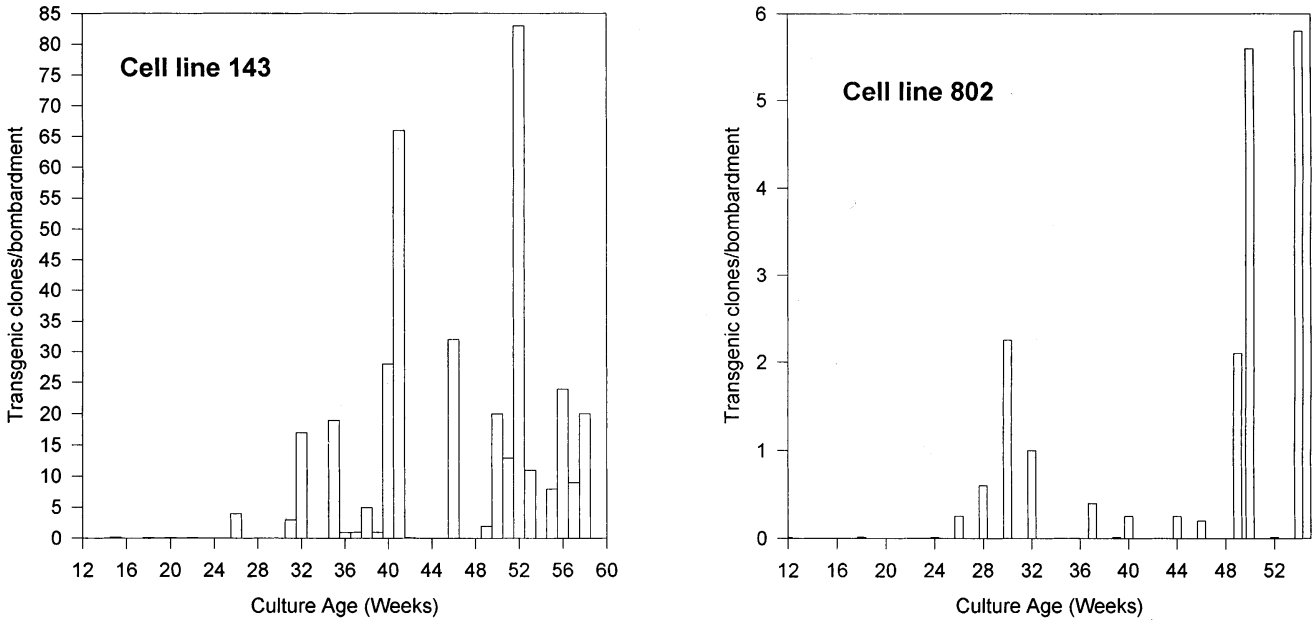

Fig. 1 The effect of culture age on the transformability of two cell lines of cultivar A2872, cell line 143, which is highly transformable, and cell line 802, which has a low transformation capacity

viously described (Bailey et al. 1993). Cultures were maintained in 50 ml FN medium in Erlenmeyer flasks capped with Bellco (Vineland, NJ) silicone enclosures, or with aluminum foil covered with Parafilm, and subcultured biweekly. Flasks were maintained at 28 °C and shaken at 125 rpm, under a 16-h photoperiod, unless specified otherwise, which was provided by cool white fluorescent tubes (50 μE m^{-2} s^{-1}). Somatic embryo differentiation, maturation, desiccation, and germination were also as described by Bailey et al. (1993).

Tissue growth

The tissue growth between subcultures was analyzed for Jack soybean by inoculating approximately 35 mg of tissue (six uniform embryogenic clumps) into each of 16 flasks with fresh medium. This permitted 2 flasks from each time point to be harvested at 0, 2, 4, 6, 8, 10, 12, and 14 days post-inoculation. At each sample date, the contents from one flask were weighed and subjected to analysis of the mitotic index (*MI*), while the contents of the other flask were used for microprojectile bombardment. The entire experiment was repeated three times. All cultures were maintained under continuous illumination for this analysis.

Microprojectile bombardment

DNA preparation and bombardment were as described by Browse et al. (1993), using pTRA140 (Zheng et al. 1991), or similar plasmids, which contain a 35S-*hph* gene for hygromycin resistance and 35S-*gus* as a marker (Jefferson 1989), for bombardment with a DuPont PDS 1000/HE system. Briefly, a 5-μl sample of plasmid DNA (1 μg/μl) was added to 50 μl of gold particles (Biorad 1 μm, at 0.06 mg/500 μl), followed by the addition of 50 μl of 2.5 M $CaCl_2$ and 20 μl of 0.1 M spermidine. The mixture was agitated for 3 min, spun down in a microfuge for 10 s at 5,000 *g*, and the supernatant removed. The DNA-coated particles were washed once in 400 μl of 70% ethanol, resuspended in 100% ethanol, and subjected to three 1-s sonications, after which 5 μl of the suspension was loaded onto each macrocarrier. For bombardment, six embryogenic clumps of soybean were placed in the center of a Petri dish, and residual liquid was removed with a pipet. The plate was placed 5 cm below the stopping screen, the chamber evacuated to 27 mm Hg, and bombarded three times using the 1,100-psi rupture disk and a 1-cm flying distance.

Effect of culture age on transformability

Two cell lines, labeled 802 and 143, were established from Asgrow cultivar A2872 as described previously. These were bombarded at 1- to 3-week intervals as described, beginning from the time the cell lines were 3 months old until they were 15 months old. The exact length of the interval between bombardments was determined by growth of the cell lines, which fluctuated sufficiently to make it impossible to bombard on a regular schedule. Tissue collected from four to eight flasks was bombarded at any given date, again, depending on the growth of the cell lines during the previous culture interval. Eleven to 14 days after shooting, the cultures were exposed to selection (hygromycin at 50 μg ml^{-1}) for 6 weeks, with transfers to fresh medium at weekly intervals, after which the final number of transgenic cell lines was recorded. This level of hygromycin permits no escapes, but nevertheless, cell lines were sampled randomly and their transgenic status verified using the polymerase chain reaction and/or Southern analysis (data not shown).

Histology

Histology was performed on four cell lines, all from Asgrow cultivar A2872, which differed in their transformability. The two transformable cell lines were designated “17” and “143,” while the non-transformable lines were designated “802” and “806.” Tissues were fixed in 2.5% glutaraldehyde/1.5% paraformaldehyde (pH 7.0) and dehydrated in a graded ethanol series to 100%. The tissues were embedded in Historesin (Leica, Heidelburg, Germany) at room temperature under a vacuum, and 2-μm sections prepared. The sections were stained with either 0.5% toluidine blue or periodic acid-Schiff’s reagent and 1.0% aniline blue black (Feder and O’Brien 1968).

Determination of MI and its effect on transient gene expression

The MI was determined in two ways. Jack soybean was subjected to cytogenetic analysis, using tissues harvested at 0, 2, 4, 6, 8, 10, 12, and 14 days after transfer to fresh medium. Tissues were pretreated in 2 mM 8-hydroxyquinoline for 4 h at 4°C, fixed in glacial acetic acid and ethanol (1:3 vol/vol) for 24 h at room temperature, and

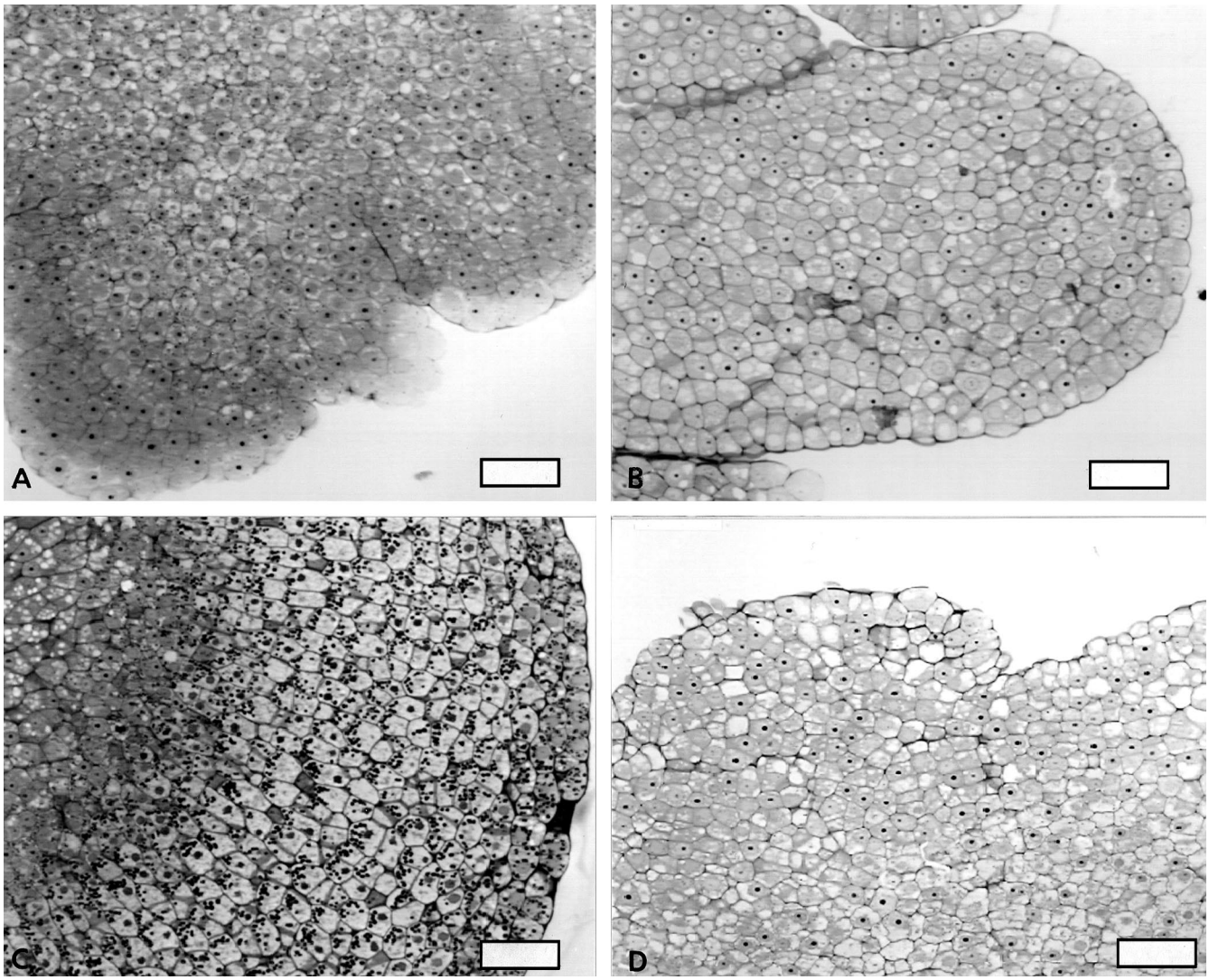

Fig. 2A–D Overall view of thin sections of embryo clusters of four cell lines of A2872 (*bar* 0.05 mm). Cell lines 17 (**A)** and 143 (**B)** are highly transformable. Cell lines 806 (**C)** and 802 (**D)** are recalcitrant to transformation

stored in 70% ethanol at 4 °C. Tissues were then hydrolyzed in 1 M HCl for 10–15 min at 60°C and squashed in 2% acetocarmine. Between 1,000 and 1,500 cells were observed per sampling date. The MI was calculated as the percentage of observed cells undergoing mitosis. At each sample date, half of the tissue was subjected to microprojectile bombardment as described. Following bombardment, the tissues were returned to FN medium for 48 h, at which time tissues were assayed for β-glucuronidase (GUS) expression using standard histochemical procedures (Jefferson et al. 1987), and the number of blue spots counted. The experiment consisted of six replications.

Alternatively, tissues of cell lines 802 and 143 were subjected to 4,6-diamino-2-phenylindole (DAPI) (Sigma, St. Louis, M.) staining and quantification. Tissues were fixed in 2.5% glutaraldehyde/1.5% paraformaldehyde for 2 h under vacuum. Tissues were stained with DAPI (0.01 $\mu g\ ml^{-1}$) for 15 min. The tissues were rinsed with deionized distilled water for 5 min, and observed under epifluorescence microscopy with a ×40 objective. For each sampling date, at least five clumps of embryogenic tissue were sampled, and the MI calculated based on observations of 1,500–2,000 cells.

Results and discussion

Effect of culture age on transformability

Cultures 143 and 802, both derived from genotype A2872, were bombarded at different times following their initiation. No stable transformants were obtained from either cell line until they were about 6 months old (Fig. 1). After that, cell line 143 became much more transformable than cell line 802, with cell line 143 yielding about ten-fold more transformants per bombardment, although the number of transgenic cell lines recovered fluctuated widely from week to week for both cell lines. The ability to obtain highly transformable cell lines with culture age had been previously reported in soybean (Hadi et al. 1996). Although these two cell lines were of the same genotype, they behaved very differently, suggesting that traits acquired during culture have a large effect on the transformability of a cell line.

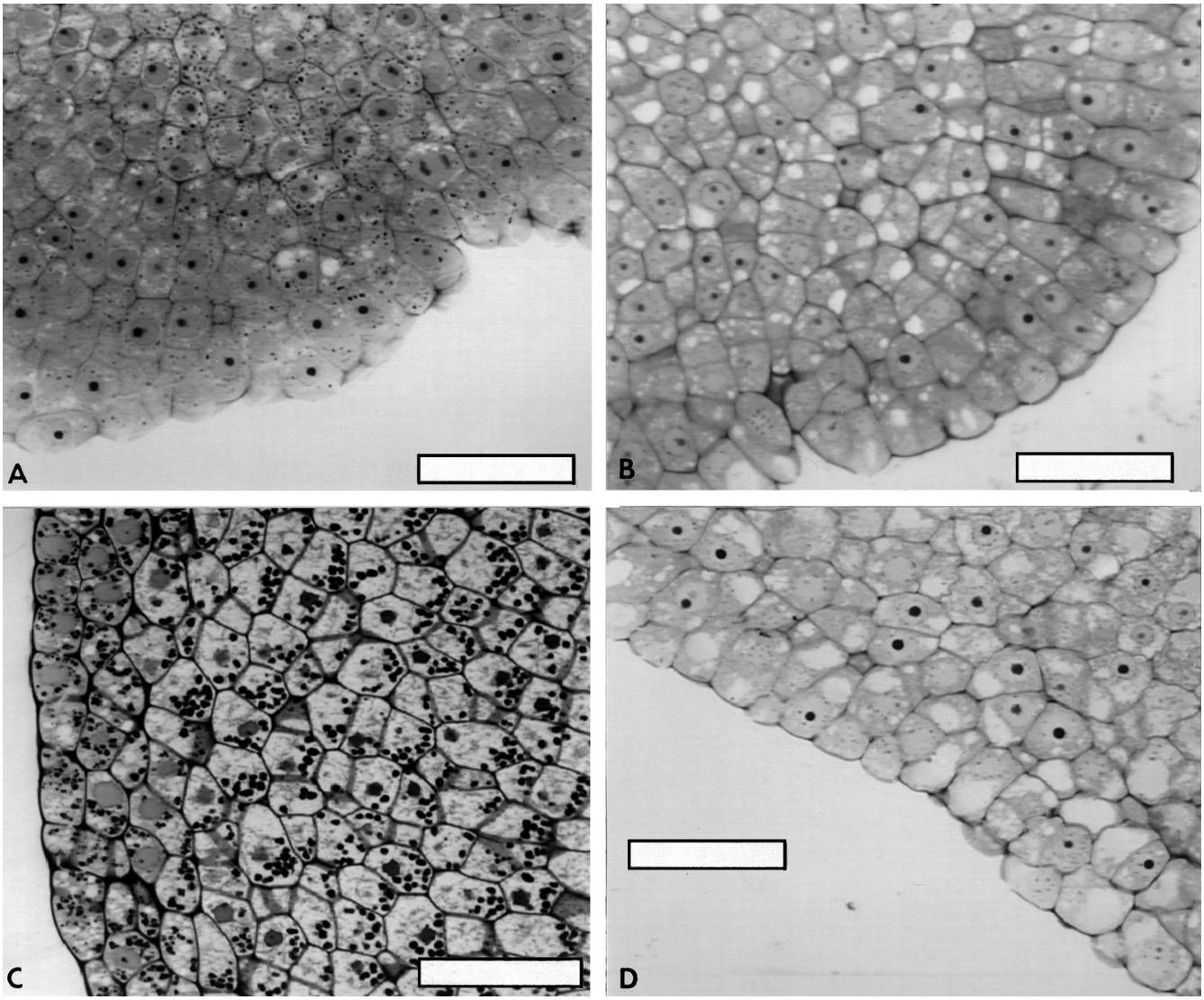

Fig. 3A–D High magnification of thin sections from embryogenic tissues from four cell lines of A2872. Cell lines 17 (**A)** and 143 (**B)** are highly transformable. Cell lines 806 (**C)** and 802 (**D)** are recalcitrant to transformation (*bar* 0.05 mm)

Morphological and histological characterization

Micrographs of readily transformable cultures (17 and 143) and poorly transformable cultures (806 and 802) are shown in Figs. 2 and 3. At the time histological observations were made, the transformable cell lines (17 and 143) were 8 and 30 months old, respectively, while the less transformable cultures (806 and 802) were 22 and 26 weeks old, respectively, highlighting the effect of age on culture phenotype and transformability. In general, the more readily transformable cultures were characterized by tightly packed globular structures, while the less transformable cultures were more lobed in appearance (Fig. 4).

Observations on thin sections also revealed differences between transformable and poorly transformable cell lines. Figures 2A and 3A (culture 17) and 2C and 3C (culture 806) show liquid-cultured embryogenic tissue stained with periodic acid-Schiff's and 1% aniline blue black. This staining procedure reveals starch granules and nuclei with prominent nucleoli. These sections show that the outer layers of culture 17 are comprised of cytoplasmic-rich cells. Cells deeper within the tissue contain prominent vacuoles. Culture 806 displays a cell organization that is the inverse of that of culture 17. Relatively small, cytoplasmic-rich cells are prominent in an interior layer of the tissue. This layer of cells with dense cytoplasm resembles cambial-like tissue that has also been observed in embryogenic tissue of *Panicum* (Lu and Vasil 1985). The cells in the peripheral layers of culture 806 are relatively more vacuolated than those in the peripheral layer of culture 17.

The presence of starch granules in peripheral layers is a characteristic of embryogenic suspension cultures as described by Halperin and Jensen (1967), Thomas et al. (1972), and Williams and Maheswaran (1986). A prominent difference between culture 17 and 806 (Figs. 3A and 3C, respectively) is the number and size of the starch granules. Starch granules in the peripheral cell layers of 806

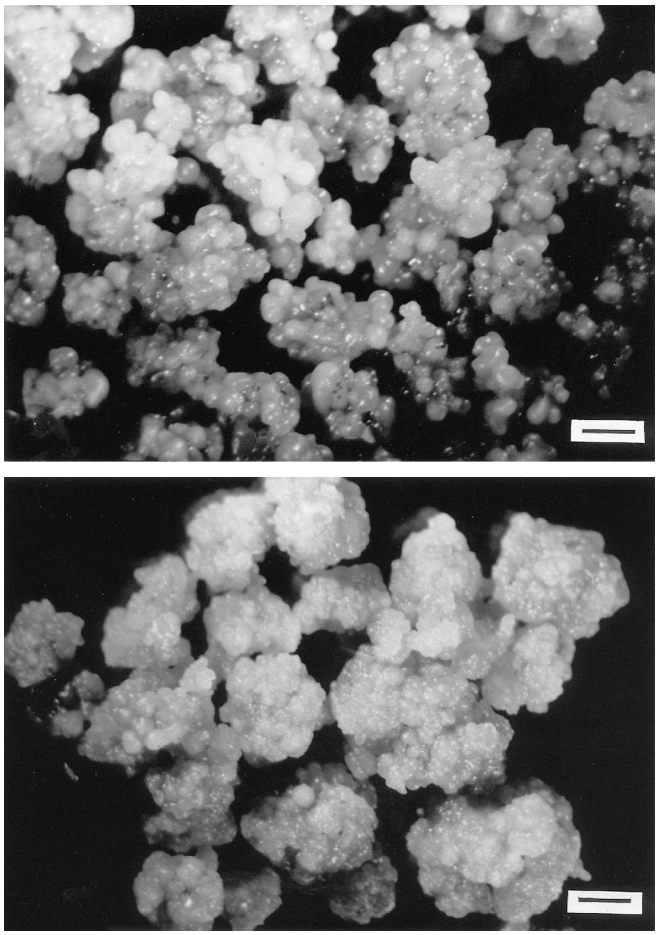

Fig. 4 Macroscopic view of cell lines 802 (*top*) and 143 (*bottom*). The less transformable line 802 consists of clusters of relatively large individual embryos, giving a lobed appearance, while those of the more transformable line 143 are much smaller (*bar* 1 mm)

(the less transformable culture) are relatively large and more numerous. Cells in the cambial-like layer and those more interior to the cambial layer have fewer starch granules. The starch granules in culture 17 (more transformable) are less numerous and much smaller than those in culture 806.

Figures 2B and 3B (culture 143) and 2D and 3D (culture 802) show tissue stained with 0.5% toluidine blue. The outer layers of culture 143, which is readily transformable, are rich in cytoplasm, while the outer layers of culture 802 are less rich in cytoplasm and have prominent vacuoles. These observations are similar to those made by Halperin and Jensen (1967) in carrot, Thomas et al. (1972) in *Ranunculus*, and Vasil and Vasil (1982) in *Pennisetum*. The outermost cell layers in embryogenic cultures consist of cytoplasm-rich cells that are meristematic, while interior cells are more vacuolated and less cytoplasmically dense. Presumably the cytoplasmic-rich cells are capable of division, while the more vacuolated cells may be terminal and capable of cell expansion only. The phenotypical and histological differences in morphology between transformable cultures (17 and 143) and less transformable cultures (802 and 806) may help explain the differences in transformability, even when these differences appear subtle.

Tissue growth after subculture and effect on transformability

To further evaluate factors affecting the behavior of cell lines, the effect of subculture on growth and transient gene expression was studied. A constant increase in fresh weight was obtained for soybean cultivar Jack (Fig. 5) over the normal 2-week culture period. Growth was curvilinear during this period, and fit a second-order curve very closely (r^2=0.93). However, there was significant variation in the MI over this same time period (Fig. 5). The MI was highest 2–6 days after subculture to fresh medium, with a peak on the fourth day. The patterns of GUS expression tended to reflect the MI (Fig. 5). Although the MI did not appear to peak until the 4th day for soybean, GUS expression was significantly greater for cultures shot on the 2nd day after subculture, with frequency of transient expression dropping continuously for tissues shot on subsequent days. Similarly for alfalfa, transient expression of GUS was highest 3–6 days after transfer to fresh medium (Brown et al. 1994), a period that was previously identified as having the highest frequency of cells undergoing division (Atanassov and Brown 1984).

To more easily verify the effect of subculture on MI, DAPI staining was used to quantify the MI of cell lines 17, 143, and 802. In all cases, the MI peaked on the 4th day after subculture. Cell line 143, which was the most transformable, had the highest MI throughout the entire subculture period, while cell line 802, which was the least transformable, had the lowest MI (Fig. 5). It must be pointed out that the Jack soybean line in Fig. 5 had an even higher MI than line 143. Yet, while 143 was highly transformable, the Jack line was not transformable, and had a culture phenotype like that of line 806 (Figs. 2C, 3C). Thus, a high MI appears to be a necessary but not sufficient factor for high transformation competence, and has to be coupled to the proper culture phenotype before it can contribute to a high transformation ability.

The MI for the soybean cultures tended to peak every 4 days for two of the five lines studied, suggesting that there may be a periodicity to mitosis in these cultures. The highest MI observed at any time was only 9%. By comparison, the highest MI reported for unsynchronized alfalfa cultures was only about 3%, while MIs of 30–70% have been reported for synchronized tobacco cells (Okada et al. 1986; Iida et al. 1991). This suggests that synchronization of the embryogenic cultures may result in a greater MI than has currently been observed. The large standard error bars reflect the fact that this burst of mitotic activity does not always occur, and may help explain the wide fluctuations seen between different bombardments.

Thus far, several studies agree that transformation frequency is greatest during the M phase of the cell cycle (Meyer et al. 1985; Iida et al. 1991; Pijnacker and Ferweda 1994). It has been suggested that the nuclear membrane is

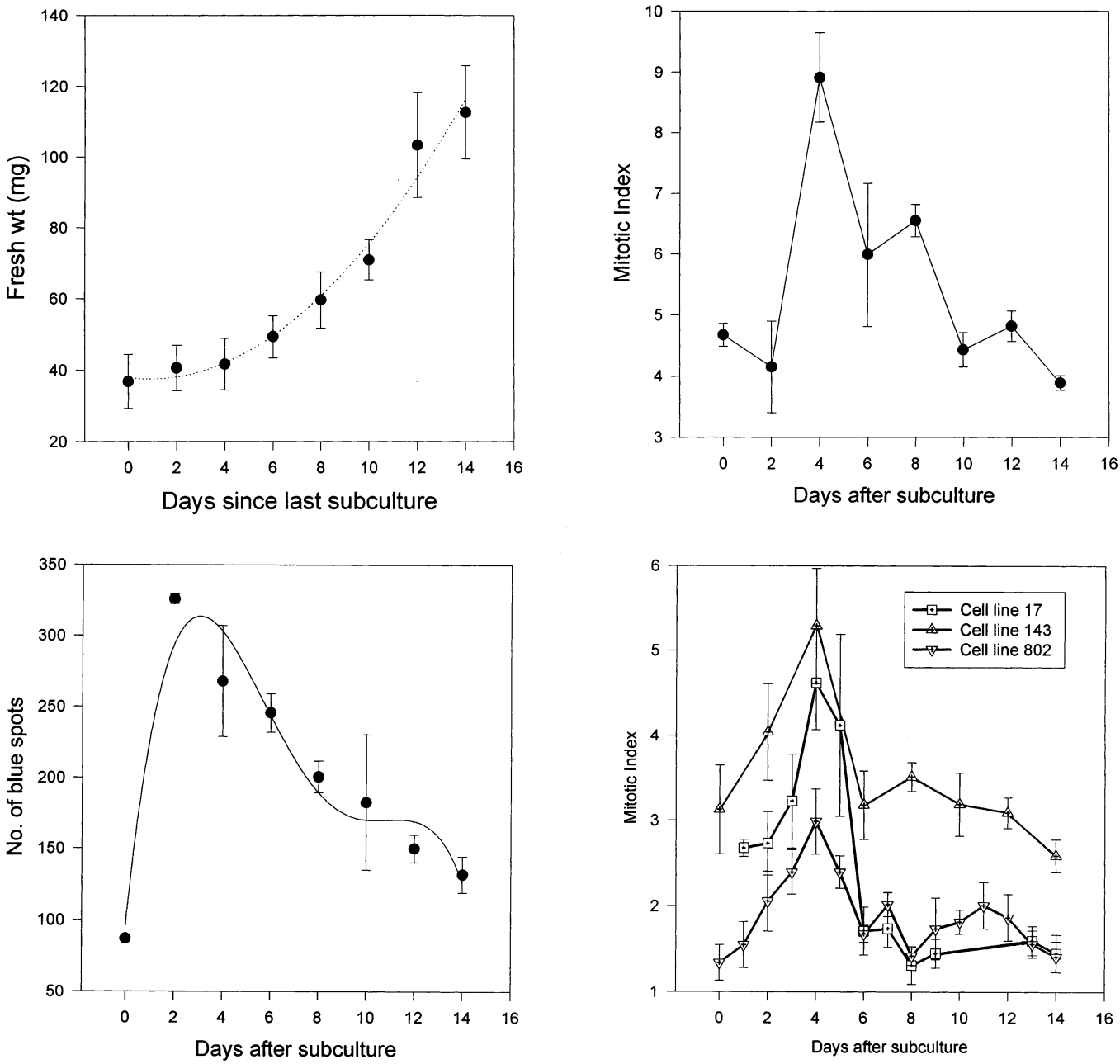

Fig. 5 Growth traits of soybean embryogenic cultures. *Upper left*: growth of Jack soybean over a 2-week culture period within a given flask. *Bars* represent standard errors based on six replications per time point. *Upper right:* MI of Jack soybean over a 2-week period. *Bars* represent standard errors based on a minimum of five replications per time point. *Lower left:* transient GUS expression in Jack soybean over a 2-week period. *Bars* represent standard errors based on six replications per time point. *Lower right:* MIs over a 2-week period of three A2872 soybean cell lines differing in transformation ability. Line 143 is the most transformable, and line 802 is the least transformable. *Bars* represent standard errors based on a minimum of five replications per time point

an effective barrier for the entry of DNA into the nucleus from the cytoplasm (Meyer et al. 1985), and almost all cells transiently expressing GUS in tobacco leaves after particle bombardment have the particle in the nucleus rather than the cytoplasm (Hunold et al. 1994). However, since the nuclear membrane is not present during the M phase, it could be that bombarded particles may land anywhere in the cell, and thus have a larger target than just the nucleus.

It has also been suggested that DNA synthesis or repair is necessary for successful integration of exogenous DNA (Köhler et al. 1989; Mayerhofer et al. 1991), which could help explain why higher-than-average transformation frequencies were reported during the S phase (Meyer et al. 1985). Similar reports during the G_2 stage (Iida et al. 1991) may be due to difficulties in separating this relatively short phase from the preceding S or subsequent M phase. In addition, all reports are consistent in that lowest transformation frequencies are obtained in the G_1 phase, which immediately follows mitosis, and during which no DNA synthesis occurs. The occurrence of crossing-over between sister chromatids in cultured tissues (Evans 1989) and its

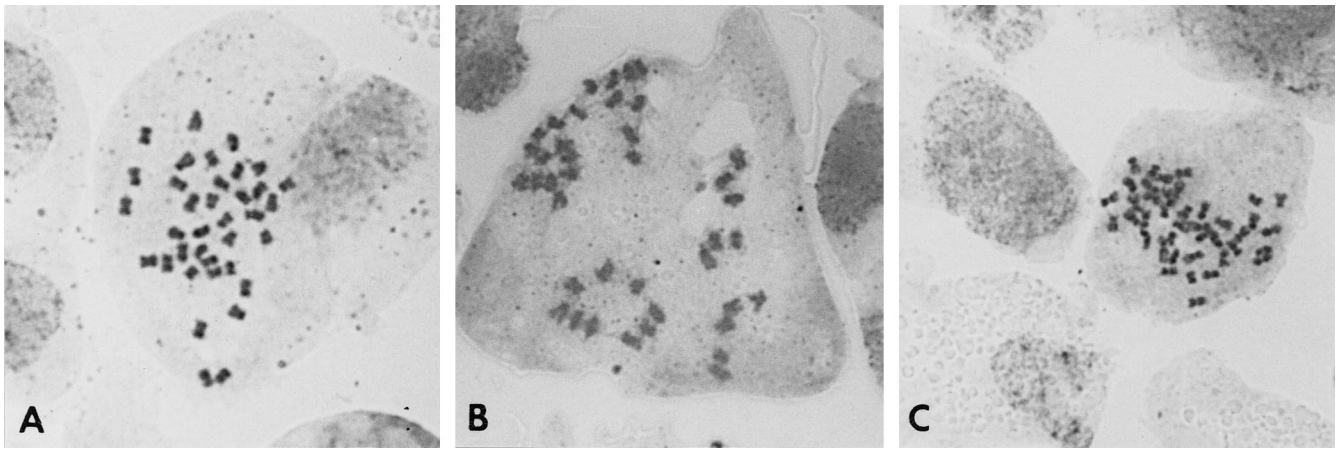

Fig. 6 Embryogenic cells of Jack soybean showing altered (**A**, **C**) and normal (**B**) chromosome numbers (**A** $2n=4x+2=42$, **B** $2n=4x=40$, **C** $2n=4x-4=36$ chromosomes)

promotion by 2,4-dichlorophenoxyacetic acid and sucrose (Pijnacker and Ferweda 1994) suggest that DNA synthesis for repair must also occur during mitosis.

Studies of tobacco protoplasts mitotically synchronized through the use of amphidicolin and transformed via electroporation (Okada et al. 1986) or subjected to polyethylene-glycol-mediated transformation (Meyer et al. 1985) found that transformation frequencies were highest when the protoplasts were in the M phase, or the S or M phases of the cell cycle, respectively. Particle bombardment of similarly synchronized tobacco suspension-cultured cells identified the G_2 and M phases as giving the highest rate of transformation.

Meristematic tissues, which presumably have a large amount of mitotic activity, have shown higher levels of transient GUS expression (Serres et al. 1992), as has tissue in which cell division was stimulated with plant growth regulators (McCown et al. 1991). Despite the availability of such information, optimization of particle-bombardment-mediated transformation procedures generally has not considered the effect of the cell cycle, focusing instead on a wide variety of other factors, such as osmotic treatments and physical bombardment parameters (Russell et al. 1992).

The number of stable transformants recovered in this system is but a fraction of the cells which transiently express a reporter gene. Hunold et al. (1994) attributed this to the damage caused by penetration of a microprojectile. The damaged cells appeared to have a limited capacity to survive and go on and divide. Russell et al. (1992) demonstrated that treatments that mitigate damage can also increase stable transformation rates. The data presented here indicate that cell division also plays a critical role in transformation efficiency, and that the MI is related to both rates of transient and stable transformation. Morphology is also important in that in some cultures (such as 802 and 806) there may be a limited number of cells in the outermost cell layer that are capable of division, or at least of division as an independent cell rather than as part of a coordinated group (Williams and Maheswaran 1986). This latter capacity is essential if a transformed cell is to divide into a transgenic embryo.

While studying the MI, a low percentage (~5%) of cells with chromosome numbers other than the expected $2n=40$ were detected (Fig. 6), ranging from 32 to 42. Such aneuploidy is not unusual in cultured tissues, being attributed to a wide variety of factors ranging from cell age to deficiencies or excesses of minerals in the culture medium (d'Amato 1991). This aneuploidy can survive the regeneration process, and has been reported in plants regenerated from this protocol, where it possibly contributes to sterility (Parrott et al. 1994). Nevertheless, recent results by Singh et al. (1998) indicate that the occurrence of chromosomal aberrations is genotype specific.

With the current protocols, transformability of the cell lines is inversely correlated to their competence for conversion into plants. While plants from the first transformation events were fertile, fertility was reduced in plants obtained from subsequent transformation events (Singh et al., 1998). This highlights the need to develop culture protocols for soybean which will permit the efficient transformation of cell lines less than 6 months old.

In conclusion, changes in the culture phenotype which occur with age appear to greatly enhance the ability to recover transgenic cell lines following microprojectile bombardment. However, the use of older cell lines is clearly not desirable, as chromosomal and perhaps genic abnormalities may occur with age, which may contribute to the difficulty in plant recovery and sterility frequently observed in transgenic plants recovered from this process. Hence the key to making this a viable protocol for transformation may lie in the ability to obtain a culture phenotype amenable to transformation while the cell lines are still young. Likewise, any changes in the culture protocols that help reduce the occurrence of aneuploidy or favor the growth of euploid cells in the tissues may increase the chances of recovering plants with the correct chromosome number.

Even within a given cell line, other factors determine the ability to recover transgenic cell lines. Embryogenic tissues of soybean frequently undergo a burst of mitotic activity shortly after transfer to fresh medium. As the tis-

sues appear to be particularly amenable to transformation via particle bombardment during this period of high mitotic activity, any culture manipulation that can increase mitotic activity at any given moment may help increase the frequency with which stable transformation events are recovered, as long as the proper culture phenotype is also present.

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