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EMBRYOGENIC RESPONSE OF MULTIPLE SOYBEAN [*GLYCINE MAX* (L.) MERR.] CULTIVARS ACROSS THREE LOCATIONS

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Summary

Nine soybean [*Glycine max* (L.) Merr.] cultivars representing midwestern, mid-south, and southern US growing regions were evaluated at each of three locations (Athens, GA; Lexington, KY; and Wooster, OH) using uniform embryogenic induction and proliferation protocols in order to evaluate the portability of soybean somatic embryogenic protocols to different locations. The experimental design minimized variation between locations by having all cultivars present at all locations on all days. A quantitative weighted score for primary embryo induction was developed on average embryo number per explant and was used to describe non-embryogenic, poorly embryogenic, moderately embryogenic, and highly embryogenic responses. Ranking of cultivars remained similar across all locations, indicating a uniform transportability of the protocol, at least as far as embryo induction is concerned. Continued proliferation of embryogenic cultures was also measured using a repetitive growth measure but few meaningful conclusions could be made due to the high level of variability including inconsistent growth of cultures between each subculture. Overall, several cultivars were identified as being uniformly embryogenic or non-embryogenic at the primary induction phase at all locations, and we predict that those embryogenic cultivars could be used by any laboratory for high-efficiency induction of embryogenesis. The best of these cultivars, 'Jack', was uniformly responsive across all locations and should be selected as the genotype most likely to yield positive results when attempting to culture and genetically engineer soybeans via embryogenic protocols.

Key words: tissue culture; somatic embryogenesis; environmental effects.

Introduction

Tissue culture and regeneration protocols still remain the cornerstone for transformation of all crops. Soybean has remained particularly recalcitrant to highly-efficient transformation, and recovery of large numbers of transgenic plants remains far from routine, despite the efforts of many research groups (Trick et al., 1997). Organogenic systems have been studied extensively (Cheng et al., 1980; Barwale et al., 1986; Wright et al., 1986; Meurer et al., 1998) and used in combination with transformation protocols to recover transgenic plants (Hinchee et al., 1988; Di et al., 1996; Zhang et al., 1999; Donaldson and Simmonds, 2000).

Embryogenic soybean regeneration systems have also been studied extensively (Lazzeri et al., 1985; Ranch et al., 1986; Finer and Nagasawa, 1988) and have been recognized for their potential to produce large numbers of independently transformed plants (Finer and McMullen, 1991). Embryogenic soybean tissue was first identified as a target for transformation by Parrott et al. (1989a) using *Agrobacterium tumefaciens*-mediated transformation. Additional research showed embryogenic tissues to be amenable to transformation via biolistic particle delivery (Stewart et al., 1996; Maughan et al., 1999; Simmonds and Donaldson, 2000). Recently, Yan et al. (2000) used *Agrobacterium tumefaciens*-mediated transformation of zygotic cotyledons to recover stable transgenic primary somatic embryos, which gave rise to transformed plants expressing a 15-kDa zein protein. Only the cultivar 'Jack' was used in this *Agrobacterium tumefaciens*-mediated transformation of zygotic cotyledon-derived somatic embryos.

The large amount of variation encountered from laboratory to laboratory when using embryogenic soybean cultures for transformation has hindered the identification of optimal conditions for transformation and regeneration of transgenic soybean plants. An optimized embryogenic system could increase the potential for production of large numbers of independent transgenic lines. At present, a limited number of soybean cultivars have shown good response in embryogenic systems (Parrott et al., 1989a; Simmonds and Donaldson, 2000). A majority of these genotypes, identified as embryogenic, have come from maturity groups (MG) 00–III, primarily encompassing Canadian and midwestern US-adapted cultivars (Parrott et al., 1989b; Delzer et al., 1990; Bailey et al., 1993a; Santarem et al., 1997; Simmonds and Donaldson, 2000).

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In the present research, previously identified embryogenic cultivars and additional cultivars representing other maturity groups, particularly those from later maturity groups (later than MG III) were evaluated for their comparative embryogenic response at three locations (Athens, GA, Lexington, KY, and Wooster, OH). Cultivars selected were those previously recognized as highly embryogenic, related to embryogenic genotypes, were mid-south US, southern US, and South American-adapted cultivars, and a variety identified as amenable to *Agrobacterium* transformation (Mauro et al., 1995). The specific goal of this study was to evaluate the reproducibility of results, obtained with uniform protocols, across different laboratories. Failure of results to coincide across locations would then identify particular steps in the regeneration protocol that would benefit from added research to improve their reproducibility across locations.

Materials and Methods

Initiation, induction, and proliferation of cultures. Plants of soybean varieties Benning (MG VII), Defiance (MG III), Fayette (MG III), IAS-5 (MG V), Jack (MG II), Kunitz (MG III), Peking (MG V), Pennyrile (MG IV), and Stonewall (MG VII) were grown in greenhouses at all locations under a 14.5 h/9.5 h photoperiod. Due to wide maturity group differences, seed were planted at 10-d intervals for 4 mo. to ensure that pods of the appropriate stage for all cultivars were available on each harvest date. Immature pods were harvested on the day of use and brought to the laboratory for surface sterilization.

Harvested pods, containing embryos 4–6 mm in length, were rinsed briefly in soapy water, drained, then immersed in 30% bleach solution for 10 min. After immersion, the bleach was decanted and pods were rinsed three times with sterile water. Immature zygotic cotyledons were isolated as in Trick et al. (1997) and placed abaxial side down on D40 induction medium [MS (Murashige and Skoog) salts, B5 vitamins, 3% sucrose, 181 μ*M* 2,4-dichlorophenoxyacetic acid (2,4-D), 0.2% Gelrite, pH 7.0]. Twenty-five explants were placed on each 100×15 mm Petri plate. Plates were wrapped with Nescofilm (Karlan Research Products, Santa Rosa, CA) and placed at 24–27°C and 25–40 μmol m^{-2} s^{-1} light on a 23/1 h light/dark cycle.

At 30 d after culture initiation, each cotyledon was visually scored using the index shown in Fig. 1 and described below. Cotyledons were then cultured on the same plates for an additional 15 d and scored again using the same index.

Scoring and statistical analysis of primary induction. Each zygotic cotyledon explant was scored using the rating system shown in Fig. 1. All visual scoring was conducted with dissecting microscopes [Zeiss Stemi SV8 (University of Kentucky), Leica model M8 (Ohio State University), and Zeiss SZ40 (University of Georgia)]. No assessment was made on embryo quality with all embryos being counted. The score for each experimental unit (i.e. each plate) was calculated by summing the score for each explant on the plate and then dividing by the total number of explants.

The primary induction experiment was designed and analyzed as a completely randomized split-plot design with location as the main effect (whole plot) and genotype by location interaction as the split plots in SAS using the Proc GLM procedure (SAS Institute, 1988). The statistical design had five replications of each cultivar at each location. Duncan's mean separation test was used to separate cultivar by location means. A standard error was determined and also allowed both Akaike's Information Criterion and Schwarz's Bayesian Criterion tests for homogeneity of variance to be calculated (SAS Institute, 1988).

Proliferation and scoring of proliferating embryos. At the completion of scoring the primary induction phase of the experiment, promising cultures were selected for continued proliferation studies. Embryogenic cultures were

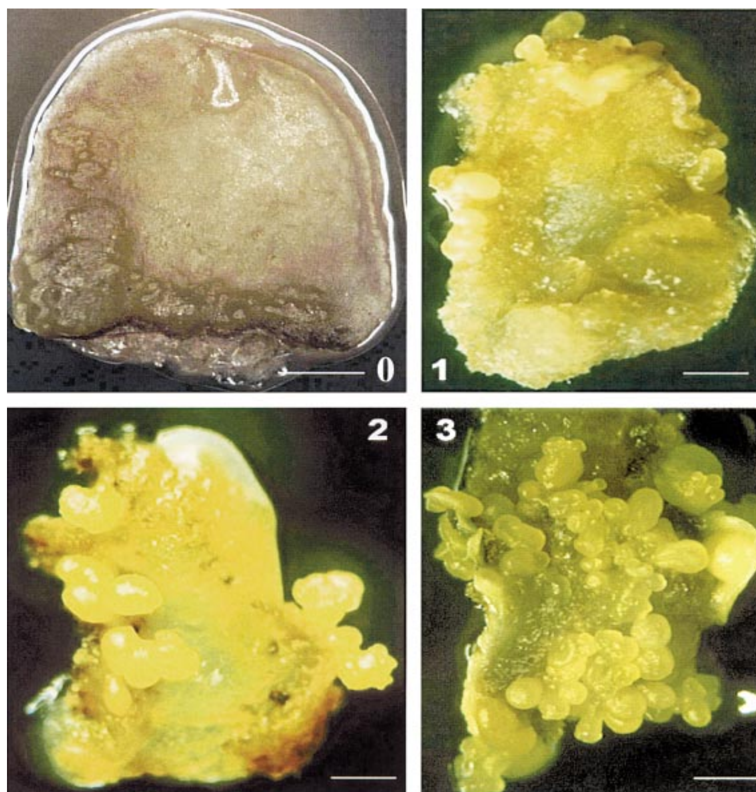

Fig. 1. Visual scoring system of primary embryo induction of each soybean explant. 0 = non-embryogenic with no visible embryos, 1 = moderately embryogenic with 1–5 embryos visible, 2 = embryogenic with 6–15 embryos produced, 3 = highly embryogenic with more than 15 embryos produced.

considered promising if they were of a uniform morphology and had demonstrated evidence of repetitive embryogenesis in preliminary experiments. All cultivar and location combinations were not carried into the proliferation phase due to insufficient embryo induction of some cultivars at some locations. For those genotypes that were selected at each location, 25 embryogenic clumps per genotype were selected, combined, weighed and placed in 100 × 15 mm Petri plates containing D20 proliferation medium (MS salts, B5 vitamins, 3% sucrose, 90 μ*M* 2,4-D, 0.2% Gelrite, pH 5.8). Plates were wrapped in Nescofilm and cultured as previously described for induction. At 30 d after transfer, 25 clumps of promising material were again selected from the entire plate for continued subculture. The total weight of the 25 clumps was recorded, and the tissue was subcultured onto D20 proliferation medium for an additional 30 d. As in the previous subculture, the total weight of 25 selected clumps was recorded again at the end of 30 d. This provided a total of three weights, one for each subculture. The recorded response variable for proliferability was weight gain in mg from initial weight to 30-d weight over each subculture period, with the average gain being the summary weight gain or loss for each period divided by the number of subcultures.

Results and Discussion

Statistical analysis and evaluation of primary induction clearly showed that location (i.e. state) was not a significant factor for either the 30-d or 45-d measurement dates (Table 1). This demonstrates that the existing embryogenic induction protocol is portable across multiple locations. Variability across locations has often been blamed for failure of establishment of a soybean regeneration protocol at other locations. However, we have clearly demonstrated that the use of appropriate protocols should permit the replication of embryogenic induction at any location, as long as an appropriate genotype is used.

The ranking system based on somatic embryo number was sufficient to allow a quantifiable comparison of soybean somatic embryogenesis across locations and cultivars. Additional selection criteria such as amount of green color or color density could be added by scanning images of the plates but the correlation between these traits, or others, and embryogenic potential are not clearly defined (Fig. 2). Initial tissue culture proliferation, however, does not completely reflect transformation or regeneration potential.

Using the 30- and 45-d statistical data analyses, cultivar was a significant factor but cultivar effects were confounded with location due to significant location by cultivar interactions. These interactions only permit analysis of the location by cultivar means, as presented in Table 2. Clear trends were evident for both sets of primary induction data, with cultivars routinely referred to as embryogenic, such as Jack, Kunitz and Fayette, showing up repeatedly with higher means. Likewise, our experiments also demonstrate that certain cultivars such as Stonewall and Defiance were identified as unlikely candidate genotypes for a successful embryogenic system.

Selection of cultivars such as Jack, Kunitz and Fayette should provide a reasonable likelihood of success at any laboratory initiating soybean embryogenic protocols. Although, Kunitz is a near isogenic line of Williams (Bernard et al., 1991), Williams has been difficult to culture in our laboratories. A genetic basis for the somatic embryogenic response has not been clearly demonstrated in spite of studies comparing genetically related cultivars (Bailey *et al.*, 1993b; Tian et al., 1994; Simmonds and Donaldson, 2000). Hence, Williams and Kunitz might be useful cultivars with which to study the genetic aspects of somatic embryogenesis and regeneration in soybean.

TABLE 1

ANALYSIS OF VARIANCE FOR PRIMARY INDUCTION OF SOYBEAN SOMATIC EMBRYOGENESIS

Source	df	30 d		45 d	
		Mean square	P > F	Mean square	P > F
State	2	7.8	0.6	16.57	0.37
Rep(State)	12	88.84	92.25		
Cultivar	8	412.44	0.001*	558.78	0.001*
State × Cultivar	16	254.39	0.001*	192.44	0.002*
Cultivar × Rep(State)	96	308.76	352.23		

* Significant at the 0.05 level of probability.

TABLE 2

MEAN PRIMARY SOYBEAN SOMATIC EMBRYO INDUCTION SCORE OF ALL LOCATION BY GENOTYPE COMBINATIONS

Location	Cultivar	30-d mean	Location	Cultivar	45-d mean
OSU	Peking	2.78 a	UGA	Jack	2.85 a
UGA	Jack	2.74 ab	OSU	Peking	2.80 ab
UK	Kunitz	2.55 abc	OSU	Jack	2.67 abc
UGA	Kunitz	2.55 abc	UGA	Kunitz	2.67 abc
OSU	Jack	2.54 abc	UK	Kunitz	2.64 abc
UK	Fayette	2.37 abcd	UK	Fayette	2.48 bcd
OSU	IAS-5	2.33 abcde	OSU	IAS-5	2.38 bcde
UGA	Fayette	2.27 bcdef	UGA	Fayette	2.37 bcde
UK	Jack	2.22 cdefg	UK	Jack	2.35 bcde
OSU	Kunitz	2.19 cdefg	OSU	Kunitz	2.30 bcde
UK	Pennyrile	2.11 cdefgh	UK	Pennyrile	2.20 cdef
UGA	IAS-5	1.99 defgh	UGA	IAS-5	2.10 def
UGA	Pennyrile	1.95 efgh	UGA	Pennyrile	2.09 defg
OSU	Pennyrile	1.93 fgh	UGA	Benning	2.01 defg
UGA	Benning	1.90 fgh	OSU	Pennyrile	1.96 efgh
UGA	Defiance	1.84 fghi	UGA	Defiance	1.90 efgh
OSU	Fayette	1.66 ghij	UK	Peking	1.80 fgh
UK	Benning	1.65 ghij	UGA	Peking	1.78 fgh
UK	Peking	1.62 ghij	OSU	Fayette	1.77 fghi
OSU	Defiance	1.62 ghij	UK	Benning	1.69 fghi
UGA	Peking	1.54 hij	OSU	Defiance	1.64 fghi
UK	Defiance	1.54 hij	UK	Defiance	1.61 ghi
UK	IAS-5	1.34 ij	UK	IAS-5	1.36 hi
UK	Stonewall	1.27 ij	UK	Stonewall	1.31 hi
OSU	Stonewall	1.22 ij	UGA	Stonewall	1.22 hi
UGA	Stonewall	1.13 j	OSU	Benning	1.20 hi
OSU	Benning	1.13 j	OSU	Stonewall	1.15 i

Means followed by the same letter are not statistically significant at the 0.05 level.

TABLE 3

ANALYSIS OF VARIANCE FOR CONTINUED PROLIFERATION OF SOYBEAN SOMATIC EMBRYOS

Source	df	Mean square	P > F
State	2	66607.79	0.012*
Rep(State)	10	7555.85	
Cultivar	7	6831.95	0.0021*
State × Cultivar	12	3485.98	0.0331*
Cultivar × Rep(State)	22	1423.79	

* Differences are significant at the 0.05 level of probability.

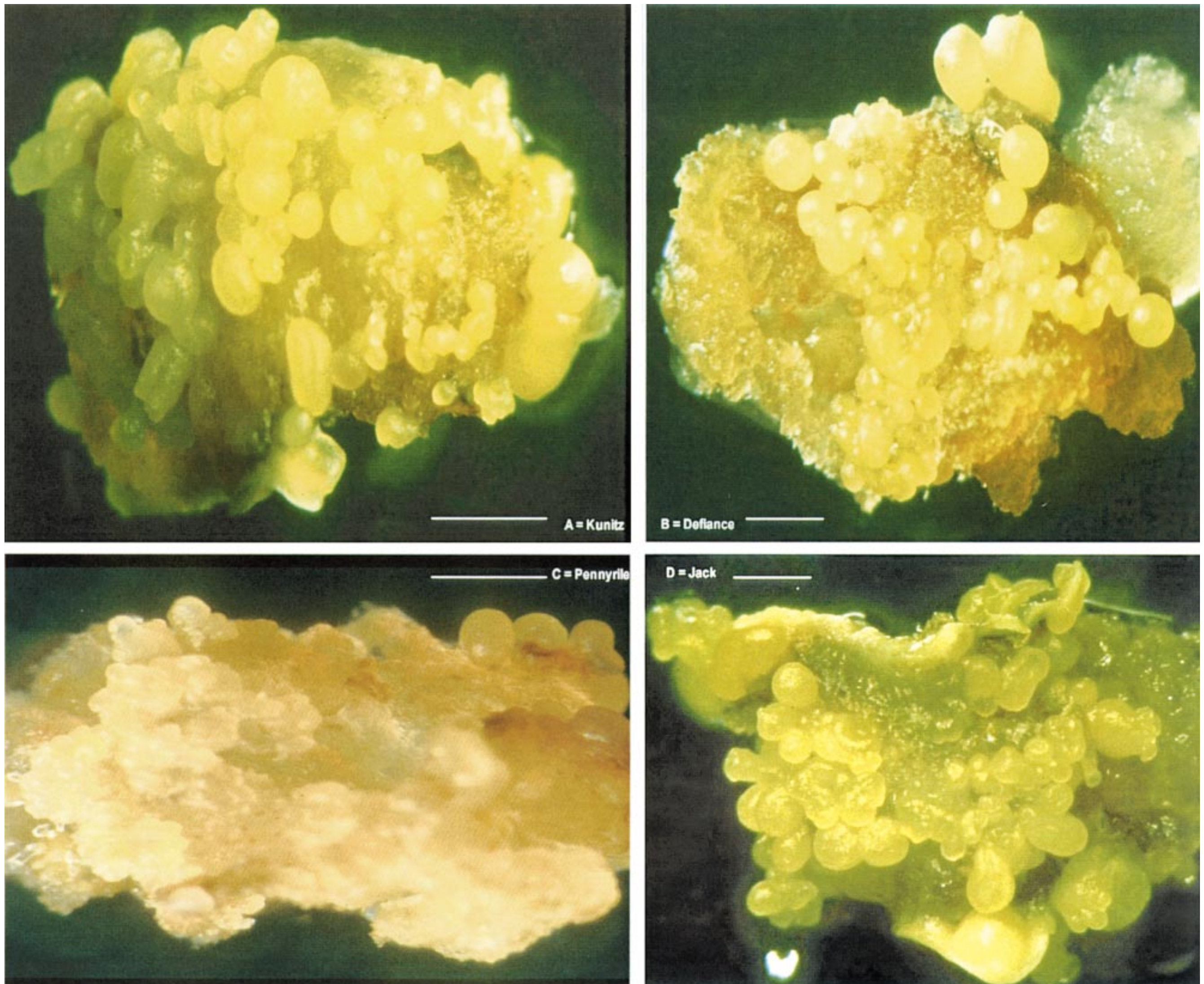

FIG. 2. Explants of four different cultivars chosen for continued proliferation studies demonstrate the level of non-uniformity among explants classified as highly embryogenic.

Measures of repeated embryogenesis were confounded. Statistical analysis showed that location, cultivar, and location by cultivar effects were all significant (Table 3). Due to extremely high standard deviations, it was not possible to obtain means separations. To demonstrate the variability of this data, average means of cultivars by location combinations for continued proliferation over subcultures are presented in Fig. 3. Means were highest, often by a factor of 10, at the Kentucky location and this may reflect a difference in sampling or subculture protocol rather than actual growth gain. This result reflects the variability in soybean embryo proliferation. Many genotypes displayed a negative or divergent growth trend with each subsequent transfer, and no clear pattern was evident across locations. After three subcultures, the only genotypes that displayed stable or increasing growth at all locations were Jack, Kunitz, and Fayette (Fig. 3). The cultivars Pennyrile, Stonewall, and Defiance displayed continued divergent growth patterns indicating a loss of consistent embryogenic proliferation, perhaps due to subtle, but apparently significant differences in culture conditions or subculture technique.

Continuous proliferation of embryogenic cultures is much less precise and more difficult to measure than initial proliferation. Whereas initial proliferation can be described adequately by counting embryo numbers, an accurate descriptor for continued proliferation requires pre-existing skill in soybean embryogenic systems. Continual selection at each subculture for embryogenic masses was required in order to maintain an acceptable morphology. In spite of large efforts, proliferative cultures of many cultivars could not be established (Fig. 3). The absence of a correlation between somatic embryo initiation and continued proliferation of cultivars, or pedigrees, which we observed has been previously reported (Bailey et al., 1993b; Simmonds and Donaldson, 2000). Due to the difficulties in identifying genotypes or genetic backgrounds that respond similarly to tissue culture regimes, soybean somatic embryogenesis has been, and continues

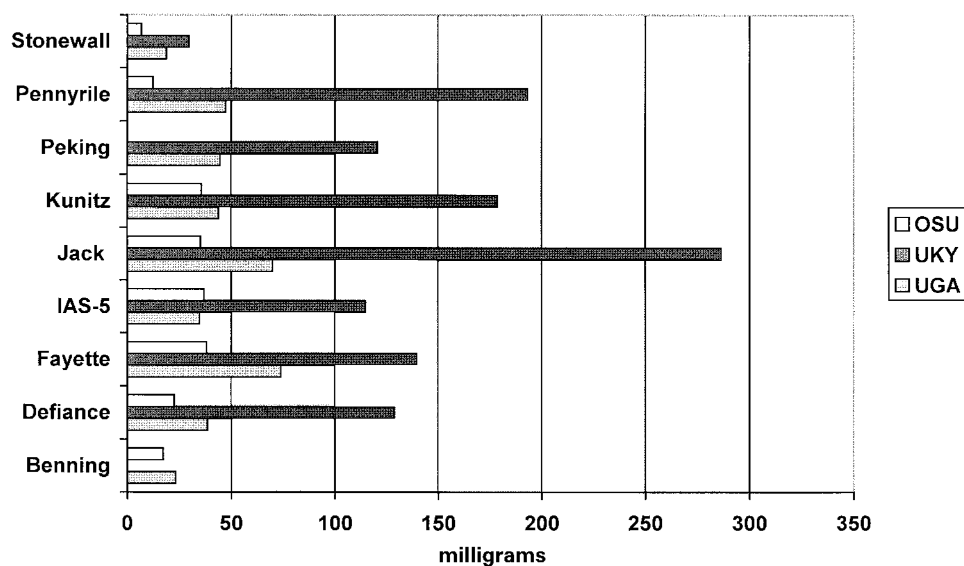

FIG. 3. Graphical representation of mean proliferative growth of cultivar by location combinations.

to be, a difficult protocol to reproduce without using previously identified embryogenic cultivars such as Jack in well-established protocols.

There are two major routes evident to improve embryogenic culture-based soybean regeneration and transformation protocols with the goal of increased recovery of transgenic fertile lines. Either significant time and resources will be needed to screen large numbers of new soybean cultivars and genotypes for embryogenic potential, or existing protocols using cultivars such as Jack will need to be coupled with traditional breeding programs for the express purpose of introgressing transgenic traits into other genotypes. The second option is presently feasible for regeneration of transgenic plants, as the authors have used embryogenic cultures of Jack to genetically engineer traits for insect resistance (Stewart et al., 1996) and protein modification (Yan et al., 2000) into soybean, and have also back-crossed these transgenic traits into other germplasm. Any laboratory desiring to start a soybean embryogenic protocol should be able to realize acceptable levels of success in embryo initiation with genotypes such as Jack, and marker-assisted selection can be used to cross transgenes into additional cultivars, with minimal linkage drag.

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