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Improved tissue culture response of an elite maize inbred through backcross breeding, and identification of chromosomal regions important for regeneration by RFLP analysis

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Summary. The frequency of initiation of friable, embryogenic callus from immature embryos of the elite maize inbred line B73 was increased dramatically by introgression of chromosomal segments from the inbred line A188 through classical backcross breeding. Less than 0.2% of the immature B73 embryos tested (5 of 3,710) formed embryogenic callus. The breeding scheme consisted of six generations of backcrossing to B73 with selection at each generation for high frequency initiation of embryogenic cultures. BC_6 individuals were selfed for four generations to select homozygous lines. The average embryogenic culture initiation frequency increased to 46% (256/561). Nearly all (91%) of the embryos from one BC_6 S_4 plant formed embryogenic cultures.

RFLP analysis was used to determine the locations and effects of the introgressed A188 chromosomal segments. Five segments were retained through at least the fifth backcross generation. The hypothesis that one or more of these five regions contains genes controlling somatic embryogenesis in maize was tested using an F_2 population of the cross A188 X Mo17. A set of five DNA markers (three of them linked) explained 82% of the observed phenotypic variance for percentage of immature embryos forming embryogenic callus. Four of the five markers were located in or near introgressed A188 chromosome segments.

The region marked by probe c595 on the long arm of chromosome 9 was highly associated with several measures of in vitro culture response (percent embryogenic embryos, plants per embryo, and plants per embryogenic embryo). We propose that there is a major gene (or genes) in this region in A188 that promotes embryogenic callus initiation and plant regeneration in B73, Mo17, and probably many other recalcitrant inbred lines of maize.

Key words: *Zea mays* L. – Restriction fragment length polymorphisms – Tissue culture – Plant regeneration – Backcross breeding

Introduction

Genotype, explant source, and environment are all critical factors in the establishment of regenerable tissue culture systems. The discovery of the immature embryo as an explant tissue was a major breakthrough in cereal tissue culture and led to the development of reasonably efficient regeneration systems for some genotypes of most cereal crops on standard MS-based (Murashige and Skoog 1962) media formulations (for recent review, see Bhaskaran and Smith 1990). A better understanding of environmental factors, including growth conditions of donor plants, culture media, and culture incubation conditions, have led to further improvement in culture response. In particular, media modifications have greatly expanded the range of genotypes that can be regenerated after in vitro growth (e.g., Duncan et al. 1985; Close and Ludeman 1987). Unfortunately, even under the best environmental conditions currently known, many agronomically elite genotypes are difficult to manipulate in vitro. Although most genotypes can be regenerated, the systems commonly used are plagued by low initiation frequencies or poor culture quality (i.e., extremely organized callus that is difficult to maintain). While our understanding of optimum environmental conditions for in vitro growth and regeneration of cereal crops is im-

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proving, it is still clearly insufficient to totally overcome the recalcitrance of some genotypes.

Numerous studies have been conducted on the genetic control of plant tissue culture responses. While additive gene effects appear to predominate, dominant, cytoplasmic, maternal, and paternal effects have also been reported (e.g., Nesticky et al. 1983; Tomes and Smith 1985; Hodges et al. 1986; Mathias et al. 1986; Willman et al. 1989; Peng and Hodges 1989). Heritability estimates have been consistently high (Keyes et al. 1980; Lazar et al. 1984; Charmet and Bernard 1984; Koornneef et al. 1987), and attempts to improve response through recurrent selection (Bingham et al. 1975) or limited backcrossing (Koornneef et al. 1987) have been successful. Consistent with the high heritability estimates and ease of breeding, most studies aimed at determining gene number have concluded that relatively few genes are involved (Reisch and Bingham 1980; Miah et al. 1985; Koornneef et al. 1987; Hernandez-Fernandez and Christie 1989; Nadolska-Orczyk and Malepszy 1989; Peng and Hodges 1989; Willman et al. 1989).

Relatively little has been reported on attempts to improve the tissue culture response of maize through classical breeding and selection. Notable exceptions are the reports by Petolino et al. (1988) and Morocz et al. (1990) in which anther and protoplast culture responses, respectively, were dramatically increased by breeding and selecting for highly responsive genotypes. In this paper, we describe the successful transfer of genes involved in the establishment of regenerable tissue cultures from A188 (high responder; agronomically poor) into B73 (low responder; agronomically elite) by backcross breeding. We also report on the identification by RFLP analysis of chromosomal regions that appear to be important for tissue culture regeneration in both B73 and Mo17 (an unrelated, agronomically elite, poor tissue culture response line).

Materials and methods

Culture initiation and plant regeneration

For the A188/B73 backcrossing study, cultures were initiated and plants regenerated as described by Armstrong and Green (1985). Briefly, immature embryos approximately 1.0–2.0 mm in length were aseptically isolated from surface-sterilized half-ears and cultured with the scutellum-side up on N6 medium (Chu et al. 1975) supplemented with 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 100 mg/l casein hydrolysate, and 25 m*M* L-proline (abbreviated N6 1-100-25), and solidified with either 7 g/l Difco-Bacto agar or 2 g/l Gelrite. The remaining half-ear was left on the plant for mature seed development. Cultures were scored for the presence of somatic embryos or embryo-like structures at approximately 14 and 28 days following embryo isolation. Cultures were maintained by subculturing every 2 weeks onto fresh medium of the same composition. Plants were regenerated as previously described (Armstrong and Green 1985).

For the A188/Mo17 study, cultures were initiated and plants regenerated from embryos isolated from self-pollinated ears from F_2 plants and from plants backcrossed to either A188 or Mo17 as described by Willman et al. (1989). Briefly, embryos 1.3–1.8 mm long were aseptically isolated from surface-sterilized ears and placed scutellum-side up on modified MS medium (Murashige and Skoog 1962) containing 0.5 mg/l 2,4-D. Cultures were visually scored for the presence or absence of at least one somatic embryo 3 weeks later. Undivided calli were transferred to regeneration medium, and plantlet regeneration was scored after 3 and 6 weeks (Willman et al. 1989). The culture response of each F_2 plant was then summarized into three classes of phenotypic data: (1) percent embryogenic embryos; (2) plants per embryo; and (3) plants per embryogenic embryo.

Backcrossing method

The backcrossing program was initiated by establishing Type-II (friable, embryogenic) cultures from immature F_2 embryos from a sib-pollinated A188 × B73 plant. A regenerated plant from one F_2 culture was crossed with B73 to produce the BC_1 population. Ten BC_1 plants were selfed and immature embryos from half-ears placed into culture. Plants were regenerated from one BC_1S_1 culture from the BC_1 plant with the highest embryogenic culture initiation frequency, and one of these plants was crossed to B73 to produce the BC_2 population. This procedure was repeated to produce the BC_3 population. Beginning with the BC_3 population plants were simultaneously self-pollinated and backcrossed. The plant with the best tissue culture response from each backcross population (BC_n) was determined by culturing immature embryos from half-ears from the selfed plants, and the corresponding BC_{n+1} population produced from this plant was used to continue the breeding program. After six backcrosses, plants were selfed for four generations with selection for percentage of immature embryos forming embryogenic cultures in an effort to produce homozygous sixth-backcross recoveries of B73 with approximately 100% culture response.

DNA isolation and RFLP analysis

For the A188/B73 study, seed from half-ears remaining on selfed plants was germinated to produce seedling tissue for DNA extraction. Tissue from about 10- to 20- 14-day-old seedlings was pooled for each sample. For the A188/Mo17 study, second earshoots from each F_2 plant in the study were harvested for DNA extraction and analysis.

DNA samples were digested with *Eco*RI (and in some cases also a separate digest with *Eco*RV), then electrophoresed through 0.85% agarose gels using a TRIS-acetate running buffer (pH 8.1). Following electrophoresis, DNA was blotted onto Genetran nylon membranes. The membranes were dried by baking under vacuum, and then blocked before hybridization. Membranes were probed with ^{32}P-labeled RNA prepared using maize DNA probes cloned into Riboprobe vectors (Promega; Madison, Wis). Following exposure to X-ray film, membranes were stripped, regenerated, and stored frozen for later re-use.

Statistical analysis

To determine which RFLP markers were linked to loci contributing to regenerability in the A188/Mo17 F_2 population, the data were first analyzed by single factor analysis of variance. In this analysis the effect of each probe is examined individually, assuming that each probe is a genetic treatment and that each of the three genetic states produced (maternal homozygotes, heterozygotes, and paternal homozygotes) are "levels" within the

Table 1. Percentage of immature embryos forming regenerable cultures on N6 1-100-25 medium for B73 (recurrent parent), FRB73 (an independently maintained source of B73; Illinois Foundation Seeds), and each backcross generation

Generation[a]	Number of ears tested	Total number embryos tested	Percentage of embryos forming regenerable cultures on N6 1-100-25 medium		
			Mean of all ears	Lowest response	Highest response
B73	21	2,248	0.1	0.0	2.0
FRB73	10	1,462	0.1	0.0	1.4
BC_1	10	1,441	4.7	0.0	18.3
BC_2	18	1,904	13.5	0.0	37.0
BC_3	17	1,603	1.3	0.0	6.7
BC_4	18	1,800	9.9	0.0	31.2
BC_5	17	2,477	10.2	0.0	39.3
BC_6	10	1,001	0.7	0.0	3.0
BC_6 S_1	4	384	11.0	0.0	40.0
BC_6 S_2	9	1,153	8.4	0.0	35.4
BC_6 S_3	13	1,289	23.5	0.7	75.7
BC_6 S_4	9	561	45.6	0.0	91.0

[a] Under these conditions about 60% of immature A188 embryos will form regenerable callus (Armstrong and Green 1985)

treatment (Edwards et al. 1987). The data were then analyzed by a variation of multiple regression by leaps and bounds (Furnival and Wilson 1974), a least squares method that evaluates the effect of different combinations of probes and selects the set that explains the most phenotypic variance with the least number of probes (Romero-Severson, unpublished). Both linear and quadratic effects were considered in the regression analysis.

Results

Culture response data for the A188/B73 backcross study is summarized in Table 1. Only 3 out of 2,248 immature embryos of the recurrent parent, B73, formed regenerable cultures when explanted onto N6 1-100-25 medium. These embryos were obtained from 21 different plants grown at three different locations and in different years, and thus represent a reasonable sampling of environmental conditions. The same response frequency (0.1%; 2 out of 1,462 embryos) was observed using a second, independently maintained seed source of B73 (FRB73; Illinois Foundation Seeds, Champaign, Ill).

The percent response averaged across ears for each of the six backcross generations varied considerably, from a low of 0.7% for BC_6 to a high of 13.5% for BC_2 (Table 1). Averaged across all 90 ears, the percentage of BC_1 through BC_6 immature embryos forming regenerable cultures was 7.5%, which is 75-fold higher than the average response for the recurrent parent. Response frequencies for each of the backcross generations were determined at different times, and therefore much of the variation observed between generations is probably due to differences in environmental conditions. Despite the low response of the BC_6 generation, no trend was observed for a decline in culture response with increasing generations of backcrossing. Further evidence that no favorable alleles were lost during later backcross generations is provided by the BC_6 S_1 through S_4 data. As expected, it was possible to quickly and dramatically improve the culture response frequency by selfing and selecting (presumably fixing favorable alleles in a homozygous state). Nearly half (45.6%) of all embryos cultured from BC_6 S_4 plants formed regenerable cultures. Almost all (91.0%) of the embryos from one BC_6 S_4 plant formed regenerable cultures, which could indicate that this plant was homozygous for all favorable culture alleles transferred into B73 from A188. Note that relatively small numbers of plants were examined in this part of this study. Theoretically, similar progress could have been made in fewer generations if larger sample sizes had been used. The culture response from immature embryos of a BC_6 plant compared to the parental B73 line is shown in Fig. 1.

The preceding results demonstrate the effectiveness of backcrossing in improving the culture response of B73. The effectiveness of the breeding program in recovering the genotype of the recurrent parent was addressed using RFLP analysis. A total of 73 probes, distributed reasonably uniformly across the ten chromosome complement of the maize genome, were examined (Fig. 2). The maximum distance of any chromosomal segment to the nearest RFLP marker was 36 map units, and most regions were within 20 map units of a marker. After five backcrosses, the RFLP marker-based estimate of percent recovery of B73 was 95%. Assuming no further improvement in recovery with the sixth (final) backcross and complete fixation of all A188 segments during the subse-

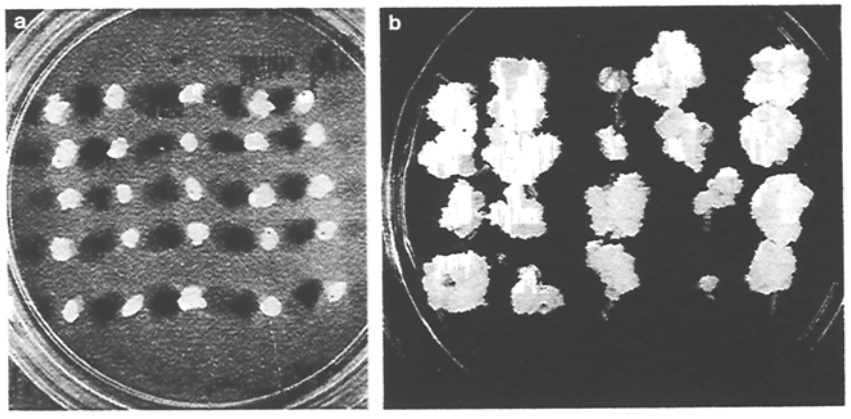

Fig. 1 a, b. Culture response of immature embryos from B73 (**a**) and B73 BC6 (**b**) isolated onto N6 1-100-25 medium. Photograph taken approximately 4 weeks after embryo isolation

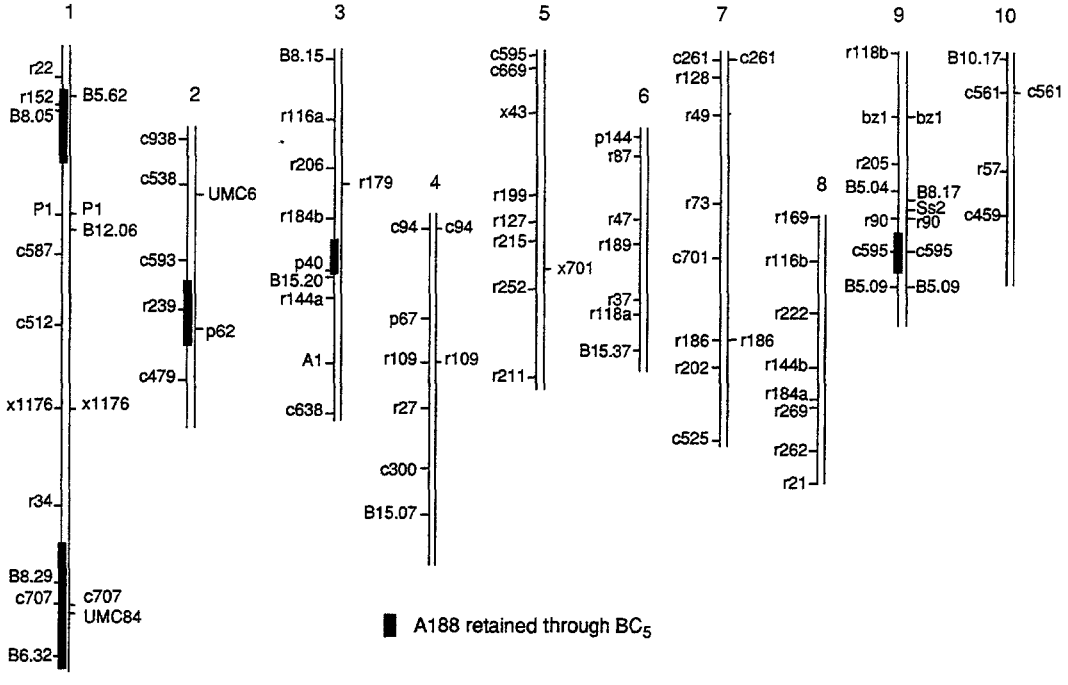

Fig. 2. Linkage map of DNA markers used on the A188 × B73 BC_5 population (*left-side labels*) and DNA markers used on the A188 × Mo17 F_2 population (*right-side labels*). The prefixes *c*, *p*, *r*, and *x* indicate markers from the Agrigenetics Company (Madison, Wis., USA). The prefixes *B* and *UMC* indicate markers from Brookhaven National Laboratory (Long Island, N.Y., USA) and the University of Missouri (Columbia, Mo., USA) respectively

quent selfing and selection, the minimum percent B73 recovery in the BC_6 S_4 material is 90%.

The locations of chromosomal segments from A188 that were maintained through at least the fifth backcross generation are shown in Fig. 2. One or more of these five chromosomal segments (hereafter referred to as "conserved regions") are likely to contain genes important for the establishment of regenerable cultures under the conditions employed in this study. Additional data supporting this hypothesis were obtained from RFLP analysis of an A188/Mo17 F_2 population that had been previously scored for embryogenic culture initiation and plant regeneration (Willman et al. 1989). DNA extracted from second earshoots from 86 plants was analyzed using 21 RFLP markers. Thirteen of the markers were chosen because they reside in or near the "conserved regions", while the 8 remaining probes mark random unlinked regions of the genome (Table 2 and Fig. 2). Three sets of phenotypic data were analyzed: (1) percent embryogenic embryos; (2) plants per embryo; (3) plants per embryo-

Table 2. Results of single factor analysis of variance on RFLP data for 86 individuals from the A188/Mo17 F_2 population. Angular transformation was applied to all phenotypic data before analysis

Probe	Chromosome	% embryogenic embryos	Plants per embryo	Plants per embryogenic embryo
B5.62	1	ns	ns	ns
P1	1	ns	ns	ns
B12.06	1	ns	ns	ns
x1176	1	ns	ns	ns
UMC84	1	ns	ns	ns
c707	1	ns	*(Q)	ns
UMC6	2	ns	ns	ns
p62	2	ns	ns	ns
r179	3	ns	ns	ns
c94	4	ns	ns	ns
r109	4	*(L)	ns	ns
x701	5	ns	ns	ns
c261	7	ns	ns	ns
r186	7	ns	ns	ns
bz1	9	ns	ns	ns
B8.17	9	ns	ns	ns
Ss2	9	ns	ns	ns
r90	9	ns	ns	ns
c595	9	*(L)	*(L)	ns
B5.09	9	ns	*(L)	ns
c561	10	ns	ns	ns

ns, Not significant; *significant at $P<0.05$
L, Linear; Q, quadratic effect

Table 3. Regression statistics for the "best set" of probes as determined by leaps and bounds analysis for three sets of phenotypic data for the A188/Mo17 F_2 population

Probe	Chromosome	Effect	Partial regression coefficient[b]	Standard error	*t*-value
A. Percent embryogenic embryos (multiple $R^2=0.82$)[a]					
c595	9	Linear	0.257	0.052	4.91**
r109	4	Linear	0.212	0.037	5.70**
P1	1	Quadratic	0.104	0.027	3.81**
ss2	9	Linear	0.192	0.076	2.53*
B8.17	9	Linear	0.267 (neg.)	0.083	3.23**
B. Plants per embryo (multiple $R^2=0.70$)[a]					
c595	9	Linear	0.143	0.036	3.99**
c707	1	Quadratic	0.072	0.026	2.79**
gp62	2	Linear	0.095	0.033	2.89**
P1	1	Quadratic	0.068	0.026	2.64*
C. Plants per embryogenic embryo (multiple $R^2=0.34$)[a]					
c595	9	Linear	0.321	0.086	3.75**
UMC84	1	Quadratic	0.197	0.073	2.68*

* $P<0.05$; ** $P<0.01$
[a] The multiple R^2 statistic is an estimate of the proportion of the phenotypic variance jointly explained by the select probe set
[b] The partial regression coefficient is a measure of the degree of phenotypic variance which would be explained by the probe if the effect of the other probes were held constant

genic embryo. Following angular transformation (Steel and Torrie 1980), the data were analyzed using single factor analysis of variance (Table 2) and multiple regression by leaps and bounds (Table 3).

RFLP markers lying in or near four of the five conserved regions identified in the A188/B73 backcrossing study were also found to explain a significant portion of the variation in culture response in the A188/Mo17 F_2 study (Tables 2 and 3). The remaining conserved region, located on chromosome 3, was inadequately tested in the A188/Mo17 study as only 1 marker was used (r179), and it was only loosely linked (approximately 30 map units) to the region of interest. Several other probes in this region were tested but were not polymorphic for A188 and Mo17 using *Eco*RI or *Eco*RV digests. Of the 8 probes (x1176, c94, r109, x701, c261, r186, bz1, c561) marking chromosomal regions unlinked (at least 50 map units) to the "conserved regions", only 1, r109 on chromosome 4, was found to explain a statistically significant portion of the variation in culture response among F_2 plants in the A188/Mo17 population.

A set of 5 DNA markers (c595, r109, P1, ss2, B8.17) representing three separate chromosomal regions explained 82% of the observed phenotypic variance for percentage of immature embryos forming embryogenic callus (Table 3 A). Four of the 5 markers (c595, P1, ss2, B8.17) were located in or near introgressed A188 chromosome segments.

The region marked by probe c595 on the long arm of chromosome 9 is of particular interest. This probe marked a conserved region in the A188/B73 backcrossing study and was found to have a significant linear effect in the single factor analysis of variance for both percent embryogenic embryos and also plants per embryo in the A188/Mo17 study. c595 was also the first probe selected by the leaps and bounds regression analysis for explaining the variation observed between A188/Mo17 F_2 plants for each of the three sets of phenotypic data. Three other markers on the long arm of chromosome 9 were also found to have significant linear effects in one or more of the statistical tests (B5.09, ss2, and B8.17). It seems likely that there is a major gene (or possibly genes) in this region in A188 that promotes embryogenic callus initiation and plant regeneration in both B73 and Mo17.

Discussion

Our results are consistent with at least two other studies (Tomes and Smith 1985; Lowe et al. 1985) in which it was shown that the frequency of initiation of friable, embryogenic (Type-II) cultures from immature B73 embryos on standard MS- or N6-based media formulations containing 2,4-D as the auxin source and 2–3% sucrose was a rare event (less than 1%). Manipulation of growth regulators, osmotic conditions, and other culture medium components has resulted in higher embryogenic culture initiation frequencies, but the cultures are non-friable and difficult to maintain, and therefore unsuitable for some applications (Lu et al. 1983; Duncan et al. 1985; Close and Ludeman 1987). Through backcross breeding we have been able to introgress regions of the A188 genome into B73 that result in up to 91% Type-II culture initiation frequencies from immature embryos on a standard N6-based medium. The backcross-derived lines have a close phenotypic resemblance to the parental B73 line, but careful agronomic evaluations have not been conducted.

At the molecular level RFLP analysis has demonstrated that our backcross-derived lines are at least 90% recoveries of B73. Five chromosomal segments from A188 were conserved through the backcrossing program – two unlinked regions on chromosome 1 and one region each on chromosomes 2, 3, and 9. The hypothesis that one or more of these regions contains genes that are important for tissue culture response was tested using an F_2 population from the cross A188 × Mo17. All but one of the chromosome regions was also associated with tissue culture response in the A188/Mo17 population. The remaining region, on chromosome 3, was inadequately tested (nearest marker 30 map units away). The region marked by probe c595 on the long arm of chromosome 9 was strongly associated with several measures of in vitro culture initiation and plant regeneration. We propose that this region contains a gene or genes that strongly influences the initiation of regenerable cultures from immature embryos of maize.

It is important to note that with the backcrossing and selection scheme used, the probability of recovering five unlinked alleles important for regeneration through repeated generations of backcrossing is low. It is quite possible that only the region on chromosome 9 and perhaps one or two additional loci were actively selected and required for regenerability. Additional data are needed to firmly establish which of these chromosomal regions actually contain genes that control regenerability in corn.

The correspondence observed between the chromosomal regions identified as potentially important in the control of in vitro regeneration in the two different studies of this report (A188/B73 backcross study and A188/Mo17 F_2 population study) is of interest for several reasons. First, B73 and Mo17 have highly divergent genetic backgrounds (Smith et al. 1990). Therefore, it appears that the same genetic factors in A188 can have a positive influence on in vitro culture response regardless of genetic background. Second, different media were used for the B73 and Mo17 studies (N6- and MS-based, respectively). This indicates that the same genetic factors can positively influence culture response irrespective of the basal media used (but note that the plant growth regulators and osmotic conditions in the media for the two studies were roughly equivalent). Third, the studies were conducted under different environmental conditions, indicating that these genetic factors function independently of environmental influences. Finally, different individuals in different laboratories scored the culture response, using somewhat different criteria, in the two studies. This indicates that the genetic influence observed is independent of specific subjective scoring criteria used to describe "regenerability".

Recent studies have shown that the addition of silver nitrate to the culture medium can dramatically improve the frequency of initiation of Type-II cultures from immature maize embryos, presumably by blocking the action of ethylene (Vain et al. 1989a, b; Songstad et al. 1991). Up to 20% of the immature B73 embryos cultured on N6 1-100-25 medium further supplemented with 10 μM silver nitrate formed Type-II callus (Songstad et al. 1991). Since both A188 and the B73 BC_6 lines generated in this study also showed a strong positive response to the addition of silver nitrate (Songstad et al. 1991), it seems likely that the genetic contribution of A188 to the culture response of B73 and Mo17 is mediated through some process other than response to ethylene.

Further study is needed to determine whether genetic factors which affect regenerable culture formation from immature embryos also control the regeneration response from other somatic tissues of maize. Previous studies in both wheat and rye have demonstrated that genes that control regeneration in anther cultures are independent of those that control regeneration from somatic cells (Agache et al. 1988; Lazar et al. 1987). Therefore, it is likely that a separate set of genes than those identified in this study control regeneration from gametophytic tissues of maize.

Additional work is also needed to more accurately locate and identify specific genes controlling regenerability in maize. With more precise mapping, it should be possible to use molecular markers such as RFLPs to facilitate the transfer of regenerability into elite, recalcitrant germ plasm. Perhaps more importantly, however, the development of near-isogenic lines differing widely in culture response should facilitate the study of the physiological and biochemical basis of regenerability. Clearly, there is no inherent "genetic lesion", at least in most maize germ plasm that has been studied, that absolutely

precludes embryogenesis or organogenesis from somatic cells of the scutellum (Duncan et al. 1985; Close and Ludeman 1987). Just as clearly, however, our current understanding of the physiological and biochemical basis of "genetic recalcitrance" is insufficient to allow high frequency establishment of long-term regenerable cultures, suitable for mutant selection or gene transfer experiments, from any desired genotype.

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