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Mapping genes conditioning *in vitro* androgenesis in maize using RFLP analysis

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Summary. This research was designed to map the genes in maize which condition a high response to anther culture using RFLP analysis. A set of 98 S_1 families were developed from the cross of B73 × 139/39-05. In vitro-cultured anthers of 139/39-05 produce numerous embryo-like structures while anthers cultured from B73 produce none. Plants from each of the families were grown in the greenhouse. Tassels were harvested from ten individual plants within each family and pretreated prior to culture. Up to three Petri dishes with 60 anthers each were cultured from each tassel. Response was measured as the number of embryo-like structures per 100 anthers cultured. In excess of 105 RFLP clones were screened to detect polymorphism among the parents. A subset of 75 widely distributed clones were scored in the 98 families. Based on the analysis of the resulting genetic data set, the high anther culture response observed in 139/39-05 is conditioned by two major recessive genes, which are epistatic, and two minor genes. One of the two major loci resides in the proximal region of the long arm of chromosome 3 near the indeterminate gametophyte (ig1) gene. The second major locus maps to the centromeric region of chromosome 9. The minor genes reside on chromosomes 1 and 10. Fifty seven percent of the variability among the 98 family means is explained by a genetic model which includes these four chromosomal regions. Moreover, segregation at these loci explains much of the variability observed within the families.

Key words: RFLP – Anther culture – In vitro androgenesis – Linkage – Epistasis

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Introduction

The maize pollen grain represents the beginning of a short-lived male gametophytic phase during which the vegetative and generative nuclei are delivered to the vicinity of the egg prior to fertilization. Although this stage of the life cycle consists of only a few cell divisions, under certain experimental conditions, immature male gametophytes can be induced to undergo an altered development leading to the production of haploid, embryo-like structures directly, without an intervening fertilization (Raghavan 1990). This remarkable process, known as androgenesis, is the biological basis for a collection of in vitro technologies which show potential for genetic manipulation (i.e., haploid breeding, gamete selection, microspore transformation). Immature maize pollen grains, the so-called micropores, have been used to produce tissue cultures capable of regenerating haploid and doubled haploid plants (Ku et al. 1978). By encouraging abnormal pollen development, culturing the resulting cells and tissues, doubling their chromosome complement, and regenerating plants, one can advance, in a very short period of time, from genetically heterozygous material to completed inbred lines ready for evaluation (Petolino 1990). These tissue cultures have also been used to produce haploid suspension cultures from which totipotent protoplasts have been obtained (Morosz et al. 1990; Mitchell and Petolino 1991).

Unfortunately, only certain maize genotypes have been found to undergo in vitro androgenesis to any measurable degree (Dieu and Beckert 1986; Petolino and Jones 1986). These significant genotypic effects suggest that genetic factors are particularly important in determining the potential degree of in vitro androgenesis. Indeed, progress has been made in developing highly androgenic germplasm by inter-mating microspore-derived

doubled haploids (Petolino et al. 1988; Barloy et al. 1989). Although it would be most desirable to remove the genotype limitation altogether, a more realistic approach to broadening the currently limited germplasm-base which has high androgenic potential, is to transfer androgenic capacity via genetic means. Studies designed to explore the inheritance of androgenic capacity in maize indicated that parents which give rise to androgenic hybrids can be identified (Petolino and Thompson 1987). General combining ability was found to be highly significant such that the average value of a parental line was important in predicting the level of in vitro androgenesis expressed in a given cross. This pattern is usually a function of additive gene effects and their interactions and is typical for quantitatively inherited traits. Although progress can be made by selecting for highly-androgenic genotypes, labor-intensive tissue culture-based screening, coupled with high environmental variation and low heritability, make it inefficient to manipulate in vitro androgenesis.

The resolution of quantitatively inherited traits into their single gene components via linkage with RFLP markers has recently become possible in maize. Genetic linkage maps containing large numbers of markers at close intervals have been constructed (Helentjaris et al. 1985). Associations between the segregation of a given trait and a set of RFLP markers corresponding to known chromosomal positions allow important loci to be identified and open up the potential for marker-based selection (Cowen 1988). The identification of RFLP probes linked to genes conferring high-androgenic capacity would be useful for broadening the germplasm-base amenable to this type of cultural manipulation. The present study was designed to identify loci associated with high in vitro androgenic capacity in maize. The approach involved generating a series of S_1 lines from a cross between a high- and non-androgenic genotype, evaluating the progeny for in vitro androgenic capacity, and searching for associations with segregating RFLP markers.

Materials and methods

Plant material

A total of 98 S_1 families were generated by selfing individual F_2 plants derived from the cross B73 × 139/39-05. Seed of the inbred B37 was obtained from Holden's Foundation Seed, Williamsburg, IA, USA. This genotype, related to the Iowa Stiff Stalk Synthetic, had been previously found to be non-androgenic as evidenced by its total lack of responsiveness to anther culture (Petolino and Jones 1986). The genotype 139/39-05 is an S_4 line derived from a cross between anther-derived doubled haploids (Petolino et al. 1988). This genotype, related to the Lancaster Sure Crop, is highly-androgenic in vitro and typically produces hundreds to embryo-like structures per 100 anthers cultured. Donor plants were grown in a greenhouse during April to May, 1991 in Champaign, IL, USA. Seed was sown individually in pots containing approximately 18 kg of dry soil mix # 3 (Conrad Fafard, Inc., Springfield, MA, USA.) moistened and adjusted to pH 6.0. Plants were maintained under a 16/8 h light/dark photoperiod. Ambient daylight was supplemented with a combination of high pressure sodium and metal halide lamps such that the minimum light intensity 2 m above ground level was 1,500 ft-candles. Greenhouse temperature was maintained within 3 °C of 28 °C during the day and 22 °C at night. The plants were irrigated as needed with a solution containing 400 mg/L of 20-20-20 fertilizer (W. R. Grace & Co., Fogelsville, P.) plus 8 mg/L chelated iron (Ciba-Geigy, Greensboro, NC, USA).

Cultural procedures

Tassel harvest, pretreatment, and surface sterilization procedures were identical to those previously described (Petolino and Jones 1986). Briefly, between 40 and 50 days after planting, tassels containing microspores at the mid- to late-uninucleate stage were excised from the whorl, wrapped in moist paper towels and aluminum foil, and placed in an incubator maintained at 8 °C. After 14 days, the outer leaf sheaths were removed and the tassels were incubated for 20 min in a 0.5% sodium hypochlorite solution and then rinsed with sterile distilled water. Anthers containing early- to mid-binucleate microspores were excised, placed in 20 × 60 mm Petri dishes (60 anthers per dish) containing 10 mL of medium and maintained in the dark at 28 °C. The medium consisted of YP major nutrients (Ku et al. 1978) and N6 minor nutrients (Chu et al. 1978), 27.9 mg/L $FeSO_4 \cdot 7H_2O$, 37.3 mg/L Na_2EDTA, 60 g/L sucrose, and 5 g/L activated charcoal (which was filtered out after autoclaving) adjusted to pH 5.8. After 21 days, embryo-like structures (ELS) appeared. These structures were yellowish-white with diverse shapes ranging from normal bipolar or globular to multi-lobed or otherwise abnormally configured embryos. At 21 days, and weekly thereafter, all ELS larger than 0.5 mm were removed and counted. The total number of ELS produced after 6 weeks was expressed per 100 anthers cultured. Tassels were harvested from ten individual plants within each S_1 family and three dishes of 60 anthers were cultured from each tassel.

RFLP procedures

DNA for each S_1 family was prepared from lyophilized leaf tissue of 25 greenhouse-grown plants as described by Saghai-Maroof et al. (1984). Eight micrograms of each DNA was digested with either *Eco*RI, *Hin*dIII or *Sst*I as suggested by the manufacturer (Bethesda Research Laboratory, Gaitherburg, MD, USA) and separated by agarose-gel electrophoresis. The DNA was blotted onto nylon membranes as previously described (Southern 1975; 1980).

Genomic and cDNA clones were selected from collections of mapped clones kindly provided by B. Burr (Brookhaven National Laboratory, Upton, NY, USA), D. Hoisington (University of Missouri, Columbia, MO, USA), and CERES-NPI, Inc. (Salt Lake City, UT, USA). Probe DNA was prepared using an oligo labeling kit purchased from Parmacia LKB Biotechnology, Inc. (Piscataway, NJ, USA) with 50 microCuries ^{32}P-dCTP (3,000 mCi/mM, NEN). Probes were hybridized to the DNA on the blots. Blots were then washed at 60 °C in 0.25 × SSC and 0.2% SDS for 45 min, blotted dry, and exposed to XAR-5 film overnight with two intensifying screens. One hundred and seven probes, covering all ten chromosomes were used to detect polymorphism between the parents. Of these, 75 (70%) were polymorphic. All polymorphic clones were used to screen the population.

Statistical analysis procedures

Because of the unbalanced nature of the data set, the generalized linear model procedure of the SAS statistical package (Anony-

mous 1991) was used for all analyses. Neither the parents, nor the F_1 were included in the study. Thus, the variability could be subdivided into two sources: among S_1 families and within families. Analysis proceeded in a stepwise fashion. Once the effects of one or more genes were detected further analysis was carried out on the residuals from the previous genetic model. The process ceased when the effects of additional genes could no longer be detected at the 0.01 probability level.

Results and discussion

A minimum of five plants per family were included in the final data set, with most families being represented by eight to ten plants. Ninety-five of the ninety-eight families had at least one plant which produced no ELS. The mean number of ELS per 100 anthers cultured for the entire experiment was 4.5 (Fig. 1). The maximum individual plant response observed in this study was 247.5 ELS per 100 anthers cultured. Four families had at least one plant which produced more than 100 ELS per 100 anthers cultured. Half of the families in the study responded with fewer than one ELS per 100 anthers cultured. Approximately 30% of the variability in the data set was expressed among the family means. Since S_1 lines were used, variability both within and among families arises from genetic as well as non genetic sources.

The preliminary genetic analysis identified two chromosomal regions, each containing two probes, with highly significant effects on in vitro androgenesis. The two chromosomal regions reside on the long arms of chromosomes 3 and 9 (Fig. 2). Either region considered separately explained only 4% of the variability in the data set. Fitting a model which included both chromosomal regions and their interaction resulted in a much more extensive explanation of the data than would have resulted from ignoring the interaction. This model explained more than 47% of the variability among the family means. The means for the nine genetic classes, considering the most informative clone in each region, are given in Table 1. The average number of ELS per 100 anthers cultured for the 139/39-05 double homozygous class was more than 6.5 times the response for any other genetic class. As expected, low frequencies of high responding individuals were observed in families which were heterozygous for both chromosomal regions.

Because of the large effects of genes in these two interacting chromosomal regions, the effects of other genes were difficult to detect. Further genetic analysis proceeded on the residuals from this genetic model. Eighteen percent of the variability in the residual data set was expressed among the family means. Two additional chromosomal regions were identified which jointly explain 3% of the variability in the residual data set, or 17% of the remaining variability among the family means. There was no significant interaction between them. The first of the these regions is on chromosome 1 (Fig. 2). In this region, the heterozygous class has the highest response, followed by the 139/39-05 homozygote. This region was marginally significant in the complete data set, disregarding the effects of the two major genes. The heterozygote advantage was observed in the complete data set as well. This chromosomal region probably has an independent effect on the response since its impact can be detected either by accounting for the major genes or by ignoring them. The second chromosomal region identified in the residual data set is on chromosome 10 (Fig. 1). In this region the 139/39-05 homozygote produced the highest response. This chromosomal region was not identified as contributing to the response in the original data set. Its effect was only detected by accounting first for the effects of the two major interacting genes. This region probably has its effect on the trait by interacting with the two major genes affecting the trait. Both additional regions, on chromosomes 1 and 10, were detected by a single clone each. No other chromosomal regions were found which explain more than 0.8% of the remaining variability, regardless of whether analysis proceeded on the residual data set or on a data set of residuals from fitting a model including the second set of chromosomal regions.

Genes affecting developmental processes at the gametophytic (haploid) level, when examined in a population of microspores, would appear to display additive

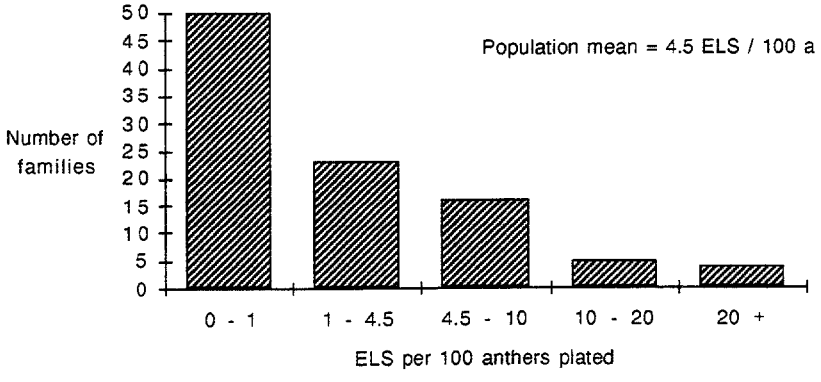

Fig. 1. Frequency table of mean numbers of ELS per 100 anthers plated

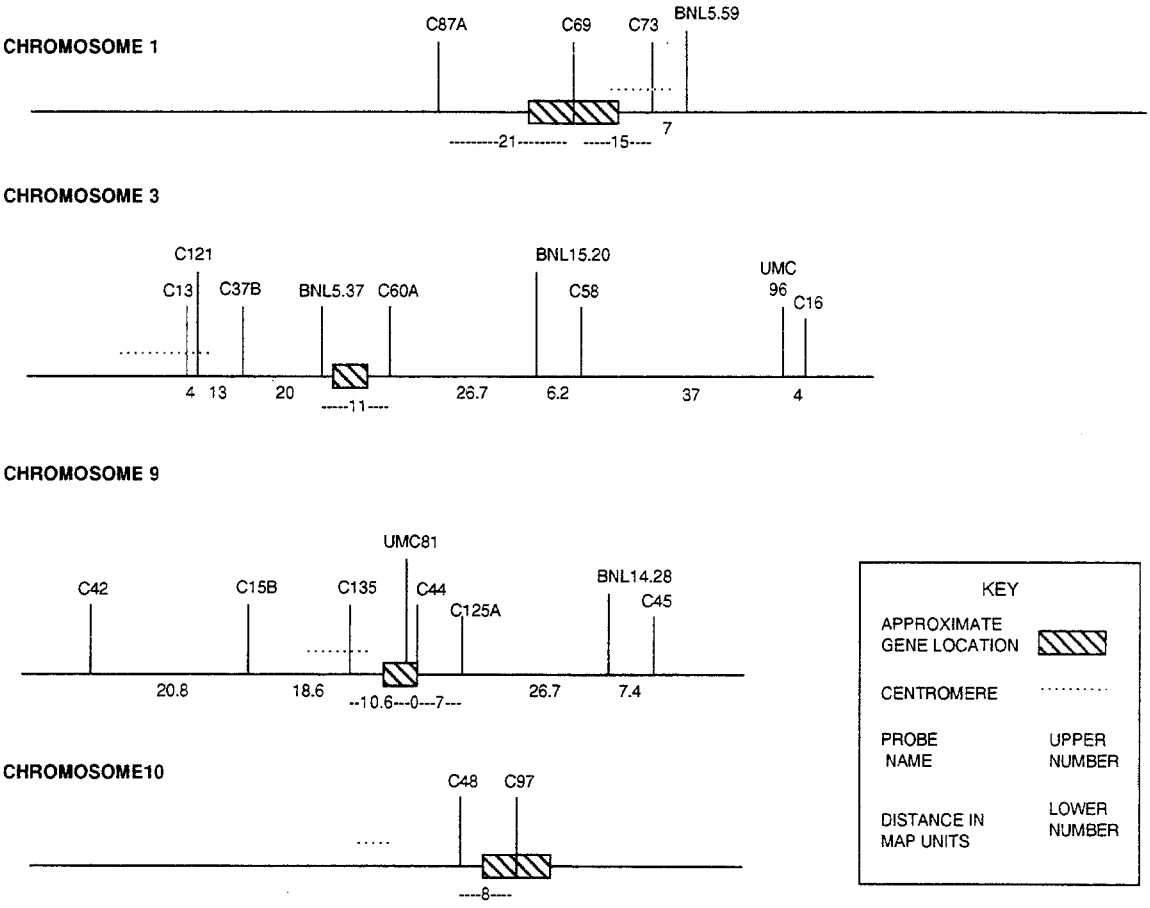

Fig. 2. Approximate chromosomal locations of genes affecting in vitro androgenesis

Table 1. Embryo-like structures per 100 anthers plated for different genetic classes using probes on chromosome arms 3L and 9L

Chromosome 9L probe UMC81	Chromosome 3L probe BNL 5.37		
	B73 homozygote	Heterozygote	139/39 homozygote
B73 homozygote	1.64	3.40	2.66
Heterozygote	1.31	2.04	3.97
139/39 homozygote	3.17	6.62	38.67

gene action. In the present study, the only gene which displayed this type of gene action was on chromosome 10 (Fig. 2) and its effects were not detectable until after the effects of the other loci were taken into account. On the other hand, the identification of two interacting loci on chromosomes 3 and 9 (Fig. 2) suggests that these genes from 139/39-05 conditioning high anther culture response are functioning at the sporophytic (diploid) level (Table 1). Interestingly enough, in the identified region of chromosome 3 (Fig. 2), there exists a known mutant, indeterminate gametophyte (ig1), which affects in vivo androgenesis (Kermickle 1969). This same chromosomal region has also been identified in recent studies designed to map genes conditioning type II callus formation from immature embryos (Armstrong et al. 1992) and haploid callus production from cultured anthers (Y. C. Wan, personal communication).

In summary, there are four chromosomal regions which condition high anther culture response characteristic of 139/39-05 and its derivatives. Having located the genes affecting the response, much of the variability within families was attributable to segregation of these loci. Genes in three of the regions interact, all of which require the 139/39-05 homozygous genotype to maximize response. The identified chromosomal regions acount for more than 57% of the variability among family means.

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