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RFLP analysis to identify putative chromosomal regions involved in the anther culture response and callus formation of maize

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Summary. RFLP analysis was performed with anther culture-derived callus lines developed from the maize F_1 hybrids Pa91 × FR16 (PF), H99 × Pa91 (HP) and H99 × FR16 (HF). Relatively evenly spaced RFLP markers were selected to cover the maize genome with 52, 58 and 35 RFLP markers for the PF, HP and HF callus lines, respectively. The results from populations PF and HP combined with limited information from HF showed that six chromosomal regions on chromosomes 1, 2 (two regions), 3, 6 and 8 appear to be associated with the formation of embryo-like structures (ELSs) from microspores or the subsequent formation of regenerable callus from the ELSs. Regions at the end of the long arm of chromosome 2 and on the long arm of chromosome 8 appear to be associated with ELS formation, and the other regions appear to be associated with either ELS or regenerable callus formation or both. Certain regions that we have identified are the same as those found in other studies to be important for friable, embryogenic callus formation (chromosomes 1 and 3 and near the centromere of 2) and for ESL formation (chromosomes 1 and 3). This study has provided evidence for the genetic basis of the maize anther culture response and callus formation.

Key words: *Zea mays* L. – Embryo-like structure – Callus line – Restriction Fragment Length Polymorphism – RFLP marker

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Introduction

The development of maize inbreds in a conventional breeding program is a time-consuming process. Anther culture offers an attractive alternative for the development of maize inbreds in one generation by producing first haploid plants and then doubled haploid plants through either spontaneous or artificial chromosome doubling at the whole plant (Wu et al. 1983) or callus culture (Wan et al. 1989, 1991) stage. However, the application of this technique to maize breeding has been limited by many factors, in particular by genotype specificity. The screening of a wide range of genotypes for their responses to anther culturing revealed a relatively small proportion of genotypes that were responsive (Miao et al. 1978; Petolino and Jones 1986; Dieu and Beckert 1986; Petolino and Thompson 1987). The anther culture response frequencies of the responsive genotypes are also usually unsatisfactorily low (Petolino and Jones 1986; Dieu and Beckert 1986). The highest anther response frequencies (% anthers which respond) of commercial genotypes are about 18% (Genovesi and Collins 1982) and usually only 1 or a few of about 2,000 microspores per anther can form embryo-like structures (ELSs). This suggests that the anther culture response is a highly selective trait where only those microspores bearing certain favorable genetic factors may respond. The capacity to respond in anther culture has been suggested to be a dominant trait (Sheridan 1982; Petolino and Thompson 1987; Barloy et al. 1989). The results from the evaluation of a large number of anther-derived doubled haploid lines indicated that the genetic determinant may be relatively simple (Barloy et al. 1989). Studies have also suggested that anther culture response and plant regeneration ability are not correlated (Dieu and Beckert 1986; Barloy et al. 1989), indicating that they may be con-

trolled by different genetic factors. Since genetic factors are involved in the anther culture response, the regenerants from anther culture should have the favorable genes that can be accumulated by selection. On the basis of this strategy, lines with much higher response frequencies have been developed (Petolino et al. 1988; Barloy et al. 1989).

In order to utilize anther culture techniques more effectively, a better understanding of the genetic basis of the anther culture response and plant regeneration is desirable. The objective of the study presented here was to identify chromosomal regions which may be associated with the anther culture response or plant regeneration from callus using RFLP analysis of maize anther culture-derived callus lines from F_1 plants. Since the anther culture-derived callus lines should presumably carry the minimum number of genetic factors essential for ELS formation (anther culture response) from microspores and for regenerable callus formation (plant regeneration) from the ELSs, the chromosomal regions bearing these genetic factors should be selectively transmitted. These regions should then be identifiable by RFLP analysis of the anther culture-derived callus lines.

Materials and methods

Anther culture and anther culture-derived lines

Three field-grown maize inbred lines (H99, FR16 and Pa91) were used for 2 years as donor plants. The three F_1 hybrids (H99 × Pa91, Pa91 × FR16 and H99 × FR16) were also anther cultured for 3 (H99 × FR16) or 4 (H99 × Pa91 and Pa91 × FR16) years, respectively. Tassel harvest, cold treatment of the tassel and anther culture on semi-solidified or in liquid medium were according to previously published procedures (Petolino and Thompson 1987). The embryo-like structures which developed from some microspores in the responsive anthers were transferred to callus induction medium (Pescitelli et al. 1989) in an attempt to initiate regenerable callus. Some ELSs developed into compact, nodular, regenerable callus, which was propagated selectively. All of the callus tissue from one ELS is referred to as one callus line. The callus lines of H99 × Pa91 and H99 × FR16, which were developed in 1988, 1989 and 1990, and the randomly selected callus lines of Pa91 × FR16, which were developed in 1988 and 1989, were used for RFLP analysis. The three callus line populations, each of which consists of the callus lines from the F_1 plants of one cross, are designated as PF (Pa91 × FR16), HP (H99 × Pa91) and HF (H99 × FR16) and consist of 50, 23 and 10 callus lines, respectively. The numbers of callus lines in the HP and HF populations were adjusted later to 15 and 9 however since the RFLP pattern indicated that some lines in these two populations had originated from the same microspore.

DNA isolation, Southern blots

Genomic DNA for Southern analysis was isolated from each anther culture-derived callus line and from 2-week-old seedings of each parental inbred line growing in the greenhouse using a protocol developed by Dr. R. M. Hauptmann [described in Menancio et al. (1990)]. Ten micrograms of DNA was digested with restriction endonucleases *Bgl*II, *Eco*RI, *Eco*RV or *Hin*dIII (BRL, Bethesda Research Laboratories Life Technologies). Digested DNA was fractionated by gel electrophoresis on 0.8% agarose gels in TBE buffer and Southern transferred (Southern 1975) to MagnaGraph nylon membranes (MSI, Micron Separations) in 10 × SSC. The DNA was immobilized on the membrane with a UV Stratalinker (Stratagene).

Preparation of radioactive probes

The maize RFLP probes, both UMC and BNL probes from the University of Missouri-Columbia and Brookhaven National Lab at Upton, N. Y., respectively, cloned into the *Pst*I site of the pUC 19 plasmid were kindly supplied by J. Gardiner, University of Missouri-Columbia. The insert fragments were obtained either by amplification of the plasmid (Maniatis et al. 1982) followed by *Pst*I digestion, fractionation on 1% low-melting agarose gel and cutting out of the gel band containing the insert fragment, or by polymerase chain reaction (PCR)-based amplification. For PCR amplification the sequences of 20-base forward and 21-base reverse primers in the universal primer regions were supplied by Dr. T. Helentjaris, University of Arizona (personal communication) and were synthesized in the Genetic Engineering Laboratory of the University of Illinois. The PCR reaction mixture was set up as described by the manufacturer's instructions and amplified with a thermal cycler for 30 cycles. The amplification profile involved denaturation at 95 °C for 30 s, primer annealing at 50 °C for 1 min and extension at 72 °C for 3 min. The probe DNA was radioactively labeled by random-primed DNA labeling according to the manufacturer's instructions (United States Biochemical Corp). The labeled probe was separated from unincorporated nucleotides by column chromatography on Sephadex G-50 in TE.

Hybridization and autoradiography

The blot hybridizations were performed using a protocol of Drs. F. Belanger and A. Kriz (personal communication). The blots were prehybridized for at least 4 h at 42 °C in 0.1 ml/cm^2 of a prehybridization solution [50% formamide, 5 × SSC, 100–200 µg/ml salmon sperm DNA, 5 × Denhardt's solution, 50 m*M* phosphate buffer (pH 6.8), 1% SDS, 2.5% dextran sulfate] and hybridized overnight in a hybridization solution [50% formamide, 5 × SSC, 100–200 µg/ml salmon sperm DNA, 1 × Denhardt's solution, 20 m*M* phosphate buffer (pH 6.8), 1% SDS, 5% dextran sulfate] with the heat-denatured radioactive probe. The blots were then washed in 2 × SSPE containing 0.5% SDS for 15 min at room temperature and 15 min at 68 °C and then in 0.2 × SSPE containing 0.2% SDS for 15 min at 68 °C. The blots were exposed to X-ray film with intensifier screens at −70 °C for 24 h or longer. The probes were stripped from the membranes after autoradiography by washing the membranes in 0.1 *N* NaOH containing 0.2% SDS and then neutralizing them in 0.2 *M* TRIS-Cl (pH 7.5), 0.1 × SSC and 0.2% SDS at room temperature for 10 min. The membranes could be rehybridized about 10 times.

Marker selection and χ^2 test

The RFLP markers used in this study were selected to cover the whole genome with the map distance between two adjacent markers within 50 c*M* so that the maximum distance between a putative gene and a nearby RFLP marker would be about 25 c*M*, a distance within which a linkage relation between the gene and marker may be detected. However, in some chromosomal regions the distance between two adjacent markers used was more than 50 c*M* due to the lack of polymorphic probes (Fig. 1). The parental inbred lines, H99, FR16 and Pa91, were screened for polymorphism with certain combinations of restriction endonuclease enzymes and RFLP markers. The enzyme-marker

combinations which detected polymorphisms between the two parental inbred lines were used to test the haploid callus lines derived from the F_1 cross of those two lines. Thus, three to eight RFLP markers from each chromosome were used for the PF and HP populations (Fig. 1). The segregation ratio in each haploid callus line population for a given RFLP marker was determined from the results obtained on the autoradiographs and analyzed with the χ^2 test for goodness-of-fit to the expected 1:1 ratio.

Because only 35 selected markers were used for the HF lines, the data from this population will be used only as a reference and will not be presented in this report.

Results and discussion

Anther culture and regenerable callus formation responses of three inbred lines and their F_1 hybrids

The three inbred lines (FR16, H99 and Pa91) and their F_1 hybrids (Pa91 × FR16 or PF, H99 × Pa91 or HP and H99 × FR16 or HF) were tested for anther culture and callus formation responses for 2 (inbred lines), 3 (HF) and 4 (PF and HP) years. The pooled response data presented in Table 1 show that FR16 is capable of forming ELSs from some anthers, but none of the 349 ELSs formed regenerable callus, which indicates that this line carries favorable allele(s) of the gene(s) for ELS formation but not for regenerable callus formation. Pa91 was capable of forming both ELSs and regenerable callus from some of the ELSs (3.27%), so this line should have favorable alleles of the genes controlling both of these steps. We were not able to obtain ELSs from the anthers of H99, so no regenerable callus could be obtained. However, results from other studies have indicated that H99 should have favorable allele(s) of the gene(s) involved in the anther culture response (Petolino and Thompson 1987; Wan and Widholm 1992), so the nonresponsiveness of this line is likely due to poor microspore development, which is characteristic of this inbred line.

These results also suggest that different genetic factors control the formation of ELSs and the subsequent formation of regenerable callus, the latter being a trait related to plant regeneration. In addition, when fertilized, immature, diploid embryos of these inbred lines are cultured, H99 and Pa91 embryos are capable of forming regenerable callus at high frequencies, while FR16 embryos do not form any regenerable callus (Duncan et al. 1985 and unpublished data). Thus, the formation of regenerable callus from anther culture-derived ELSs and from immature embryos appears to be similar, suggesting possible common genetic control mechanisms.

The three F_1 hybrids were capable of forming both ELSs and regenerable callus from the ELSs (Table 1). The results suggest that H99 should also carry the favorable allele(s) of the gene(s) for the formation of regenerable callus from the ELSs, since the hybrid H99 × FR16 was capable of forming regenerable callus although

Table 1. Anther culture response and the capacity to form regenerable callus from ELSs of three inbred lines and the three F_1 hybrids between them[a]

Inbred lines or hybrids	Number of anthers cultured	Number of ELSs formed		Number of callus lines formed	
		Total	Per 100 anthers	Total	Per 100 ELSs
FR16	6,480	349	5.39	0	0
H99	7,200	0	0	–	–
Pa91	8,792	153	1.93	5	3.27
Pa91 × FR16 (PF)	47,460	3,603	7.59	231	6.41
H99 × Pa91 (HP)	38,400	241	0.63	26	10.78
H99 × FR16 (HF)	44,640	248	0.56	19	7.66

[a] The data are pooled from the results obtained in 1989 and 1990 for inbred lines, in 1987, 1988, 1989 and 1990 for HP and PF and in 1987, 1988 and 1990 for HF

FR16 itself was not. These results indicate that H99 and Pa91 but not FR16 carry favorable alleles for regenerable callus formation and that all three inbreds carry favorable alleles for ELS formation even though H99 does not express this due to poor pollen development.

RFLP analysis of the anther-derived callus lines of the F_1 hybrids

A total of 75 RFLP probes were used to screen for polymorphism among the three parental inbred lines using the restriction endonucleases *Bgl*II, *Eco*RI, *Eco*RV and *Hin*dIII. Of these, 58 probes revealed polymorphism between H99 and Pa91 with at least one of the enzymes and were subsequently used for analyzing the HP callus lines (Fig. 1). In the case of Pa91 × FR16, 56 probes revealed polymorphism and 52 were used with the PF callus lines (Fig. 1). The frequencies of probes which revealed polymorphism between H99 and Pa91 and between Pa91 and FR16 were 77.3% and 74.7%, respectively.

The segregation ratios of RFLP markers used in the two callus populations, HP and PF, are given in Fig. 1. The markers which showed segregation ratios significantly different from 1:1 could be identified, and most of them were concentrated on certain chromosomal regions (Fig. 1), which indicates that these regions may bear the genes associated with ELS formation (anther culture response) or regenerable callus formation (plant regeneration). Four markers from chromosomes 2 (UMC6, UMC34, UMC131 and UMC5) and 3 (BNL5.37, UMC60, UMC16 and UMC96), respectively, showed segregation ratios highly skewed in favor of H99 alleles in both the HP population (Fig. 1) and the HF population (data not presented). The evidence strongly suggests that H99, but not Pa91 or FR16, may carry favorable alleles for the formation of either ELS or regenerable callus in these regions. Pa91 appears to have favorable

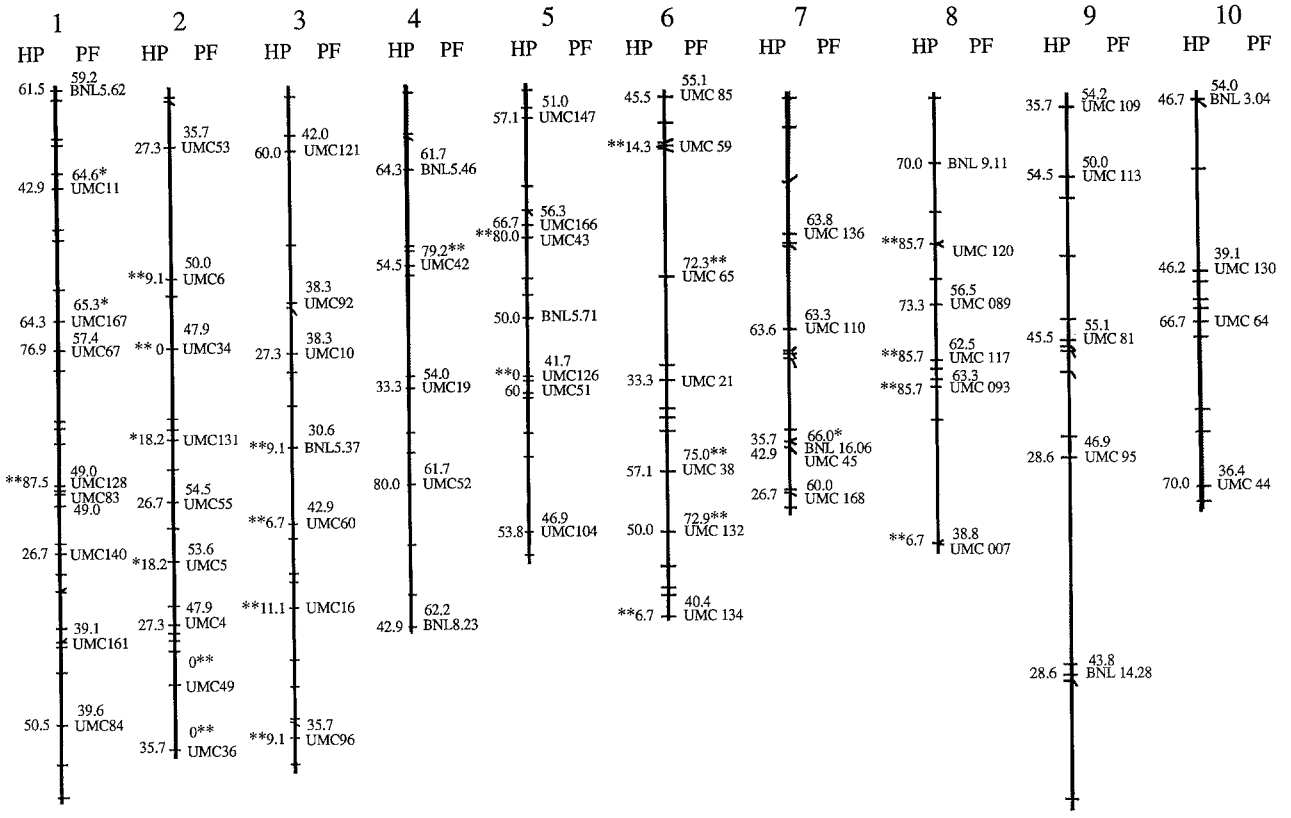

Fig. 1. Maize RFLP map (University of Missouri-Columbia, 1991, copied from the Maize Genet Newsl, vol 65. Note that for reproduction purposes chromosomes 1–5 have been reduced 20% in size.) showing the probes used in this study to measure segregation ratios in two callus populations. The data for the HP population is to the *left* of the chromosome and the data for the PF population is to the *right* of the chromosome. The *numbers* that are immediately *above* or *below* the probe designation are for the PF population and the numbers that are to the *left* of the probe are for the HP population. These numbers are the percentage of callus lines that carried the allele from Pa91. Due to differences in number of callus lines used for the Chi-square tests, the actual numbers that are associated with a significantly deviant ratio will vary. *, ** Significant at the 0.05 and 0.01 probability levels, respectively

Table 2. Possible chromosomal regions of each inbred line carrying favorable alleles for ELS formation or callus formation.

Inbred line	Chromosome number
Pa91	1, 6, 8
H99	2[a], 3, 6
FR16	2[a], 8

[a] There are two regions on chromosome 2, one near the centromere where H99 has the favorable alleles and another at the end of the long arm where FR16 has the favorable alleles

alleles for the formation of either ELS or regenerable callus in these regions. Pa91 appears to have favorable alleles on chromosomes 1, 6 and 8 (Fig. 1). Three markers on chromosome 1 (BNL5.62, UMC11 and UMC128) had skewed ratios toward Pa91 alleles in both the HP and PF populations. Pa91 alleles of 3 clustered markers on chromosome 6 (UMC65, UMC38 and UMC132) were predominant over FR16 alleles in the PF population, but not over H99 alleles in the HP population, which suggests that the H99 and Pa91 alleles are equally favorable while FR16 alleles are detrimental. This conclusion is supported by the data collected on the HF population, where the segregation ratios of all these markers favored the H99 alleles although due to the small population size the results were not significant. The segregation ratios of 3 markers on chromosome 8 (UMC120, UMC117 and UMC93) were in favor of Pa91 alleles in the HP population but not in the PF population. FR16 showed a strong predominance at the end of the long arm of chromosome 2 where 2 markers (UMC49 and UMC36) showed 1:0 (FR16 allele: Pa91 allele) segregation ratios in the PF population (Fig. 1).

In this study we also found that some RFLP markers segregated in a ratio significantly different from 1:1 in one or the other callus line population, but the markers are scattered on different chromosomes, such as BNL 5.37 (chromosome 3), UMC42 (chromosome 4), UMC43, UMC126 (chromosome 5), UMC59, UMC134 (chromosome 6), BNL16.06 (chromosome 7) and UMC7 (chromosome 8) (Fig. 1). Although these markers showed skewed segregation ratios, the markers near some of them had ratios close to 1:1 (such as UMC126)

or ratios that deviated in the opposite direction (i.e. the segregation ratios were in favor of the alleles of other parent, such as UMC7). The regions where these markers are located may bear the genes associated with ELS or regenerable callus formation, but it is also possible that other factors such as genetic background or microenvironment caused the distortion. Also, the small size of the HP population (15 lines) could lead to false distortions.

The chromosomal regions possibly related to ELS formation or regenerable callus formation

As shown in Fig. 1 and summarized in Table 2, H99 may have favorable alleles for ELS or regenerable callus formation in the proximal region of chromosome 2 and long arm of chromosome 3. Apparently H99 also has favorable alleles on the long arm of chromosome 6 since the Pa91 alleles of the RFLP markers in this region also showed predominance over the FR16 alleles, but not those of H99. Pa91 may have favorable alleles on chromosome 1 and the long arms of chromosomes 6 and 8. FR16 may have favorable alleles at the end of the long arm of chromosome 2 and the long arm of chromosome 8. Thus, a total of six regions on chromosomes 1, 2 (the region near the centromere and that near the end of the long arm), 3 (the long arm), 6 (the long arm) and 8 (the long arm) appear to be involved in the ELS or regenerable callus formation. However, as mentioned earlier RFLP data also indicates that other regions may also be involved, but this data is not as firm.

The formation of ELSs from microspores and regenerable callus from the ELSs is apparently under different genetic controls. By combining the information from the anther culture studies and the RFLP analysis, we can reason that FR16 should carry the favorable alleles of the genes for ELS formation, which may be located at the end of the long arm of chromosome 2 and on the long arm of chromosome 8 (Fig. 1, Table 2). In the same region of chromosome 8, Pa91 also carries favorable alleles that may also contribute entirely or partially to the capability of Pa91 to form ELSs. For the other chromosomal regions in which H99 and Pa91 carry favorable alleles (Fig. 1, Table 2), the information available is insufficient to draw conclusions about whether they bear the genes for the ELS or regenerable callus formation.

The same regions on chromosomes 1 (on the short arm) and 3 which we have identified were also shown by Cowen et al. (1992) to be important in ELS formation. Cowen et al. (1992) performed RFLP analysis on 98 S_1 families that were generated by selfing individual F_2 plants derived from the cross of a non-androgenic inbred (B73) and a highly-androgenic genotype (139/39-05). The genotype 139/39-05 is an F_1 hybrid of two anther-derived plants from (H99 × FR16) × Pa91 (Petolino et al. 1988). They identified four regions on chromosomes 1, 3, 9 and 10 as the locations that possibly carry genes affecting in vitro androgenesis.

Our results are also consistent with those of Armstrong et al. (1992) who successfully transferred genes involved in the establishment of friable, embryogenic cultures from immature embryos from a high responder (A188) to a low responder (B73) by backcross breeding. Five regions located on chromosomes 1 (two unlinked regions), 2, 3 and 9, which may bear the genes controlling this response, were identified by RFLP analysis (Armstrong et al. 1992). The regions on chromosomes 1 (the one on the short arm), 2 and 3 appear to be the same or close to those we have identified.

These three studies (Armstrong et al. 1992; Cowen et al. 1992 and the data presented here) have all implicated similar regions on chromosomes 1 and 3 even though Armstrong et al. (1992) was measuring friable, regenerable callus formation, Cowen et al. (1992) ELS formation, while we measured regenerable callus and ELS formation. One might conclude that these chromosomal regions have more than one gene or that certain genes may be important for both responses. The region on chromosome 2 implicated by both Armstrong et al. (1992) and ourselves is likely associated solely with callus formation or plant regeneration.

There are, however, several regions that were not implicated as being important in all of the studies. The most critical is the region on chromosome 9, which was found by Cowen et al. (1992) to be most important for androgenesis and by Armstrong et al. (1992) to be most important for friable, regenerable callus formation. We found no evidence for the importance of this region in our studies. The reason for this discrepancy could be that the studies of Armstrong et al. (1992) and Cowen et al. (1992) used B73, which is a poor responder in both anther culture and tissue culture, so detrimental genes may be located on B73 chromosomal regions including chromosome 9. Armstrong et al. (1992) also used A188 as the high responder, which could give results different from those found with the H99, Pa91 and FR16 genotypes used in the other two studies. We used vigorous F_1 hybrid plants while the other two studies used less vigorous backcross conversions or S_1 families, which could also lead to different results.

The information presented here should help determine the genetic mechanisms involved in the maize anther culture response and plant regeneration ability and help in the selection of anther culture responsive germ plasm. To eliminate the possible effect of gametic selection, which can also result in the deviation of segregation ratios, as observed in studies with rice (Guiderdoni et al. 1989; Guiderdoni 1991), studies on the segregation of the markers in F_2 progeny, which had skewed ratios in this study, may be needed. Further studies can also be carried out to finely map the regions that are highly associated

with the anther culture response by performing more detailed molecular analyses. Further genetic and molecular characterization of the regions identified in the three mapping studies should be carried out to gain more information concerning the biological significance of the genes involved.

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