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DIFFERENTIAL ALLELIC EXPRESSION AT A LOCUS CONTROLLING AN ENDOSPERM PROTEIN IN TETRAPLOID WHEAT (Triticum turgidum)[1]

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ABSTRACT

Two hydrophobic endosperm proteins, designated CM3 and CM3', have been purified from appropriate cultivars of tetraploid wheat (T. turgidum) and characterized. They are inherited as though controlled by alleles at a single locus, designated CM3 and CM3' respectively. The net output of protein molecules has been measured for each of the alleles at 1, 2 and 3 doses. The output of CM3' is 50%-65% of that found for CM3 . For both there is a linear gene dosage response. These effects were observed for the parental material, the reciprocal F_1 generations and the segregating F_2 generation.

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Several cases of differential allelic expression have been investigated in higher plants and animals. These studies offer some insights into certain aspects of gene regulation in eucariotes and are also relevant in connection with the evolutionary significance of protein polymorphism.

Two general types of differential allelic expression may be considered: i) the amount of gene product is the same for two given alleles when these are in homozygosis, but one of them predominates in the heterozygote, and ii) the amount per gene dose of one of the two allelic products is greater both when the homozygotes are compared and in the heterozygote.

The first type is best exemplified by the genetic control of alcohol dehydrogenase in maize. This has been extensively investigated by SCHWARTZ (1966, 1971, 1973, 1976), who has also formulated a competition model for the regulation of gene expression. The inheritance of acid phosphatase activity in maize (EFRON,1973) and to a lesser extent that of peroxidase in Oryza perennis (ENDO, 1971), have also been explained in terms of this model.

A number of cases can represent the second type of differential allelic expression: E_1 esterase in maize (SCHWARTZ, 1962), alpha-amylase (Amy-1) in maize (CHAO and SCANDALIOS, 1971), beta-hemoglobin (β^A, β^{RB}) in man (FARACE and BANK, 1973) and possibly alcohol dehydrogenase in Drosophila (GIBSON 1972; DAY, HILLIER and CLARKE 1974).

Often, the amounts of allelic products have been inferred by measuring enzyme activities, due to the difficulty of measuring an individual protein in a mixture. In some cases the problem has been overcome . Thus, SCHWARTZ (1973) has elegantly confirmed by immunoelectrophoresis his previous results based on densitometric enzyme activity measurements. In other cases, however, an agreement has not been reached with respect to whether the observed differences in activity between alleles are due to different

number of enzyme molecules or to different specific activity of the allelic products. This is the case of Adh (alcohol dehydrogenase) in Drosophila (GIBSON 1972; DAY, HILLIER and CLARKE 1974).

We report here on the differential net accumulation of two allelic variants of a hydrophobic endosperm protein, designated CM3 and CM3' (RODRIGUEZ-LOPERENA et al. 1975). These have been purified from tetraploid wheat (Triticum turgidum) and characterized.

MATERIALS AND METHODS

Biological materials

Triticum turgidum cultivars Senatore Capelli, R. Iznalloz, and Hibrido D (carrying protein CM3) and Ledesma and Fartó B (carrying CM3') were from the Centro de Cerealicultura, INIA, Madrid. Reciprocal crosses and F_2 generations were obtained for the following pairs of cultivars: Ledesma x S. Capelli, Fartó B x R. Iznalloz, Fartó B x Hibrido D.

Electrophoresis and electrofocusing

Horizontal starch gel electrophoresis in aluminium lactate/lactic acid buffer, pH 3.2, 3M urea was carried out as previously described (ARAGONCILLO et al., 1975a).

Combined electrofocusing x electrophoresis was performed by a modification of the method of WRIGLEY (1970): polyacrylamide gels for electrofocusing were 2 x 60 mm, ampholine pH range was 7-9, voltage was brought up to 260 V, never exceeding 0.5 mA/tube, and then kept for 6 hr. Electrophoresis, in the second dimension, was carried out in 27 x 18 x 0.2 cm starch gels at 14 V/cm for 1 hr 30 min. Four electrofocused samples were inserted in each of these gels.

Staining of separated proteins was carried out with 0.05 % water soluble Nigrosine (Fluka catalog no. 1167) in methanol-water-acetic acid (5:5:1) for 16 hr, and destaining with 70 % ethanol after rinsing with tap water (ARAGONCILLO et al., 1975a).

Purification of proteins

A full description of the purification of proteins CM3 and CM3' will be published elsewhere and only an abbreviated account will be given here. Protein CM3 was purified from S. Capelli flour and CM3' from that of

Ledesma. Flours were partially delipidated with petroleum ether (bp 35-60º) and then extracted with chloroform-methanol (2:1). After evaporation of the solvent, the extracts were dispersed in 3M urea, dialyzed against H_2O, and freeze-dried. The freeze-dried material was fractionated in Sephadex-G-100 and the gel filtration peak with mol wt under 25.000 was recovered after dialysis against H_2O,and freeze-dried. Proteins CM3 and CM3' were obtained from this fraction by preparative electrofocusing in polyacrylamide columns (FINLAYSON and CRAMBACH, 1971). Contaminating ampholines in the CM3 preparation were eliminated by trichloroacetic acid precipitation of the protein and acetone extraction of the ampholines. The CM3' preparation was contaminated with some low mol wt gliadins and with ampholines, which were eliminated in a further step of preparative electrophoresis at pH 3.2 and repeated dialysis against H_2O. Both proteins appeared completely pure when subjected to high resolution combined electrofocusing x electrophoresis at overloading conditions.

Aminoacid analysis and molecular weight determinations

Aminoacid analysis of the purified proteins were carried out essentially following MOORE and STEIN (1963). Appropriate aliquots were hydrolized for 24, 48 and 72 hr in a Thermoblock at 110±1º. A Jeol JLC-6AH autoanalyzer was used.

Calculation of the minimum molecular weight, based on the aminoacid analysis, and of the set of analytical values best adjusted to it, was carried out according to DELAAGE (1968), using a Hewlett-Packard electronic calculator (model 9810A) with a function inscriptor (model 9862A).

Molecular weights were also determined by sodium dodecyl sulfate (SDS) electrophoresis as described by WEBER, PRINGLE and OSBORN (1972).

Protein quantitation procedures

Protein in solution were quantitated by micro biuret method, as described by WILLIAMS and CHASE (1968), by the method of LOWRY et al (1951), and by the molar yield of aminoacids after hydrolysis, as indicated in the previous section.

The quantitation of proteins CM3 and CM3', in the different genetic stocks and in individual kernels, was carried out by reflectance densitometry (Chromoscan densitometer, Joyce & Loebl, 654 nm filter) of the stained gels. Densitometric peak height was found to be more reproducible (var. coeff. $s/\bar{x} < 0.05$ for triplicates) than peak area. Peak height varied linearly in two-dimensional maps with the amount of extract up to that corresponding to 3 mg of ground kernel per tube, or with the amount of purified protein up to 10 µg per tube. In some experiments, extracts of the different genetic stocks to be compared, corresponding to a constant amount of ground kernel, were fractionated in parallel, stained with the same Nigrosine solution, and then subjected to densitometry. In these cases, more than 50 kernels (1.5-2.0 g) of each stock were milled in a Culatti mill and a greater amount than that finally needed was delipidated with petroleum ether (bp 35-60º) and extracted 3 times with 70 % ethanol (10 + 10 + 10 v/w). The solvent was eliminated in vacuo from the combined extracts, redissolved in a known volume of 9M urea, and an appropriate aliquot was incorporated into the electrofocusing polymerization mixture.

Individual kernels were handled in a similar manner, after crushing them between two polished metal plates with the aid of a hammer. To quantitate proteins CM3 and CM3' in the F_2 generation, two components of the map, designated R_1 and R_2, which were found not to differ significantly in intensity in the parents and in the reciprocal hybrids, were used as internal standards.

No traces of CM3, CM3', R_1 and R_2, were found in the ground

kernel residue after three extractions with 70 % ethanol.

RESULTS

Aminoacid composition, molecular weight and solubility properties of proteins CM3 and CM3'

The purification of proteins CM3 and CM3' from tetraploid wheat is reported here for the first time, although some of their properties had been previously investigated (GARCIA-OLMEDO and GARCIA-FAURE 1969; GARCIA-OLMEDO and CARBONERO 1970; REDMAN and EWART 1973; ARAGONCILLO et al. 1975b , RODRIGUEZ-LOPERENA et al. 1975). Most T. turgidum cultivars carry protein CM3 and only a few carry CM3', instead (Figure 1). All T. aestivum cultivars analyzed have the CM3 phenotype which, in that case, was found to be controlled by more than one locus (ARAGONCILLO et al. 1975b). This protein has been previously purified from hexaploid wheat (ARAGONCILLO 1973, REDMAN and EWART 1973).

The aminoacid compositions and molecular weights of proteins CM3 and CM3' from T. turgidum are presented in Table 1. Their close relationship is evident from the aminoacid data, which are also quite similar to those for CM3 from T. aestivum (ARAGONCILLO 1973, REDMAN and EWART 1973). The good agreement between the minimum molecular weights, calculated from the aminoacid composition, and the molecular weight determined by SDS electrophoresis, as well as the fact that each of these proteins give only one band by SDS electrophoresis, indicate that we are dealing with only one type of protein chain in each case. REDMAN and EWART (1973) arrived at the same conclusion for CM3 from T. aestivum.

Both CM3 and CM3' have peculiar solubility properties, being soluble in chloroform-methanol mixtures (from 1:7 to 7:1 v/v), slightly

soluble in methanol, and quantitatively extracted with 70 % ethanol. Furthermore, they can be made water soluble after certain treatments (RODRIGUEZ-LOPERENA et al. 1975).

Inheritance of proteins CM3 and CM3'

Kernels from reciprocal crosses between one CM3' cultivar (Fartó B) and two carrying CM3 (R. Iznalloz and Hibrido D) were analyzed by one-dimensional electrophoresis. Both proteins were detected when the CM3 cultivars were used as polen donors, but only CM3 seemed to be present when polen from Fartó B was used. The phenotypes CM3, CM3' and CM3-CM3' segregated in a 2/1/1 ratio in the F_2 generations: 58/28/25 ($\chi^2 = 0.36$, $p < 0.80$) for the F_2 from the cross Fartó B x R, Iznalloz and 54/33/27 ($\chi^2 = 0.94$, $p < 0.50$) for the F_2 from the cross Fartó B x Hibrido D. To ascertain whether CM3' was actually absent from the crosses in which Fartó B was the polen donor, kernels from Fartó B, R. Iznalloz and their reciprocal F_1 generations were subjected to combined electrofocusing x electrophoresis. The four phenotypes observed are shown in Figure 2. Protein CM3' was detected at a low level, by this technique, in kernels from the (R. Iznalloz x Fartó B) F_1. The reciprocal cross yielded a phenotype CM3' > CM3. In the F_2 generation, the phenotypes, CM3, CM3 > CM3', CM3' > CM3, CM3', were easily identified and segregated 1/1/1/1 (36/22/38/32, $\chi^2 = 4.4$, $p = 0.20$). The above data indicate that proteins CM3 and CM3' are inherited as though determined by alleles at a single locus, designated CM3-CM3'. The endosperm is triploid, with two chromosomal complements maternally derived, and thus, the phenotypes CM3 > CM3' and CM3' > CM3 would correspond to the genotypes CM3/CM3/CM3' and CM3'/CM3'/CM3, respectively.

The lower apparent levels of CM3' with respect to CM3 could be due to differential allelic expression or to different specific staining, so this matter was further investigated.

Staining response of proteins CM3 and CM3'

Two types of experiments were performed to compare the staining response (Nigrosine binding capacity) of the purified CM3 and CM3' proteins. In the first type, solutions were made of approximately the same concentration of both proteins. Appropriate aliquots from these solutions were used for aminoacid analysis and for densitometric determinations, after combined electrofocusing x electrophoresis. The molarity of the protein solutions was calculated from the aminoacid analysis data and the staining response from the densitometric readings. The response per mole of CM3' was 1.29 ± 0.16 times greater than that of CM3. In the second type of experiment, solutions of the two proteins were made at exactly the same concentration, as judged by the biuret and by the LOWRY'S methods, and appropriate aliquots were used for densitometric determination, after one-dimensional electrophoresis. The staining response per unit weight of CM3' was greater than that of CM3 by a factor of 1.10 ± 0.06.

Since the molecular weights of the two proteins do not differ significantly (Table 1), both factors have the same meaning. The second one is probably more reliable, because, in the first experiment, aminoacid destruction during acid hydrolysis could be somewhat different for the two proteins. The higher Nigrosine binding capacity of CM3' is consistent with its higher content of basic aminoacids (12 versus 11, see Table 1), because Nigrosine is an anionic dye.

Differential allelic expression at the CM3-CM3' locus

To demonstrate, on a quantitative basis, the differential allelic expression at the CM3-CM3' locus, reciprocal crosses between cultivars S. Capelli (CM3) and Ledesma (CM3') were obtained. The levels of the two proteins were determined densitometrically in the parental material and in the hybrids, after combined electrofocusing x electrophoresis, both on a dry

matter basis and with respect to a reference protein. More than 50 kernels were pooled for each genetic stock and milled. Results of this experiment, represented in Figure 3, clearly demonstrate differential expression. The level of CM3' in Ledesma was 0.65 times that of CM3 in S. Capelli ($0.65 \neq 1.00$, $p < 0.001$), and the CM3/CM3' ratio corresponding to the CM3/CM3/CM3' genotype (3.01 ± 0.24) greater than the CM3'/CM3 ratio in the CM3'/CM3'/CM3 (1.76 ± 0.19; $3.01 \neq 1.76$, $p < 0.01$). In both cases, the amount of gene product increased in an essentially linear manner with respect to gene dosage.

Two-dimensional maps corresponding to individual kernels of the F_2 from the cross Fartó B x R. Iznalloz were also scanned. Results are represented in Figure 4. Since sample loads were not standardized, the levels are expressed relative to a reference protein. Again, the differential allelic expression is demonstrated. The apparent deviation from linearity of gene dosage responses is due to the fact that sample loads were in the upper limit of the linearity range of the densitometric method and, thus, values for the higher dosages, specially those for CM3, were underestimated.

Time of synthesis of proteins CM3 and CM3'

The date of anthesis was tagged to spikes of cultivars of S. Capelli (CM3) and Ledesma (CM3'). Kernel samples were taken periodically during their development, freeze-dried, and analyzed. Both proteins could be barely detected at about 25 days after anthesis and their net accumulation seemed to proceed throughout the rest of the development period.

DISCUSSION

The above results demonstrate differential allelic expression at the CM3-CM3' locus: the net output of protein molecules for allele CM3' is 65 % that of the allele CM3, if the uncorrected densitometric data are used for the calculation,and could be as low as 50 %, if the higher staining response of protein CM3' is taken into account. This lower net output of the CM3' allele is attained both in homozygosis and heterozygosis (allele CM3 absent or present), so it seems we are dealing with a case of the second type of differential expression described in the introduction of this paper.

The present results are similar, in some respects, to those obtained by CHAO and SCANDALIOS (1971) for the Amy-1 locus in maize. The apparent absence of the Amy-1^B product in some tissues with the Amy-1^A/Amy-1^A/Amy-1^B genotype could be the result of the same technical problem faced by us in the study of CM3-CM3' segregations by one-dimensional electrophoresis. These workers were somewhat at a disadvantage having to deal with enzyme activities, instead of protein levels.

In our case, the differential expression does not seem to be based on the late switching on of the CM3' allele, as described for some eucariotic systems (WRIGHT and MOYER 1966; COURTRIGHT 1967; OHNO et al. 1968, 1969; CASTRO-SIERRA and OHNO 1968; HITZEROTH et al. 1968; GOLDBERG, CUERRIER and WARD 1969; KLOSE and WOLF 1970), because synthesis of both CM3 and CM3' seems to be started at about the same time.

SCHWARTZ (1962) has described the regulatory mutants $E_1^{F'}$ and $E_1^{S'}$, at the E_1 locus in maize, which are switched off earlier than the normally active alleles, E_1^F and E_1^S, from which they were derived. Again, this does not seem to be the case for the CM3-CM3' system, because net accumulation of these proteins occurs in the late stages of kernel development.

In the case of β^{RB} hemoglobin (FARACE and BANK 1973), the lower

expression of this allele has been tentatively ascribed to either reduced transcription, an unstable mRNA, or to a deffect in the initiation of translation. The linear gene dosage response found for CM3 and CM3' suggests that transcription is limiting gene expression. This would point to reduced transcription or more unstable messenger as plausible causes of the differential expression, but does not exclude differences at the translation level as an explanation.

Modifying or regulatory genes have been proposed to explain the apparent discrepancies between the results obtained by GIBSON (1972) and by DAY, HILLIER and CLARKE (1974) with respect to the net output of alleles Adh-F and Adh-S in Drosophila. EFRON (1973) has also presented evidence for a gene that controls the level of expression of the AP_1 (acid phosphatase) locus in maize. Whatever regulatory elements could be responsible for the observed differential expression of alleles CM3 and CM3', would have to be closely linked to the structural genes, because this phenomenon is also observed in the segregating F_2 generation.

SCHWARTZ (1976) has obtained a mutant of the Adh-F (alcohol dehydrogenase) allele, designated Adh-F278, that is expressed at half the level of Adh-F in certain tissues. He has suggested that the Adh-F allele is associated with two promoter-like sites, one of which has been eliminated or rendered non functional in the Adh-F278 mutant. The present results, where one of the alleles is expressed at about half the level of the other, could be perhaps explained in these terms, if competition with other genes is postulated (SCHWARTZ 1971) or if no competition takes place.

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TABLE 1

Aminoacid composition and molecular weight of proteins CM3 and CM3'

Aminoacid	CM3		CM3'	
	Found	Nearest integer	Found	Nearest integer
Lysine	2.5	3	2.4	2
Histidine	1.7	2	1.7	2
Arginine	5.9	6	7.9	8
Aspartic acid	7.4	7	7.1	7
Threonine	6.3	6	5.3	5
Serine	7.9	8	7.9	8
Glutamic acid	14.3	14	13.6	14
Proline	13.0	13	12.5	13
Glycine	8.6	9	8.6	9
Alanine	8.3	8	8.1	8
Cystine/2	> 6.8	> 7	> 5.4	> 5
Valine	7.7	8	7.7	8
Methionine	1.1	1	1.0	1
Isoleucine	4.4	4	4.5	5
Leucine	12.2	12	12.4	12
Tyrosine	4.8	5	4.1	4
Phenylalanine	3.9	4	4.0	4
Total nº of residues *		110		110
Minimum mol wt*		11.983		12.016
SDS mol wt †		13.000		13.300

* Tryptophan was not analyzed. This aminoacid and cystine/2 were not included in the calculations

† Molecular weight determined by sodium dodecyl sulfate electrophoresis.

FIGURE 1. - One-dimensional electrophoresis of: 1) Chloroform-methanol extract of Ledesma endosperm (CM3'). 2) Purified CM3' protein. 3) Purified CM3 protein. 4) Chloroform-methanol extract of S. Capelli endosperm.

FIGURE 2. - Combined electrofocusing (I) x electrophoresis (II) of 70 % ethanol extracts from (Fartó B x R. Iznalloz) F_2 kernels. The observed phenotypes are shown: CM3, CM3 > CM3', CM3', and CM3' > CM3. Map components R_1 and R_2 were used as reference proteins in experiments of Figures 3 and 4, respectively.

FIGURE 3. - Levels of proteins CM3 (▲ Δ) and CM3' (● O) as a function of gene dosage, on a dry matter basis (▲ ●) and relative to map component R_1 (Δ O). S. Capelli (CM3/CM3/CM3); Ledesma (CM3'/CM3'/CM3'), and their reciprocal hybrids (CM3/CM3/CM3' and CM3'/CM3'/CM3) were analyzed. Equal amounts of each genetic stock were extracted and aliquots representing equal proportions of the extracts were subjected to combined electrofocusing x electrophoresis. Each point represents the average of six determinations.

FIGURE 4. - Levels of proteins CM3 (Δ) and CM3' (O) as a function of gene dosage in kernels of (Fartó B x R. Iznalloz) F_2. The levels were expressed relative to map component R_2. The amounts of ground kernel corresponding to the extracts inserted in each gel was not standardized, but were about 4-5 mg. These are somewhat outside the range of linear relationship between densitometric peak height and extract load. Map component R_1 could not be measured in these gels because of overloading. Each point represents the average ± SEM of 16 kernels.

Running title:

DIFFERENTIAL ALLELIC EXPRESSION IN TETRAPLOID WHEAT

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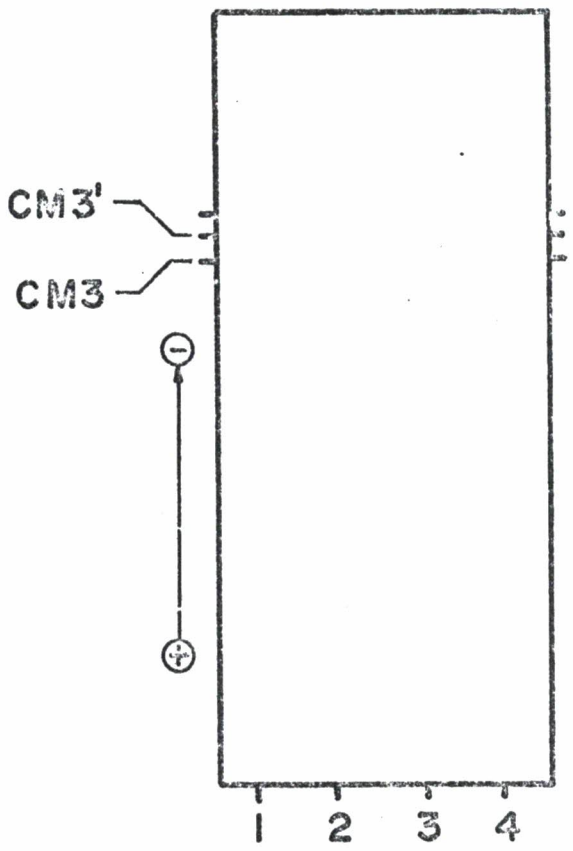

Figure 5: Salado et al

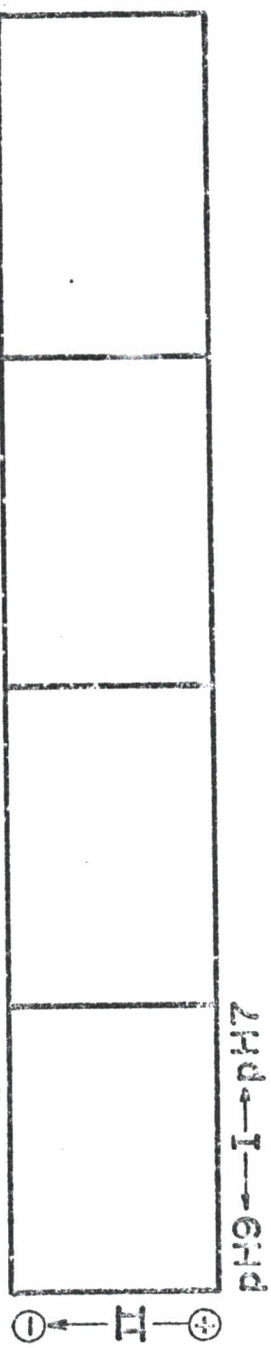

Figure 2. Salcedo et al

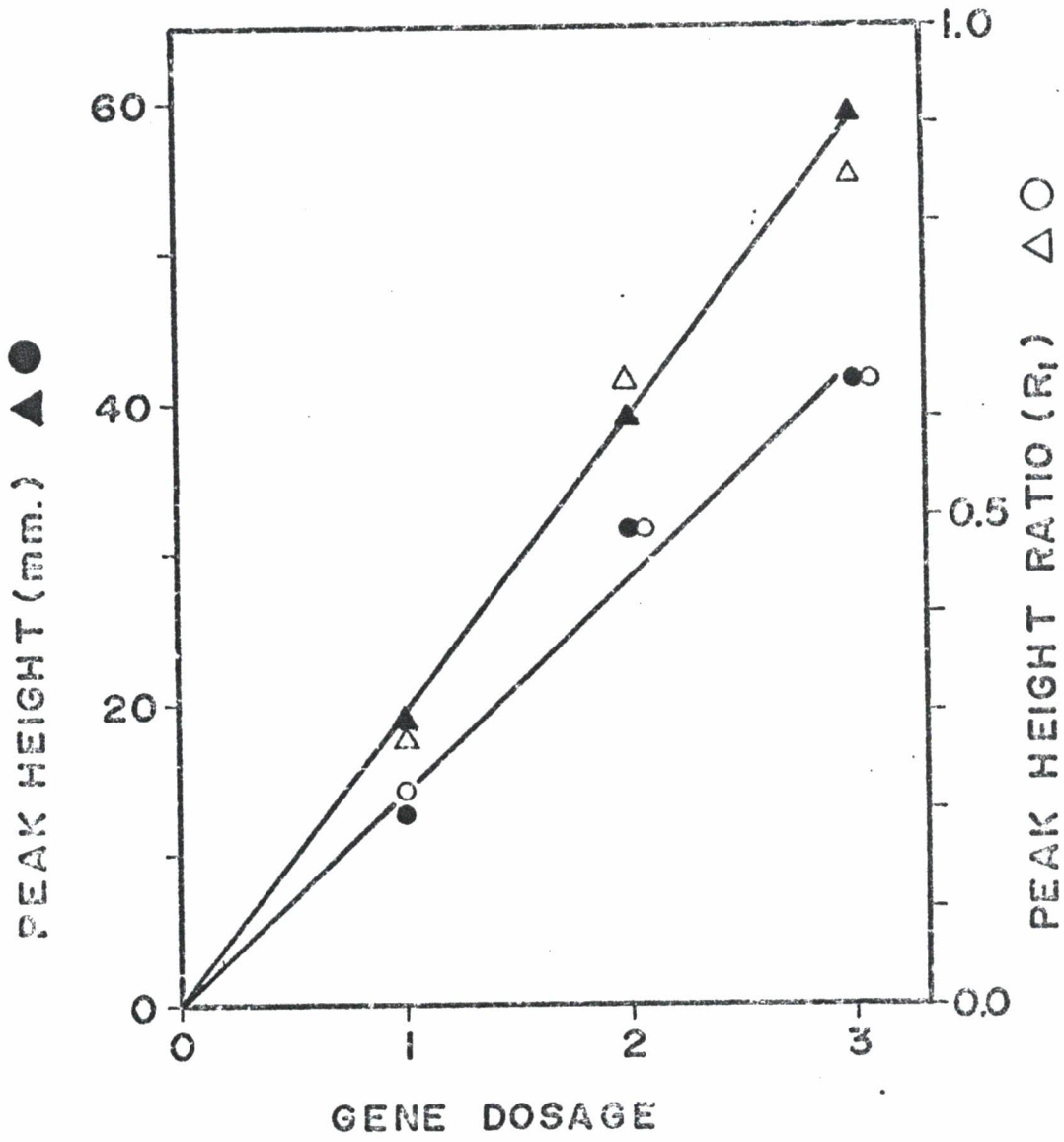

Figure 3 Sakaguchi et al

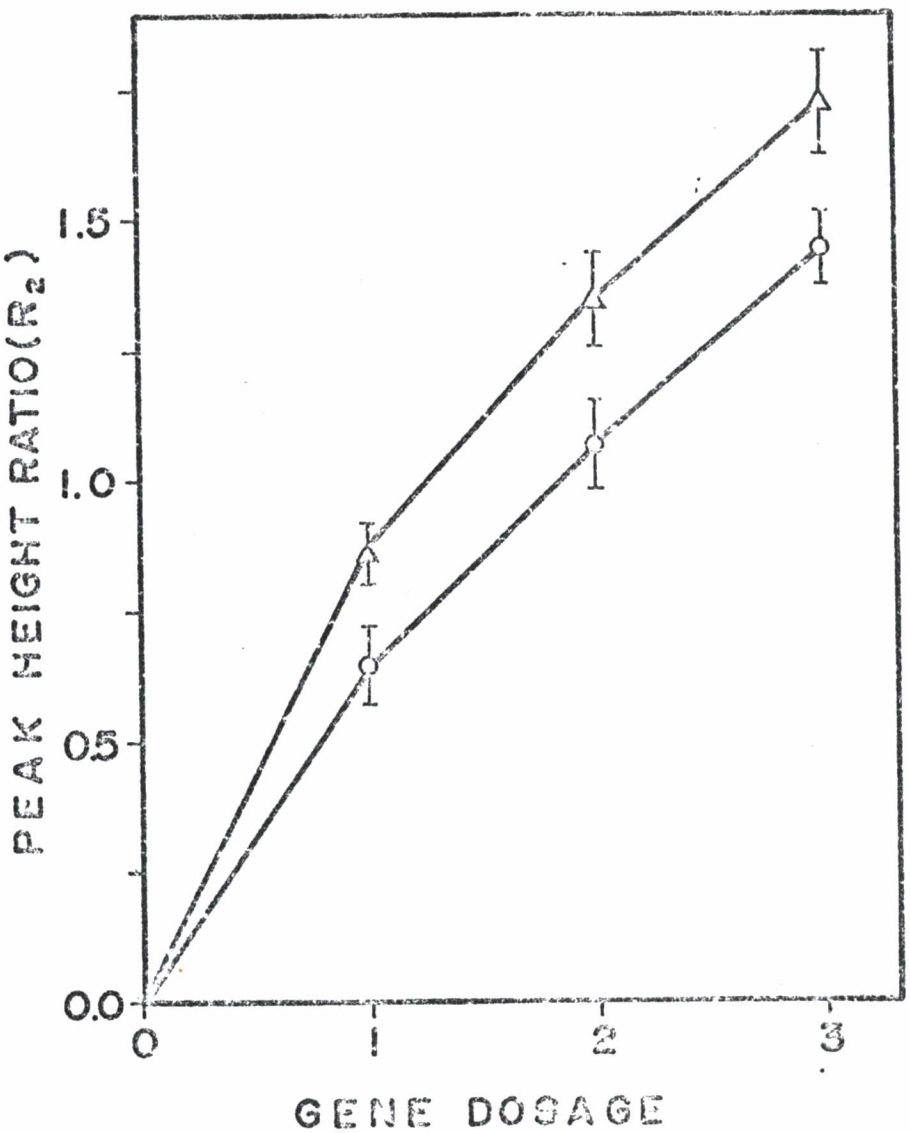

Figure 4 - Selection Trial