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Evidence for Triplicate Genes for Alcohol Dehydrogenase in Hexaploid Wheat*

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Abstract. The alcohol dehydrogenase zymogram phenotypes of 40 wheat strains, each possessing a distinctive chromosomal constitution, were determined. Genes involved in the production of alcohol dehydrogenase were located on the beta arm of chromosome 4A and on chromosomes 4B and 4D. The results support the hypothesis that the active alcohol dehydrogenase isozymes are dimers composed of the six possible combinations of subunits coded by triplicate structural genes.

Introduction. A long series of cytogenetic and genetic studies of tobacco,[1,2] cotton,[3] and wheat[4,5] have convincingly demonstrated that the possession of an extensive quantity of duplicated genetic material is both a characteristic and significant feature of allopolyploids. A generalized understanding of the genetic and evolutionary consequences of this extensive duplication, in terms of its effects upon specific genes, has not yet been derived. This is largely because the analysis of individual genes in allopolyploids by classical genetic techniques has been restricted to the morphological and physiological levels of gene expression.

By employing the zymogram technique, the analysis of individual genes may be carried to the level of their catalytically active enzymatic products. Significant results have been obtained by the recent application of this technique to problems of chromosome homoeology and genetic control in allopolyploids.

This paper reports the results of a study of the genetic control and subunit structure of the enzyme alcohol dehydrogenase (E.C.1.1.1.1.) in wheat, utilizing principally a nullisomic-tetrasomic series of hexaploid wheat (*Triticum aestivum* L. cultivar Chinese Spring) developed by Sears.[5] Brewer *et al.*[6] have used this material to demonstrate that a gene (or genes) for alkaline phosphatase is located on chromosomes 4B and 4D, and Barber *et al.*,[7] using similar material, have reported obtaining evidence which suggests that each of the homoeologues of group 3 is involved in the production of certain esterase isozymes. An earlier genetic study of the dicoccon group (formerly *T. dicoccum*) of *T. turgidum*, a tetraploid wheat, provided evidence that alcohol dehydrogenase is a dimer and led to the postulation that duplicate structural genes are involved in its production.[8]

Materials and Methods. The 21 different chromosomes of hexaploid wheat have been classified into seven homoeologous (related) groups of three,[4] based on the finding that the deleterious effects of nullisomy for each chromosome of each genome

can be reduced or eliminated by making tetrasomic a specific (homoeologous) chromosome belonging to either of the two other genomes.[5] There are 42 possible compensating nullisomic-tetrasomic combinations, six for each of the seven homoeologous groups. All have been studied except four (nulli-2A tetra-2B, 2A-2D, and 4A-4D, which are sterile or nearly so, and 4A-4B). Also studied was a strain monoisosomic for the alpha arm of chromosome 4 (that is, a strain deficient for the beta arm of 4A and possessing two representatives of the alpha arm in the form of an isochromosome) and the variety Chinese Spring.

Vertical polyacrylamide slab electrophoresis (24-slot gels) was employed in these studies, using modifications of methods previously described.[8] The acrylamide slab was composed of 12.0 g Cyanogum-41, 0.3 ml *N, N, N', N'*-tetramethylethylenediamine, 1.2 mg riboflavin, and 228 ml Tris-HCl buffer (0.38 M Tris, titrated to pH 8.55 with HCl). A 1:19 dilution of a stock solution of pH 8.3 Tris-glycine buffer (6.0 g Tris and 28.8 g glycine/liter) was used in the reservoirs. Electrophoresis was performed for 6 hr at 150 V with the apparatus maintained at 7°C by a circulating refrigerant. Each gel was stained for alcohol dehydrogenase activity by incubation in a solution composed of 72.0 ml Tris-HCl buffer (0.069 M, pH 7.5), 4.0 ml $MgCl_2$ (0.1 M), 4.0 ml NAD (0.005 M), 4.0 ml Nitro Blue Tetrazolium (0.006 M), 4.0 ml NaCN (0.03 M), 4.0 ml phenazine methosulfate (0.002 M), and 8.0 ml ethanol (44%).

Alcohol dehydrogenase is a component of the embryo and the starchy endosperm and aleurone of the wheat grain. The alcohol dehydrogenase zymogram phenotypes obtained for each of these tissues and for whole grains are indistinguishable.[9] Further, no variation has been detected in the zymogram phenotypes, in terms of either the presence or absence or the relative staining intensities of the alcohol dehydrogenase bands, during the period from 4 days after pollination through full grain maturity, nor during a period of water imbibition by mature grains of up to 48 hr.

For this study, mature grains were soaked in water for 18 hr before being individually macerated in 0.3 ml of a 12.5% (w/v) sucrose solution. After centrifugation the supernatant was used for electrophoresis. In any one slot, material from only one grain was used. A minimum of three grains of each of the strains was studied, and the extract from each grain was placed in each of two slots. Since hexaploid wheat is self-fertilizing, no intergrain variability was expected nor found within any strain.

Results. Three distinct alcohol dehydrogenase zymogram phenotypes, differing with respect to the presence or absence and the relative staining intensities of their bands, were observed among the 40 strains examined (Fig. 1). Chinese Spring produces a zymogram phenotype on which three bands are observable (I, Figs. 1 and 2). The two more cathodic bands (bands 2 and 3) stain much more intensely than the anodic band (band 1). Phenotypes indistinguishable from that of Chinese Spring were produced by each of the nullisomic-tetrasomic combinations of homoeologous groups 1, 2, 3, 5, 6, and 7.

Differences between the zymogram phenotypes occurred when the chromosomes of group 4 were varied. When the beta arm of chromosome 4A is absent from the grain, bands 1 and 2 are missing (III, Figs. 1 and 2). This indicates

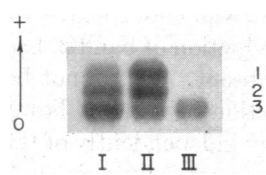

FIG. 1.—Photograph of the three ADH zymogram phenotypes observed among the 40 strains examined. *I:* Phenotype of the strains Chinese Spring, nulli-4D tetra-4B, nulli-4B tetra-4D, and each of the nulli-tetra combinations of groups 1, 2, 3, 5, 6 and 7. *II:* Phenotype of the nulli-4D tetra-4A and nulli-4B tetra-4A strains. *III:* Phenotype of the strain monoisosomic for the alpha arm of chromosome 4A. The bands of ADH activity are identified by the numbers on the right. Migration was toward the anode from the origin, as indicated by the arrow.

that 4A (specifically, the beta arm of 4A) contributes to the production of both bands 1 and 2. That 4A alone of the three homoeologues contributes to band 1 is indicated by the finding that tetrasomy for 4A, combined with nullisomy for either 4B or 4D, produces a phenotype in which the intensity of band 1, relative to that of bands 2 and 3, is greatly increased over that which occurs when there is disomy for each of the homoeologues of group 4 (II, Figs. 1 and 2).

Band 3 of the phenotype produced by grains nullisomic for either 4B or 4D, and tetrasomic for 4A, is much less intense, relative to the other bands, than in Chinese Spring (II, Figs. 1 and 2). This indicates that 4B and 4D are both involved in the production of band 3, and that they make an approximately equal contribution. The finding that nulli-4B tetra-4D and nulli-4D tetra-4B grains each produce a phenotype indistinguishable from that of Chinese Spring is consistent with this observation.

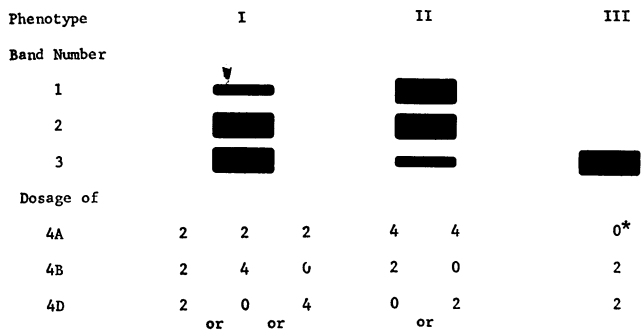

FIG. 2.—Diagram showing the relationships between dosages of the group 4 homoeologues and the ADH zymogram phenotypes produced.

Isozymes	Chinese Spring	nulli-4D tetra-4B	nulli-4B tetra-4D	nulli-4D tetra-4A	nulli-4B tetra-4A	monoiso-4A alpha
ADH-1	1/9 $\alpha\alpha$	1/9 $\alpha\alpha$	1/9 $\alpha\alpha$	4/9 $\alpha\alpha$	4/9 $\alpha\alpha$	
ADH-2	4/9 $\alpha\beta$,$\alpha\delta$	4/9 $\alpha\beta$	4/9 $\alpha\delta$	4/9 $\alpha\beta$	4/9 $\alpha\delta$	
ADH-3	4/9 $\beta\beta$,$\delta\delta$,$\beta\delta$	4/9 $\beta\beta$	4/9 $\delta\delta$	1/9 $\beta\beta$	1/9 $\delta\delta$	$\beta\beta$,$\delta\delta$,$\beta\delta$
Dosage of						
Adh_A	2	2	2	4	4	0
Adh_B	2	4	0	2	0	2
Adh_D	2	0	4	0	2	2

FIG. 3.—Schematic model for the subunit composition of the ADH isozymes produced by Chinese Spring and by each of the strains possessing group 4 aneuploid combinations. Dimers on the same line in the figure have coincident electrophoretic mobility. The expected quantitative distribution of the isozymes is indicated by the ratios preceding the dimers.

It was noted above that the beta arm of chromosome 4A contributes to the production of band 2, based on the absence of this band when the beta arm of 4A is absent. 4A cannot be the only chromosome of group 4 involved in the production of band 2, however, for the intensity of band 2 has been observed to vary independently of that of band 1 (increasing the dosage of 4A from disomy to tetrasomy, while either 4B or 4D is decreased from disomy to nullisomy, results in a large increase in the intensity of band 1 relative to that of band 2) and it was

shown above that 4A is the only chromosome of group 4 involved in the production of band 1. It may thus be concluded that chromosomes 4B and 4D also contribute to the production of band 2. They must both contribute, and contribute equally, since, as previously noted, nullisomy or tetrasomy for 4B produces an effect indistinguishable from nullisomy or tetrasomy for 4D.

Discussion. The results of this study clearly demonstrate that each of the chromosomes of homoeologous group 4 possesses a gene (or genes) involved in the production of alcohol dehydrogenase (ADH). In an earlier study[8] of an allotetraploid wheat, evidence was obtained in support of the postulation that the active ADH enzymes are dimers. The simplest hypothesis which is in full agreement with the observed relationships between the chromosomal constitution and the zymogram phenotype of each of the strains examined in this study is one which assumes, (1) that each homoeologue of group 4 possesses a gene that codes for an ADH polypeptide chain, or subunit, (2) that the active ADH enzymes are dimers produced by the random association of subunits, and (3) that each ADH gene produces an approximately equal quantity of its respective ADH subunit.

A schematic model for the subunit composition of the ADH isozymes of Chinese Spring and of the strains possessing group 4 aneuploid combinations based on the dimer hypothesis is summarized in Figure 3. The ADH structural genes located on chromosomes 4A, 4B, and 4D are designated, respectively, as Adh_A, Adh_B, and Adh_D, and the subunits for which they code as α, β, and δ, respectively.

The hypothesis predicts that in strains in which each of the three group 4 homoeologues is present (e.g., Chinese Spring), random association of the α, β, and δ subunits results in the production of six possible dimers, namely, $\alpha\alpha$, $\beta\beta$, $\delta\delta$, $\alpha\beta$, $\alpha\delta$, and $\beta\delta$. The three forms of ADH of *T. aestivum*, composed of these dimers, are designated ADH-1 ($\alpha\alpha$ dimer), ADH-2 ($\alpha\beta$ and $\alpha\delta$), and ADH-3 ($\beta\beta$, $\delta\delta$, and $\beta\delta$). These isozymes compose bands 1, 2, and 3, respectively.

The association of the $\alpha\alpha$ dimer with band 1 is consistent with the finding that chromosome 4A is involved in the production of band 1. Likewise, the designation of the $\beta\beta$, $\delta\delta$, and $\beta\delta$ dimers as the enzymes responsible for the production of band 3 is in agreement with the finding that chromosomes 4B and 4D are involved in the production of band 3. Finally, the association of the $\alpha\beta$ and $\alpha\delta$ hydrid dimers with band 2 is based on the observation that each of the three group 4 homoeologues contributes to its production.

This study has shown that nullisomy for either chromosome 4B or 4D does not produce any detectable change in terms of the presence or absence of zymogram bands, since 4B and 4D make an equal and interchangeable contribution to bands 2 and 3. The model is consistent with this finding. The $\alpha\delta$ and $\delta\delta$ isozymes cause the production of bands 2 and 3, respectively, when the β subunit is absent as a result of nullisomy for 4B, while nullisomy for 4D results in the production of bands 2 and 3 by the $\alpha\beta$ and $\beta\beta$ dimers, respectively. The strain which possesses the isochromosome, 4A alpha, produces only band 3, however. This is in agreement with the linkage of Adh_A with the beta arm of 4A, and with the α-subunit being a necessary component of the isozymes which produce both bands 1 and 2.

Further support for the dimer hypothesis, and particularly for the assumptions of random association of subunits and of production of an approximately equal amount of subunits by each of the ADH structural genes, comes from observations on the relative staining intensities of the zymogram bands. Under the assumption that each ADH gene produces an equal amount of its respective enzyme subunit, the expected distribution of the six possible dimeric enzyme molecules that result from random associations of the subunits will be based on $(p + q + r)^2$, where p, q, and r represent, respectively, the frequencies of the α, β, and δ subunits. In the case of Chinese Spring, which is disomic for each of the homoeologues of group 4, $p = q = r = 1/3$, and the expected trinominal proportions are $1/9\ \alpha\alpha : 1/9\ \beta\beta : 1/9\ \delta\delta : 2/9\ \alpha\beta,\ 2/9\ \alpha\delta : 2/9\ \beta\delta$. Combining the proportions for those dimers which have coincident electrophoretic mobility, the expected distribution of the isozymes that are assumed to be responsible for the production of bands 1, 2, and 3 is 1:4:4, respectively. This proportion is in good agreement with the observed staining intensities of the zymogram bands of Chinese Spring and the other strains disomic for 4A, 4B, and 4D (I, Figs. 1 and 2).

For those strains which are nullisomic for either 4B or 4D, the expected distribution of the three possible dimers will be based on $(p + q)^2$, where p represents the frequency of the α-subunit and q the frequency of either the β- or δ-subunit, depending upon which ADH gene is absent. $p = 1/3$ and $q = 2/3$ for the strains in which 4A is disomic and either 4B or 4D is tetrasomic, and $p = 2/3$ and $q = 1/3$ when 4A is tetrasomic and either 4B or 4D is disomic. In the former case, the expected distribution of the isozymes assumed to produce bands 1, 2, and 3 is 1:4:4, respectively (as for Chinese Spring), but in the latter case it is 4:4:1, respectively. For each of the four strains possessing different nulli-tetra combinations of group 4, the observed staining intensities of the zymogram bands are in good agreement with the expected distribution of the isozymes that are assumed to be responsible for the production of the bands (I and II, Figs. 1 and 2).

The ADH zymogram phenotypes of the nulli-4D tetra-4B and the nulli-4B tetra-4D strains are indistinguishable, as are the phenotypes of the nulli-4D tetra-4A and nulli-4B tetra-4A strains, in agreement with the quantitative distribution of isozymes predicted by the model. However, the model does predict differences between the members of these pairs in terms of the specific subunit composition of the isozymes that produce bands 2 and 3. But, since the β- and δ-subunits are differentiated solely on the basis of their genetic site of origin, it is clear that the model does not predict a structural difference between these subunits. No evidence has been obtained to date to suggest such a structural difference. It is thus quite possible that the Adh_B and Adh_D genes are identical so that in turn the ADH isozymes within each of the pairs of strains mentioned above are structurally and functionally identical.

It is probable that the ADH genes which have been localized to the homoeologues of group 4 are structural genes. *T. monococcum*, *T. speltoides* (*Aegilops speltoides*), and *T. tauschii* (*Ae. squarrosa*), or their close relatives, are the sources of the A, B, and D genomes, respectively, of *T. aestivum* (for reviews, see Morris and Sears[10] and Sears[11]). A basis for the presence of a minimum of one ADH structural gene in each genome of hexaploid wheat comes from the finding that

zymograms made with extracts of grains of several contemporary representatives of each of these three species each show ADH activity.[12] The location of the genes on homoeologous chromosomes and the interaction of their products is consistent with the presumed origin of the A, B, and D genome diploids from a single primordial diploid. It is thus reasonable to conclude that *T. aestivum* possesses triplicate ADH structural genes, the evolutionary descendants from the primordial diploid of a single ADH structural gene.

Most studies of the homoeology of wheat chromosomes have utilized cytogenetic (e.g., see Sears[5]) or cytological (e.g., see Riley and Kempanna[13]) techniques. The analysis of individual genes by the methods used in this study may have wide applicability. Barber *et al.*[14] have successfully used similar methods to demonstrate that genes on wheat chromosome 3A and its rye homoeologue are involved in the production of certain esterase isozymes, and later reported[7] obtaining evidence for the involvement of the B and D genome homoeologues also. Brewer *et al.*[6] demonstrated that a gene (or genes) for alkaline phosphatase is located on chromosomes 4B and 4D. This study has located ADH genes on each of the chromosomes of group 4 and, in the case of 4A, specifically on the beta arm of the chromosome. These methods have applicability for studies beyond that of chromosome homoeology, however. Barber *et al.*[14] obtained evidence on the subunit structure of the esterase isozymes. The present study has contributed information on the genetic control and the subunit structure of alcohol dehydrogenase. Given knowledge of the genetic control of an enzyme, the genetic and evolutionary consequences of the duplication in an allopolyploid of the gene which produces the enzyme may be examined. Such an examination of alcohol dehydrogenase in *Triticum*, based partly on the results of this study, will be presented elsewhere.[12]

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