*STUDIES OF ISOZYME PATTERNS IN NULLISOMIC-TETRASOMIC COMBINATIONS OF HEXAPLOID WHEAT**

By George J. Brewer,† Charles F. Sing,† and E. R. Sears‡

UNIVERSITY OF MICHIGAN, ANN ARBOR, AND U.S. DEPARTMENT OF AGRICULTURE, COLUMBIA, MISSOURI

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Abstract.—Thirty-eight wheat strains, each nullisomic for one chromosome and tetrasomic for a homoeologous (related) one, were analyzed electrophoretically for 12 different enzymes, and a gene (or genes) for alkaline phosphatase was localized to chromosomes 4B and 4D. The other 11 enzymes showed no significant electrophoretic variation. The fact that the only mutants observed affect the same enzyme favors the concept that rigorous selection operates in the polyploids both to preserve certain types of variants and to eliminate others.

The probable evolutionary history of polyploid wheat[1, 2] is reconstructed in Figure 1. It is thought that a number of diploid species originated from a single primordial diploid, designated "PP" in the figure. At some unknown time the AA and BB diploids spontaneously hybridized to form a tetraploid, AABB. Archeological evidence[3] suggests that at least 8000 years ago the AABBDD hexaploid was formed under primitive cultivation by natural hybridization of AABB with a diploid species designated DD. The hexaploid comprises a group of cultivated wheats grown around the world. The tetraploid wheats (AABB) are less widely cultivated, and the diploids (AA) almost not at all. Representatives of AA, DD, AABB, and possibly BB still exist in the wild.

In an earlier study of leaf material from contemporary species, Sing and Brewer[4] found no significant differences in the mean number of isozyme bands among the diploid, tetraploid, and hexaploid species for eight enzyme systems. Material from seeds revealed a significantly greater number of bands in the AA, AABB, and AABBDD species than in the BB and DD species, but no significant differences among the AA, AABB, and AABBDD species. These findings are surprising, especially in view of the fact that the isozyme patterns of the diploids differed markedly one from another. Further, there was almost no additivity of isozyme patterns; i.e., the pattern in AABBDD was usually not the combined pattern of AA, BB, and DD. This contrasts with the report of Johnson and Hall[5] in which additivity of patterns was found in seed-storage proteins.

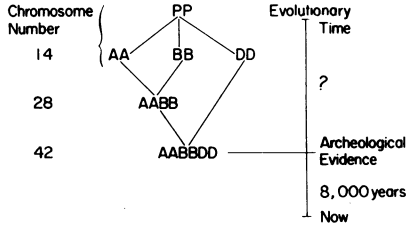

FIG. 1.—Reconstruction of the probable evolutionary history of wheat. The diploid chromosome number is shown at the left and a time scale at the right.

Since the considerable variation in the isozyme patterns of the contemporary diploid species did not seem to be reflected in the patterns of the hexaploid, we studied the variation among the A, B, and D genomes at specific loci in the hexa-

ploid, utilizing a nullisomic-tetrasomic series of hexaploid wheat (*Triticum aestivum* L. cv. Chinese Spring) developed by Sears.[6] This material had been used successfully to locate genes for seed-protein bands on particular chromosomes by Shepherd,[7] who observed not only disappearance of certain bands when a critical chromosome was absent, but also intensification of the same bands when the chromosome was present in quadruplicate. He found that all but two of the 17 major, so-called gliadin proteins were dependent, at least in part, on chromosomes of homoeologous groups 1 and 6.

Materials and Methods.—Within each genome of hexaploid wheat, each chromosome has a close relative (homoeologue) in each of the other two genomes.[8] The 21 chromosomes thus comprise seven homoeologous groups of three. The deleterious effects of nullisomy for each chromosome can be reduced or eliminated by making either of the two homoeologues tetrasomic.[6] The possible nullisomic-tetrasomic combinations for each homoeologous chromosome group are six, as illustrated in Figure 2. This generates 42 possible nulli-tetras, all of which have been studied but four (nulli-2A tetra-2B, 2A-2D, and 4A-4D, which are sterile or nearly so, and 2B-2A).

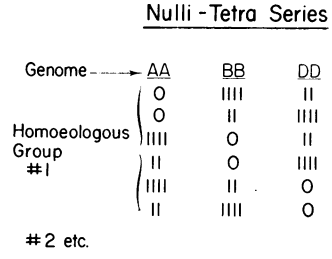

FIG. 2.—The six possible nullisomic-tetrasomic combinations of hexaploid wheat involving chromosomes of group 1. An equal number of combinations can theoretically be generated for each of the other six chromosome groups, making 42 possible combinations. In this paper 38 of the 42 have been studied.

Vertical starch-gel electrophoresis (30-slot gels) was employed throughout all studies. Details of the alkaline-phosphatase starch-gel method are given in the legend to Figure 3*a*. Most methods used have been previously reported.[9] Modifications for application of the methods to wheat will be reported elsewhere.[10]

Seeds were planted and the resulting seedlings allowed to grow for approximately 35 days, by which time the shoots were about 15 in. in height. Approximately 120 mg of leaf material from individual plants was then ground in about 0.2 ml of the gel buffer to be used for the particular enzyme system. After centrifugation the supernatant was used for electrophoresis. Material from only one plant was used in each slot. Two plants of each of the nulli-tetra types were studied in each enzyme system. Differences from plant to plant were not anticipated because of the long-term inbreeding of this wheat cultivar, and none were found. All enzyme systems were studied on the same plants.

Results.—With one system, alkaline phosphatase, differences were observed in the isozyme patterns among the A, B, and D genomes when the chromosomes of homoeologous group 4 were varied (Fig. 3). No effect of varying the chromosomes of the other six groups was detected. The original study of group 4 is shown in Figure 3*a*, and a repeat study along with an explanatory diagram is shown in Figure 3*b*. It is apparent that bands 5 and 6 are missing when the plant is nullisomic for chromosome 4D (slots 1 and 2 of Fig. 3*b*). In keeping with 4D specifying bands 5 and 6, when this chromosome is tetrasomic, the 5,6-band area tends to be more intense (slots 6 and 7 of Fig. 3*a*). Band 6, but apparently not 5, is also more intense in Figure 3*b*, slot 4.

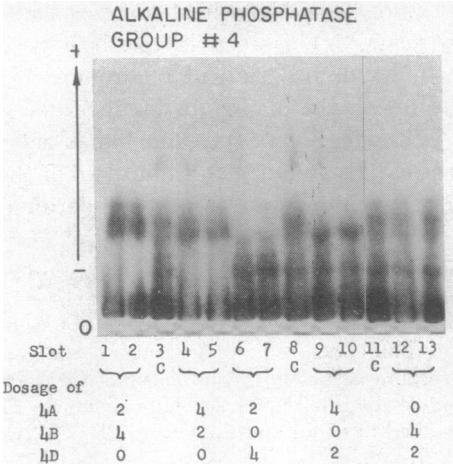

FIG. 3—(*a*) The first study of isozyme patterns for alkaline phosphatase involving combinations of group 4 chromosomes. Three different *control 'Chinese Spring' hexaploids* are present in three slots marked by *C*. Each nulli-tetra combination is represented by two individual plants, with the chromosome combinations indicated. *O* indicates the origin. The alkaline phosphatase method employs a gel buffer of 0.005 *M* histidine, pH 7.0, and a bridge buffer of 0.41 *M* sodium citrate, pH 7.0. Electrophoresis is carried out for 4 hr at 2°–4°C at 10 v/cm. The isozyme bands are stained by incubation with 50 mg of sodium-alpha-naphthyl-phosphate, 500 mg of polyvinylpyrolidone, and 50 mg of Blue RR salt per 100 ml of staining solution in 0.001 *M* Tris buffer pH 8.5 also containing 0.3 *M* sodium chloride and 0.0005 *M* magnesium chloride.

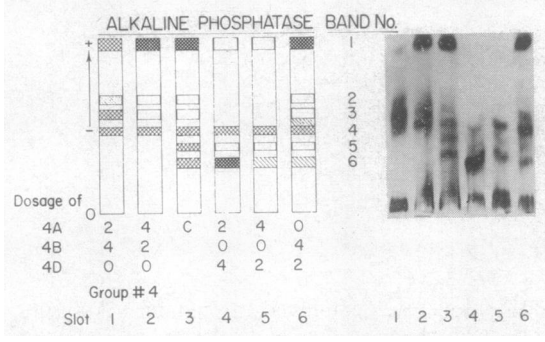

FIG. 3—(*b*) The repeat study of alkaline phosphatase involving group 4 combinations. The electrophoretic method was identical to that described above and the figure markings are the same. Slot and band numbers are indicated. An interpretive diagram is shown at the left. See text for discussion of results.

Removal of chromosome 4B causes disappearance of alkaline-phosphatase bands 1, 2, and 3 (slots 4 and 5 of Fig. 3*b*). In keeping with 4B specifying bands 2 and 3, when 4B is tetrasomic, bands 2 and 3 tend to be somewhat darker. This is best illustrated in slot 1 of Figure 3*b* (nulli-4D tetra-4B) but is also clear in slot 6 (nulli-4A tetra-4B) for band 3. In Figure 3*a* the 4D-4B combination (slots 1 and 2) is again more intense in bands 2 and 3 than is 4A-4B (slots 12 and 13). Unlike bands 2 and 3, band 1 shows no intensification when the dosage of 4B is increased to 4. In fact, band 1 of slot 1 of Figure 3*b* (tetra-4B) seems to be less intense than band 1 of slot 2 (disomic 4B). Further, in the original study (Fig. 3*a*) band 1 was less intensely stained in general, and removal of chromosome 4B did not seem to affect the intensity of band 1.

The absence of chromosome 4A had little effect on the alkaline-phosphatase banding patterns (Figs. 3*a* and *b*).

The other 11 isozyme systems all showed bands of activity, but there was no variation among the chromosome combinations. Results with a typical system, malic dehydrogenase, are shown in Figure 4. The other systems were glucose-6-phosphate dehydrogenase, phosphoglucomutase, hexokinase, acid phosphatase, esterase, adenylate kinase, isocitric dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, leucine amino peptidase, and 6-phosphogluconate dehydrogenase.

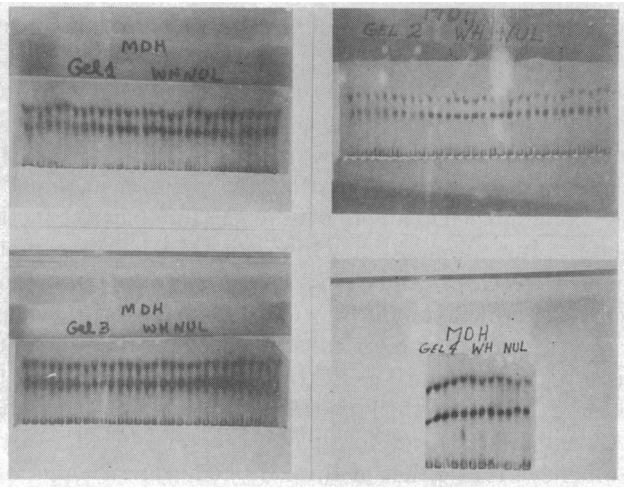

FIG. 4.—Isozyme patterns for malic dehydrogenase in the 38 nulli-tetra plants and controls. The individual plants are not indicated, since all patterns were identical. The markings on the gels are for laboratory identification. The malic dehydrogenase method employs a gel buffer of 0.005 *M* histidine, pH 8.0 and a bridge buffer of 0.41 *M* sodium citrate, pH 8.0. Electrophoresis is carried out for 4 hr at 2°–3°C at 10 v/cm. The isozyme bands are stained by incubation with 0.2 *M* DL-malic acid, 0.001 *M* DPN, 0.00016 *M* phenazine methosulfate, and 0.00043 *M* nitro blue tetrazolium in 0.05 *M* Tris buffer, pH 7.0.

Discussion.—It is quite clear that a gene (or genes) for alkaline phosphatase is present on each of chromosomes 4B and 4D. Presumably these are structural genes, although they could conceivably be some type of regulatory gene. Since chromosomes 4A, 4B, and 4D have a common origin, it is reasonable to assume that they have a common locus concerned with alkaline phosphatase. It then follows that all three chromosomes are different, indicating at least two mutants. The findings in the case of alkaline phosphatase make it apparent that this approach is valid for localizing genes to specific chromosomes.

The data also suggest an increased effect of extra dosage on alkaline phosphatase. That is, quadruplication of genes specifying certain bands of alkaline phosphatase tends to result in darker bands on the gel, although this is not always apparent. Obviously, quantitative methods would be desirable to further evaluate this point.

It is somewhat surprising that only one of the 12 isozyme systems showed variation. The 12 systems evaluate at least 12 genetic loci, and probably 20 or more, because some isozyme systems reveal more than one gene product. Of these 12 to 20 triplicated loci within this hexaploid, only one shows variation. The others, within the limits of the electrophoretic detection technique, are identical in all three genomes. This is surprising because in an early study[4] we had seen much variation in patterns among contemporary diploids. We did not anticipate less variation among diploid genomes merely because they were embedded in a hexaploid plant. Further, we anticipated that the polyploids would be a storehouse of variation, in keeping with the generally accepted hypothesis

that polyploidization allows retention of the original function at one locus while allowing experimentation with new functions at the homoeologous loci.

In seeking an explanation for finding so few variants, we unfortunately cannot assume that the parental diploids, which now differ strikingly in their banding patterns, were identical at the time of origin of AABB and AABBDD. The diploids may well have resembled each other more closely at that time than they do now, for they have since had an opportunity to differentiate further from their common ancestor. But this additional differentiation has presumably amounted to relatively little in the case of the DD species, *Triticum tauschii* (Coss.) Schmalh. (*Aegilops squarrosa*), which combined with AABB to form hexaploid wheat a mere 8,000 or so years ago. Cytological studies[11] show that the chromosomes of the D genome of hexaploid wheat are little, if any, different from those of present-day *T. tauschii*, as far as their pairing affinities are concerned. Although the union of AA and BB to form tetraploid wheat occurred earlier, there is no good reason to believe that either of these diploids was a young species at the time.

Studies of the variation in protein banding within each of the diploid species have not involved large enough samples to rule out that for each enzyme the same pattern may occur in representatives of all three species. However, since 12 enzymes were studied, all presumably varying independently, it is unreasonable to suppose that the three diploid strains involved in the synthesis of AABBDD happened by chance to be identical in pattern for 11 of 12 enzyme systems.

This does not rule out the possibility that the amphiploids concerned were produced many times from different AA, BB, and DD strains, and that only the rare combination involving identical isozymes for 11 of the 12 systems was fit enough to survive. It seems probable that the production of AABBDD from AABB $\times$ DD did occur many times, for *T. tauschii* presumably grew as a weed in fields of AABB wheat, and most hybrids of AABB with DD are found to set selfed seeds which give rise to plants with the doubled chromosome number. Recurrent production of AABB is much less likely, for spontaneous selfed fertility is seldom observed at this ploidy level, and, perhaps more important, crossing and chromosome doubling would have had to be followed almost at once by a mutation to suppress homoeologous pairing; otherwise the new tetraploid would have been too poorly fertile to survive.[12]

If, as seems most likely, the diploids from which the polyploids were produced had different banding patterns, we must assume that the newly formed polyploids had a relatively great multiplicity of bands, for other work[13, 14] has indicated that hybrids in the wheat group express all the enzyme bands of both parents. Such an augmented number of bands was not shown, however, by Chinese Spring nor by the cultivar used by Sing and Brewer.[4] Some variation in banding pattern doubtless occurs among the cultivars of *T. aestivum*, but there is no obvious reason to assume that Chinese Spring is grossly atypical of the species.

The existence of intergenomic differences in the isozymes of ancestral AABB and AABBDD and the absence of such differences in present-day forms would mean that evolution at the tetraploid and hexaploid levels modified the contributions of the different genomes to make them identical (except for the alkaline phosphatase system). This implies a strong selection pressure against un-

bounded multiplicity of gene products, that is, against a multiplicity of gene products beyond some optimal limit. An even stronger selection pressure must be assumed if we accept the alternative explanation that all amphiploids were eliminated except those produced from parents with identical isozymes.

What the basis for the indicated selection pressure may have been can only be conjectured at present, but one likely possibility is that most of the isozymes of the diploids were inefficient in the polyploids and hence were eliminated. In any case a means for elimination of the genome differences was available through the rare pairing of homoeologous chromosomes of the A, B, and D genomes. The resulting recombination, with selection for identity of bands, could have brought the homoeologues to carry the same genes for banding.

Once identical gene content of the different genomes had evolved, selection must have continued to operate against changes in the isozymes. Even with minimal mutation rates, many mutations to isoalleles must have occurred in the indicated 8000 years since the origin of AABBDD and the presumably much longer period since the origin of AABB; yet all have been eliminated except two in the alkaline phosphatase system.

It seems clear that evolution has operated very selectively and very strongly, both to remove and to preserve variation. Alkaline phosphatase, for which variation has been preserved, may be of considerable importance in the plant's capacity to survive and grow in a variety of soils containing various amounts of phosphate. Hence a high degree of fitness may pertain to having a variety of molecular forms of alkaline phosphatase.

Selection in favor of homogeneity of forms apparently has not been so great for the seed proteins, for Shepherd[7] found differences in the A, B, and D genomes for almost all the seed-protein bands.

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† Department of Human Genetics, University of Michigan Medical School, Ann Arbor, Michigan 48104.

‡ Department of Genetics, Curtis Hall, University of Missouri, Columbia, Missouri 65201.

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