TRENDS IN HYBRID AND PURELINE WHEAT YIELD AND STABILITY IN OKLAHOMA AND AN ANALYSIS OF THE VERNALIZATION CHARACTER WITH RFLP PROBES IN HEXAPLOID WHEAT (*TRITICUM AESTIVUM* L.)

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PREFACE

Little margin for error can be tolerated in breeding methodologies needed for plant improvement to satisfy the burgeoning human population, described by the Nobel laureate Norman Borlaug as the "hunger monster." The current logarithmic growth in population requires tremendous improvement in yield of major cereal food crops such as wheat. Although yield increases have occurred in most wheat producing countries[1], the advancements generally have been linear over the last 100 years with an estimated 1% genetic improvement in yield per year[2], despite the efforts of distinguished breeders and geneticists. Improvement in the world's food cereal crops must continue at unprecedented levels to avoid catastrophe. Since wheat is the most important cereal in world agriculture, providing approximately 30% of the world's grain[3], its improvement is imperative.

The next generation plant breeder must be able to incorporate the traditional methods of plant selection and data analysis, and molecular techniques for selections and transformations, yet remain cognizant of economic, social and legal considerations. Foremost, the breeder must be a geneticist, with the ability to make artful selections to advance plant improvement by traditional selection techniques. Collaterally, he must also be acquainted with the applications of biotechnology and gene manipulation and evaluate feasibility in a breeding program. Finally, he must remain knowledgeable of the ever advancing fields of physiology, pathology, and entomology as well as trends in agronomic practices.

A wide range of expertise requires a divergent background with a range of research expertise. This dissertation has incorporated two areas. First, the field based component analyzes trends in performance by reviewing numerous years of hybrid and pureline performance data to criticize the future of pureline and hybrid wheat improvement programs. Second, the laboratory based problem focused on establishing and utilizing molecular techniques to identify markers which may be of use in identifying a major vernalization gene involved in adaptation. This study was a sequel to the cold hardiness study completed as part of the Master's project using near-isogenic lines of winter wheat with different vernalization genes. As part of this objective, it was necessary to refine procedures for DNA extraction and develop a radiolabeled system for analysis.

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INTRODUCTION

Each chapter in this dissertation conforms to the *Publications Handbook and Style Manual* of the American Society of Agronomy. Chapter 2 will be submitted for publication in *Crop Science*, a Crop Science Society of America publication. An appendices has also been provided at the end of this dissertation including modeling of the grain yield data and comments on the check cultivars used in calculating the relative indices, and also an elaboration of the protocols and reagents used in the molecular analysis.

TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

Table Page

CHAPTER II

CHAPTER III

LIST OF FIGURES

Figure Page

CHAPTER II

CHAPTER III

CHAPTER 1

HYBRID WHEAT PRODUCTION AND POTENTIAL FOR THE SOUTHERN GREAT PLAINS

HYBRID WHEAT PRODUCTION AND POTENTIAL

FOR THE SOUTHERN GREAT PLAINS

The priorities of the wheat breeding program of the Oklahoma Agricultural Experiment Station (OAES) have been to develop pureline cultivars with high grain yield and test weight, acceptable maturity, breadmaking quality, and resistance to prevalent diseases and insects (Khalil et al., 1995). The generally long-term, presumably steady, progress associated with most traditional breeding programs is frequently the subject of criticism, where prevalent producer demands require immediate and accelerated improvement. A shift to hybrid wheat breeding objectives offers promise, noting that the advent of hybrid seed revolutionized the seed industry for several agronomic crops, prominent among them hybrid sorghum (*Sorghum bicolor* L.) and hybrid maize (*Zea maize* L.). However, the use of hybrid wheat seed on a commercial basis has been limited. For hard red winter wheat (HRWW), the reasons are disguised as an assortment of factors, including the lack of heterosis sufficient to justify the additional seed expenditure, the practical difficulties in producing hybrid wheat seed from the seedsman's perspective, and the relatively high seeding rates necessary to establish acceptable plant populations.

HYBRID WHEAT SEED PRODUCTION

There are many practical constraints on wheat seed production systems that make it difficult to achieve low-cost high-quality F_1 seed, creating a serious obstacle to the exploitation of hybrid wheat (Pickett and Galwey, 1997). Plant population and seed multiplication ratio are the functional constraints that most affect the utility of hybrid wheat breeding and seed production (Lucken, 1986). Hybrid wheat seed may be produced through cytoplasmic sterility systems (CMS) or through chemical hybridizing agents (CHAs). Cytoplasmic male sterility and a male fertility restoration system come from *T. timopheevi* Zhuk. (Lucken, 1986). A major concern in the production of hybrid

wheat seed is the degree of seed set on male-sterile parents following cross-pollination. Wheat is normally a self-pollinating plant, but appears to have a latent ability to cross-pollinate which can be enhanced by selecting from the considerable variation in diversity of floral traits that exists among wheat lines (Lucken, 1986). Cross pollination and seed set are a function of environment and genotype, both of which can have large effects (Lucken, 1986). A further difficulty in the use of CMS concerns the restoration of male fertility. The inability of a system capable of restoring fertility of a hybrid across all environmental ranges has been noted (Pickett and Galwey, 1997). These practical difficulties, and the necessity of dedicating one-third to one-half of the field to be sown to the male parent thwart the hybrid seedsman's ability to produce low cost seed using CMS (Pickett and Galwey, 1997).

The use of chemical hybridizing agents (CHAs) provides an alternative method of producing hybrid wheat seed. When applied to the growing wheat plant these chemicals can selectively induce male sterility. If the female parent is treated with a CHA at some stage before anthesis, the resulting male-sterile plants are pollinated by wind-born pollen. However, the use of CHAs are influenced through genotype x chemical, environment x chemical, and genotype x environment x chemical interactions. Additionally, optimum application rates and treatment timing may be crucial to maximize seed production of certain exceptional hybrid combinations (Lucken, 1986). CHAs offer an advantage over male-sterile systems in that there is no need to increase the number of male-sterile lines, thereby eliminating a costly component of hybrid seed production systems. The use of CHAs over male sterility systems also reduces the need for good anther extrusion in maintainer lines which would allow many lines with poor anther extrusion to serve as

female parents (Lucken, 1986). However, the application of CHAs requires cooperative weather, including periods without extended rainfall or prolonged wind, during a narrow window for effective application (Lucken, 1986). Advancements continue to be made in CHAs allowing the production of hybrid wheat seed. A new CHA (SC2053) was able to effect sterility close to 100% for the first three tillers when applied at rates of 700 to 1000 g ha^{-1} when the primary tiller was from 11 to 20 mm in length (Wong et al., 1995).

Outcrossing ability in wheat can be improved. Selection can be made for plants which perform well in a system for hybrid seed production. A recurrent selection system using gridded mass selection was effective after four cycles in shifting the population of spring wheat to those individuals that increased seed set when CHAs were applied (Kofoid, 1991). Plants in the treated area were allowed to pollinate at random with adjacent untreated plots. Plants were selected within a grid based on average seed set per spike and a selection intensty of 16.67%. A group of 32 random half-sib selfed families were chosen from both the C_0 and the C_4 cycles of selection and were evaluated, along with 32 random inbreds of the original population and 32 conventional inbred lines from several wheat breeding programs. In replicated tests at two locations, differences were found among entries for all traits evaluated (days to head, plant height, grain yield, test weight, kernel weight, grain protein, spike length, spikelets per spike and kernels per spike) when averaged across treatments. A significant rate x entry interaction was found among both the conventional and developed inbred lines, but not for the half sib families of either the C_0 or C_4 cycles, for grain yield, test weight, grain protein concentration, kernel weight, and kernels per spike. The families from the mass-selected population had a 20% greater seed set than the families from the original population and a 73% greater seed set

than the inbred lines when treated with the CHAs. Inbred lines developed from the population had a 45% greater seed set than the conventional inbred lines when treated. The increased seed production appeared to be due to increases in spike length and the number of spikelets per spike. Interestingly, when the mass-selected group was not treated with CHAs it maintained a slightly greater grain yield than the original population.

IMPACT OF CULTURAL PRACTICES

Farmers in the Southern Great Plains have frequently saved seed from harvested crops to sow the following season. What will be the effect of planting the segregating progeny of the F_1 hybrid plant? Recently, a comparison was made of CHA F_1 hybrids of soft red winter wheat with their respective F_2 populations and parents (Kratochvil and Sammons, 1990). When compared with the F_1 hybrids, the F_2 populations revealed a significant 8.3% yield decline averaged over environments and families, although seven of the 12 family-environment combinations tested had no significant differences for yield between the F_1 and F_2 populations. High-parent heterosis of 4.5% for yield was observed when averaged over all families and environments, although only four of 12 family-environment combinations had significant high-parent heterosis. They concluded that the F_2 population derived from an F_1 wheat hybrid wheat could be successfully used by a farmer for crop production when given current high hybrid seed costs and low market prices. However, under the conditions studied, and given the relatively low levels of high-parent heterosis, pureline cultivars would probably provide the most economical results for the farmer.

Seeding rate is another concern. Assuming a higher cost of hybrid seed wheat relative to pureline seed or farmer saved seed, one could anticipate that the seeding rate

will be reduced in the planting of hybrid seed to offset the additional cost of seed. However, grain yields are typically reduced by lowering seeding rates. In a study on the effects of seeding rates on harvest index, grain yield, and biomass yield, Sharma and Smith (1987) found lower grain yield and biomass yield for ten genotypes tested, including three hybrid lines, at lower seeding rates. Interpretation should be limited, however, as the analysis noted the presence of highly significant genotype x environment and seeding rate x environment interactions.

HETEROSIS AND PERFORMANCE

Increase in yield due to heterosis appears prominent in the utility of hybrid wheat.[1] The term heterosis was coined by Shull (1948) as a shortened form of heterozygosis and was used to described the phenomenon where the union of unlike gametes had a stimulating effect upon the physiological activities of an organism as manifested in its rapidity of growth, height and general robustness and increased vigor, size, fruitfulness, speed of development, resistance to disease and to insect pests, or to climatic rigors of any kind (Shull, 1952). The term is now used generally to describe the increases resulting from hybridity (Pickett, 1993). Although heterosis by definition requires differences between parents, diversity measurements do not necessarily predict F_1 performance (Martin et al., 1995). The situation is particularly complicated with allopolyploids, where the species is sometimes referred to as a permanent heterozygote, or fixed heterozygote, since it is possible to have a large degree of intergenomic heterosis. Indeed, this theory has often been used to explain the performance in yield of purelines or inbreds being close to that of hybrids (Pickett and Galwey, 1997). The complexities of heterosis are not easily

understood, and knowledge about the combining ability and heterotic patterns among wheat populations will need to be extensively analyzed in identifying parents for superior hybrids as they are in developing maize hybrids (Vasal et al., 1992).

Two methods are frequently used to express hybrid advantage. The first is termed mid-parent advantage and describes the increase in yield of the hybrid over the mean yield of the parents. The second is frequently termed heterosis and describes the increase in yield (or other character) of the hybrid compared to that of the superior parent (Morgan, 1998). In general, hybrid wheat tends to express a midparent advantage for a character, particularly when there are large phenotypic differences between parents for that character. Conversely, heterosis is often exhibited and tends to be greatest when the phenotypic differences between the parents are slight (Morgan, 1998). Plant height, grain yield, grains per ear, mean grain weight, ears per unit area, and biomass were evaluated in the Morgan (1998) study. Of the characters studied, positive heterosis for mean grain weight resulting in heavier seeds was the most important yield component contributing to greater grain yield. Heterosis for number of grain seeds per ear was not significantly different from zero and negative heterosis occurred for number of ears per unit area. Additionally, height reducing genes (*Rht*) have been found to have pleiotropic yield effects, resulting in plants of intermediate height having maximum yields (Flintham et al., 1997). This heterosis associated with the *Rht* genes combined additively with other yield component traits to generate the highest overall grain yields in hybrids. Further analysis of the *Rht-B1c* allele suggested a pattern of single gene overdominance in increased grain

[1] It is noted that the emergence of the transgene industry and concerns over the protection of intellectual property suggest the possible importance of hybrid wheat as a deployment vehicle.

yield, and also an advantage of the heterozygote in at least one combination with an alternate allele for some grain quality traits influencing pre-harvest sprouting.

The degree of realized yield advantage of hybrids over traditional pureline cultivars, and of both relative to long-term checks, is an important consideration. Early tests indicated a 32% advantage of hybrids over parents and a 31% advantage of hybrids over the best cultivar (Livers and Heyne, 1968). However, in testing of experimental hybrids, the grain yields relative to check cultivars were often disappointing (Johnson, 1977). The future of hybrid wheat in the USA remains unclear. Admittedly, substantial progress has been recognized with hybrids evidencing yield advantages over standard cultivars in the range of 10% and as high as 20% (Johnson, 1986). However, it is performance at 20% or higher that is commonly identified as the probably level required for hybrids to be commercially competitive with traditional cultivars (Johnson, 1986). Many questions also remain as to the magnitude of heterosis that is available in wheat (Johnson, 1986). In general, hybrids have frequently outyielded pureline cultivars (Smith et al., 1985; 1986), but with periods of time evidencing no advantage over the best purelines (Smith et al., 1988). Unfortunately, the level of realized heterosis has not been consistent with optimistic expectations.

Genotype x environment (GE) interactions exist in analysis of yield for hybrids as they do for regular cultivars. Trials must be conducted over several years and locations to definitively establish hybrid adaptation and performance (Smith et al., 1986; Lucken, 1986). Additional consideration must be given to the analysis of quality characteristics. In general, no particular quality advantage appears to be associated with the hybrids, and low protein content continues to be a problem at high yields (Lucken, 1986).

Currently the OAES is not pursuing the development of inbred lines for use in a hybrid wheat breeding program. The most recent releases of hard red winter wheat (HRWW) by land grant institutions for the Southern Great Plains have been pureline cultivars. However, pursuant to statutory requirements under Oklahoma law, numerous wheat hybrids and purelines have been tested in performance nurseries. Until recently Oklahoma seed law statutes (2 O.S. §8-21 et seq.; 2 O.S. §788.1 et seq.) required that hybrid wheat be tested before being sold in the state. Oklahoma State University was responsible for conducting these tests through an arrangement with the State Department of Agriculture. Experimental lines were entered in the Variety-Hybrid Performance Nursery (VHPN). Over the past 21 years, 226 genotypes have been tested. Of these, 104 were hybrid cultivars, and 122 were pureline cultivars. Results of the hybrid testing have been reported frequently by the OAES (Smith et al., 1985; 1986; 1988).

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CHAPTER 2

TRENDS IN HYBRID AND PURELINE WHEAT YIELD AND STABILITY IN OKLAHOMA

TRENDS IN HYBRID AND PURELINE WHEAT YIELD AND STABILITY IN OKLAHOMA

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ABSTRACT

Pureline wheat cultivars (*Triticum aestivum*) continue to dominate production fields on the Southern Great Plains despite the availability of hybrids. This study was to analyze yield trends and stability of yield in both hybrid and pureline entries in the Oklahoma Variety-Hybrid Performance Nursery. Grain yield data from 1975 to 1995 from four locations were selected and analyzed using relative yield indices. Regression equations over time were calculated for both hybrids and purelines relative to the mean performance of long-term check cultivars. Both hybrids and purelines evidenced yield improvement, with the yield of hybrids, in general, increasing at a greater rate than that of purelines. Predicted values in the last year tested indicated a 10.9% advantage of hybrids over purelines. Stability parameters were compared by regressing hybrid and pureline yields on an environmental index based on location mean yields for checks. Stability slopes of hybrids and purelines were not significantly different from one, nor from each other. Confidence intervals for hybrid and pureline performance generally overlapped throughout the observed yield ranges, evidencing no divergence in predicted grain yield as environmental yield potential increased. The variances of pureline entries were not significantly greater than hybrid entries. Hybrid wheat offers opportunity for increased grain yield on the Southern Great Plains, but without a stability advantage over pureline cultivars.

INTRODUCTION

Grain yield and grain yield stability in differing environments are two important factors concerning wheat production in Oklahoma and the Southern Great Plains. Advantages of hybrids over purelines have been suggested. However, only a relatively small acreage is devoted to hybrid wheat production in the Southern Great Plains. Continuing inquiry is made regarding increased production of hybrid wheat when considering additional grain yield and stability that hybrid lines may have over their pureline counterparts. The objectives were to study yield trends and stability of yield in both hybrid and pureline entries in the Variety-Hybrid Performance Nursery (VHPN). The analysis focused on analyzing the differences in grain yield between pureline and hybrid entries, and evaluating the stability of grain yield performance in different production environments. There were two hypothesis: (1) The improvements made in grain yields for purelines and hybrids, for the years analyzed, are significantly different from zero, and significantly different from each other; and (2) Hybrids and purelines exhibit different levels of stability for grain yield for the environments analyzed, a) with the regressions significantly different from a slope of one, and significantly different from each other; and, b) with the variances associated with pureline entries significantly greater than the variance associated with hybrid entries.

Genetic Gain and Yield Performance

Various procedures have been employed to estimate genetic gain in wheat improvement. Estimation of genetic gain for cultivars of different eras can be made by growing all in a common nursery and evaluating grain yield against year of release (Cox et al., 1988). For data taken in performance nurseries over years, a typical approach has

been to analyze performance by evaluating yield through multiple year moving averages and as a percentage change from the yield of a long-term check (Schmidt, 1984; Schmidt and Worral, 1983), or the use of moving means and least squares smoothing (Clarke et al. 1994). Relative yield is another method for analyzing grain yield performance and requires each entry in the nursery to be given a performance index based on its yield relative to the mean yield of all entries in the nursery (Yau and Hamblin, 1994). The use of relative yield as a measure of performance against all entries has several advantages including: (1) conversion of simple entry variance across sites to a practical, agronomic stability measure, (2) the giving of equal weight to each site when calculated means across sites, and (3) ease in comparing large numbers of entries tested in different experiments at the same site (Yau and Hamblin, 1994). However, the use of a relative yield index would not account for improvement over multiple years if a nursery index is used and the entries used to calculate the nursery index are not constant. This study used relative yield indices calculated from the mean yield of long-term check cultivars.

Stability Analysis

Of importance to an agronomic society is the stability of yield of a cultivar over a range of production environments. The cultivar must have the genetic potential for superior performance under ideal growing conditions, and yet also be able to produce acceptable yields under less favorable environments. Sharma et al. (1987) suggested that the regression of an ideal cultivar should have a slope of one and a positive intercept when yield is regressed on the yield index of increasingly favorable environments.

There are also many methods for analyzing yield stability (Lin et al., 1986). The stability analysis proposed by Perkins and Jinks (1968), partitions significant genotype x

environment (GE) interaction into a component due to heterogeneity between regressions and a remainder component, to determine if GE interactions of either or both of these components were significantly greater than the experimental error.

It has long been suggested that heterozygous and heterogeneous populations offer the best opportunity to produce cultivars which show small GE interactions (Eberhart and Russell, 1966). It has further been suggested that wheat hybrids and purelines appear to differ in their responsiveness to improving production conditions (Guenzi et al., 1985). Carver et al. (1987) confirmed the disparity in responsiveness when comparing 30 wheat genotypes in six environments over four years and found that the yield advantage of hybrids over semidwarf purelines decreased from 7.4% in the most productive environment to 5.0% in the least productive environments. A 10.8% yield advantage was calculated for hybrids over purelines in the preliminary performance nursery from the Agripro Standard Variety Trial, and a 13.5% average hybrid yield advantage was observed in the advanced trials, with the yield advantage of the hybrids being significant in all four years as well as for the data pooled over years (Bruns and Peterson, 1998). Evaluations of yield and stability suggest that hybrids show significantly higher mean yields and that the yield advantage generally increases with increasing environmental yield potential (Peterson et al., 1997). They also found that deviations from regressions for both the hybrids and the purelines were of similar magnitude, suggesting no evidence that hybrids provided an additional component of yield stability over purelines.

MATERIALS AND METHODS

Yield Data

For twenty-one years, yield data have been recorded on hard red winter wheat hybrids and purelines entered in the Variety-Hybrid Performance Nursery (VHPN) at Oklahoma State University pursuant to statutory testing requirements in unpublished annual reports of the VHPN. In general, six environments were used to test the hybrid and pureline cultivars. Field stations near Stillwater, Lahoma, Woodward, Goodwell, and Altus were used for the VHPN. The Goodwell location included both irrigated and dryland experiments. The Goodwell dryland site would be representative of extreme water stress in most years. However, data from this site were excluded from this analysis since data were missing in more than half of the test years, and had a high coefficient of variation for the years in which they were available. Data from the Woodward location were also excluded since this location was discontinued in 1992.

A core set of data from the remaining four locations, Goodwell irrigated, Stillwater, Lahoma, and Altus, were used for these analyses (Fig. 1). Data for all years were available for this analysis, except for three years (1979, 1991, 1993) at the Altus location. The Goodwell irrigated site should be representative of a favorable environment with highly productive soils and supplemental irrigation to allow the test cultivars to reach their genetic potential. The Altus location would be representative of an unfavorable environment, typically with moderate drought and heat stress, primarily late in the growing season. The locations at Stillwater and Lahoma generally receive adequate moisture but are influenced strongly by disease pressure, particularly fungal foliar pathogens and insects.

Thirty to forty entries were included each year in the VHPN. Typically, this number was comprised of an assortment of prominent hybrid and purelines, including recent state agricultural experiment station releases in the region, and two long-term check cultivars, Triumph 64 and TAM W-101. In any given year, the nursery entries represented the most advanced genetic materials available in the region. For each year and location, the entries were grown in a randomized complete block design with four replications at each location. All locations were fertilized at levels consistent with good management in the area and generally seeded at normal planting dates for the area, given proper soil moisture conditions and weather conditions (Smith et al., 1985; 1986; 1988). Plot size was 1.2 by 3.1 meters, consisting of either 4 or 5 rows. All plots were harvested with a Hege 125B combine harvester. Harvest dates typically ranged from mid June until early July. Data used for statistical analyses consisted of entry mean yields at each location.

Genetic Gain Analysis

All twenty-one years of grain yield performance data were analyzed in evaluating improvement in hybrid and pureline wheat performance. No adjustments were made for conventional versus semi-dwarf lines. Data for a particular line were limited to the first five years from the year that particular line was first entered in the nursery. The five year limitation was to prevent bias to performance yield of the purelines, which were occasionally included for more than five consecutive years, even though more advanced genetic material was being entered in the nursery. Similarly, data from entries with a release date in 1971 or earlier were excluded from the analysis. In total, 122 different purelines and 104 different hybrid entries were included for analysis. On average, each

pureline entry was included in the nursery for 2.6 years, and each hybrid entry included for 1.7 years.

The grain yield performance of hybrids and purelines was analyzed over time by comparing the grain yield of each relative to the mean of the long-term check cultivars on the assumption that mean grain yield of the long-term checks remained constant over time. Effects of class (hybrid or pureline), environment, and class x environment (GE) interactions were analyzed. A linear model was assumed after preliminary modeling. Year and location effects were assumed to be random and class effects were assumed to be fixed in developing the statistical model (Sharma et al., 1987; Peterson et al., 1997). All trends in grain yield were analyzed by year, after noting large annual effects. Relative yield indices were calculated for both hybrids and purelines at each location by dividing the mean grain yield for each, by the mean grain yield of the check cultivars. Relative yields for hybrids and purelines were plotted by location for the years tested. Indicator variable regression analysis (SAS Institute, 1996) was used to test the rate of gain of hybrids against purelines.

Stability Analysis

Yield stability was analyzed similar to that suggested by Eberhart and Russell (1966) from the regression analysis. Stability was defined as a function of slope and deviations from the regression of nursery entries on an environmental index. First, the linear regression coefficients of the hybrids and purelines (b_H and b_P, respectively) were calculated by using the mean yield of hybrids and purelines by location against an environment index based on the mean yield of the check genotypes in that environment. Differences in the calculated slopes for hybrids and purelines were tested for significance

from a slope of one, and from each other. Pooled deviations from the linear response, in terms of SE(b), were compared.

Each year of the VHPN was then analyzed separately using PROC MIXED (SAS Institute, 1996) to test the heterogeneity of slopes between hybrids and purelines, and among entries within these two genotypic classes. Under the model assumed, environments was considered a random effect and class (hybrid or pureline) a fixed effect. Entries within genotypic classes were considered random. Variance components of the random effects and interaction terms were estimated and tested for significance. The fixed effect (class) was analyzed by comparing the estimated mean grain yield and standard error by year. Variance components of hybrid entries and pureline entries were also estimated for each year of data using PROC MIXED (SAS Institute, 1996). One-tailed F tests were constructed to declare whether entry variances for purelines were greater than entry variances for hybrids.

RESULTS AND DISCUSSION

Genetic Gain Analysis

In general, the yield trends across locations were similar for both wheat hybrids and purelines. A large proportion of variability was due to the effects of location and years, but without a significant year x location interaction. The regression components by location for both hybrids and purelines are presented in Table 1.

The mean yields of the long-term check cultivars were used to estimate relative yield. The check means evidenced no significant linear change over the 21 year period (Fig. 2). If a trend in the long-term checks exists, the regression analysis suggests a slight annual decline in yield of 0.0208 Mg ha^{-1} (0.310 Bu ac^{-1}). A true decline in check mean over time would generate an upward bias in the estimation of genetic gain of hybrids and purelines. Such a decline would also not readily be explained by genetic causes. However, there could be environmental reasons, such as a check cultivar succumbing to new virulent strains of pathogens, or a decline in native fertility associated with the loss of organic matter from continuously tilled plots, or effects caused by changes in agronomic practices. Note however, that if genetic gain were measured by a pureline or nursery index, then performance would be evaluated against a moving standard, possibly with abrupt fluctuations with periodic releases of superior purelines or as new hybrids were developed. The use of a pureline or nursery environmental index, while eliminating much of the year to year environmental effects, would fail to compensate for changes in entry performance over time due to continual improvement of nursery entries.

The regression components for a linear model by location using calculated relative indices are presented in Table 2 and plotted in Fig. 3. In general, genetic gain was realized

in both hybrid and pureline performance. For all locations, the realized genetic gain was slightly higher for hybrids than purelines. However, when evaluated on a location basis maximum annual improvement in the mean performance of hybrids occurred at the Lahoma and Stillwater locations, intermediate gain at the Goodwell irrigated location, and questionable gain if at all, particularly for pureline performance, at the Altus location.

The hybrid rates of improvement were significantly different from zero at all locations except for Altus. The pureline rates of improvement were significantly different from zero only at Stillwater and Lahoma (Table 2). In general, the rate of improvement in hybrid performance exceeded that for pureline performance. Over locations, the rate of improvement for hybrids was 1.546% and for purelines was 0.844%, evidencing a significant difference between the two (P=0.0198). These rates of improvement were generally consistent with earlier reported estimates of genetic gain in pureline wheat breeding of 1% per year (Borojevic, 1986). When the data were considered on a per-location basis, the differences in rates of improvement were not found to be significant at any location based on the probabilities for heterogeneity of slopes (Goodwell irrigated, P=0.172; Stillwater, P=0.226; Lahoma, P=0.171; and Altus, P=0.294).

Rates of improvement based only on grain yield may require additional interpretation. Breeding efforts may contribute to factors whose improvement is not necessarily reflected in grain yield, such as improvement in grain quality or increased forage production. Some factors such as continual emphasis on disease resistance may be particularly evident in having a positive influence on yield at Stillwater and Lahoma locations. The presumption of high environmental stress at the Altus location suggests

that the more recent entries fail to improve grain yield over that of the long-term check cultivars.

In the last year of testing (1995) the predicted values evidenced a 26.3% advantage for hybrids, and a 13.9% advantage for purelines, on average, over the long-term checks. Additionally, the predicted yield advantage of the hybrids over the purelines was 10.9%, on average (Table 3). This relative advantage of hybrids over purelines is consistent with the 10.8% advantage reported in preliminary trials and the 13.5% advantage reported in advanced trials by Bruns and Peterson (1998). A heterotic yield advantage of 20% has been suggested (Johnson, 1986) as the minimal advantage required to economically justify the production of hybrid wheat over pureline wheat when considering the additional costs associated with production, particularly seed costs. Other considerations may also be relevant, such as the use of hybrid wheat in the deployment of transgenes. The results suggest that the heterotic advantage of hybrids over purelines is approaching this range for each of the locations analyzed.

The largest advantage of grain yield of hybrids over purelines did not occur at the Goodwell irrigated location (7.14%), despite suggestions that maximum benefit of hybrids over purelines is realized in high yield environments. On the other hand, considerable literature has postulated that an inherent advantage of hybrids should be a superior buffering to the environment. Hybrids excelled (>10%) over purelines in the three remaining stressful environments (Table 3).

Stability Analysis

In analyzing yield stability, annual mean yield of hybrids and purelines by location were plotted against an environmental index (Fig. 4). The environmental index was

determined from the annual mean yield of the long-term check cultivars for each location. By definition, the regression of the mean yield of the long-term checks in the stability analysis would have a slope of one (b=1.000).

The regression equations for hybrids and purelines were similar, when calculated using the annual mean yields by location of the hybrids and purelines against the corresponding mean check yield. The hybrid stability slope (b_H) was virtually identical to that for the purelines (b_P) (P [b_H=b_P]=0.9714) (Table 4). In both cases the slopes were not significantly different from one. For comparison purposes, when the same analysis was performed utilizing the pureline mean as the environmental index, rather than the check mean, little adjustment occurred and the b_H was approximately 0.997, with the b_P being one by definition.

Deviations from regression of hybrid entries were slightly higher than for purelines, but were not declared to be significant, upon a review of the standard errors associated with the regressions (Table 4). This is similar to that reported by Peterson et al. (1997), and suggests no stability advantage of hybrids over differing environments. However, the SE(b) estimates were extremely small, and a comparison did not compensate for the increase in variability explained by the regression of the purelines over that of the hybrids.

When comparing differences between hybrids and purelines for predicted grain yield in a specific environment, consideration was given to variability associated with both regressions. The confidence bands for both hybrids and purelines were interpreted simultaneously to test differences in predicted yield at a given environmental index. Significant differences can be declared if the confidence bands fail to overlap. Since both confidence bands require a confidence limit to be set, these limits should be set in a way to

assure that the desired error rate for the test is attained. An α=0.05 test is approximated by plotting and comparing 85% confidence bands, and a more conservative α=0.01 test is approximated by the 95% confidence bands (Payton et al., in review). In plotting these bands and making tests between two populations, there was no divergence in performance between hybrids and purelines when comparing the 95% confidence bands (Fig. 5A). There was also no divergence in performance between hybrids and purelines when comparing the 85% confidence bands, except for a few intermediate production environments (Fig. 5B). The overlapping confidence intervals show that there was no advantage for hybrids in high yield environments or in low yield environments over that of purelines. Stated conversely, there was no disadvantage for hybrids in any production environment.

The variance components estimated by PROC MIXED for the random effects are presented by year in Table 5, with environment estimating the effect (linear) of the four different locations, and entry estimating the effect of the different cultivars within a class. The mean grain yield for the fixed effect are presented by year in Table 6, with class estimating the effect of hybrid entries versus pureline entries. There was a significant entry effect declared at α=0.10 in a majority (13 of 21) of the test years for purelines, but in only three years for the hybrids. No significant differences in stability between hybrids and purelines were declared by testing variance represented by the pooled deviations from the linear response as evidenced by the SE(β). However, significant differences were declared at α=0.10 between the estimated variance components of hybrids and purelines in three years (1976, 1988 and 1992). Pureline variance components were equal to or greater than hybrids variance components in all but one year (1984) and were significantly

larger at α=0.10 than hybrid variances in four years (1976, 1982, 1988 and 1992). The lack of significance in the differences between variance components suggests no hybrid buffering advantage across environments.

CONCLUSIONS

Long-term progress continues to be made in both wheat purelines and hybrids. An analysis of data for 21 years of hybrid and pureline grain yield in Oklahoma provides a good indication of the continuing improvements made in grain yield for both. The genetic gain made in hybrids was increasing at a greater rate than that of purelines. However, no differences in stability of grain yield were detected across different environments for either purelines or hybrids, and the average regression slopes of each were similar. Deviations of mean yield data from the stability regressions were of similar magnitude for hybrids and purelines, and confidence intervals for hybrid and pureline stability regressions generally overlapped. At its current rate of improvement in grain yield, hybrid wheat remains a tool to maximize wheat production, but from the data analyzed there does not appear to be a stability advantage.

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Table 1. Regression analyses for grain yield by location for entries grown in the variety-hybrid performance nursery during years 1975-1995.

Location	Mean	Intercept	Slope†	r^2
	Mg ha^{-1}			
Goodwell irrigated				
Hybrids	4.83	3.32	0.018	0.010
Purelines	4.77	5.22	-0.005	0.001
Checks	4.52	6.76	-0.026	0.029
Stillwater				
Hybrids	3.13	2.05	0.013	0.010
Purelines	2.88	2.93	-0.001	< 0.001
Checks	2.70	4.90	-0.026	0.051
Lahoma				
Hybrids	3.18	1.12	0.024	0.029
Purelines	3.03	2.72	0.004	0.001
Checks	2.94	5.49	-0.030	0.048
Altus				
Hybrids	2.90	1.90	0.012	0.007
Purelines	2.74	3.04	-0.004	0.001
Checks	2.67	2.99	-0.004	0.001
Pooled				
Hybrids	3.53	1.95	0.019	0.009
Purelines	3.38	3.34	0.000	< 0.001
Checks	3.22	4.99	-0.021	0.013

† Regression year is the no. of years after 1900.

Table 2. Regression analyses for grain yield by location for relative indices calculated from the mean grain yield of check cultivars for the variety-hybrid performance nursery during years 1975-1995

Location	Mean	Intercept	Slope	r^2
Goodwell irrigated				
Hybrids	1.073	0.155	0.0108**	0.329
Purelines	1.058	0.668	0.0046	0.131
Stillwater				
Hybrids	1.169	-0.548	0.0202***	0.560
Purelines	1.082	-0.057	0.0134**	0.407
Lahoma				
Hybrids	1.110	-0.896	0.0236***	0.541
Purelines	1.054	-0.221	0.0150***	0.470
Altus				
Hybrids	1.075	0.642	0.0051	0.087
Purelines	1.021	1.123	-0.0012	0.005
Pooled				
Hybrids	1.112	-0.206	0.0155***	0.364
Purelines	1.054	0.337	0.0084***	0.201

*, **, *** Significantly different from a zero slope at the 0.05, 0.01 and 0.001 levels, respectively.

† Regression year is the no. of years after 1900.

Table 3. Summary of improvement for hybrids and purelines by location from predicted values of relative yield for entries grown in the variety-hybrid performance nursery during years 1975-1995.

Location	Predicted Value		Annual Progress	Advantage Over	
	1975	1995		Pureline	Check Mean
			---------- % ----------		
Goodwell irr.					
Hybrids	0.966	1.183	1.07	7.14	18.3
Purelines	1.012	1.104	0.43	---	10.4
Stillwater					
Hybrids	0.966	1.370	1.99	12.94	37.0
Purelines	0.946	1.213	1.35	---	21.3
Lahoma					
Hybrids	0.871	1.342	2.58	11.83	34.2
Purelines	0.901	1.200	1.58	---	20.0
Altus					
Hybrids	1.024	1.126	0.47	11.55	12.6
Purelines	1.033	1.009	(-0.11)	---	0.9
Pooled					
Hybrids	0.954	1.263	1.54	10.88	26.3
Purelines	0.970	1.139	0.83	---	13.9

Table 4. Linear regression analyses of hybrid and pureline mean yield against a check environmental index for entries in the variety-hybrid performance nursery during years 1975-1995.

Class	Intercept	Slope (*b*)	SE(*b*)	r^2
	Mg ha^{-1}			
Hybrids	0.24	1.02	0.0420	0.882
Purelines	0.09	1.02	0.0322	0.926

Table 5. Estimated variance components (VC) of random effects from regressions of hybrid and pureline grain yields on a check environmental index for entries in the variety-hybrid performance nursery during years 1975-1995. (p. 1 of 3)

	1975		1976‡		1977		1978		1979		1980		1981	
Source	df	VC	df	VC	df	VC	df	VC	df	VC	df	VC	df	VC
All Entries														
Environment (E) (linear)	1	0.697	1	0.516	1	0.380	1	2.168	1	1.727	1	1.036	1	0.611
Class x E (linear)	1	0.000	1	0.004	1	0.023	1	0.001	1	0.015	1	0.000	1	0.117
Entry(class)	20	0.036*	24	0.089**	24	0.035*	24	0.015	23	0.043†	19	0.034	21	0.133*
Entry x E(class) (linear)	20	0.000	24	0.000	24	0.000	24	0.000	23	0.000	19	0.000	21	0.000
Deviations	44	0.057	52	0.105	52	0.100	52	0.107	25	0.114	42	0.151	46	0.313
Hybrids (H)														
Environment (E) (linear)	1	0.626	1	0.536	1	0.285	1	2.012	1	2.218	1	0.982	1	0.268
Entry(H)	8	0.034	15	0.037†	9	0.028	5	0.000	7	0.023	4	0.026	5	0.000
Entry x E(H) (linear)	8	0.000	15	0.000	9	0.000	5	0.000	7	0.000	4	0.000	5	0.000
Deviations	18	0.047	32	0.078	20	0.087	12	0.133	8	0.095	10	0.045	12	0.214
Purelines (P)														
Environment (E) (linear)	1	0.744	1	0.496	1	0.509	1	27.461	1	1.694	1	1.054	1	1.097
Entry(P)	12	0.036†	9	0.175†	15	0.039	19	0.184	16	0.029	15	0.035	16	0.175†
Entry x E(P) (linear)	12	0.000	9	0.000	15	0.000	19	0.057	16	0.000	15	0.000	16	0.000
Deviations	26	0.066	20	0.148	32	0.107	40	2.757	17	0.047	32	0.183	34	0.344

†, *, ** Variance components significant at the 0.10, 0.05 and 0.01 levels, respectively

‡, By year, variance component of purelines significantly greater than variance component of hybrids at the 0.10 level

Table 5. Estimated variance components (VC) of random effects from regressions of hybrid and pureline grain yields on a check environmental index for entries in the variety-hybrid performance nursery during years 1975-1995. (p. 2 of 3)

	1982‡		1983		1984		1985		1986		1987		1988‡	
Source	df	VC	df	VC	df	VC	df	VC	df	VC	df	VC	df	VC
All Entries														
Environment (E) (linear)	1	1.471	1	2.494	1	2.970	1	1.446	1	1.087	1	2.274	1	0.258
Class x E (linear)	1	0.035	1	0.000	1	0.009	1	0.000	1	0.004	1	0.000	1	0.000
Entry(class)	19	0.088*	19	0.029	22	0.004	30	0.133**	23	0.062*	23	0.034*	20	0.116**
Entry x E(class) (linear)	19	0.000	19	0.000	22	0.000	30	0.000	23	0.000	23	0.000	20	0.000
Deviations	42	0.091	42	0.149	48	0.066	64	0.106	50	0.077	50	0.074	44	0.092
Hybrids (H)														
Environment (E) (linear)	1	1.768	1	2.475	1	3.130	1	1.528	1	1.148	1	2.324	1	0.257
Entry(H)	13	0.058†	16	0.027	14	0.008	17	0.119*	8	0.054	8	0.026	5	0.011
Entry x E(H) (linear)	13	0.000	16	0.000	14	0.000	17	0.000	8	0.000	8	0.000	5	0.000
Deviations	28	0.073	34	0.175	30	0.063	36	0.072	18	0.061	18	0.054	12	0.058
Purelines (P)														
Environment (E) (linear)	1	1.195	1	2.562	1	2.794	1	1.336	1	1.052	1	2.243	1	0.259
Entry(P)	6	0.153	3	0.028	8	0.000	13	0.151*	15	0.067*	15	0.038†	15	0.151*
Entry x E(P) (linear)	6	0.000	3	0.000	8	0.000	13	0.000	15	0.000	15	0.000	15	0.000
Deviations	14	0.130	8	0.050	18	0.068	28	0.154	32	0.086	32	0.089	32	0.104

†, *, ** Variance components significant at the 0.10, 0.05 and 0.01 levels, respectively

‡, By year, variance component of purelines significantly greater than variance component of hybrids at the 0.10 level

Table 5. Estimated variance components (VC) of random effects from regressions of hybrid and pureline grain yields on a check environmental index for entries in the variety-hybrid performance nursery during years 1975-1995. (p. 3 of 3)

	1989		1990		1991		1992‡		1993		1994		1995	
Source	df	VC	df	VC	df	VC	df	VC	df	VC	df	VC	df	VC
All Entries														
Environment (E) (linear)	1	0.447	1	0.984	1	1.942	1	4.773	1	4.464	1	0.026	1	0.210
Class x E (linear)	1	0.000	1	0.007	1	0.100	1	0.016	1	0.000	1	0.002	1	0.003
Entry(class)	28	0.030†	25	0.082*	23	0.000	22	0.072*	21	0.132*	18	0.111**	20	0.087*
Entry x E(class) (linear)	28	0.000	25	0.000	23	0.000	22	0.000	21	0.000	18	0.000	20	0.000
Deviations	60	0.115	54	0.138	25	0.143	48	0.165	23	0.108	40	0.064	44	0.116
Hybrids (H)														
Environment (E) (linear)	1	0.524	1	0.702	1	1.548	1	4.701	1	4.782	1	0.000	1	0.158
Entry(H)	6	0.000	4	0.000	4	0.000	3	0.003	2	0.000	1	0.089	6	0.043
Entry x E(H) (linear)	6	0.000	4	0.000	4	0.000	3	0.000	2	0.000	1	0.000	6	0.000
Deviations	14	0.057	10	0.139	5	0.210	8	0.088	3	0.056	4	0.129	14	0.090
Purelines (P)														
Environment (E) (linear)	1	0.425	1	1.099	1	2.441	1	4.833	1	4.415	1	0.033	1	0.244
Entry(P)	22	0.038†	21	0.104*	19	0.000	19	0.083*	19	0.147*	17	0.113**	14	0.105*
Entry x E(P) (linear)	22	0.000	21	0.000	19	0.000	19	0.000	19	0.000	17	0.000	14	0.000
Deviations	46	0.132	44	0.131	20	0.129	40	0.177	20	0.115	36	0.056	30	0.128

†, *, ** Variance components significant at the 0.10, 0.05 and 0.01 levels, respectively

‡, By year, variance component of purelines significantly greater than variance component of hybrids at the 0.10 level

Table 6. Analysis of class (hybrid or pureline) effects from regressions of hybrid and pureline grain yields on a check environmental index for entries in the variety-hybrid performance nursery during years 1975-1995.

	Hybrids		Purelines	
Year	Mean	SE	Mean	SE
	---------- Mg ha^{-1} ----------			
1975	2.71	0.42	2.80	0.42
1976	2.84	0.37	3.04	0.38
1977	2.96	0.33	3.04	0.32
1978	3.03	0.74	3.13	0.74
1979	4.58	0.77	4.54	0.76
1980†	3.54	0.52	3.29	0.51
1981	3.56	0.47	3.41	0.44
1982†	4.58	0.62	4.13	0.63
1983†	4.19	0.79	3.90	0.80
1984	3.48	0.86	3.34	0.86
1985	4.00	0.61	3.82	0.61
1986	3.52	0.53	3.29	0.53
1987	2.98	0.76	3.04	0.76
1988	3.74	0.30	3.54	0.27
1989**	3.35	0.35	3.05	0.34
1990	3.34	0.52	3.04	0.50
1991	3.48	0.83	3.60	0.83
1992†	3.54	1.11	3.15	1.10
1993	3.72	1.24	3.79	1.22
1994*	4.74	0.26	4.08	0.12
1995	2.61	0.26	2.39	0.25

†, *, ** Means of hybrids and purelines significantly different at the 0.10, 0.05 and 0.01 levels, respectively

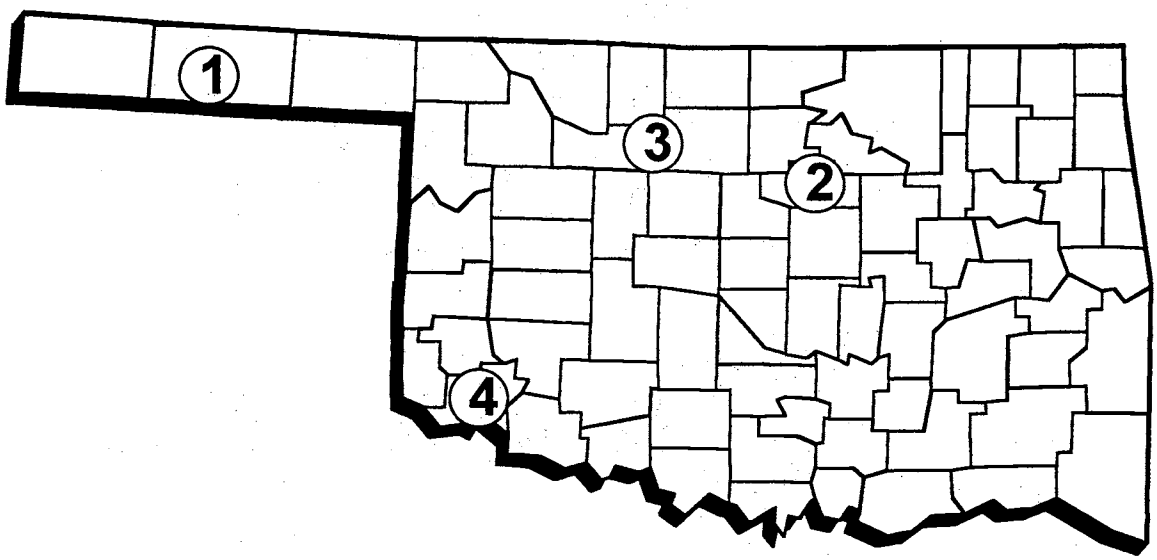

Fig. 1. Map of nursery locations included in data analysis. By location: Goodwell irrigated (1), Stillwater (2), Lahoma (3), and Altus (4). (Courtesy of Okla. Ag. Exp. Sta.)

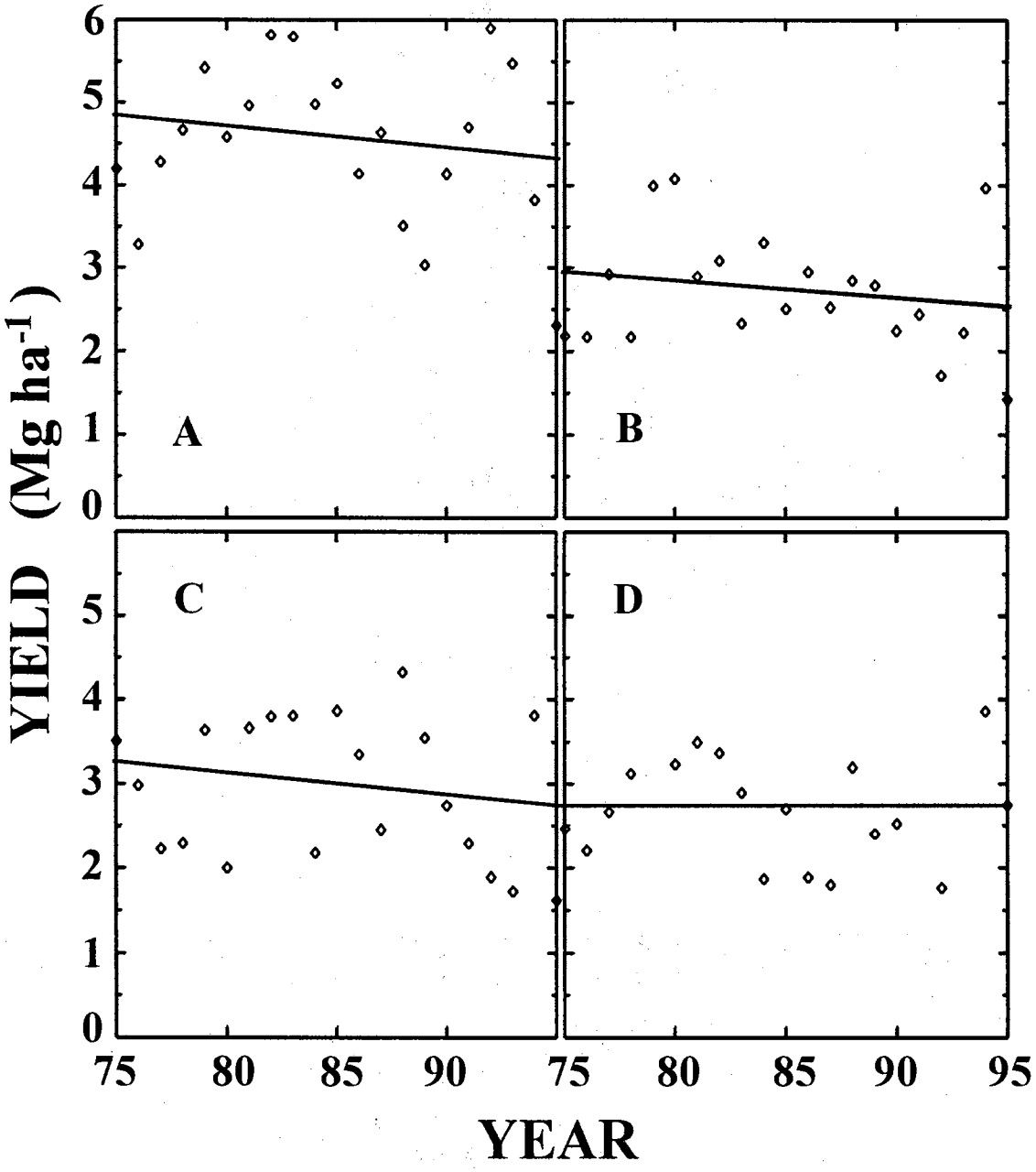

Fig. 2. Mean grain yield of long-term check cultivars (TAM W-101 and Triumph 64) by year and location with linear regressions plotted for Goodwell irrigated (A), Stillwater (B), Lahoma (C), and Altus (D).

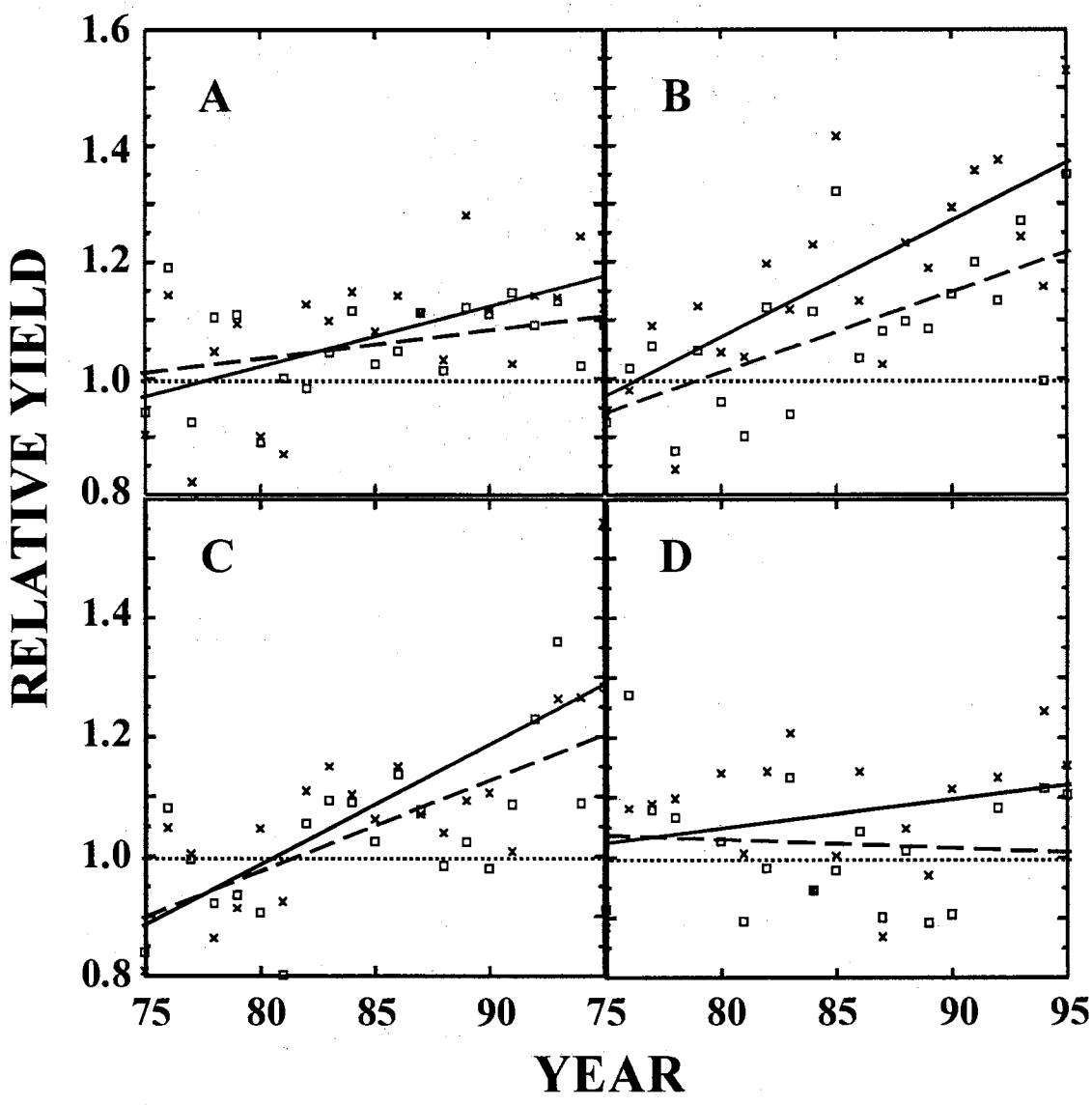

Fig. 3. Linear regressions for relative yield of hybrids (solid line with x data) and purelines (dashed line with □ data) by year for Goodwell irrigated (A), Stillwater (B), Lahoma (C) and Altus (D). The check mean reference (dotted line) was equal to 1.000 by definition.

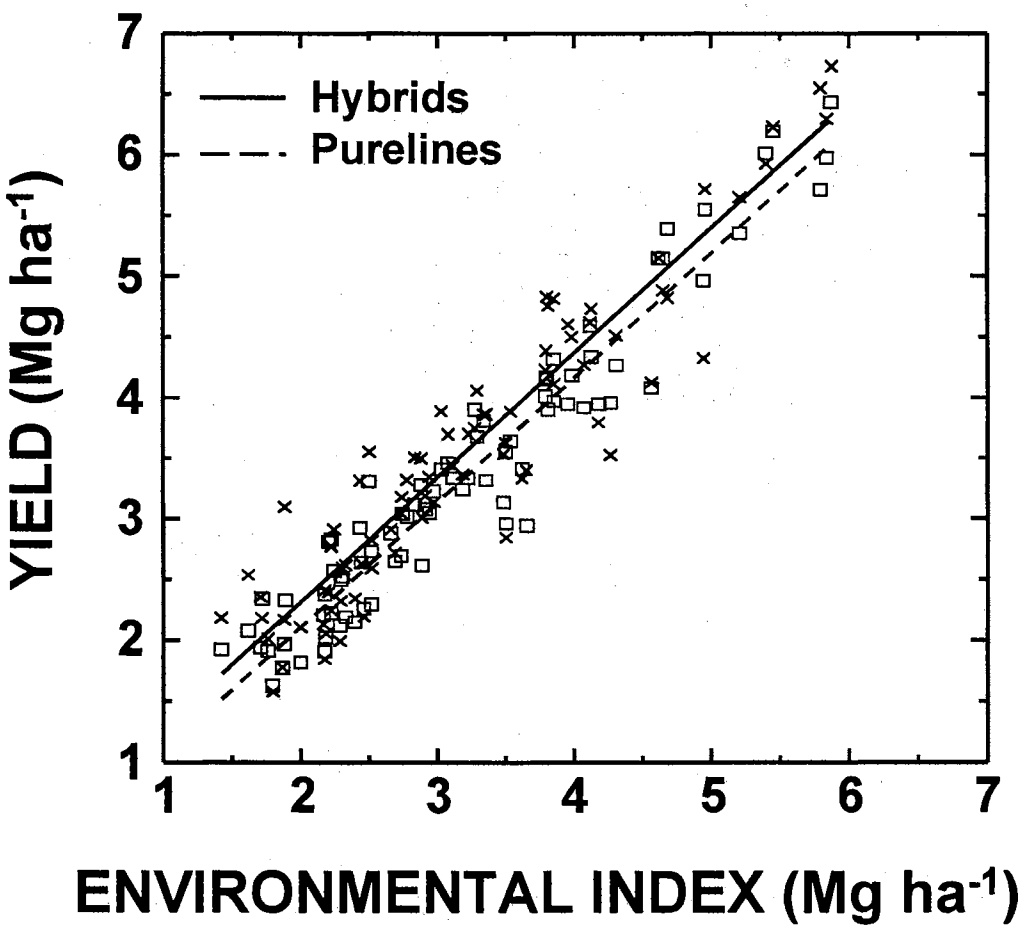

Fig 4. Mean grain yield of hybrids (solid line with X data) and purelines (dashed line with □ data) against a check environmental index.

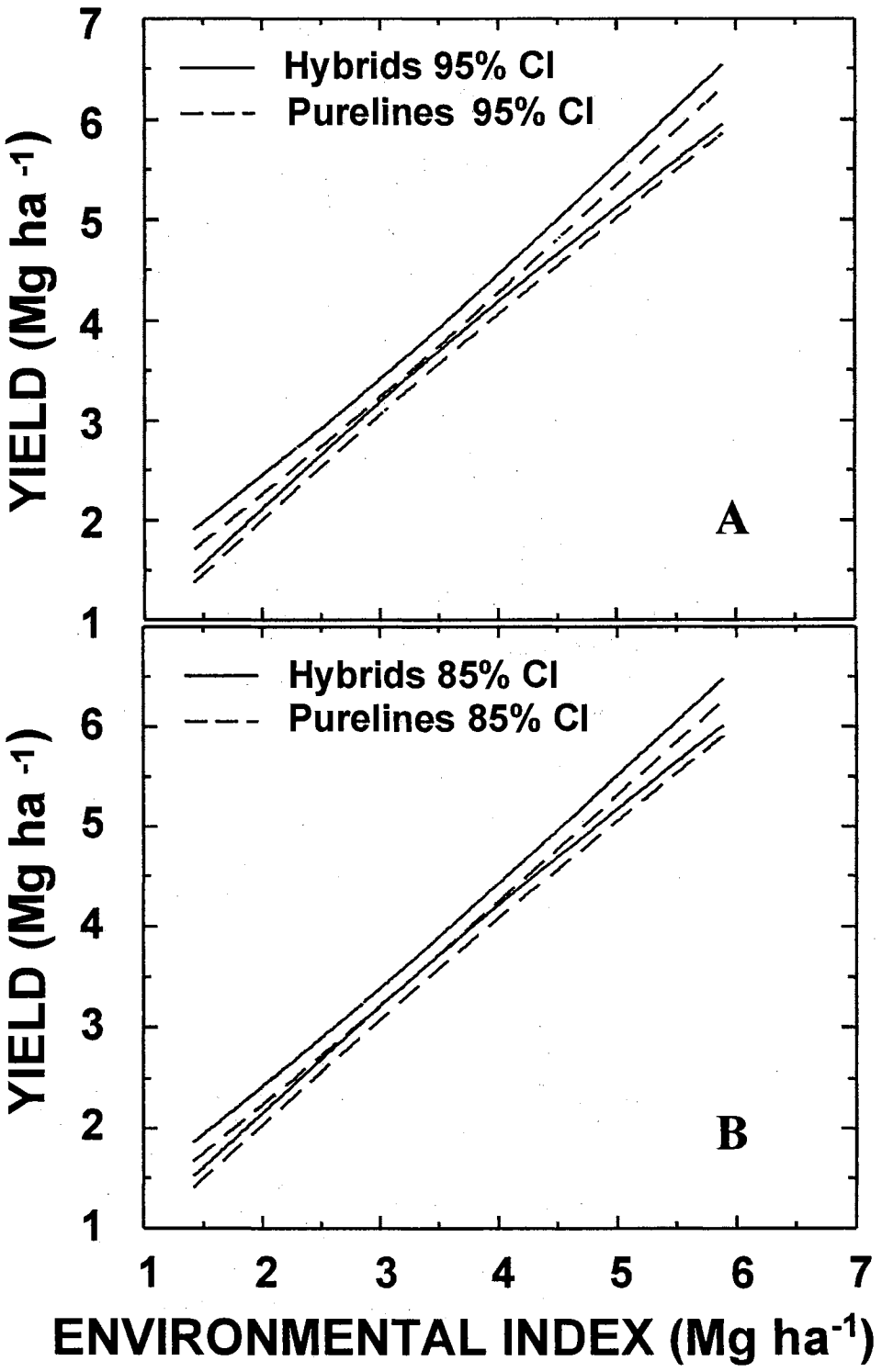

Fig. 5. Comparison of 95% (A) and 85% (B) confidence intervals of hybrids (solid lines) and purelines (dashed lines) to approximate a test of differences between the two populations at α=0.01 and 0.05, respectively.

CHAPTER 3

ANALYSIS OF THE *VRN3* CHARACTER WITH RFLP PROBES IN HEXAPLOID WHEAT (*TRITICUM AESTIVUM* L.)

ABSTRACT

The use of molecular techniques is becoming increasingly important in plant breeding programs and the physiology of the vernalization response is important to adaptation in wheat. A molecular mapping study was initiated to identify molecular markers linked to the *Vrn3* locus on chromosome arm 5DL in hexaploid wheat. Near-isogenic lines in a 'Triple Dirk' genetic background were used as parents. Crosses were made and the F_1 was selfed to create an F_2 population segregating for *Vrn3* alleles. The segregating population was grown in a controlled environment without vernalization and heading dates were recorded to determine the growth habit phenotype. Tissue samples were harvested from each plant for DNA analysis. Molecular differences between the parental lines were analyzed by Restriction Fragment Length Polymorphisms with a ^{32}P radiolabeled detection system using probes of known homology to 5DL. Two probe-restriction enzyme combinations evidenced useful polymorphisms of 14 probes initially tested with five different restriction enzymes. Probe Xbcd450, with *Hind*III digestion or with *XbaI* digestion, yielded unique banding patterns between the two parents. However, an analysis of the molecular marker and the phenotypic characterization were not linked when evaluated on 90 F_2 individuals segregating for the growth habit trait.

INTRODUCTION

Differences in response to vernalization and photoperiod cause the primary physiological responses of wheat that affect adaptation (Hoogendoorn, 1985b). In Oklahoma, winter wheat is grown as a cool season annual, with spike initiation in the spring and harvest occurring in early summer. By refining the vernalization requirement, cultivar development should be possible so that an optimum spike emergence date is targeted with floral development after the danger of a late freeze, but early enough that the reproductive cycle can be completed before the onset of typically hot and dry summer weather. The vernalization genes are of particular importance due to their effect on reproductive physiology and adaptation. Although the many major vernalization genes appear too strong to be of utility in a winter wheat breeding program in Oklahoma, minor vernalization genes may be important in the adaptation of winter wheat cultivars grown on the Southern Great Plains. The objective of this research was to identify probes currently on the chromosomal map of wheat which could be used to select for growth habit at the molecular level. Particular focus was given to the *Vrn3* locus.

The *Vrn3* locus was selected for this molecular mapping study. *Vrn3* is important as a major adaptation gene and typically gives a strong phenotypic response to environmental temperatures. In general, it operates qualitatively as a single major gene, which allows segregating progeny in a mapping study to be easily characterized by phenotype based on the number of days to spike emergence of non-vernalized plants. The *Vrn3* allele may also have utility in a wheat breeding program producing cultivars with a low vernalization requirement and also with a large degree of cold hardiness, noting that

previous research suggested that alleles for this gene, or genes closely linked to them, are associated with an increase in the level of cold hardiness (Koemel, 1996).

Although this study focuses on a qualitative trait with large effects, molecular breeding techniques may be most useful in selection for quantitative trait loci, traits with low heritability, or traits which are profoundly influenced by environmental factors. The potential of molecular markers to be utilized in a traditional breeding program could represent a tremendous savings in time and labor.

The Vernalization Response

Vernalization in the context of winter wheat means the acquisition, or acceleration, of the ability to flower by chilling treatment (Flood and Halloran, 1986; Salisbury and Ross, 1985). Vernalization is generally defined as the promotion of flowering by a period of low temperatures. The low temperature response that promotes flowering was first termed jarovization by the Russian geneticist T. D. Lysenko, and is now referred to as vernalization (Flood and Halloran, 1986). Vernalization implies "springization" (Salisbury and Ross, 1985). Spring wheat generally refers to a cultivar unresponsive to vernalization, facultative wheat to one with an intermediate response, and winter wheat to one with a strong response (Flood and Halloran, 1986). Since the relationship to time of year has caused confusion, it has been suggested that these terms be redesignated vernal wheat, semi-vernal wheat, and non-vernal wheat (Pugsley, 1982).

Vernalization is affected by temperatures of 10°C or less (Flood and Halloran, 1986), with temperatures above 10°C generally inhibitory of vernalization (Flood and Halloran, 1984). The optimum temperature for maximum rate of vernalization is 7°C, but this may be dependent on the cultivar, and lower rates of vernalization occur at

temperatures both higher and lower than 7°C (Flood and Halloran, 1986). However, vernalization ceases when the temperature becomes too low, with no vernalization occurring at temperatures below -2°C (Aherns and Loomis, 1963). The nature and interaction over different temperatures in the growth range, as it influences time of flowering, is not understood clearly (Flood and Halloran, 1984). Additionally, the responsiveness of wheat to vernalization varies with age (Gott, 1957), and interactions can occur with other physiological mechanisms such as photoperiod response (Flood and Halloran, 1986; Halloran and Boydell, 1967).

The vernalization response in the field is satisfied as the summation of many individual vernalization and devernalization reactions that occur with fluctuating temperatures (Trione and Metzger, 1970). Generally the longer the period of cold treatment, the less reversible is the vernalized condition. The devernalization action increases both with temperature and with the duration of the treatment.

Vernalization Genes

Five major vernalization genes have been mapped by cytogenic analysis to particular chromosomes, and for some, chromosome arms. *Vrn1* has been mapped to chromosome 5AL (Law et al., 1976; Flood and Halloran, 1986), *Vrn2* to chromosome 2B (Flood and Halloran, 1986), *Vrn3* to chromosome 5DL (Law et al., 1976; Flood and Halloran, 1986), *Vrn4* to chromosome 5B (Flood and Halloran, 1986), and *Vrn5* to chromosome 7BS (Law et al., 1966; Cahalen and Law, 1979; Flood and Halloran, 1986; Zemetra and Morris, 1988, Chao et al., 1989). *Vrn1*, *Vrn3* and *Vrn4* are on the homoeologous group five chromosomes in wheat, and it has been proposed that these are

part of a homoeoallelic series across the A, B, and D genomes of hexaploid wheat, and extending across species to include *Sp1* in rye and *Sh2* in barley (Plaschke et al., 1993).

The group 5 chromosomes are also frequently implicated in cold hardiness response (Sutka and Kovacs, 1985), and chromosomes 5A and 5D appear to carry major genes influencing cold hardiness (Sutka and Snape, 1989; Galiba et al., 1995). Early studies possibly confounded the relationship between vernalization and cold hardiness. The most recent research suggests that the genes influencing the vernalization and cold hardiness are separate responses. This is the case for at least one major vernalization gene, *Vrn1*, and a major cold hardiness gene, *Fr1*, on 5AL. Using recombinant inbred lines, Galiba et al. (1995) were able to break the linkage between *Vrn1* and *Fr1*, and demonstrate that these two genes are controlled by separate and distinct loci, but that these two loci (*Vrn1* and *Fr1*) are closely linked at 2.1 cM.

The combination of recessive vernalization alleles at all five loci confers winter habit. However, the presence of even one dominant allele gives a spring or facultative habit (Flood and Halloran, 1986; Zeven et al., 1986). Typically, low response to vernalization is partially dominant and epistatic to high response (Klaimi and Qualset, 1974; Law et al., 1976; Ward et al., 1983). Generally, *Vrn1* gives complete insensitivity to vernalization (Pugsley 1972). A wheat cultivar carrying *Vrn1* is a true spring wheat, whereas one carrying *Vrn2*, *Vrn3* or *Vrn4* is of facultative habit (Zeven et al., 1986). However, in progenies segregating for vernalization response, part of the variation in the rate of development is due to dosage effects of the vernalization genes. For example, the *Vrn1Vrn1* genotype flowers distinctly earlier than the *Vrn1vrn1* genotype (Flood and Halloran, 1986; Pugsley, 1972).

Stelmakh (1990) compared vernalization response of cultivars from around the world using Pugsley's near isogenic lines. In his analysis of 647 accessions belonging to cultivars from different agricultural zones of the world, Stelmakh concluded that the frequencies of *Vrn1, Vrn2,* and *Vrn3* were 81.0, 62.4, and 19.6%, respectively. The *Vrn3* allele appears to have been introgressed into many cultivars around the world from Japanese cultivars (Stelmakh, 1990, 1993), while the *Vrn2* allele prevails in Mediterranean winter wheat cultivars (Hoogendoorn, 1985a).

Adaptation Research

There has also been a resurgence of interest in refining the adaptation of soft red winter wheat grown in the Southeast USA to its environment by altering the vernalization response (Gardner and Barnett, 1990), primarily to influence heading dates. Additionally, Phillips (1992) worked directly to evaluate adaptation of spring versus winter habit cultivars. Six photoperiod insensitive spring wheat cultivars were converted to a winter wheat by introducing genes for vernalization requirement through backcrossing. The resulting winter types, requiring vernalization, were grown at four different locations, and compared to the spring types. When planted in early spring, the winter types had a significantly later heading date than the spring types, allowing the opportunity to escape damage from late winter freezing temperatures. In addition to adaptation, the vernalization genes appear to influence other agronomic characteristics and yield components (Stelmakh, 1987, 1993; Zeven, 1986).

Genetic Stocks

Pugsley (1971, 1972, 1973) developed several near-isogenic experimental lines (NILs) isolating dominant alleles for four different vernalization genes in a Triple Dirk

genetic background given in Table 1. Triple Dirk (TD) was derived from the cross Uruguay 1084/NX Dirk 48 and developed in New Zealand (Zeven and Zeven-Hissink, 1976). Five NILs were developed, each exhibiting different physiological behavior with respect to the vernalization response. Although Triple Dirk contains dominant alleles for *Vrn1* and *Vrn2*, and is photoperiod sensitive, the genetic lines developed from it are distinct in that each is homozygous dominant for a different vernalization gene, with the exception of the true winter type which is homozygous recessive for all vernalization genes. Each of the NILs is also photoperiod insensitive preventing daylength interaction on spike emergence. The stocks were developed through a minimum of three or four backcrosses allowing recovery of 94 to 97% of the Triple Dirk genetic background, although the breeding history is not well documented (Zeven et al., 1986). Triple Dirk D (TDD) has homozygous dominant vernalization alleles for the *Vrn1* locus, Triple Dirk B (TDB) for the *Vrn2* locus, Triple Dirk E (TDE) for the *Vrn3* locus, and Triple Dirk F (TDF) for the *Vrn4* locus. Triple Dirk C is homozygous recessive at each vernalization locus. TDE is of spring or facultative habit and was derived from a cross with the spring habit parent 'Loro', which presumably contributed the dominant allele for the *Vrn3* locus. TDC represents the true winter type and derives its recessive vernalization alleles from the cultivar 'Winter Minflor'.

Effect of Vernalization on Spike Emergence

When a line's genotype includes a dominant vernalization allele, the number of days to reach spike emergence is reduced. The degree of acceleration depends on which dominant vernalization gene is expressed. This gene is expressed with or without cold treatment. In a study evaluating four of the Triple Dirk NILs, Salisbury et al. (1979)

concluded that the lines reacted in different ways to increases in the vernalization treatment given to growing plants. The *vrn1vrn2* type showed a graded response while the *vrn1Vrn2* genotype exhibited no response. The *Vrn1vrn2* and *Vrn1Vrn2* genotypes exhibited a threshold response. The production of a compound, frequently termed florigen, has been suggested which promotes floral initiation. The production reaction proceeds only at low temperatures, or compound accumulation occurs only at low temperatures, and the floral initiation is triggered upon accumulation of a threshold level of the compound (Sachs and Hackett, 1969).

The spike emergence dates of each line of spring (or facultative) habit are significantly different from the true winter type, and there also appears to be a dosage effect of dominant alleles allowing the heterozygotes to be distinguished from either parent. Thus, by growing the different genotypes under conditions where no cold treatment is given, the identity of a spring, heterozygote, or winter type can be determined. In this manner the vernalization genotype of a segregating population such as the F_2 can be determined.

Probes

Numerous probes are available for wheat and they have been mapped to chromosomes and detect polymorphisms in wheat (Gill et al., 1990; Gill et al., 1993). Single locus or low copy number probes particular to chromosome arm 5DL have been identified from maps of previous work (Figs. 1 and 2). M.E. Sorrells (Cornell University, New York) is developing and refining molecular maps for many of the wheat chromosomes as part of the Triticeae Mapping Initiative. Other probes mapped to group

5 chromosomes are available from M.D. Gale (Plant Science Research Ltd., UK) (Xie et al., 1993).

Probes that hybridize to regions near the *Vrn3* gene, include Xpsr426 (Xie et al., 1993), Xrz395 (Nelson et al., 1995) and Xbcd450 (Nelson et al., 1995). Xpsr426 is a genomic wheat DNA probe and linked to *Sp1*, the spring habit in rye. Plaschke et al. (1993) suggested the possibility of a homoeoallelic series of vernalization genes on the Group 5 chromosomes of wheat, and inferred that Xpsr426 might be linked to one or more of the vernalization genes of this set, including *Vrn3* on chromosome arm 5DL in wheat. Galiba et al. (1995) subsequently reported Xpsr426 as being closely linked to *Vrn1* on chromosome arm 5AL. Nelson et al. (1995) also identified two probes, Xrz395 and Xbcd450, that may be closely linked to *Vrn3*. As part of his dissertation research, Nelson (1994b) ordered several barley and oat clones on chromosome arm 5DL near the *Vrn3* locus, and the results of this research have been entered in the Graingenes database (Nelson, 1995). Xrz395 is a rice genomic DNA clone which cosegregated with the *vrn* trait in the wheat mapping population studied by Nelson et al. (1995) and hybridized to sequences on the 2A, 5A and 5D chromosomes of wheat. Nelson (1995) also suggested that Xbcd450, which is a barley genomic probe, mapped near *Vrn3* and was 5 cM distal of Xrz395. Xbcd450 hybridized to sequences on chromosomes 5D and 5B. The position of Xpsr426 relative to the other two probes is not known, but Xie et al. (1993) reported that it hybridized to wheat chromosome arms 5AL, 5BL, 5DL, in addition to 5RL.

Analysis of Linkage

In general, 100 to 200 plants should suffice for statistical certainty when segregation of one gene is involved (Sedcole, 1977; Steel and Torrie, 1980; Wu et al.,

1991). It was assumed that the *Vrn3/vrn3* alleles would segregate 1:2:1 in the F_2. Without vernalization treatment, the phenotypic ratio of spike emergence dates would be 1/4 early (*Vrn3 Vrn3*), 1/2 intermediate (*Vrn3 vrn3*) and 1/4 late (*vrn3 vrn3*).

MATERIALS AND METHODS

The Triple Dirk NILs provided an excellent opportunity to investigate molecular markers that might be linked to the respective vernalization genes. For this study particular attention was given to the isolines differing at the *Vrn3* locus. One method of analyzing molecular differences is through restriction fragment length polymorphisms (RFLPs). The creation of a mapping population and mapping strategy is outlined in Fig. 3. For a mapping population created using NILs as parents, one would anticipate few genetic differences between the parents, thereby increasing the likelihood that any polymorphisms would be caused by DNA sequences implicated in the vernalization response, or closely linked sequences. The F_1 heterozygotes were produced by crossing a homozygous dominant parent with the homozygous recessive winter type parent and would have one dominant vernalization allele and one recessive allele at the *Vrn3* loci. An F_2 population was created by selfing of the heterozygotes. Linkage of an RFLP to a vernalization allele was evaluated by comparing RFLPs in the segregating F_2 population.

Mapping Population

Triple Dirk E (TDE) and Triple Dirk C (TDC) of the NIL's developed by Pugsley were used as parents to create a mapping population segregating for the *Vrn3* allele. Spikes of TDC were emasculated and pollen of TDE was used to fertilize to create F_1 seed. Crossed seeds were then grown and protective pollination bags were placed over emerging spikes to ensure self pollination in producing the F_2 seeds. F_2 individuals were grown under controlled environmental conditions without vernalization (22° C /14 h day and 18° C /10 h night) and scored as to phenotype. Three classes of phenotype were recognized: 1) early maturity, representing the spring growth habit, 2) intermediate

maturity, representing the heterozygotes and 3) late maturity, representing the winter habit.

A total of 144 F_2 plants were grown in two growth chambers. Each chamber included a set of 72 randomized F_2 individuals, and six plants each of TDC, TDE, and the F_1 as checks. The checks were included for comparison in evaluating the phenotype of the F_2s. Ten centimeters of space was allocated between pots to prevent interaction due to shading by neighboring individuals with early maturity. All plants were grown without vernalization.

Phenotypic Classification

The number of days from seedling emergence to spike emergence was recorded for each F_2 individual and the checks. The relative heading dates for the checks of known genotype served to insure that each F_2 was classified correctly. The vernalization genotype of each F_2 was inferred based on days to spike emergence. Plants which reached reproductive maturity at an early date inferred a homozygous spring genotype (*Vrn3 Vrn3*). Plants intermediate in the number of days to spike emergence were presumably heterozygotes (*Vrn3 vrn3*). Finally, plants which were late to reach spike emergence were assumed to be homozygous recessive vernalization genotype (*vrn3 vrn3*). Due to some difficulties in distinguishing between all F_2 individuals based on phenotype, it was necessary to grow F_3 families to confirm segregation within individuals for the F_2 plants classified as heterozygotes. F_3 families consisting of 20 plants were also grown in a late spring field plantings in 1995, being late enough to preclude the saturation of the vernalization requirement of winter types. Field rows were scored for one of three spike emergence maturity groups: 1) individuals all uniformly early, 2) individuals segregating

for maturing 3) individuals all uniformly late. F_3 families, with a minimum of twenty individuals, were also grown in the greenhouse without vernalization (approximately 25° C/14 h day) in 1997, and again scored as to phenotype to eliminate ambiguities seen in growth habit under field conditions. Segregation for number of days to spike emergence was noted for individuals within families, and provided evidence of heterozygousity at the *Vrn3* gene.

Molecular Analysis and Genotypic Classification

Initial efforts were made to identify polymorphisms between TDC and TDE. Plant leaves were harvested from each F_2 plant and the DNA extracted using an SDS extraction technique (Nelson, 1994a). DNA of each individual F_2 plant was extracted and analyzed for the RFLPs identified between the parents. Leaf tissue was harvested by removing mature leaves without the stem from the plants subsequent to flowering. DNA samples were not taken prior to flowering to prevent tissue sampling from influencing the number of days to spike emergence. Due to the minimal amount of DNA recovered from the F_2 leaf samples, a second set of tissue samples was taken from the F_3 families grown in 1997 using leaf tissue pooled from 20 F_2 individuals.

For initial surveys, the parental DNA was separately digested using five restriction enzymes, *Eco*RI, *Eco*RV, *Hind*III, *Dra*I and *Xba*I, and fragments separated by gel electrophoresis (Sambrook et al., 1989). The DNA was then transferred to a nylon membrane (Hybond N+, Amersham) by Southern (1975) blotting and probed by hybridization with radiolabeled probes with sequences of known homology to chromosome arm 5DL. A set of probes previously designated within 50 map units of the estimated location of *Vrn3* was tested. These included probes kindly provided by M.E.

Sorrells (Cornell University) and M.D. Gale (Cambridge Laboratory). Probes were labeled by the random primer method using dCTPs with incorporated ^{32}P (Amersham). Unincorporated nucleotides were removed by passage through sephadex (G-50) size exclusion columns. Southern hybridizations were performed (Nelson, 1994a). Following hybridization, membranes were wrapped with Saran Wrap® and exposed to X-ray film. Differences in banding patterns were noted between digested parental DNA samples using the selected probes. The RFLP genotype of each F_2 plant (or F_3 family) was then determined for the two polymorphisms identified and linkage evaluated.

RESULTS

Phenotypic Characterization

The parents and F_1 individuals grown in a controlled environment without vernalization had different phenotypes evidenced by differences in the number of days to spike emergence. Homozygous dominant (*Vrn3 Vrn3*) individuals reached spike emergence significantly earlier (67.9 days) than homozygous recessive (*vrn3 vrn3*) individuals (166.3 days) as shown in Table 2. The heterozygote was intermediate in response, although skewed toward the early parent. The three genotypes fell into three distinct groups, with an early, intermediate and late class.

When evaluating the F_2 individuals (Fig. 4A) the three classes could no longer be discriminated. Indeed, the early and intermediate classes overlapped. The correct characterization of individuals in the overlapping range of days was made by growing F_3 families and identifying which F_3's included individuals segregating for number of days to spike emergence. Segregates in the F_3 generation inferred heterozygousity in the F_2 generation. Spring field evaluations (no vernalization) were not conclusive as most plants were not well adapted to Oklahoma growing conditions and heavy infestations of disease and insects affected growth. However, by also growing F_3 families under greenhouse conditions (no vernalization) the genotypes of the F_2 were confirmed in all cases. The number of days to spike emergence was consistent with numerous checks of dominant and recessive homozygotes and also F_1 heterozygotes. After growing the F_3 families it was apparent that overlap had occurred between the late individuals that were homozygous dominant and early heterozygotes (Fig. 4B). The classification of the F_2 individuals is summarized in Table 3. Of 144 individuals, 28 were homozygous dominant (early

maturity), 71 were heterozygous and 45 were homozygous recessive (late maturity). This gave a χ^2 value of 4.04 for a goodness of fit test to a 1:2:1 segregation ratio, with a probability of 0.1465 of obtaining a greater value of χ^2 that could explain the deviations from the expected observations due to chance alone. As the χ^2 test was not significant at the α=0.05 level of significance, a 1:2:1 segregation ratio was not rejected. It is noted, however, that this probability was lower than expected, and some inadvertent selection may have occurred by selecting large viable seed for the growing of F_2 individuals.

Genotypic Characterization

The following probes were used in hybridization reactions to assay molecular differences: Xbcd450, Xrz395, Xprs426, Xbcd1103, Xcdo1326, Xpsr911, Xpsr79, Xpsr637, Xcdo1508, Xbcd1874, Xcdo346, Xpsr912, Xpsr360, and Xpsr580, although the latter three did not hybridize well. Only Xbcd450 revealed a polymorphism using digestions with *Hind*III and with *Xba*I (Fig. 5). Interestingly, Xrz395 did not reveal a polymorphism (Fig. 6). Differences in fragment sizes between the parents were apparent after evaluating the banding patterns following hybridization.

The polymorphisms were then evaluated on the F_2 individuals. In cases where DNA for an F_2 individual was not available, or was limiting for the F_2 individuals, DNA samples were taken from an F_3 family and pooled with a minimum of 20 individuals sampled. Both the Xbcd450 combinations with *Hind*III digestions and *Xba*I digestions were evaluated. The polymorphic bands were approximately the same size, complicating the certainty of classification in many instances, in which case the data were excluded from the analysis. To accommodate, a high percentage agarose gel (1%) was used to maximize the resolution, and DNA samples were electrophoresced for a longer period of time to

separate the targeted bands further in the gel. Band analysis was confirmed by measuring the optical intensity of the images formed on the autoradiogram after hybridization (Figs. 7A and 7B). These graphs evidence three peaks, indicating fragments of DNA to which the labeled probe hybridized. The three different bands presumably were the result of digestion on the three homologous group 5 chromosomes, representing the three different genomes of wheat. The polymorphic band was inferred to correspond to differences in the fragments from chromosome 5D.

DNA was also extracted from tissue of the parental and F_1 heterozygous checks grown concurrently with the F_2 individuals and samples randomly included on blots in lanes adjacent to F_2 samples. Each of the parental and F_1 heterozygous checks scored confirmed the expected parental and heterozygous banding patterns. For each parent the polymorphic banding pattern using *Hind*III digestion always corresponded with an analogous polymorphic banding pattern when the sample was digested with *Xba*I. Each of the heterozygous checks confirmed the expected banding pattern in the heterozygotes, with the contribution of bands from both parents (Fig. 8). Each F_2 sample evaluated yielded a banding pattern of one of the parents or the heterozygote. Additionally, a polymorphism detected using the *Hind*III digestion always corresponded with the expected polymorphic banding pattern when digested with *Xba*I. The fact that the polymorphisim with *Hind*III and *Xba*I digestions give the same banding pattern with every different individual as expected, as the probe is hybridizing to the same region of the digested DNA in each case and the cut site is at a region near the site of DNA homologous to the probe sequence. However, the banding patterns of the segregating F_2 individuals (or F_3 families) failed to produce correlated matches between phenotype and

inferred genotype. Indeed, it was apparent that the degree of recombination was highly significant, suggesting that the banding polymorphism was not linked to the vernalization gene of interest. The fact that the molecular marker did not cosegregate with the phenotypic trait is evidence that the homologous sequence to the probe, and the DNA within the identified fragment, does not include the DNA sequence influencing the number of days to spike emergence. Double digests of parental samples produced banding patterns similar to the individual parental digests (Fig. 8), but of lower molecular weight. This pattern suggests different areas of non-homology for the identified fragments of the single digests, and presumes that the smaller severed fragment did not include a sequence of homology to the probe, or, if it did, the fragment was so small that it was electrophoresced beyond an area of the gel blotted and surveyed (less than 500 bp).

The DNA of all 144 F_2 individuals was evaluated, but due to difficulties in scoring some samples, and in order to avoid misclassification of samples, only 90 F_2 individuals (or F_3 families) were included in the segregation analysis. A goodness of fit test on a 1:2:1 segregation ratio gave a χ^2 value of 9.09 (Table 4) suggesting that this fit should be rejected, since the probability of obtaining a higher value of χ^2 by chance alone was 0.01098. These deviations from the expected ratio were difficult to explain, with four possible suggestions being postulated: 1) The genetic stocks are contaminated, 2) All alleles are not fixed, 3) The identified fragments may be subject to a restriction enzyme inhibition process, such as a methylation response as was proposed by Burn et al. (1993), or 4) The sample size was too small.

Linkage Analysis

The linkage analysis was initially evaluated by Chi-square analysis as summarized in Table 5. The statistical linkage analysis program MAPMAKER (version 3.0) was used to examine the linkage relationship between *Vrn3* alleles (based on phenotypic classification) and the genotypic classification based on banding patterns. The results indicated that the *Vrn3* alleles and the sequence homologous to Xbcd450 are not linked.

Nelson et al. (1995) suggested that the *Vrn3* allele was linked to both Xrz395 and Xbcd450. Based on the inferred location of the *Vrn3* allele on the consensus map prepared by Nelson et al. (1995) one would anticipate that the *Vrn3* allele would be approximately 60-70 cM distal of the centromere, and the region of homology to Xbcd450 approximately 80-90 cM distal. An estimated distance of 20 cM would suggest a cross over frequency of approximately 20%. Even though the location of Xbcd450 has been postulated to be relatively close to the *Vrn3* loci, the fragment identified by the labeled probe does not include the gene of interest. Surprisingly, it did not even appear to be linked. Indeed, the results in this study suggest that the *Vrn3* allele and Xbcd450 are further apart than that postulated by Nelson et al. (1995). The lack of identification of polymorphisms other than with Xbcd450 was also discouraging, as no additional map refinement can be made concerning the relative position of probes to each other. No conclusions could be reached as to whether the gene was distal or proximal of Xbcd450.

In attempting to scrutinize these results, the NILs must first be criticized. Differences in the NILs other than the *Vrn3* allele are probable. It is possible that a polymorphic region of DNA occurs near a region of homology for Xbcd450, and that this region is a considerable distance from *Vrn3* allele, or even on a different chromosome.

This DNA could produce a different banding pattern, but the polymorphism is not linked to *Vrn3* allele due to the distance between them. As the distance between the probe sequence and agronomic gene increases, so does the number of cross-over events and the utility of the probe decreases. There are two basic scenarios to explain a polymorphic event identified between two near-isogenic lines (Fig. 9), which could explain the identity of the polymorphism and its lack of linkage to the *Vrn3* allele which are further described below:

In **Scenario I** the probe hybridizes to a region of the DNA which contains no other cleavage sites between the region of homology and the DNA coding for the phenotypic response. Additionally, there are different DNA cleavage sites at or near this region of homology or the coding sequence for the gene of interest. Fortuitously, one of the survey enzymes cleaves the DNA differently to yield an identifiable polymorphism. In this case, the molecular marker and the phenotypic marker will co-segregate. Additionally, this leads into a map-based cloning project which can then be used to recover the gene of interest. There are four potential crossover sites at points a-d on Fig. 9. However, minimal crossover events should be observed as the fragment length should be relatively small. The banding pattern and the trait of interest should co-segregate. This is the ideal situation which is not supported by the results observed.

In **Scenario II** the gene of interest is not included within the excised DNA. The probe hybridizes to a sequence of DNA that could be a considerable distance from the gene of interest. The phenotypic trait and molecular marker will no longer co-segregate because cross-over events occur between the gene of interest and the region of homology to the probe designated as point e on Fig. 9. The distance between them can be estimated

by the number of cross-over events. The distance shown by point e determines the number of potential cross-over events and thus, the utility of the probe. The utility of the probe as a molecular marker for breeding purposes diminishes greatly as the number of potential crossover events increases, until there is no associated linkage. Cross-over events may also occur at points f, g, and h, similar to b, c, and d in the first scenario.

The second scenario most likely fits the results observed, with a long distance inferred at point e due to the failure to identify any linkage. The distances for points f-h are relatively small, as evidenced by the size of the bands (approximately 6 kb) and the lack of any noted cross-over events within the cut region, since the polymorphic banding patterns using *Hind*III and then using *Xba*I always produced the expected results. Note, however, that it may be difficult to detect this event for a crossover at point h as a distal restriction site for a 6 base pair enzyme will by probability occur 4096 bp from the original restriction event, but closer restriction sites may be difficult to detect due to band migration discrimination on the gel.

By the results observed, the relative position of probes and genes can not be determined. Interestingly, no other probe tested detected a polymorphism even though the previous linkage map evidence relative proximity of probes to each other. Both Xrz396 and Xcdo1326 were estimated to be within a few cM of Xbcd450. However, neither evidenced a polymorphism with the survey digestion. If Nelson's map is correct and other probes such as Xpsr79 and Xcdo1326 are of homology to sequences closer to the *Vrn3* loci than Xbcd450, then presumably a cleavage site must occur between these sites and the region of homology for Xbcd450.

Based on the observations, there appears to be a genetic difference between the near-isogenic lines at a sequence distal to the gene of interest which is unrelated to the phenotypic response. This molecular difference, although unfortunate for yielding results in the mapping project, is not precluded through the development of isogenic lines. Each successive backcross should decrease, on average, 50% of the genetic differences between the recurrent parent and the nonrecurrent parent. Inevitably, some differences will persist. For instance, after 8 backcrosses, and assuming no other selection pressure, the recovery of the genetics of the recurrent parent should be 99.21875%. Potential differences in the DNA sequence still exist in 0.78125% of the DNA. If one considers that there are approximately 16 billion base pairs in wheat, then potentially 1.25 billion base pairs of the recurrent parent have not been recovered, allowing for molecular differences between the lines which are unrelated to the gene of interest. By screening with probes of known homology to a particular chromosome, the number of potential unrelated polymorphisms is greatly reduced, but still exists.

This comment is of particular importance when considering the close linkage between the *Fr1* and *Vrn1* alleles recently identified by Galiba et al. (1995), and the results of Koemel (1996) in a cold hardiness evaluation which found significant differences in the levels of cold hardiness to suboptimal temperatures between some of the NILs. There also exists a possibility that the lines are not identical for all cold hardiness alleles, particularly for alleles closely linked to a vernalization gene. Additionally, Zeven et al. (1986) tested the Triple Dirk lines and found significant differences across NILs for some morphological traits. The limited number of backcrosses used in developing the NILs has probably not eliminated all linkage drag from the donors of the different vernalization alleles.

CONCLUSIONS

Through the use of the Triple Dirk genetic stocks and RFLP probes already available, it was hoped that the map could be refined to identify one or more probes closely linked to the *Vrn3* gene with a crossover frequency low enough that the marker could be useful in a breeding program. The genetic stocks available provided an excellent opportunity to refine the mapping of *Vrn3*. However, all but one probe from Nelson et al. (1995) and all probes from other sources failed to identify any polymorphisms between the parents differing at the *Vrn3* loci. Thus, a consensus map between the different groups of probes could not be established. Although two polymorphisms were identified, they were unlinked to the phenotypic trait of interest, precluding their utility at the molecular level. Future work could focus on improving the isogenicity of the lines and generating probes to identify a polymorphism closely linked to the *Vrn3* allele.

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Table 1. Triple Dirk near-isogenic lines with different dominant alleles at four vernalization loci (Zeven et al., 1986).

Experimental Line	Abbreviated Designation	Haploid Genotype†
Triple Dirk	TD-	***Vrn1 Vrn2*** *vrn3 vrn4*
Triple Dirk D	TDD	***Vrn1*** *vrn2 vrn3 vrn4*
Triple Dirk B	TDB	*vrn1* ***Vrn2*** *vrn3 vrn4*
Triple Dirk E	TDE	*vrn1 vrn2* ***Vrn3*** *vrn4*
Triple Dirk F	TDF	*vrn1 vrn2 vrn3* ***Vrn4***
Triple Dirk C	TDC	*vrn1 vrn2 vrn3 vrn4*

† Dominant alleles designated in bold type.

Table 2. Number of days to spike emergence from seedling emergence for individuals with different *Vrn3* alleles grown in a controlled environment without vernalization.

Class (genotype)†	No.	Mean	SD
		-------Days-------	
Homozygous Dominant (***Vrn3Vrn3***)	12	67.9	2.2
Homozygous Recessive (*vrn3vrn3*)	12	166.3	6.6
Heterozygous (***Vrn3****vrn3*)	12	87.7	3.8
F_2 Population (Segregating)	144	109.6	37.0

† Dominant alleles designated in bold type.

Table 3. Phenotypic classification of F_2 segregating individuals based on number of days to spike emergence.

Phenotype (genotype)†	Observed	Expected
	----------No.----------	
Early (***Vrn3Vrn3***)	28	36
Intermediate (***Vrn3****vrn3*)	71	72
Late (*vrn3vrn3*)	45	36
Total	144	144

† Dominant alleles designated in bold type.

χ^2=4.04 $P >$ value of χ^2=0.1465.

Table 4. Molecular classification by DNA analysis of F_2 individuals and F_3 families.

DNA Analysis (presumed genotype)†	Observed	Expected
	----------No.----------	
Homozygous I (***Vrn3Vrn3***)	22	22.5
Heterozygous (***Vrn3***vrn3)	57	45
Homozygous II (*vrn3vrn3*)	11	22.5
Total	90	90

† Dominant alleles designated in bold type.

χ^2=9.09 P > value of χ^2=0.01098.

Table 5. Co-segregation analysis of phenotypic class with molecular polymorphism for segregating F_2 individuals included in the mapping population.

	Phenotype			
DNA Analysis	Early	Intermediate	Late	Total
	----------No.----------			
Homozygous I	3	10	9	22
Heterozygous	12	24	21	57
Homozygous II	3	5	3	11
Total	18	39	33	90

χ^2=17.9 P > value of $\chi^2 \leq 0.0001$

Chromosome 5D

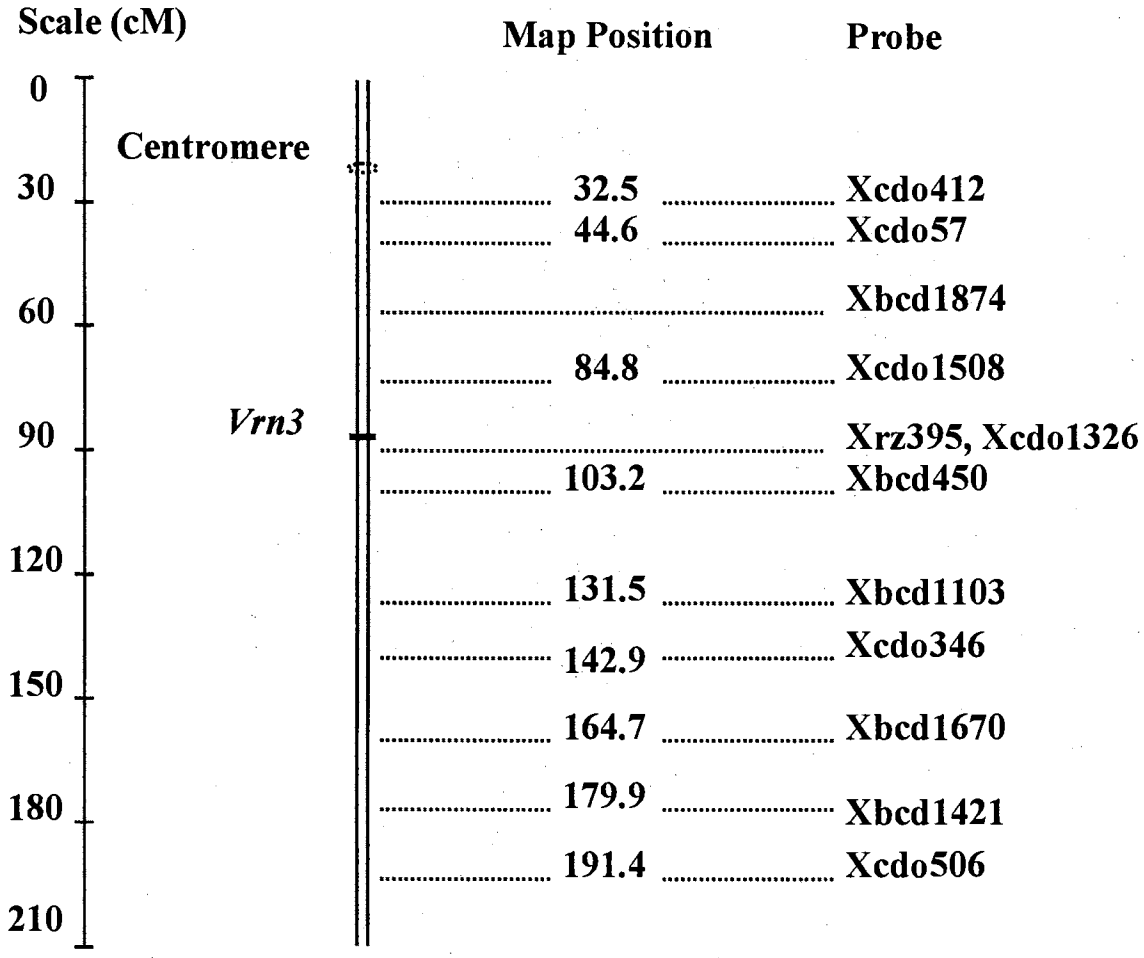

Fig. 1. Partial linkage map for chromosome 5D of hexaploid wheat, with map position identified in cM from the proximal end (Nelson, 1995).

Chromosome Arm 5DL

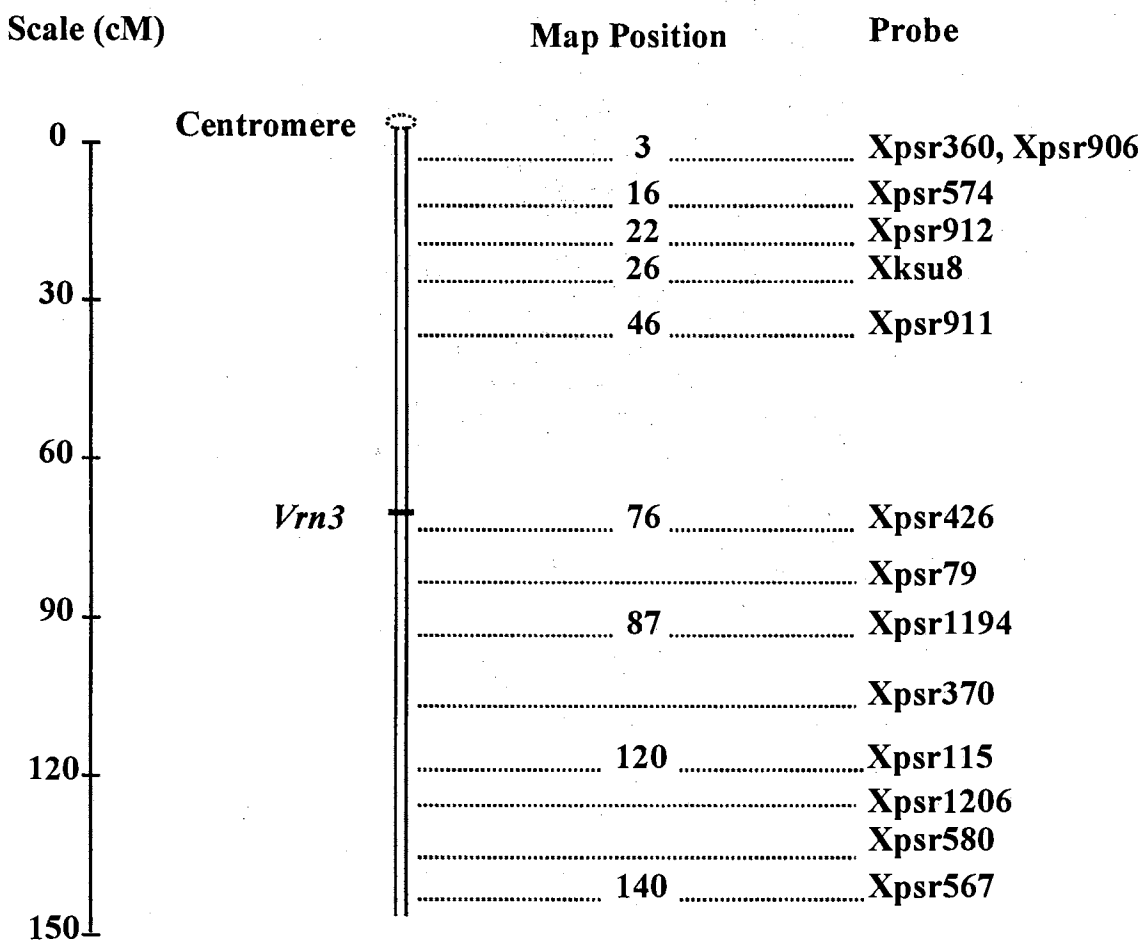

Fig. 2. Partial linkage map for chromosome arm 5DL of hexaploid wheat, including probes from consensus map for group 5, with map positions identified in cM from the centromere (Xie et al., 1993).

Mapping Strategy

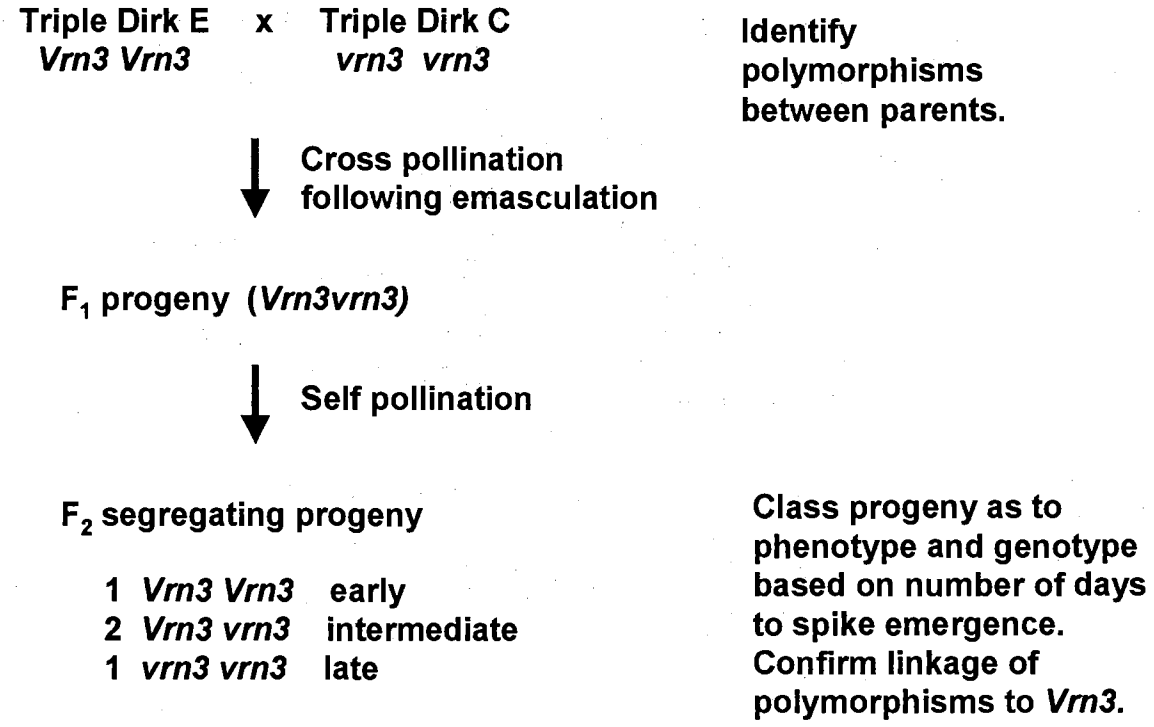

Fig. 3. Development of mapping population and strategy concerning phenotype classification and marker mapping.

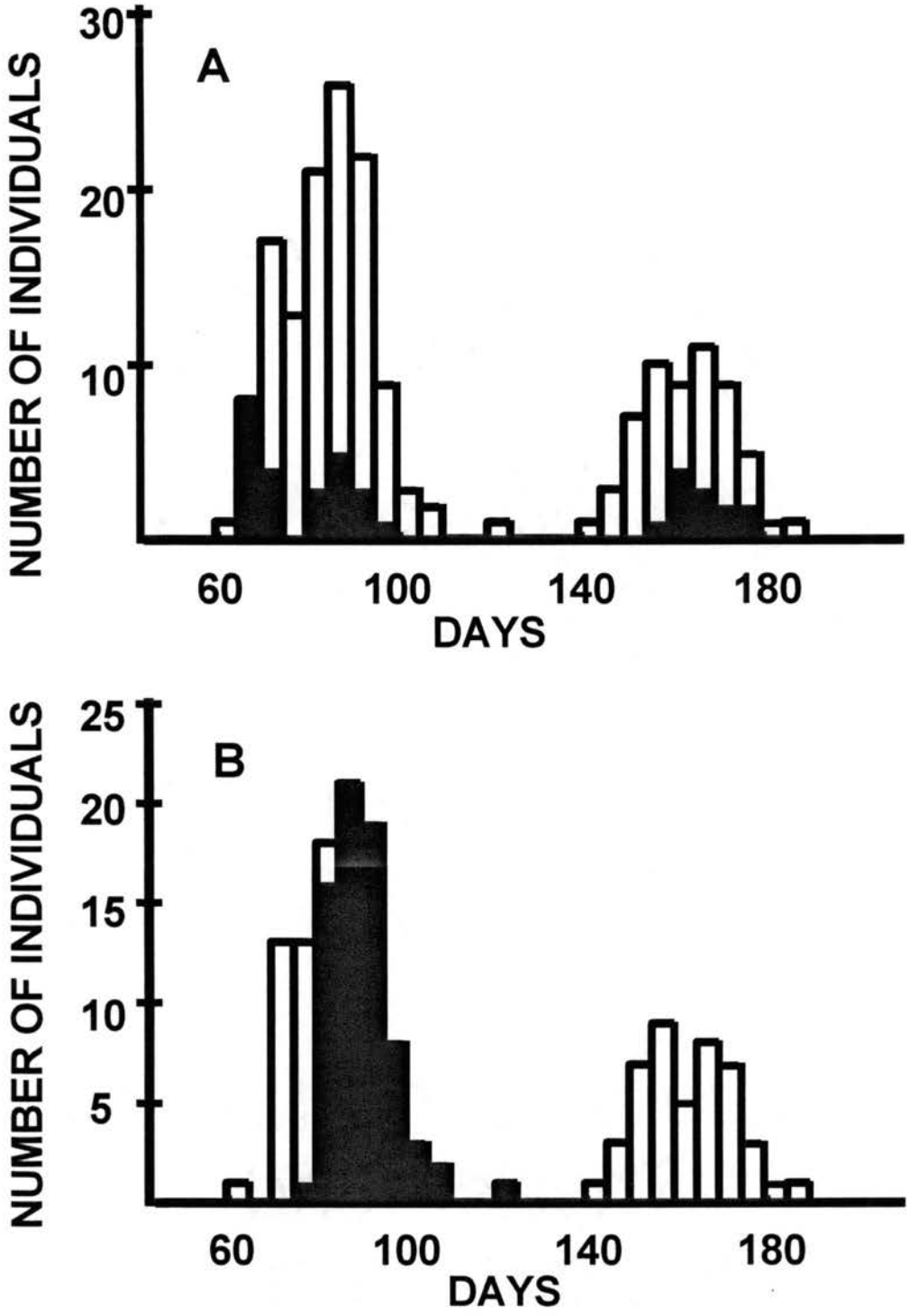

Fig. 4. The number of days to spike emergence for plants grown under controlled environment, without vernalization treatment, indicating parental and F_1 checks (blackened bars) and F_2 segregants (transparent bars) in A, and all F_2 individuals, identifying individuals (blackened bars) whose progeny segregated for growth habit in the F_3 generation in B.

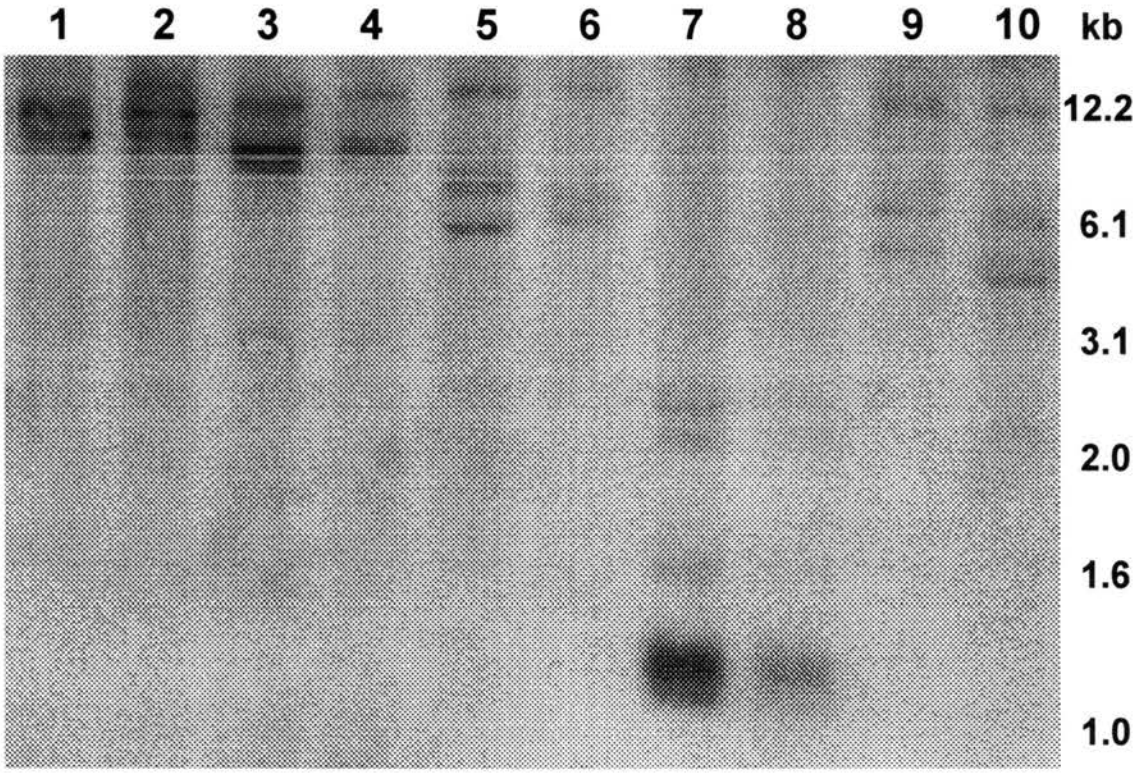

Fig. 5. Autoradiograph following Southern hybridization with ^{32}P labeled Xbcd450 as a probe with each lane containing 15 μg of digested DNA. By lanes (enzyme/sample): (1) *Eco*RI/Triple Dirk C (*vrn3vrn3*), (2) *Eco*RI/Triple Dirk E (*Vrn3Vrn3*), (3) *Eco*RV/Triple Dirk C, (4) *Eco*RV/Triple Dirk E, (5) *Hind*III/Triple Dirk C, (6) *Hind*III/Triple Dirk E, (7) *Dra*I/Triple Dirk C, (8) *Dra*I/Triple Dirk E, (9) *Xba*I/Triple Dirk C, (10) *Xba*I/Triple Dirk E.

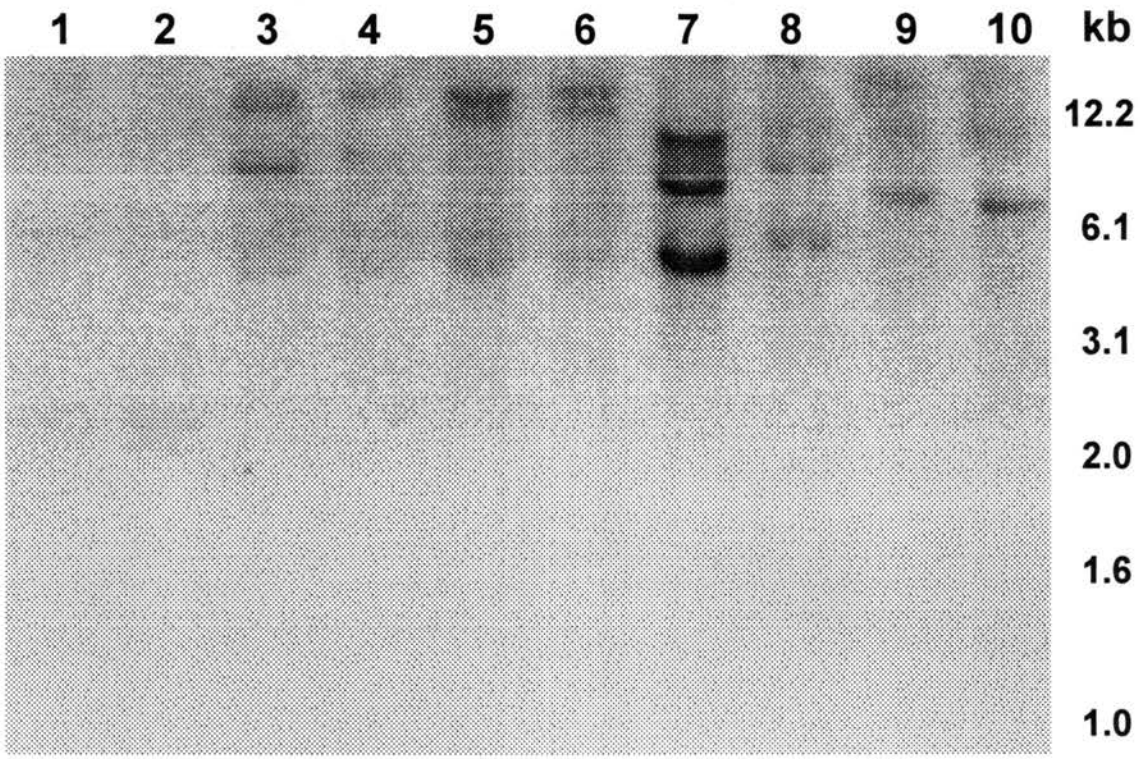

Fig. 6. Autoradiograph following Southern hybridization with ^{32}P labeled Xrz395 as a probe for lanes containing 15 μg of digested DNA. By lanes (enzyme/sample): (1) *Eco*RI/Triple Dirk C (*vrn3vrn3*), (2) *Eco*RI/Triple Dirk E (*Vrn3Vrn3*), (3) *Eco*RV/Triple Dirk C, (4) *Eco*RV/Triple Dirk E, (5) *Hind*III/Triple Dirk C, (6) *Hind*III/Triple Dirk E, (7) *Dra*I/Triple Dirk C, (8) *Dra*I/Triple Dirk E, (9) *Xba*I/Triple Dirk C, (10) *Xba*I/Triple Dirk E.

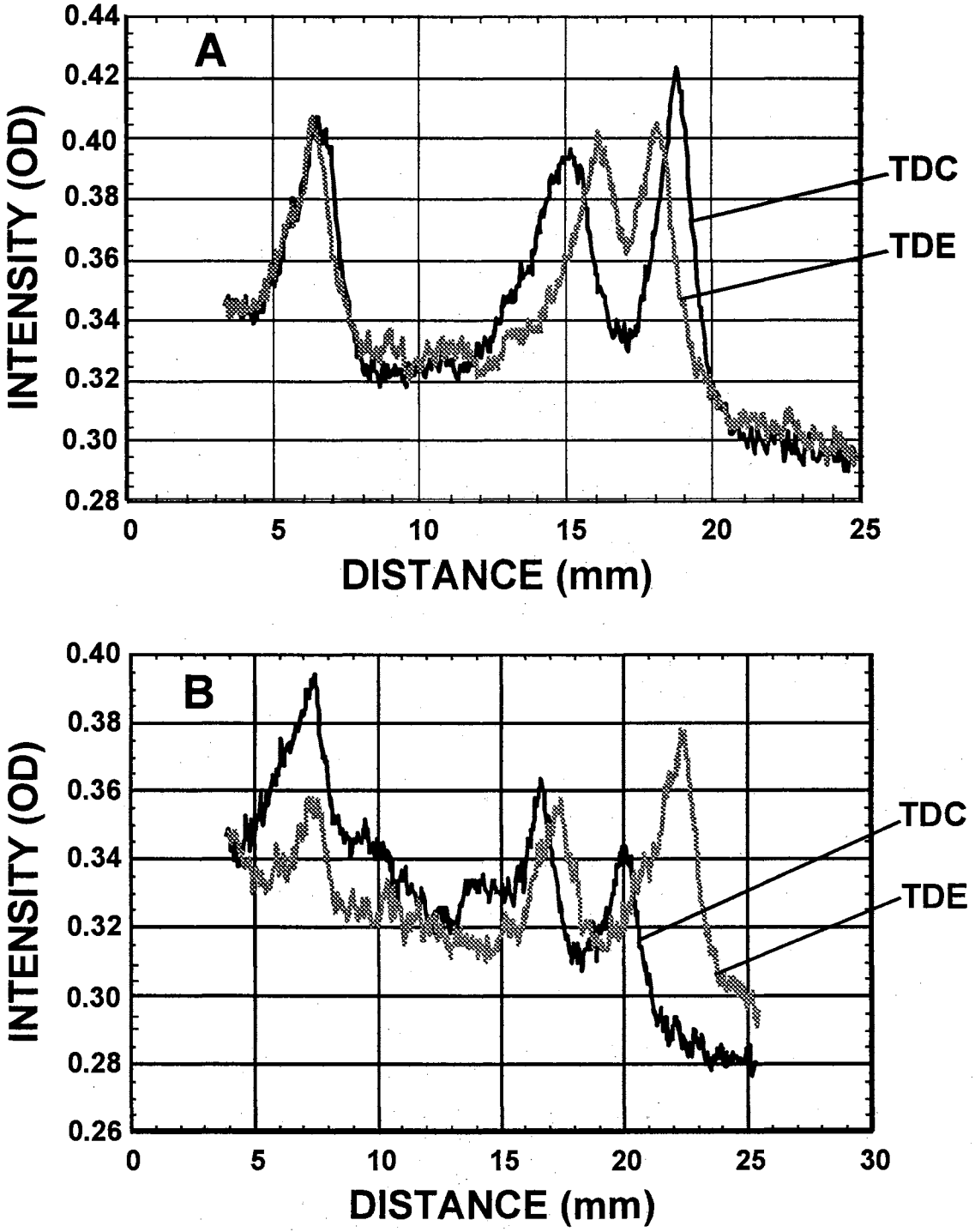

Fig. 7. Fragment analysis by measuring band intensity on autoradiogram against migration, evidencing three bands (by peaks) for *Hind*III digestion probed with Xbcd450 and polymorphism between parents for the second fragment in A, and fragment analysis for *Xba*I digestion probed with Xbcd450 and polymorphism for third fragment in B.

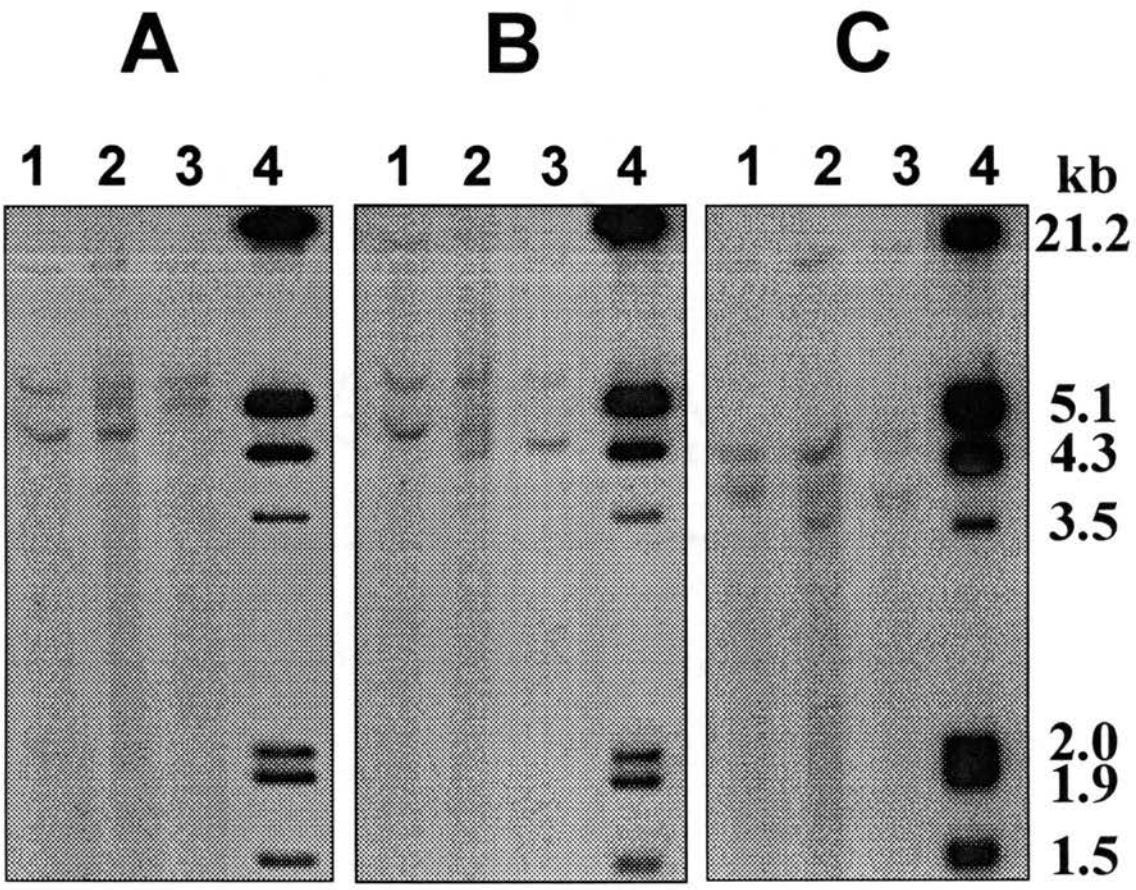

Fig. 8. Digests with *Hind*III (A) and *Xba*I (B) and the double digest (C) of samples of the winter parent Triple Dirk C (*vrn3vrn3*) in lane 1, the heterozygote (*Vrn3vrn3*) in lane 2 and the spring parent Triple Dirk E (*Vrn3Vrn3*) in lane 3, with a Lambda ladder included for size analysis in lane 4. The fragments from the double digest mirror that of the single digestion with *Xba*I , but all are of lower molecular weight, suggesting restriction site(s) for one enzyme are inclusive in the fragment from a digestion with the other.

Hypothetical Scenarios

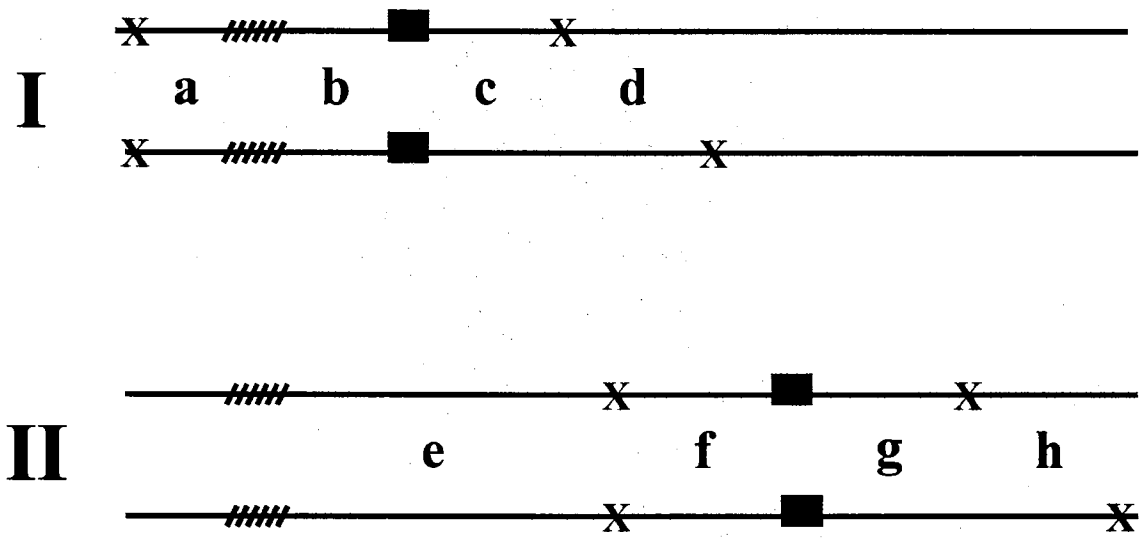

Fig. 9. Hypothetical scenarios I and II for probe hybridization to homologous DNA (black box) relative to a DNA sequence of interest (/////) following digestion by a restriction enzyme with designated cut sites (X), noting potential areas of recombination (a-h). In scenario II, the hybridization region is distanced from the gene of interest such that cross-over events frequently occur in region e preventing the gene of interest and the probe from co-segregating.

APPENDICES

Modeling for Grain Yield Data

The linear regression provided the most logical interpretation of the relative yield data when considering the year to year effects of environment. The linear, quadratic and cubic models are plotted for all locations for both hybrids and purelines (Fig. 1) and for each location (Fig. 2). As expected, addition of terms to the model continued to explain variability (Table 1), but not to the extent warranting a polynomial model rather than a linear model. One possible exception would be the model for hybrid and purelines at the Altus location (Figs. 2G and H), in which case the expansion to quadratic and cubic terms greatly increased the coefficient of determination, which were extremely low. However, there seems to be no rational basis for accepting either of these polynomial models, the cubic model tending to suggest a period of general decline in improvement in purelines or hybrids during the 1980's.

Table 1. Coefficients of determination (r^2) for variability explained by polynomial regression equations on relative indices for entries grown in the variety-hybrid performance nursery during years 1975-1995.

	Model			
Location	Linear	Quadratic	Cubic	15 degree
Goodwell irrigated				
Hybrid	0.3290	0.3396	0.3436	0.9201
Pureline	0.1307	0.1311	0.1750	0.8376
Stillwater				
Hybrid	0.5603	0.5634	0.5711	0.8693
Pureline	0.4061	0.4067	0.4067	0.7521
Lahoma				
Hybrid	0.5413	0.5791	0.6238	0.9491
Pureline	0.4704	0.5022	0.5078	0.9563
Altus				
Hybrid	0.0872	0.1197	0.3547	0.5950
Pureline	0.0050	0.2054	0.2802	0.8116
Pooled				
Hybrid	0.3638	0.3678	0.3878	0.5087
Pureline	0.2011	0.2282	0.2290	0.5178

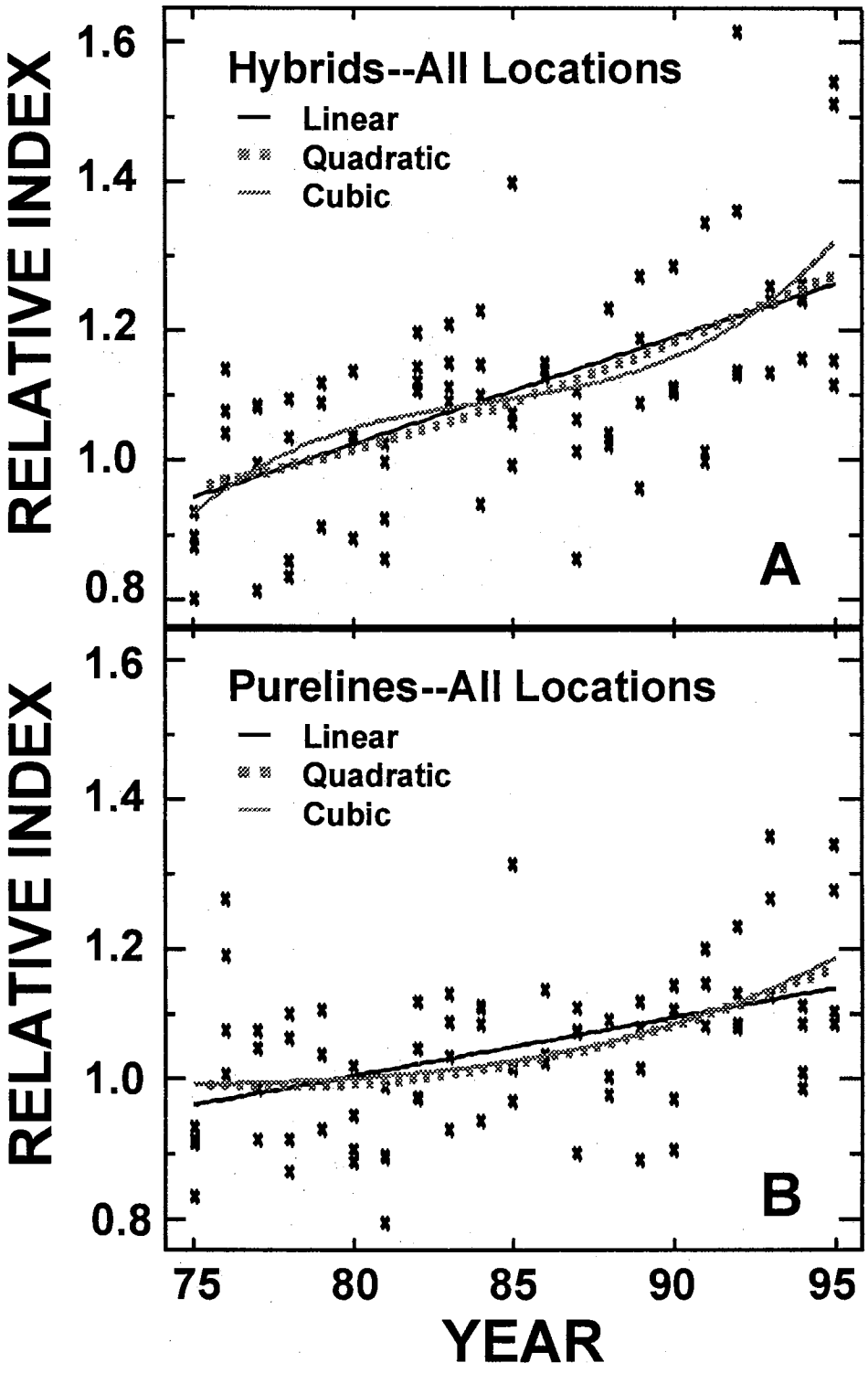

Fig. 1. Linear and polynomial modeling for hybrids (A) and purelines (B) on relative indices for all locations analyzed.

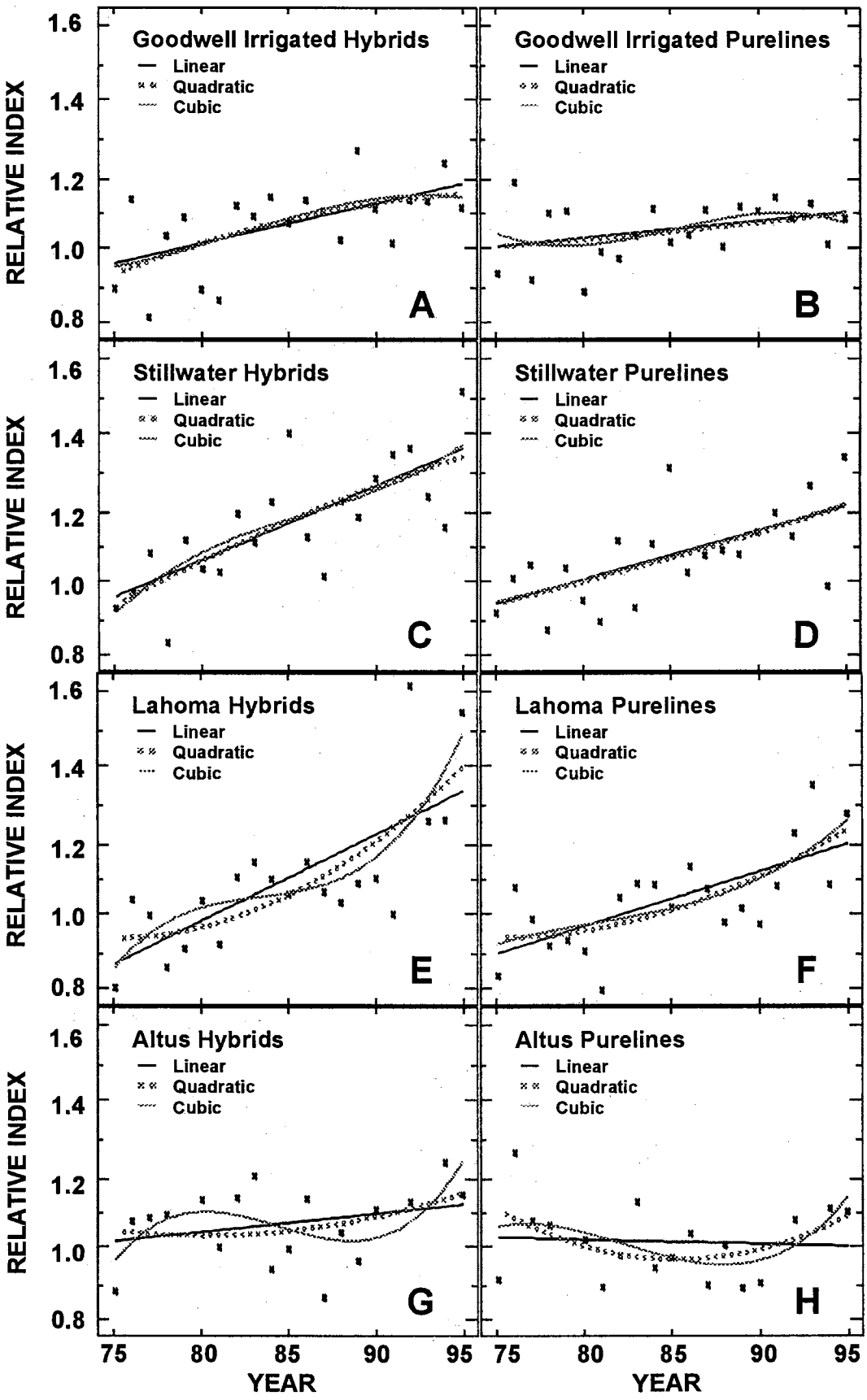

Fig. 2. Polynomial modeling by location on hybrid and pureline relative indices.

Check Cultivars Used In Calculating Relative Indices

The performance of the long-term checks was not identical in the different production environments. The data indicate that TAM W-101 offers a yield advantage in high input environments and that the yield of Triumph 64, while approximating that of TAM W-101 in poorer environments, fails to perform as well under high inputs (Fig. 1), possibly representing the superior physiology of semi-dwarfs when compared to conventional lines. This factor needs to be considered particularly when evaluating the environment for Goodwell irrigated, which, in most years, was representative of a high yield environment. However, the regression equations by location are similar for the long-term check cultivars (Table 1), all evidencing a slight trend of decrease in performance and relatively low coefficients of determination ranging from 0.0013 to 0.0845, indicating that very little of the variability was explained by the regression equation.

Table 1. Regression equations for grain yield (Mg ha^{-1}) by location for check cultivars in the variety-hybrid performance nursery during years 1975-1995.

	Check Cultivar		
Location	TAM W-101	Triumph 64	Mean
Goodwell irrigated	8.79-0.0456X†	4.92-0.00933X	6.79-0.0263X
	r^2=0.0763	r^2=0.0032	r^2=0.0286
Stillwater	4.17-0.0165X	5.63-0.0353X	4.90-0.0259X
	r^2=0.0194	r^2=0.0883	r^2=0.0514
Lahoma	6.75-0.0437X	4.22-0.0163X	5.49-0.0300X
	r^2=0.0845	r^2=0.0161	r^2=0.0484
Altus	3.31-0.00523X	4.13-0.0201X	2.99-0.00369X
	r^2=0.0021	r^2=0.0340	r^2=0.0013
Pooled	5.73-0.0273X	4.61-0.0186X	4.99-0.0208X
	R^2=0.0181	R^2=0.0116	R^2=0.0130

† The X variable is the no. of years after 1900.

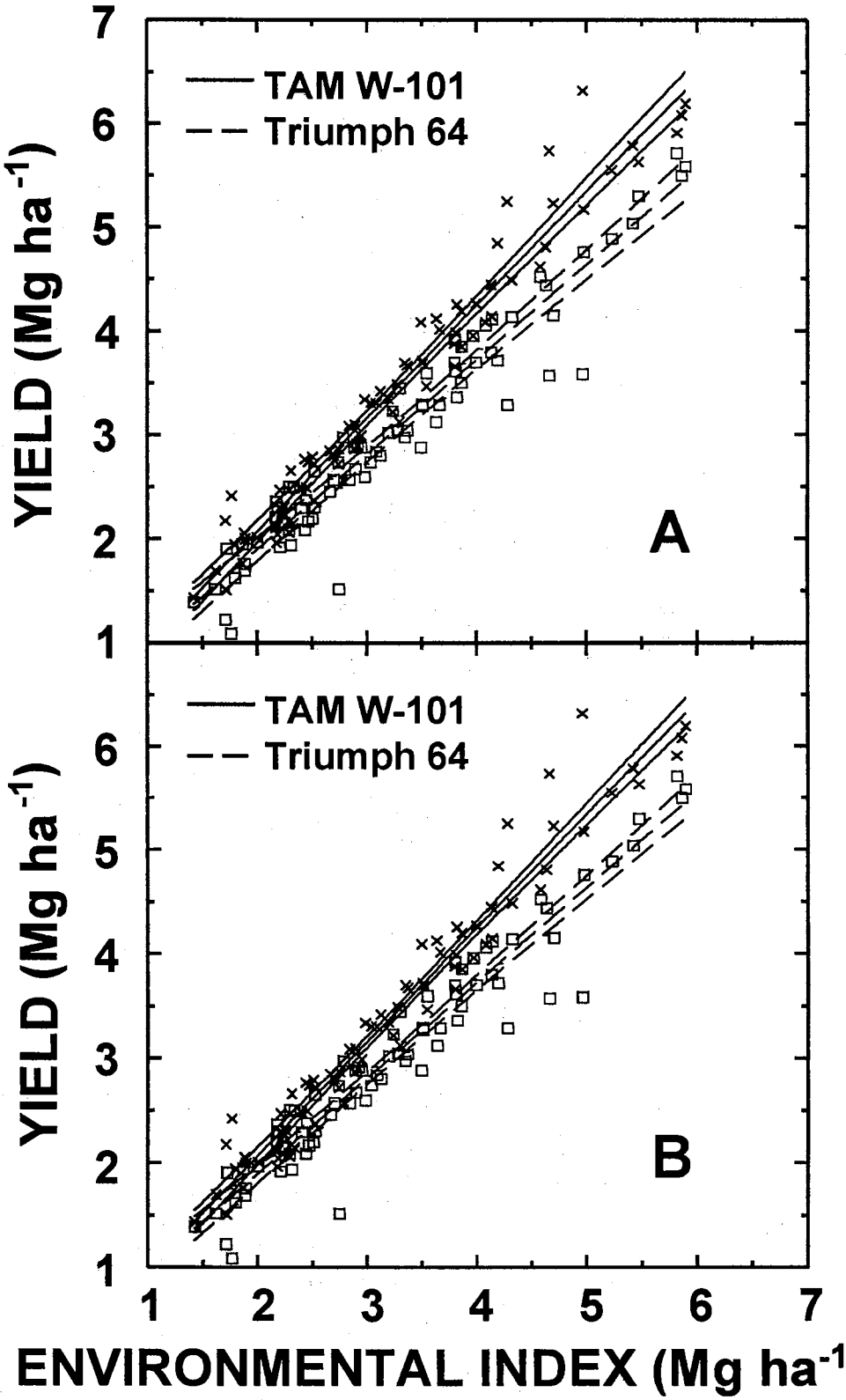

Fig. 1. Comparison of regressions of yield of long-term checks TAM W-101 and Triumph 64 in differing production environments defined by the mean check yield, with 95% (A) and 85% (B) confidence intervals plotted.

LABORATORY PROTOCOLS FOR RFLP DNA ANALYSIS

DNA EXTRACTION PROCEDURE

1. Calculate the amount of SDS extraction buffer needed for the samples at hand (25 ml for each sample). Add sodium bisulfite to the calculated volume of extraction buffer (0.19 g 100 ml^{-1}) and adjust the pH to 7.8 to 8.0 with 1 M NaOH. Small quantities of RNAseA can also be added to the extraction buffer to eliminate RNA†. Preheat the extraction buffer to 65°C in a water bath.

   † Although RNA generally does not interfere with the Southern analysis, it can affect quantification estimates and quality evaluations, particularly if large amounts of RNA are present in the sample, consuming ethidium bromide that may have been added to the gel for visualization of DNA.

2. Grind the tissue using a prechilled mortar and pestle. Grind in liquid nitrogen until the tissue sample appears as a fine green powder. Thorough grinding increases the yield of DNA. Additional liquid nitrogen may be added to prevent the sample from thawing while grinding. Pour the liquid nitrogen with care to avoid splashing the sample from the mortar. Pour the ground tissue through the chilled funnel into a prechilled 50 ml polypropylene tube, filling to the 20 ml mark. Sweep the tissue powder through the funnel with a prechilled paintbrush.

3. Extract the DNA by incubating in 25 ml of preheated extraction buffer (with sodium bisulfate) in each tube. Mix thoroughly with a spatula. Incubate for 45 minutes at 65°C in a shaking water bath. Invert the tubes every 10 minutes.

4. Remove contaminants with a chloroform: isoamyl alcohol extraction. Add 10 ml of 19:1 chloroform: isoamyl alcohol to each tube in a fume hood after allowing the tubes to cool to room temperature (approximately 10 minutes). Cap the tubes and shake vigorously for 10 seconds, or until the sample appears as a milky emulsion. Ensure that the tubes are balanced and centrifuge for 15 minutes at 1200 *x*g.

5. Recover the DNA suspended in the aqueous phase by pour through a Miracloth™ filter into a 50 ml polypropylene tube. Remove additional impurities by again adding 10 ml of 19:1 chloroform: isoamyl alcohol to each tube. Shake vigorously for 10 seconds and centrifuge for 10 minutes at 1200 *x*g.

6. Recover the DNA containing upper phase by pipetting the upper aqueous phase into a 50 ml polypropylene tube.†

   † If the aqueous phase recovered appears to have contaminants, or is greenish in color, it is preferable to repeat steps 4 and 5 to remove contaminants.

7. Precipitate the DNA by adding 25 ml of cold (-20°C) 95% ethanol. Invert the tubes several times and precipitate overnight at -20°C.

8. Recover the DNA (white globular mass) by lifting out of the tube with a hooked Pasteur pipette that has been sealed at the tip. Blot briefly on a Kimwipe™ and place in a microcentrifuge tube.

9. Wash the DNA with 1 ml of 70% ethanol. Blot briefly on a Kimwipe™ tissue.

10. Resuspend the DNA in 500 µl of TE (10 mM Tris-HCl pH 8.0, 1 mM Na_2EDTA) in a 1.5 ml nonsiliconized microtube. Allow the DNA to solubilize for at least 1 hour at 65°C, or overnight at 4°C. Gently vortex to assist resuspension. Do not despair if large amounts do not go into solution.

11. Remove contaminants by centrifuging for 10 minutes at 10,000 rpm to pellet material that did not go into solution. At this point the sample may be used directly. Quantify by comparing 10 µl of the sample on an agarose gel against a known standard and evaluating the quality by electrophoresis the sample on an agarose gel and observe the smear in each lane associated with sheared or degraded DNA.

12. The purity of the DNA may be enhanced by pouring the top 400 µl into a 1.5 ml microfuge tube. Discard the debris and lower portion of the aqueous phase remaining in the tube. Some loss of DNA will occur. Care must be taken to gently manipulate the DNA as additional mechanical degradation can become problematic.

13. Eliminate RNA by digesting with RNAse A. Add 10 µl of RNAse A stock solution (10 µg ml^{-1}) and incubate at 37°C for 1 hour.

14. Precipitate the DNA by adding 800 µl (2X volume) of 95% ethanol, and adding 300 µl of 4 M LiCl to increase the salt concentration to 0.8 M. Mix thoroughly by gentle inversion and allow to precipitate overnight at 4°C.

15. Hook out the DNA with a Pasteur pipette or toothpick into a 1.5 ml microfuge tube. Wash the pellet with 1 ml of 95% ethanol, then with 1 ml of 70% ethanol. Resuspend the pellet in 200-500 µl of TE overnight at 4°C.

16. Quantify the DNA after determining the optical densities at 260 nm (OD260) and 280 nm (OD280) on a spectrophotometer. Use a 100:1 sample dilution (990 µl TE and 10 µl of DNA sample). µg DNA=50*dilution factor*OD260/1000

17. Calculate a purity ratio. The OD 260 to 280 ratio should be 1.80-2.00. If the DNA is not in this range, it may be necessary to perform a phenol: chloroform extraction to remove contaminants.

DNA DIGESTION, ELECTROPHORESIS AND TRANSFER

1. In general, the ITMI blotting procedure (Nelson 1994a) works sufficiently well and has been adopted with few modifications. Blots may be stored between acetate sheet protectors at 4C.

2. Prepare a worksheet for adding each ingredient in the digestion (see Restriction Enzyme Digestion Worksheet, this Appendixes). Calculate the volume mix to include final concentrations of 4.0 mM spermidine and 1.0 mM DTT. Use 2.5 units of restriction enzyme for each µg of DNA to be digested.

3. For hexaploid wheat, 10-15 µg of DNA should be loaded in each lane for adequate signals. Using the sample DNA concentration, calculate the amount needed. For purposes of the digestion reaction volumes are best kept to a minimum and the restriction enzyme contribution should be less than 0.1 volume of the final reaction mix, otherwise the enzyme activity could be inhibited by glycerol (Sambrook et al., 1989). Consider that the total volume of digestion mix (plus any loading buffer) should fit inside the volume of one well (ca. 35 µl). Larger volumes may be used but the sample must be concentrated, or a larger gel well poured.

4. Keep all reagents and reaction tubes on ice. Add each ingredient according to the worksheet, preferable with the least expensive being added first and the restriction enzyme being added last. Be cognizant of the potential for loss of enzyme activity, and work quickly to add the enzyme once it is removed from the freezer.

5. Allow the reaction to digest overnight at 37°C and arrest by adding gel loading buffer to a 1X concentration.

6. Pour an 1.0% agarose gel (1.0 grams 100 ml^{-1} TBE) and allow to solidify for at least one hour prior to loading samples.

7. Add running buffer (TBE) until the top of the gel is submerged. Allocation of additional running buffer or water may be necessary to replace losses due to leaks or evaporation, particular for electrophoresis for long periods of time or at high voltages causing elevated temperatures.

8. In addition to electrical and mechanical hazards, the loss of running buffer to the extent that the gel becomes partially dried results in increases in nonspecific hybridization during Southern analysis, greatly increasing the level of background activity in radioisotope analysis.

9. Load samples in individual wells. Include a control lane such as partially digested probe and also a lane with a ladder so that the size of resulting fragments can be estimated.

10. Pull the samples into the gel by running at 100 volts for 15 minutes. Then electrophoresce at 25-40 volts until the bromophenol blue marker dye has migrated at least 3/4 the way across the gel (20 hours for 1.0 % on a 20x25 BRL Horizon).

11. Depurinate for 8 minutes by submerging gel with gently rotation in an acid wash (0.25 N HCl).

12. Rinse twice in Millipore H_2O.

13. Neutralize for 20 minutes by submerging the gel in an alkaline solution (0.4 N NaOH) with very gently rotation.

14. Transfer the DNA fragments to a nylon membrane (Amersham Hybond N+) by alkaline transfer. Prepare the blot by laying a foundation of two sheets of filter paper soaked in 0.4N NaOH. Lay the gel (upside down) on the filter paper. Lay a piece of nylon membrane (prewet with H_2O and immersed in 0.4 N NaOH immediately before use) directly on the gel. Nick the nylon membrane in the upper left side for orientation. Add 1 sheet 3MM Western blot chromatography paper (Whatman) soaked in 0.4 N NaOH. Add 2 sheets of filter paper soaked in 0.4 N NaOH. Remove air bubbles at each step by gently rolling a glass stirring rod across the top. Add 2 cm of an absorbent material such as dry paper towels to create an upward wicking transfer. Finally, apply a weight (approximately 1 kg) and allow the DNA to transfer 4-8 hours. For optimal results, ensure that the absorbent material does not contact the gel or foundational filter paper.

15. Rinse the membrane twice in 2X SSC, for 15 minutes each time.

16. Blots may be used immediately in hybridization reactions, following prehybridization, or may be stored for later experimentation between acetate sheets at 4C. Prior to storing, blot excess moisture from membranes with blotting paper and place between acetate sheets.

PREHYBRIDIZATION

1. Prior to prehybridization, raise salmon sperm (Sigma) in H_2O to a concentration of 1 $\mu g\ \mu l^{-1}$ and shear DNA passing repeatedly through a 22 gauge syringe and aliquot and store at –20°C in 1 ml aliquots. Also prepare Hybridization Buffer and store in 50 ml aliquots at –20°C.

2. Prepare Prehybridization Solution by mixing aliquot of salmon sperm with aliquot of Hybridization Buffer. Denature 1 ml salmon sperm DNA at 95°C for 10 minutes. Chill on ice for 10 minutes. Add 1 ml SS DNA to 50 ml hybridization solution preheated to 65°C in a Rubbermaid container. Mix by swirling. Add membranes one at a time to the Rubbermaid container.

3. Preheat Prehybridization Solution to 65°C. For 1-2 membranes use 50 mls. For more than 2 membranes use 100 mls.

4. Prehybridize membranes in a 50 mls of 1X prehybridization solution for 2-12 hours at 60-65°C in Rubbermaid™ or plastic trays.

^{32}P RADIOISOTOPE SAFETY

1. Dedicate an area of the lab to radioisotope work and labeling reactions. Use Beta blocking shield and the dedicated workstation at all times when handling ^{32}P isotope. Use Beta blocking container when transporting the pig and vial from storage areas to work areas. Nalgene™ finger holder racks are ideal for transporting or containing 1.5 µl centrifuge tubes during labeling reaction.

2. A Geiger counter, correctly calibrated, must be available at all times when handling radioisotopes.

3. Tubes should be opened with a dedicated opener. Pipetting should be done with aerosol barrier tips. Pipetting loading and dispensing should be performed slowly and without repeated intake and ejecting motions.

4. Contain any denaturing or boiling reactions within the dedicated work area and cover the tops of reaction tubes, water baths, heating blocks in the event of a boiling explosion.

5. Inspect waste containers prior to beginning an experiment for external contamination. Ensure ability of waste container to accept anticipated levels of waste. Segregate solid (dry) and liquid radioactive waste in appropriate containers with adequate Beta blocking protection. Discard liquid waste in secure bottles, preferably stored within another sealed container in the event of leaks or spills. Dedicate a glass funnel to assist in the transfer of liquid waste to waste bottles.

6. Log all isotope work in permanent records. Remove the label from the packing sheet and tape it to sheet in the isotope records. Include the date of use, name of user, the amount (mCu) of isotope used and information regarding waste and disposal.

7. Plan carefully in advance and order the anticipated quantity (mCu) of ^{32}P.†

   † A considerable discount is realized when ordering larger volumes of isotope. If other researchers participate in scheduling and ordering, expense efficiency of substrate can be achieved.

8. Organize expedited mail delivery. Perform swipe tests upon delivery. Swipe tests can be complete quickly using Q tips (Fisher) wet with water, and placing the swipe in a scintillation vial with 5 ml of scintillation fluid (Fisher) in each vial. Include the following:

   1. Blank
   2. Box (shipping container)
   3. Styrofoam or internal packaging
   4. Outside of the container (pig)
   5. Vial

PROCEDURE FOR LABELING PROBES

1. Label probes using ^{32}P CTP. Begin the random primer labeling procedure so that random primer incubation occurs simultaneously with prehybridization. The Procedure published by ITMI (Nelson 1994a) has been tested and works satisfactorily; however, the labeling kit by Amersham™ has produced more consistent results as provided a high labeling efficiency.

2. Set heating blocks at 37°C and at 95°C. Allow radioactive label ^{32}P CTP to thaw inside area shielded by Plexiglas. Label 1.5 µl tubes to be used in the labeling reaction. Perforate the top of tube with a 21 gauge syringe to prevent caps from popping during heating and denaturing steps.

3. Prepare labeling reaction mixture in 1.5 µl tubes. Denature the probe template before adding ingredients for the labeling reaction. A Nalgene™ holding rack works well to hold the 1.5 µl tubes during the labeling reaction.

4. Calculate the volume of probe template solution to provide 10-25 ng of probe template†. Add sterile ddw H_2O to bring to the desired reaction volume‡.

   † The probe template can be prepared in advance at a precalculated concentration and stored at –70°C so that predetermined volume (2 µl) delivers the correct amount of template. For instance, 3.0 µg (3000 ng) of excised probe (calculated based on insert size and plasmid size) can processed from a plasmid preparation and raised in 200 µl of Tris buffer. Assuming 70% efficiency in digestion, electrophoresis and yield from a Qiagen™ column, 2 µl should deliver 21 ng of purified probe as a template for each labeling reaction.

   ‡ The reaction volume depends on the procedure or kit being used. ITMI recommends a labeling volume of 28 µl. Amersham™ kits use a reaction volume of 50 µl.

5. Add the calculated volume of H_2O. Add the calculated volume of probe. Check to ensure that the ^{32}P is thawed.

6. Heat denature probe template at 95°C in heating block for 10 minutes. Chill on ice for 5 minutes.

7. Add the oligonucleotide mixture and Klenow enzyme. Finger vortex. Centrifuge briefly and place back on ice. Minimize delays once the Klenow enzyme is added.

8. Check pipette and hands for any isotope contamination. Wear double gloves. Add 5 µl ^{32}P. When adding ^{32}P, use aerosol barrier tips and avoid contacting sides of pipettor or tips with sides of vial. Check pipette and hands for isotope contamination.

9. Gently finger vortex to mix. Place in Nalgene™ rack for labeling reaction. Incubate at 37°C for 3-4 hours or overnight at room temperature.

10. Return pig and vial for storage in freezer and survey work area for contamination.

11. Following incubation, remove unincorporated nucleotides (optional step†) with Sephadex columns.

† No difference was observed in hybridization efficiency or levels of background when this step was omitted.

PREPARATION OF SEPHADEX COLUMNS

a. Prepare Sephadex solution (100 g of G50 Sephadex™ beads in 150 ml STE solution) and store at 4°C.

b. Use a 1 cc syringe to prepare the spin column. Add a small quantity (enough to prevent beads from passing through base of column, about 3mm thick) of glass wool and push to the base of the column using a Pasteur pipette or glass rod. The columns are stable for several hours following preparation. Prepare 1 spin column for each probe.

c. Shake Sephadex solution to resuspend. Fill 1 cc syringe to the top with Sephadex solution using a Pasteur pipette.

d. Centrifuge at 400g for 4 minutes. Use a 15 ml tube to hold the column and as a reservoir for solution passing through column.

e. Refill column with Sephadex solution and spin again.

f. Add 100 µl STE to top of newly prepared Sephadex column and spin at 400g for 4 minutes to test.

g. Add labeled probe mixture to tops of columns and centrifuge at 400g for 4 minutes. Add 100 µl STE and centrifuge again. A 1.5 µl tube placed at the base of the 15 ml tube assists in the capture of the labeled probe. Free nucleotides remain in the column. Place spent columns in sealed plastic bags and treat as solid radioactive waste.

h. Recover carefully the labeled probe by pipetting from the collection tube and place in a new labeled 1.5 µl tube for heat denaturization. Be extremely careful. If the syringe tip or pipette tip contact any part of the column or collection tube then there is a high potential for contamination. A sample of the recovery can be placed in scintillation cocktail and counted to determine specific activity.

12. Set a heating block at 95°C for denaturation step. Preheat hybridization fluid to 65°C.

13. Place collection tubes with labeled probe on heating block at 95°C for 10 minutes to denature. Cover all in the event of volatilization or explosion.

14. Chill tube on ice. Centrifuge briefly.

PROCEDURE FOR HYBRIDIZATION

1. Add all labeled probe to 50 ml of hybridization solution† in a Rubbermaid™ container.

   † 50 ml aliquots of hybridization buffer are prepared in advance and stored at -20°C. No formamide is used. A minimal volume of hybridization solution is optimal to maximize probe concentration; however, a sufficiently large volume must be used to avoid drying which causes areas of high background.

2. Place this Rubbermaid container inside the next larger size Rubbermaid container to minimize spread of potential leaks or contamination.

3. Place containers in hybridization oven at 65°C with gentle rotation or shaking (50 rpm) for overnight hybridization.

4. Check work area and equipment with Geiger counter. Clean any contaminated areas with Rad Con™ and Kimwipes™. Discard any waste in solid radioactive waste disposal. Soak any contaminated equipment in Lift-Away Soap™ solution in dedicated sink area.

PROCEDURE FOR WASHING AND DETECTION

1. Leave the hybridization oven on following hybridization steps. Lower stringency washes may be accomplished by lowering the oven temperature. Ensure availability of adequate wash solution. Ensure all equipment--glass funnel, waste bottle, kimwipes, Beta-blocking waste containers--are in place for proper liquid and solid waste disposal. Ensure availability of all equipment needed: Filter forceps, Blotting paper, Acetate sheets (Glad/Saran Wrap), Glass rod, Cassette holder, Film, and Autoradiography tape and pen.

2. Dispose of the radioactive hybridization solution in an appropriate liquid waste container and all solid waste in appropriate solid waste containers.

3. Fill the emptied hybridization container with wash solution sufficient to submerge the membranes (50-100 ml). Wash the labeled membranes to remove excess probe with gentle shaking or rotation (50 rpm). In general, three washes are sufficient:

   2X wash solution (2X SSC, 1.0% SDS) at 65°C for 20 minutes (Low stringency).
   1X wash solution (1X SSC, 1.0% SDS) at 65°C for 20 minutes.
   0.5X wash solution (0.5X SSC, 1.0% SDS) at 65°C for 20 minutes (High stringency).

   Dispose of all radioactive wash solution between each washing step in an appropriate liquid waste container.

4. Assay the membranes with Geiger Counter for activity. If general background is detected, continue the washing procedure with high stringency wash for up to three more hours.

5. Blot the membranes briefly between sheets of blotting paper to remove any excess fluid.

6. Position the membrane with the DNA side up (nick in the upper left side) on a clean acetate sheet (Glad or Saran Wrap). Fold the acetate sheet to encase the membrane. Roll out bubbles with a glass stir rod.

7. Load in cassette holder with DNA side up. Add labeling tape with identifying notes regarding date, membrane and probe, to preserve permanent record and orientation on film following exposure. Mark lanes or important boundaries with autoradiography pen. Expose tape to intense light before adding film in the dark room.

8. Place film and intensifying screens in cassette holder (Kodak X-Omatic®).

9. Expose X-ray film to the membrane for 3-14 days. Exposure times will vary for each hybridization and for each probe tested. Estimate length of time for exposure based on radioactivity assay following assay after final wash step. In general, assays evidencing 200 cpm should be exposed for 2 weeks and assays evidencing bands with more than 1000 cpm should be exposed 3-7 days. Expose in -70°C freezer.

10. Survey work area and all equipment with a Geiger counter to ensure no contamination in the laboratory.

11. After exposure period, develop film. Reexpose for a longer or shorter time, if necessary.

12. Membrane should be stripped following exposure by placing in a solution of 0.4N NaOH at 37°C with gentle shaking or rotation (50 rpm) for 10 minutes. Remove solution and discard as radiation waste. Repeat.

REAGENTS

SDS (Sodium Dodecyl Sulfate) extraction buffer.

Ingredient/Stock Solution†	Quantity for 1 Liter	Final Concentration
5 M NaCl	100 ml	500 mM
1 M Tris-HCl pH 8.0	100 ml	100 mM
0.25 M EDTA	200 ml	50 mM
20% SDS	62.5 ml	1.25 %
Milli Q H_2O	to volume	--
Sodium bisulfite‡	3.8 g	--

† Autoclave all stock solutions, except 20% SDS. Premix the extraction buffer, without sodium bisulfite, but stir with heating prior to use to solubilize any precipitates.

‡ Add the sodium bisulfite (0.38 g 100 ml^{-1}) immediately before use and adjust pH to 7.8-8.0. (Nelson, 1994a).

5X Gel loading buffer.

Ingredient	Amount to Add for 4 ml	Final Concentration
10 mM Tris, pH 8.0	1840 µl	4.6 mM
500 mM EDTA, pH 8.0	160 µl	200 mM
Bromophenol blue	0.005 g	0.125 %
Glycerol	2000 µl	50 %

Hybridization buffer†.

Ingredient	Amount to Add for 1000 ml	Final Concentration
1 M Na_2PO_4, pH 7.2	500 ml	0.5 M
BSA (Bovine Serum Albumin, fraction 5)†	10 g	1%
20% SDS (Sodium Dodecyl Sulfate)	350 ml	7%
Milli-Q H_2O	62 ml	--

† Suspend BSA in ddw H_2O (10 g 150 ml^{-1}) and filter sterilized by vacuum filtration. Mix the hybridization buffer well at elevated temperatures and aliquot before the solution cools to prevent precipitation.

VITA

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Doctor of Philosophy

Dissertation: TRENDS IN HYBRID AND PURELINE WHEAT YIELD AND STABILITY IN OKLAHOMA AND AN ANALYSIS OF THE VERNALIZATION CHARACTER WITH RFLP PROBES IN HEXAPLOID WHEAT (*TRITICUM AESTIVUM* L.)

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