Cause of tall off-types and estimation of genome relationships in wheat
by Eric William Storlie

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Crop and Soil Science
Montana State University

Abstract:
Wheat breeders work to develop genetically uniform wheat (Triticum aestivum L.) cultivars. Phenotypic off-types in a population may be an indication of residual heterozygosity. Tall off-types occur in some spring wheat varieties at a frequency as high as 0.20%. Cytological analysis of offspring from selfed tall off-types showed a high proportion of monosomic chromosome numbers, indicating that aneuploidy may be associated with the tall off-types. The offspring of tall off-type selections were planted and analyzed for height-segregation and production of nullisomic plants. Nullisomic 4B and 4D conditions were determined by PCR (polymerase chain reaction) using primer set G10 which amplifies distinct DNA fragments for chromosomes 4A, 4B and 4D. Nullisomic 4B and 4D offspring were identified by the absence of a DNA fragment from respective chromosomes. The offspring analysis for segregation and production of nullisomics was used to infer the chromosomal constitution of the tall off-type selection. Results of this study indicate that semidwarf wheat with height-reducing genes, Rht1 or Rht2, produces ta11 off-types when monosomic 4B or 4D conditions occur, respectively. Rhtl resides on chromosome 4B and Rht2 on chromosome 4D. Six varieties with Rht1 genotypes produced an average frequency of 0.0016 monosomic 4B wheat, and 5 varieties with Rht2 genotypes produced an average frequency of 0.0005 monosomic 4D wheat. Variation for occurrence and effect of monosomic 4B and 4D conditions between Rht1 and Rht2 may be utilized in a breeding program to minimize the problem of ta11 off-types.

In a second set of experiments, amplified DNA fragments from chromosomes 4A, 4B and 4D were used for sequence comparisons to estimate genome relationships. The DNA fragments were cloned and sequenced. Aligned sequences were compared by Parsimony and Distance matrix methods.

Parsimony analysis produced a tree that grouped, with 93% confidence, the 4A and 4D genomes on a separate clade from the B genome. Distance matrix grouped 4A and 4D genomes with 61% confidence. Results indicate the A and D genomes are more closely related than either are to the B genome.

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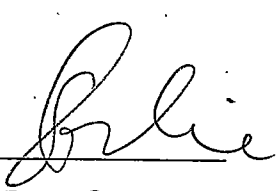
Signature 

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ABSTRACT

Wheat breeders work to develop genetically uniform wheat (*Triticum aestivum* L.) cultivars. Phenotypic off-types in a population may be an indication of residual heterozygosity. Tall off-types occur in some spring wheat varieties at a frequency as high as 0.20%. Cytological analysis of offspring from selfed tall off-types showed a high proportion of monosomic chromosome numbers, indicating that aneuploidy may be associated with the tall off-types. The offspring of tall off-type selections were planted and analyzed for height-segregation and production of nullisomic plants. Nullisomic 4B and 4D conditions were determined by PCR (polymerase chain reaction) using primer set G10 which amplifies distinct DNA fragments for chromosomes 4A, 4B and 4D. Nullisomic 4B and 4D offspring were identified by the absence of a DNA fragment from respective chromosomes. The offspring analysis for segregation and production of nullisomics was used to infer the chromosomal constitution of the tall off-type selection. Results of this study indicate that semidwarf wheat with height-reducing genes, *Rht1* or *Rht2*, produces tall off-types when monosomic 4B or 4D conditions occur, respectively. *Rht1* resides on chromosome 4B and *Rht2* on chromosome 4D. Six varieties with *Rht1* genotypes produced an average frequency of 0.0016 monosomic 4B wheat, and 5 varieties with *Rht2* genotypes produced an average frequency of 0.0005 monosomic 4D wheat. Variation for occurrence and effect of monosomic 4B and 4D conditions between *Rht1* and *Rht2* may be utilized in a breeding program to minimize the problem of tall off-types.

In a second set of experiments, amplified DNA fragments from chromosomes 4A, 4B and 4D were used for sequence comparisons to estimate genome relationships. The DNA fragments were cloned and sequenced. Aligned sequences were compared by Parsimony and Distance matrix methods. Parsimony analysis produced a tree that grouped, with 93% confidence, the 4A and 4D genomes on a separate clade from the B genome. Distance matrix grouped 4A and 4D genomes with 61% confidence. Results indicate the A and D genomes are more closely related than either are to the B genome.

ONE

INTRODUCTION

This dissertation describes three research projects which are separated into chapters two, three and four. Each project follows a progression of related ideas and hypotheses. The initial idea was provoked by an occurrence of tall off-types in a recently released Montana wheat variety, 'Hi-Line'. The persistent frequency of about 0.20% tall off-types was considered problematic and a curiosity because the frequency could not be controlled by culling the off-types. A research project was designed to explore the question of tall off-types, and subsequent projects were designed to explore questions spawned by the initial results.

Chapter two describes the analysis of tall off-types in Hi-Line. The possibility of impure genotypes was discounted by comparing glutenin banding patterns of the off-types with the Hi-Line banding pattern. A cytological analysis of the off-type offspring indicated that some of the offspring were monosomic and nullisomic. These conditions suggested an aneuploid chromosomal constitution for the tall off-types.

The next step involved identification of the chromosomes involved with the aneuploid condition. Cytological techniques such as C-banding allow the

identification of chromosomes, but these techniques are difficult and time consuming. Another approach involves the use of molecular DNA markers that are chromosome specific. The presence and absence of DNA marker bands indicate the presence and absence of chromosomes. The molecular approach can be used to identify absent chromosomes in nullisomic wheat. As indicated by the cytological analysis, some of the offspring were nullisomic. A missing chromosome 4B was identified by PCR (Polymerase Chain Reaction) using primer set G10, which amplified distinct DNA fragments for chromosomes 4A, 4B and 4D. Tall off-types that produced height-segregating and nullisomic 4B offspring were inferred to have a monosomic 4B condition.

Chapter two concludes that the monosomic condition of chromosomes 4B and 4D, containing the *Rht1* (*Rht1Rht1rht2rht2*) and *Rht2* (*rht1rht1Rht2Rht2*) genotypes, respectively, may cause tall plants. An effective methodology that relied on an offspring analysis was developed to determine a cause and effect for tall off-types in Hi-Line.

Chapter three approaches a follow-up question on variation for the occurrence and effect of tall off-types. Eleven semidwarf wheat varieties with either *Rht1* or *Rht2* genotypes were compared for frequencies and height effects of tall off-types. The same basic methodology developed in chapter two was used for the genotypic comparisons. Results

in chapter three indicate that 5 varieties with *Rht1* and 3 with *Rht2* genotypes had an occurrence of tall off-types caused by monosomic 4B and 4D conditions, respectively. Three varieties included in the analysis produced no detectable monosomics, which may be due to relatively small population sizes (*n*) in two of the populations. Comparisons indicate that *Rht2* genotypes have a lower frequency of tall off-types than varieties with *Rht1* genotypes. Comparisons within *Rht1* or *Rht2* genotypes indicate there were no genotypic influences on the occurrence of tall monosomic wheat. Comparisons for effect on height indicate significant differences between *Rht1* and *Rht2* genotypes and significant differences within the *Rht1* genotypes. This chapter concludes that *Rht1* and *Rht2* genotypes of semidwarf wheat may produce tall monosomic plants; variation was detected for monosomic conditions and for height effects between *Rht1* and *Rht2* genotypes and for height effects within *Rht1* genotypes. This variation may be utilized to minimize the occurrence and effect of tall off-types during a breeding procedure.

Chapters two and three relate to questions of tall off-types and rely on a common methodology which utilizes PCR to amplify DNA sequences from each of the 3 wheat genomes. DNA bands were associated with chromosomes 4A, 4B and 4D using tetrasomic 4 (nullisomic 4) stocks. PCR amplification of euploid wheat appears as three bands on an electrophoresis

gel. Amplification of nullisomic 4A, 4B or 4D wheat appears as 2 bands that indicate the nullisomic condition. Thus the three DNA sequences were clearly amplified from each of the genomes.

A question of evolutionary relationships between the A, B and D genomes of wheat was spawned by the identification of homologous DNA fragments amplified by PCR. Chapter 4 approaches the question of genome relationships. Each of the DNA fragments associated with chromosomes 4A, 4B and 4D were cloned and sequenced. The nucleotide sequences were aligned and compared using phylogenetic analyses. Results indicate that the A and D genomes are more closely related to each other than either are to the B genome. These results correspond with several chromosome pairing studies and provide nucleotide sequence data for more evidence on evolutionary relationships in wheat.

The three chapters approach questions related to wheat genetics and breeding. Chapter two shows that a frequency of tall off-types in a wheat variety, Hi-Line, is caused by a monosomic 4B condition. This result suggests that tall off-types may be an inherent and unavoidable feature of semidwarf wheat. Chapter 3 shows that both *Rht1* and *Rht2* semidwarf wheat may produce tall off-types and that there is some variation for occurrence and height effects between *Rht1* and *Rht2* genotypes and for height effects within *Rht1* genotypes. This variation may be used in a breeding program

to minimize the problem of tall off-types. Chapter 4 shows that DNA sequence comparisons in wheat may be an effective approach to evolutionary questions. It also bolsters evidence that the A and D genomes are more closely related. An understanding of evolutionary relationships may be important for wheat breeders to utilize ancestral germplasm for intraspecific crosses.

TWO

CAUSE OF TALL OFF-TYPES IN A SEMIDWARF SPRING WHEAT

Literature Review

Wheat cultivars are released by breeders as homozygous inbred genotypes that are genetically and phenotypically uniform. Phenotypic variation is caused by outcrossing, mechanical mixtures and aneuploid conditions (Hollingshead, 1932; Love, 1943; Myers, 1938; Powers, 1932; Riley and Kimber, 1961; Thompson and Robertson, 1930). Products of outcrossing and mechanical mixtures are easily culled, if phenotypically distinct. Occurrences of aneuploids may be difficult to manipulate because of a random and spontaneous nature.

Wheat is relatively tolerant of some aneuploid conditions because of its hexaploid (2n=6x=42) condition. Sears (1954) documented tetrasomic, trisomic, monosomic and nullisomic conditions for most of the chromosomes of wheat, and he discovered that most of these aneuploid conditions were viable. Several early studies identified an occurrence of unpaired chromosomes at metaphase I in different varieties of wheat (Hollingshead, 1932; Myers, 1938; Powers, 1932; Riley and Kimber, 1961; Thompson and Robertson, 1930). Riley and Kimber (1961) observed pollen cells of five

hexaploid wheat varieties and estimated a frequency of 0.0028 pairing failure per bivalent. The same frequency of pairing failure was estimated for five diploid ancestors of hexaploid wheat. Riley and Kimber (1961) suggest these chromosomal anomalies will produce a frequency of 0.00186 monosomic wheat from euploids, and in the diploid ancestors, the anomalies will not produce sporophytes.

Monosomic wheat was estimated by Sears (1953) to produce frequencies of 0.73 monosomics, 0.24 euploids and 0.03 nullisomic offspring. These proportions were calculated from an estimate of chromosomal constitutions of female and male gametes and respective transmissions to offspring. Seventy-five percent of female gametes are expected to have 20 chromosomes because univalents should lag and frequently become lost from the nucleus. Ninety-six percent of male gametes are expected to have 21 chromosomes because pollen with 21 chromosomes should outcompete those with 20 chromosomes for successful unions with eggs (Morrison, 1953; Sears, 1953).

Sears (1954) analyzed the offspring of monosomic Chinese Spring wheat for each chromosome and estimated frequencies of nullisomic offspring. Frequencies ranged from 0.9 percent for chromosomes 5B and 5D and 7.6 percent for chromosome 3A. Chromosomes 4B and 4D had frequencies of 6.4 and 5.9 percent, respectively (chromosomes 4B and 4D are relevant to this study). A small proportion of monosomic

offspring are isochromosomal or telocentric due to misdivision at meiosis (Love, 1943; Morrison, 1953; Sears, 1952). Sears (1954) estimated frequencies of telocentric and isochromosomal offspring produced from monosomic plants for each chromosome. Monosomic 4B and 4D wheat produced 8.4 and 0.9 percent telocentric or isochromosomal 4B and 4D offspring, respectively. The offspring of monotelocentric and monoisochromosomal plants produced offspring that were nullisomic, monotelocentric or monoisochromosomal, ditelocentric or diisochromosomal, or telocentric and isochromosomal (Sears, 1954).

Aneuploidy in wheat may result in an occurrence of phenotypic offtypes in wheat varieties (Love, 1943; Riley and Kimber, 1961; Worland and Law, 1985). Variation between varieties for the occurrence of aneuploid conditions suggests meiotic instability is heritable (Myers, 1938; Powers,1932; Riley and Kimber, 1961). Riley and Kimber (1961) further suggest the phenotypic effect of certain aneuploid conditions varies between varieties.

Worland and Law (1985) showed tall offtypes that occurred in a cultivar of spring wheat, 'Brigand', were due to a monosomic 4D condition. They estimated monosomic 4D plants occurred with a frequency of 0.13% in a particular population that had been culled of off-types in the previous generation. Monosomic conditions were associated with tall offtypes from three other varieties - 'Hobbit', 'Frontier'

and 'Guardian' - though frequencies of their occurrences were not determined. Worland and Law (1985) noted that there were significant height differences between monosomic plants of Hobbit and the other three varieties.

Chromosome 4B contains a gene, *Rht1* that confers gibberellin insensitivity and is involved with height reduction in some semidwarf and dwarf wheats (Gale et al., 1975). Another gene, *Rht2*, on chromosome 4D, serves a similar function (Gale and Marshall, 1976).

A recently released semidwarf cultivar from Montana, Hi-Line, has a frequency of 0.20% tall off-types that may be 17 to 34% taller than the population mean (Lanning et al., 1992). The objective of this investigation was to determine the genetic basis for tall off-types in Hi-Line and to determine segregation frequencies of off-type offspring, using cytological and molecular techniques and field observations for analysis.

Materials and Methods

Observations of Tall Off-Types in Hi-Line

A traditional head-row/line-row cultivar purification procedure was followed prior to the release of Hi-Line (Lanning et al., 1992). In 1988, 550 lines consisting of approximately 50 individuals per line, derived from single heads of MT8402 - subsequently named Hi-Line - were evaluated for phenotypic uniformity in Bozeman. Due to lack

of uniformity, including the presence of tall off-types, 102 lines were discarded. The remaining 448 lines were evaluated as line rows in 1989, and 208 were discarded largely due to the presence of tall off-types. Of approximately 84,000 individuals evaluated in this nursery, 199 (0.2%) were classified as tall. Seed harvested from the remaining 240 line rows were planted in 1990, and 110 rows were eliminated due to the presence of tall off-types (with an average of 22% taller than the population mean). Seed from the remaining 130 lines were bulked to form breeder seed of Hi-Line.

Segregation Analysis of *Rht1* and *Rht1* Crosses

Euploid Hi-Line was reciprocally crossed with six varieties containing known height-reducing genes. 'Siete Cerros', 'Inia' and 'Nacozari' are homozygous for *Rht1;* 'Ciano', 'Pavon' and 'Jaral' are homozygous for *Rht2* (Kihara, 1983).

Glutenin Analysis

Seeds were harvested from tall off-types and a seed from each plant was evaluated for its glutenin banding pattern using SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) to determine the genotypic purity of Hi-Line off-types.

Seed storage proteins were extracted by method of Laemmli (1970). Each seed was ground with a mortar and

pestle and suspended in 500 μL of 70% ethanol (C_2H_5OH). The samples were briefly vortexed and incubated in a 55 °C waterbath for 1 h and centrifuged for 2 min at 8,000 X g. A 250 μL aliquot of supernatant was removed from each sample and placed in a 4 °C refrigerator for 1 h. The cold supernatants were dried in a vacuum centrifuge (Savant, Farmingdale, NY). The pellets were resuspended in a cracking buffer - 23% glycerol, 80 m*M* sodium dodcyl sulfate, 145 m*M* Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol, 1.7 m*M* bromo phenol blue (3',3",5',5"-tetrabromo-phenolsulfonephthalein), 2.1 m*M* xylene cyanole FF and 30 m*M* β-mercaptoethanol.

Glutenins were separated through a 12% polyacrylamide gel - 12% acrylamide 460 m*M* Tris, 0.10% SDS, 0.01% Ammonium Persulfate, $(NH_4)_2S_2O_8$, 5 m*M* Temed, N,N,N',N'-tetramethylenediamine - using 80 volts for 16 h. Gels were stained for two h in 1.2 m*M* Coomassi Blue. The analysis for genetic purity involved a comparison of glutenin banding patterns of each tall off-type sample with a euploid Hi-Line sample.

Cytological Analysis

Ten randomly selected seeds from off-type Hi-line were germinated in petri dishes on water-soaked, 3mm filter paper (Whatman, Maidstone, England). Root-tip squashes followed the smear technique of Tsuchiya (1971). After 2 d, when

roots were at least 1 cm in length, the root tips were placed in H_2O at 4 °C for 24 h, then fixed in 45% glacial acetic acid (CH_3CO_2H) for 0.5 h. After fixing, root tips were placed in 4 °C H_2O prior to squashing and staining in acetocarmine solution (2% carmine and 45% glacial acetic acid) for cytological analysis.

DNA Extraction

Approximately 10 mg of leaf tissue was removed from wheat seedlings for DNA extraction by method of Lassner et al. (1989). Each tissue sample was ground with a glass rod in 1.5 mL microcentrifuge tubes (National Sci., San Rafael, CA) containing 100 μL extraction buffer - 0.77 m*M* sorbitol, $C_6H_{14}O_6$, 1.8 m*M* Tris, 0.06 m*M* EDTA, 13.7 m*M* $NaCl_2$, 0.06 m*M* CTAB (Hexadecyltimethyl-ammonium Bromide), $C_{16}H_{33}N[CH_3]Br$, 0.12 m*M* Sarkosyl (N-Lauroylsarcosine), $C_{15}H_{28}NO_3Na$, 1.82 m*M* β-mercaptoethanol - and 100 mg sand, and the solution was heated to 65 °C for 30 min. Samples were centrifuged for 5 min, and the supernatants were added to 60 μL isopropanol, mixed, and chilled at -20 °C for 30 min. Samples were centrifuged for 5 min to form a pellet, and the supernatants were discarded. Pellets were washed with 60 μL 70% ethanol and the previous step was repeated. The pellets were resuspended in 40 μL TE (10m*M* Tris, 1 m*M* EDTA). These DNAs were used in PCR reactions.

Polymerase Chain Reaction Primers

Two RFLP clones for chromosome 4 were obtained from B.S. Gill, Kansas State Univ. (Gill et al., 1991). These clones, labeled G10 and D21, were sequenced at both ends by the dideoxy chain termination method (Sanger et al., 1977). Primers of 20 bases in length were designed from the sequenced clones and were synthesized with a PCR-mate 391 DNA synthesizer (Applied Biosystems Inc., Foster City, CA), using standard phosphoramidate chemistry. Primer sets WG464 and ST4-6, specfic for loci on barley chromosome 4, were obtained from T. Blake (Montana State Univ., Bozeman). Primer concentrations were adjusted to 100 ng/μL with a spectrophotometer (Varian Techtron, Australia). The sequences of the primers (5'-3') are: G10L-GTGTTGATGTCCTTGAGGCC, G10R-TGTCCAGCTTCAGCGAGTAC, D21L-TCTTCCAGTTAGAGATCTCC, D21R-TCGTTCGTACTAGTAGTACC, WG464L-AGGACTGTGAAGATGCTACT, WG464R-AGTCCAAATGATGTCACAGG, ST4-ATCCACAGCGGCTGTTCCAC, ST6-CTTGGCCACCGTCATGGTCT.

Primer Evaluation

The three sets of primers were tested for PCR amplification on Chinese Spring wheat, tetrasomic-4D (nullisomic-4A), tetrasomic-4B (nullisomic-4D) and progeny of tetrasomic-4D (monosomic-4B) obtained from USDA-ARS (Columbia, MO). The progeny of monosomic Chinese Spring are expected to include a high proportion of nullisomic-

tetrasomic individuals (E.R. Sears, 1989, personal communication). Also included was *T. speltoides* (Tausch) Gren. ex Richter, a B genome relative of *T. aestivum*.

Polymerase Chain Reaction Conditions

Twenty-five μL reaction mixtures for PCR contained 2.5 μL 10x buffer (500 m*M* KCl, 100 m*M* Tris and 1% Triton X-100), 50 μ*M* each of dATP, dCTP, dGTP and dTTP, 1.5 m*M* $MgCl_2$, 400 n*M* of each primer, 0.6 unit *Taq* polymerase, and 100 ng of genomic DNA. Approximately 60 μL of light mineral oil overlaid each reaction mixture. Reactions occurred in 0.5 mL microfuge tubes (West Coast Scientific, Inc., Hayward, CA) that were thermocycled in a model 50 Tempcycler (Coy Laboratory Products Inc., Grass Lake, MI) with the following temperature conditions: 94 °C for 4 min followed by 30 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1.2 min, and ending with 72 °C for 7 min.

Polymerase Chain Reaction Product Analysis

The reaction products were digested with 1.7 units of *Hin*fI or *Rsa*I (New England Biolabs, Beverly, MA) per reaction mixture for 1 h at 37 °C. PCR products were electrophoresed through a 7% polyacrylamide gel - 7% acrylamide, 1x TBE (44 m*M* Tris, 44 m*M* boric acid and 1.0 m*M* EDTA), 4 m*M* ammonium persulfate, 3.3 m*M* temed - immersed in 0.5x TBE buffer. Gels were stained with ethidium bromide and photographed over ultraviolet light.

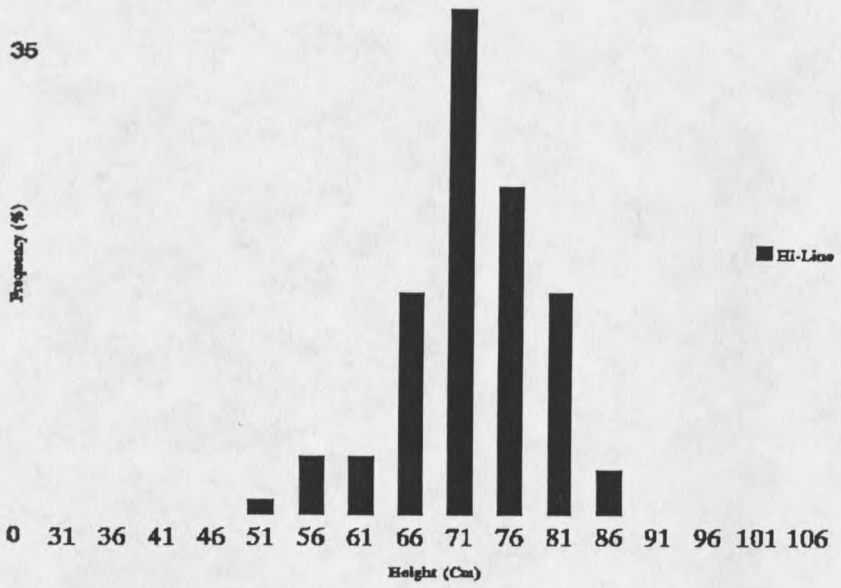

Figure 1. Height distribution of euploid Hi-Line wheat, in 1991.

Tall Off-Type Selection and Analysis

Fifty tall off-type plants were harvested from a line-row plot of Hi-Line with an estimated population size of 28,248 plants. Off-type selection was based on the height-distribution of euploid Hi-Line (Figure 1), and plants with heights taller than the euploid height-range were harvested in 1991 for further analysis. Seeds from 10 off-type Hi-Line plants were planted in pots, and 138 Gen_1 (first generation) offspring were grown to maturity in 1991 under greenhouse conditions. Second generation lines were planted in individual rows in the field in 1992 and evaluated for morphological and molecular (PCR) variation. Offspring produced from monosomic 4B Hi-Line had a height-distribution which is graphically compared with a euploid distribution in

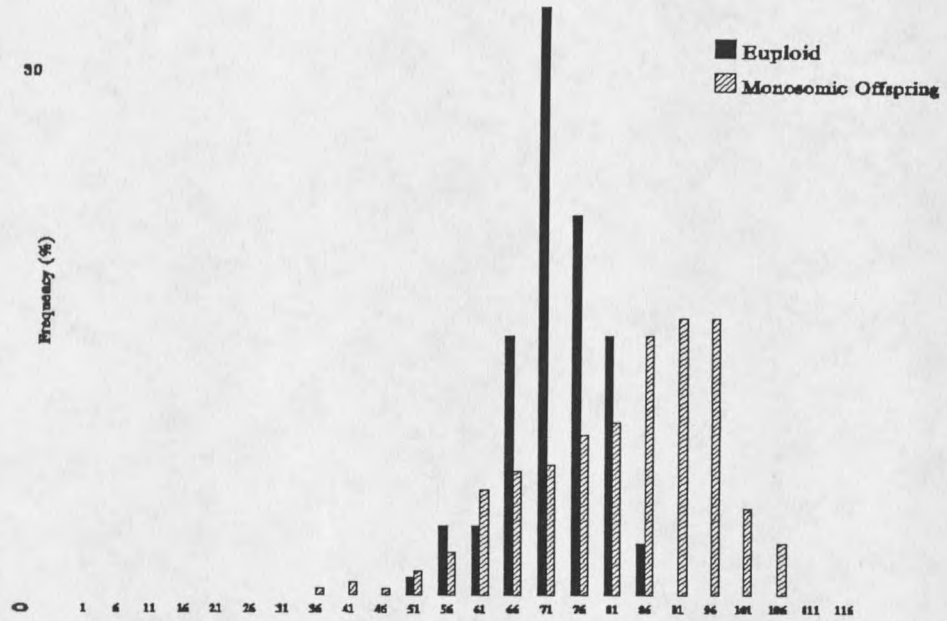

Figure 2. Height distributions of uniform euploid and segregating monosomic offspring of Hi-Line wheat, in 1994.

Figure 2. Fifty rows segregating for height, with a total 1215 plants, were selected for an evaluation of fertility and morphology. The DNA extracts from 68 randomly selected Gen_1 and 113 randomly selected Gen_2 plants were amplified for PCR analysis, using the G10 primer, when plants were at a two-leaf stage. The DNA from a subset of plants was also amplified using WG464 and ST4-6 to confirm results with G10. Plants showing a nullisomic 4B banding pattern were monitored through maturity to distinguish the nullisomic 4B morphology. Plants from Gen_1 were classified as monosomic 4B if the Gen_2 family segregated for height and as disomic 4B if the Gen_2 family was height-uniform. Plants from Gen_2 were classified as monosomic 4B if the height measured more