COLD HARDINESS EVALUATION OF SIX NEAR-ISOGENIC LINES OF HEXAPLOID WHEAT (*TRITICUM AESTIVUM* L.) AND DEVELOPMENT OF A NON-RADIOLABELED SYSTEM FOR IDENTIFICATION FOR RESTRICTION FRAGMENT LENGTH POLYMORPHISMS

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1985

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
May, 1996

OKLAHOMA STATE UNIVERSITY

COLD HARDINESS EVALUATION OF SIX NEAR-ISOGENIC LINES OF HEXAPLOID WHEAT (*TRITICUM AESTIVUM* L.) AND DEVELOPMENT OF A NON-RADIOLABELED SYSTEM FOR IDENTIFICATION FOR RESTRICTION FRAGMENT LENGTH POLYMORPHISMS

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FORWARD

The first chapter of this thesis concerns the cold hardiness evaluation of several near-isogenic lines of winter wheat with different vernalization genes. This study was incidental to a molecular mapping project which was initially planned. The cold hardiness study was initiated because of differential responses in hardiness that were noted the first year that the lines were grown, while being vernalized in cold frames at the small grains farm, Oklahoma State University, and following abnormally cold temperatures.

The second chapter concerning the development of a non-radiolabeled system for detection of restriction fragment length polymorphisms was in connection with attempting to identify molecular markers linked to *Vrn3*, and was not related to the cold hardiness study. As part of this objective, it was necessary to develop procedures for DNA extraction and analysis.

ACKNOWLEDGMENTS

I thank my understanding wife, Tina, without whose sacrifices I would not have been able to pursue this study; and I express appreciation to my family, for words of support and encouragement, and to all the residents of Stillwater, Oklahoma, who have made this stay a memorable one.

I also express appreciation to my advisors, Edward. L. Smith, who confidently enrolled me as a graduate student and guided my training as a plant breeder; and to Arron C. Guenzi, who gratuitously provided counseling and advice, and graciously extended his laboratory to accommodate my research. I thank Jeffrey A. Anderson and Charles G. Tauer for serving as members on my committee and for assistance and ideas. I also thank Glenn T. Drown for technical assistance and Jeffrey A. Anderson for help with the statistical analysis on the cold hardiness study.

I thank the Department of Agronomy, Oklahoma State University, for providing an assistantship to support this work. I also acknowledge the Australian Wheat Collection for providing the genetic stocks used in this study. Finally, I acknowledge M. E. Sorrells (Cornell University) and M. D. Gale (Cambridge Laboratory) for providing probes used in the molecular analysis to develop the non-radiolabeled detection procedure.

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INTRODUCTION

Each chapter in this thesis conforms to the *Publications Handbook and Style Manual* of the American Society of Agronomy. Chapter I will be submitted for publication in *Crop Science*, a Crop Science Society of America publication.

An appendixes has also been provided at the end of this thesis providing an elaboration of the protocols and reagents used in the molecular analysis.

CHAPTER 1

EFFECT OF VERNALIZATION GENES ON COLD HARDINESS IN NEAR-ISOGENIC LINES OF HEXAPLOID WHEAT (*TRITICUM AESTIVUM* L.)

EFFECT OF VERNALIZATION GENES ON COLD HARDINESS IN NEAR-ISOGENIC LINES OF HEXAPLOID WHEAT (*TRITICUM AESTIVUM* L.)

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Published with approval of the director, Oklahoma Agric. Exp. Stn.
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ABSTRACT

Four major genes in wheat (*Triticum aestivum* L.), with dominant alleles designated *Vrn1, Vrn2, Vrn3,* and *Vrn4,* have large effects on the vernalization response, but the effects on cold hardiness are ambiguous. Near-isogenic experimental lines in a 'Triple Dirk' genetic background with different vernalization alleles were evaluated for cold hardiness. Although Triple Dirk is homozygous dominant for *Vrn1* and *Vrn2*, four of the experimental lines are each homozygous dominant for a different vernalization gene, and one line is homozygous recessive for all four vernalization genes. After establishment, plants were acclimated for six weeks in a growth chamber. Plants were stressed in a low temperature freezer and removed over a range of temperatures as the chamber temperature was lowered 1.3°C h^{-1}. Temperatures resulting in no regrowth from 50% of the plants (LT_{50}s) were determined by estimating the inflection point of the sigmoidal response curve by nonlinear regression. The LT_{50} values were -6.7°C for the cultivar Triple Dirk, -6.6°C for the *Vrn1* and *Vrn4* lines, -8.1°C for the *Vrn3* line, -9.4°C for the *Vrn2* line, and -11.7° C for the homozygous recessive winter line. The LT_{50} of the true winter line was significantly lower than all other lines. Significant differences were also observed between some, but not all, of the lines possessing dominant vernalization alleles. The presence of dominant vernalization alleles at one of the four loci studied significantly reduced cold hardiness following acclimation.

INTRODUCTION

A long-standing major objective in wheat breeding has been to select for lines which minimize the effects of adverse environmental conditions, such as freeze damage (Hoogendoorn, 1985). Cold hardiness has proven to be an important trait in cool season cereals, particularly since the level of cold hardiness influences the area of adaptation and production (Fowler et al., 1977). The ability to survive or tolerate low temperatures is generally accomplished through cold acclimation or hardening (Levitt, 1980). However, the maximum level of cold hardiness is not uniform among the winter cereals, nor is it uniform within a species (Fowler et al., 1977). In general, cultivars of rye can tolerate the coldest temperatures (ca. -30°C), followed by winter wheat (ca. -20°C), then barley (ca. -14°C), and finally oats (ca. -10°C) (Gusta and Fowler, 1979).

LITERATURE REVIEW

Cold acclimation in winter wheat is controlled by an inducible genetic system dependent upon a source of energy and temperatures below 10°C (Chen and Gusta, 1978). A minimum of six weeks exposure to acclimating temperatures is generally necessary to develop fully the hardiness potential, with a period of continuous frost (-2 to -3°C) required at the end of this stage (Gusta and Fowler, 1979). Typically, winter cereals become hardened in the late fall, and remain hardened through the winter months, then quickly deharden upon exposure to warm spring temperatures. However, even when fully acclimated, winter cereals do not necessarily maintain one cold hardiness level for the entire winter (Andrews et al., 1974; Pomeroy et al., 1975). The cumulative effect of many environmental factors influences hardening of overwintered cereals (Gusta and Fowler, 1979). Plants rapidly lose hardiness upon exposure to warm conditions but the

rate depends on the genetic constitution of the plant and the length and intensity of the warm period (Gusta and Fowler, 1976).

Field trials are the ultimate tests of a cultivar's cold hardiness. However, these are often inconclusive due to either complete winterkill or complete survival (Gusta et al., 1982). Laboratory-based freeze tests provide an alternate method to estimate cold hardiness. Lethal temperatures (LT_{50}s) calculated from artificial freeze tests are highly correlated with field survival trials (Fowler et al., 1973; Gusta et al., 1982). The LT_{50} is defined as the lowest test temperature at which 50% of the plants survive freezing, as determined from regrowth of leaves and roots (Gusta and Fowler, 1976). Genotypes can be separated as to levels of hardiness based on estimates of their LT_{50}s in artificial freeze tests (Pomeroy and Fowler, 1973; Gullord et al., 1975; Anderson et al., 1993).

Another difficulty in studying freezing tolerance involves effects due to environmental confounding, particularly in comparisons of spring and winter wheat cultivars. Generally, spring cultivars are grown in warmer spring environments without vernalization, whereas winter wheat cultivars are grown during a cool season and require a period of cool temperatures to satisfy the vernalization requirement. The low temperatures required to fulfill the vernalization requirement in winter wheat overlap with the temperature range promoting hardiness making it difficult to ascertain which system is causing the response (Laroche et al., 1992).

When given a vernalization treatment of eight weeks or longer, spike emergence dates are expected to be uniform across genotypes (Flood and Halloran, 1986). However, when the different genotypes are grown under temperatures without cold treatment (no

vernalization), they have distinctly different floral initiation responses (Flood and Halloran 1983, 1984, 1986).

Factors Affecting Cold Hardiness in Plants

Tissue Water Content. Lee and Chen (1993) have suggested that the major strategy for cold hardiness in most plants is tolerance to freeze-induced dehydration. One of the first events to occur during cold acclimation of winter cereals is a reduction in crown moisture content. Within a species, this reduction is highly correlated ($r^2 \geq 0.9$) with an increase in hardiness (Chen and Gusta, 1978). Artificial desiccation also increases the cold hardiness in winter cereals (Gusta et al., 1982; Cloutier and Siminovitch, 1982), whereas excessive soil moisture results in a decrease in hardiness (Metcalf et al., 1970). In winter cereals, the decline in crown moisture content is also paralleled by a decrease in tissue water to dry weight ratio (Gusta et al., 1982) and a reduction of water found in intracellular spaces (Chen and Gusta, 1978). Significant correlation between the LT_{50} and percentage crown water content and dry weight have been reported by numerous researchers (Metcalf et al., 1970; Gusta and Fowler, 1976; Fowler et al., 1977). However, the causal relation with LT_{50} is not clear (Fowler et al., 1977).

Recent research has also implicated the physical state of the water as a factor in cold hardiness associated with acclimated plants. Using magnetic resonance imaging, changes in the behavior of water in biological systems have been evaluated by characterizing the spin-spin relaxation time of the water proton. In winter wheat crowns, considerable differences exist in the biophysical state of water between cold tolerant and cold susceptible lines at -4°C (Millard et al., 1995).

Freezing Rate and Duration of Exposure. Both the freezing rate (Gusta et al., 1978) and the length of exposure to low temperatures are important in determining the degree of freeze damage (Gusta et al., 1982). The freezing rate influences partitioning of ice growth. During slower cooling rates, the intracellular water is partitioned to the extracellular space, thus preventing the ice crystallization from impacting the delicate cell membrane and destroying its fluidity. A major impediment to studying freeze-thaw damage to cells involves isolation of cells without walls. In working with isolated protoplasts, Steponkus and Wiest (1979) found that the rate of cooling directly influences the probability of intracellular ice formation; however, this influence was significantly affected by the minimum temperature to which the cells were exposed. Longer exposures to low temperatures generally cause more damage as the killing temperature is approached. The degree of strain that can be tolerated by winter cereal crowns is dependent on the duration of the stress, and additional strain is caused by desiccation (Gusta and Fowler, 1979).

Composition of the Cell Membrane. The relative fluidity of the membrane is important in conferring hardiness with a large change possible depending on the percentages of short chain and saturated fatty acids (Kenrick and Bishop, 1986). As plants are hardened in response to low temperature, changes in the membrane occur to accommodate membrane fluidity (De Silva et al., 1975; Lynch and Steponkus, 1986). However, work by De la Roche (1979) suggests that changes in the fatty acid content are not necessarily crucial to the development of cold hardiness.

Proteins. De novo protein synthesis occurs at low temperatures and aids in the development of cold hardiness, possibly due to the incorporation of the proteins into

membrane structures (Trunova, 1987). There does not appear to be a single ubiquitous protein common in cryoprotection (Lyons et al., 1979). However, *Cor* genes have been implicated in the production of transcripts and proteins in response to cold acclimation (Thomashow, 1990). Low temperatures can result in protein conformational changes, and proteins induced by cold acclimation have been isolated (Houde et al., 1992). A number of enzymes show shifts in isozymic composition (Thomashow, 1990), and electrophoretic studies have shown both quantitative and qualitative differences in the protein content between nonacclimated and cold acclimated tissues (Guy, 1990).

Chemicals and Hormones. Endogenous abscisic acid (ABA) levels rise during cold acclimation (Lee and Chen, 1993), with analysis of crown tissue from acclimated winter wheat cultivars indicating a threefold higher ABA level in the hardier cultivar (Wightman, 1979). Chromosomes 5A and 5D have been implicated in cold-induced abscisic acid accumulation (Galiba et al., 1993). Applications of exogenous ABA also increase cold hardiness in winter wheat (Lalk and Dorffling, 1985). Researchers have proposed a signal transduction system for cold acclimation involving ABA. Exposure to low temperatures results in an increase in endogenous ABA levels which in turn trigger the development of freezing tolerance by altering gene expression (Lee and Chen, 1993; Nordin et al., 1993). Increased endogenous levels of ABA are also correlated with plant water stress, including freeze-induced desiccation (Davies and Mansfield, 1983). ABA may function in the upregulation of specific genes during drought stress (Lee and Chen, 1993).

Environmental and Nutritional Factors. The level of cold hardiness is dependent upon the plant's energy source and reserves, which may be influenced by light intensity,

photoperiod, and sucrose levels. Plants grown under low light do not harden as quickly, nor reach the same level of hardiness as plants grown under high light (Gusta and Fowler, 1982). Photoperiod is generally thought to have no direct effect on cold hardiness but is important indirectly in winter wheat because daylength is important in supplying light energy for photosynthesis (Chen and Gusta, 1978). However, in woody species short days have been implicated directly in induced freeze tolerance (Lee and Chen, 1993). Hardiness may also be influenced by directly altering the energy source of plants, such as by placing roots and tillering nodes in a saccharose solution prior to freezing (Trunova, 1982), although effects due to change in osmotic potential should be considered.

Differential levels of hardiness have also been noted due to other factors such as salinity levels, where there is a reduction in cold hardiness of winter wheat under conditions of salt stress (Gusta et al., 1982). The rate of cold acclimation and the maximal level of hardiness of winter wheat are influenced by mineral nutrition. Hardiness levels may be increased or decreased depending upon availability of mineral nutrients. The addition of some nutrients, such as nitrogen, generally cause a reduction in cold hardiness, whereas others, such as potassium, are generally beneficial to winter hardiness development, while the effect of some, such as phosphorous, are variable (Dexter, 1956; Freyman and Kaldy, 1979).

Mechanisms of Freeze Injury

Evidence suggests that the injury to winter wheat occurs during freezing immediately upon passing through a critical temperature, and that the primary site of injury is the plasma membrane (Lyons et al., 1979). The formation of ice in the plant tissue is the damaging factor (Ashworth, 1992). In fully hardened wheat, ice is

partitioned from the cell interior and forms first in the extracellular spaces. The freezing rate depends on many factors including supercooling, membrane permeability, the rate of heat removal, and the rate of the ice front growth. A loss of membrane semipermeability occurs upon exposure to critical temperatures and ions leak from the tissue immediately upon thawing with no measurable lag period (Gusta et al., 1982). Although cells are severely dehydrated by freezing, it appears that dehydration alone is not the sole cause of injury. It has been estimated that nearly all of the freezable water is frozen by -10° C (Burke et al., 1976). Thus, there is little additional dehydration of the cells below this temperature.

Lyons et al. (1979) described the cellular freezing process. When cells are cooled below 0° C, they initially remain unfrozen due to depression in the freezing point caused by the solutes present, and to some extent, due to supercooling. The extracellular solution freezes first, typically between -2 and -15° C. Disequilibrium results as the intracellular solution remains unfrozen and supercooled, presumably because the plasma membrane prevents the growth of ice crystals into the cell. Equilibrium may be reached either by intracellular ice formation, or by the continued extracellular ice formation. The amount of water which must be removed to achieve equilibrium by cellular dehydration will depend on the initial osmolality of the intracellular solution and the minimum temperature to which the cell suspension is cooled. This removal depends primarily on the permeability of the plasma membrane and the surface area available for efflux relative to the cell volume in relation to the cooling rate and temperature.

Low temperatures affect the physical state of membranes by affecting fluidity (Thompson, 1979) and many plants have adapted to low temperature stress by altering the

phase separation temperature. Typically, this involves increasing the number of double bonds in lipid fatty acids. Electron spin resonance spectroscopy studies suggest abrupt phase changes in membrane fluidity when the membrane passes a critical temperature. These appear to be temperature induced alterations in the molecular ordering of membrane lipids, and thought to infer "melts" in the hydrocarbon zone of the phospholipids (Gordon-Kamm and Steponkus, 1984). Two distinct alterations occur in response to temperature. These appear to correspond with two distinct changes in the membrane structure. The first occurs when changing from a fluid phase to a mixed fluid gel phase, and the second occurs from the mixed phase to a predominantly gel phase. For most species, including wheat, the membrane lipids are in the mixed fluid gel phase at the optimum temperature range for plant growth. Once the membrane passes through the lower temperature phase change, the mixed state is altered and the membrane can no longer function properly (Lyons et al., 1979). Electrolyte loss curves show damage, or lysis, of the plasma membrane on passing below the lethal temperature; but, these curves do not show when in the freeze-thaw cycle that the lysis occurs (Rajashekar et al., 1979).

Genetics of Cold Hardiness

Inheritance of cold hardiness in winter wheat is generally regarded as a quantitative trait controlled by many loci on several different chromosomes. Segregating progeny of crosses from parents of differing cold hardiness typically exhibit a continuous range of hardiness between the parental extremes, and the freezing tolerance of many F_1 hybrids typically is near the midpoint of the parents, indicating a quantitative character controlled by a number of additive genes (Guy, 1990). However, both dominant and recessive genes have been suggested as being involved in cold hardiness (Sutka, 1981).

Genes influencing cold hardiness have been identified on at least 17 of the 21 pairs of chromosomes in hexaploid wheat through the use of chromosome substitution lines (Law and Jenkins, 1970; Roberts, 1986). The group 5 chromosomes are most frequently implicated (Sutka and Kovacs, 1985) and chromosomes 5A and 5D appear to carry major genes (Sutka and Snape, 1989; Galiba et al., 1995). Interestingly, the group 5 chromosomes are most frequently implicated in the major vernalization responses in wheat.

Much research has been devoted to defining the relationship between cold hardiness and vernalization. Cahalen and Law (1979) initially found that some cultivars carried the same recessive *vrn1* allele contributing to winter habit, but differed significantly in cold hardiness. Sutka and Kovacs (1985), working with chromosome substitution lines, concluded that genes on chromosome 5A contributed to significant differences in levels of cold hardiness among the cultivars tested, with no evidence of cytoplasmic inheritance, although Aksel (1974) has identified cytoplasmic effects on some yield components using reciprocal crosses of spring by winter cultivars. Early literature was inconclusive concerning whether the relationship between cold hardiness and vernalization was pleiotropic, or the result of close linkage. Brule-Babel and Fowler (1988) concluded that a lack of vernalization requirement, as indicated by spring growth habit, did not necessarily mean a lack of cold hardiness, suggesting that the two processes were unrelated; however, there was evidence of possible genetic linkage between cold hardiness and the vernalization requirement.

Roberts (1989) investigated the linkage between the vernalization response and cold hardiness associated with chromosome arm 5AL. Lines derived from a spring by

winter cross formed a continuum of overlapping groups, rather than two major groups for hardiness. The F_2, however, segregated 3:1 for spring to winter types. Of these, the winter types were generally more cold hardy and the spring types generally more tender, with the heterozygotes generally intermediate in hardiness. Roberts concluded that there were two, and probably more, loci on 5A affecting cold hardiness. One of the loci was suspected to be, or be closely linked to, *Vrn1*.

The most recent research suggests that the two responses are the results of two separate, but closely linked genes, at least for a 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*) were closely linked (2.1 cM). The close linkage could explain Robert's (1989) failure to detect the difference due to a relatively small segregating population. The work by Galiba et al. (1995) is limited however, since the evaluation concerned only one major vernalization gene and one major cold hardiness gene, both on the same chromosome.

Objectives of Research

The objective of this study was to determine the relationship between cold hardiness and the vernalization genes in the Triple Dirk lines.

MATERIALS AND METHODS

Genetic Stocks. Pugsley (1971, 1972, 1973) developed several near-isogenic experimental lines (NILs) isolating four different vernalization genes in a Triple Dirk genetic background summarized in Table 1. 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 (Zeven et al., 1986) representing 94 to 97% of the Triple Dirk genetic background being recovered. Five NILs were developed, each exhibiting different physiological behavior with respect to the vernalization response. Triple Dirk D (TDD) has 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.

Days to Spike Emergence. Without a vernalization period, true winter types experience an extreme delay in floral induction, whereas most spring types will develop normally without the necessity of a cool period. The number of days from plant emergence to spike emergence were recorded for each genotype grown without vernalization. Plant emergence was recorded as the first day the coleoptile was visible above the soil surface. Spike emergence was recorded as the day of the first fully emerged spike. Days to spike emergence were recorded for each line grown in a growth chamber (22/18°C day/night temperature and 12 h daylength) and greenhouse (ca 25°C and 14 h daylength). Conditions in the greenhouse were somewhat variable depending on the outside climatic conditions, particularly for light intensity and temperature. Data were taken for plants grown during fall 1994, winter 1994, and spring 1995.

Growing Conditions. Seeds of the six different NILs were imbibed in petri dishes in Terraclor™ (2.5 g l^{-1}) solution for 24 hours at room temperature (25°C). Following 24 hours of imbibition, the seeds were cold-shocked at 4°C for 24 hours to overcome dormancy. Nonviable seeds were discarded, and the remaining imbibed seeds were planted to cone-tainers™ with redi-earth™ growing medium. Seven cone-tainers were used for each NIL and three seeds were planted to each cone-tainer. Seedlings were grown for 10 days (two leaf stage) at 22/18°C day/night temperature, 12 h photoperiod, with 360 μE m^{-2} s^{-1} of light intensity. Plants were then transferred to an acclimation chamber for six weeks under a 10 h photoperiod and an 8/2°C day/night temperature. Plants were watered to field capacity twice per week with a dilute Peter's solution (20-20-20) (1.25 g l^{-1}) added every fourth watering to prevent fertility problems associated with depletion of mineral nutrients. The stage of plant development was evaluated visually for all plants following six weeks of acclimation to confirm uniformity of plant growth. A random sample of six plants from each NIL was also evaluated for stage of development by dissection. Spikelet and floret development of the dissected plants were compared to growth development plates in the Cereal Development Guide (Kirby and Appleyard, 1981) to confirm that no plants had advanced beyond the vegetative stage.

Freezing Procedure. Cold hardiness was evaluated by subjecting samples to freezing temperatures over time in a low temperature freezer (Gusta and Fowler, 1977). Plants were subjected to the freeze test in the original cone-tainers in which they were grown. The procedure of Anderson et al. (1993) was used for determining LT_{50}, modified slightly to allow for trimming of the wheat plants, adjustments in the cooling rate, and the bracketing of a larger temperature range.

Vegetative tissue was trimmed one day prior to plants being placed in the low temperature freezer to allow them to be more easily maneuvered in the freezer. On the eve of the freeze test, the cone-tainers were watered to field capacity. On the morning of the freeze test the plants were randomized by genotype and thermocouples were inserted 2 cm into the growing media in each cone-tainer so that temperatures could be monitored on an individual cone-tainer basis (Anderson et al., 1993). Cone-tainers were fitted into predrilled slots in a 2 cm thick removable aluminum plate. Plants were loaded into the low temperature freezer (SureTemp CEC23, Rheem Scientific) and equilibrated overnight at -3°C. An ice chip was added to each cone-tainer as a nucleating source to prevent excessive supercooling.

The following morning the temperature in the freezer was lowered at the rate of 1.3°C $hour^{-1}$ which was slightly slower than the 1.5°C h^{-1} described by Gusta and Fowler (1977). Plants were removed from the freezer at 1°C intervals rather than 2°C intervals described by Gusta and Fowler (1977), to target a more narrow temperature range defined by preliminary estimates of LT_{50}. For each of the six lines a temperature range of at least 7°C bracketed the expected lethal temperature. For each genotype, three cone-tainers were removed from the freezer at 1°C intervals for each temperature in the targeted range. Temperatures were monitored using a data logger and cone-tainers were removed based on individual thermocouple measurements. Thermocouple wires with detachable plugs (Omega Scientific) facilitated removal of the cone-tainers, since thermocouple junctions froze firmly to the growing media in the cone-tainer. Plants were thawed slowly over a 24 hour period in an ice chest following removal from the freezer.

Plants were allowed to grow in a greenhouse (25°C; 14 h daylength), and were scored for survival after 4 to 6 weeks. The lethal temperature at which 50% of the plants failed to survive (LT_{50}) was calculated by nonlinear regression (SAS Institute, 1991). For each of the six NILs, four estimates of the LT_{50} were made by four independent freeze tests in the spring of 1994. These were treated as replications in time and an analysis of variance was performed.

RESULTS

Days to Spike Emergence. Days to spike emergence differed between growth chamber (GC) and greenhouse (GH) environments. The mean days to spike emergence for genotypes grown in the growth chamber were less variable than those from the greenhouse. Mean dates varied with replications in time, particularly for the greenhouse, and the differential responses made it difficult to distinguish genotypes by spike emergence data alone.

Homogeneity of variance for genotypic heading dates across all environments was assumed since only one experimental unit was available for each genotype by environment cell. In a combined AOV for both greenhouse and growth chamber environments, there were significant effects due to genotype by environment interaction ($P=0.0019$). An AOV was then performed by environment. In each analysis, effects due to genotype were highly significant ($P<0.0001$).

The mean days to spike emergence and values for multiple comparisons were calculated using the least significant difference (LSD) method (Table 2). There was a significant difference between the mean days to spike emergence between some, but not

all, genotypes in the growth chamber. For the greenhouse, there was a significant difference only between the true winter type (TDC) and all other NILs.

Freeze Analysis. The relationship between survival and exposure temperature was sigmoidal (Fig. 1). LT_{50}s estimated from the midpoint of response curves indicated significant differences in cold hardiness across some, but not all, of the NILs (Table 3). Since the lines should be genetically similar, except for the respective *Vrn* loci, the inference is that the differences in cold hardiness observed are due to the effect of the vernalization genes.

Stage of Development. No visual difference in the rate of growth was detected across NILs for plants subjected to the freeze test following six weeks of acclimation. Visually, plants appeared at the same level of maturity, with tillering beginning, but without evidence of reproductive growth. Additionally, random samples of apical meristems from each line were dissected and found to be in the late vegetative stage of maturity. However, it was apparent that stem elongation was beginning to occur among some plants of the Triple Dirk cultivar (TD-). For a few, the shoot apex was beginning to elongate, signaling the beginning of the floral phase when spikelets are initiated (Kirby and Appleyard, 1981). No primordia had begun to differentiate and no spikelets were visible. No plant in any line was identified with growth so advanced to conclude the initiation of floral development.

DISCUSSION

Genetic Stocks. There appeared to be some contamination of TDC (winter type) in the TDB (*Vrn2* NIL) seed stocks in the preliminary heading date evaluation, as some plants failed to head within 100 days. These were discarded and seeds for the TDB used in the freeze test were from plants with heading dates consistent with those reported by Flood and Halloran (1983, 1984). The days to spike emergence were uniform within NIL for plants grown to increase the seed source for the freeze test, suggesting no further contamination.

Zeven et al. (1986) tested the Triple Dirk lines to evaluate the extent of their similarity. Significant differences were found across NILs for some morphological traits, and some lines appeared to be a mixture of types. 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. 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.

Days to Spike Emergence. There was initial concern over the degree of variation within the true winter type (TDC). Occasionally, some plants would flower earlier than expected, and some later. Selections were made from some of the early heading plants and progeny grown from selfed heads. No segregation resulted. All were uniformly near the expected mean for the true winter type. Similarly, a late selection was isolated from TDC with the same response.

By evaluating the ranks of the mean days to spike emergence between growth chamber and greenhouse environment, it was possible to identify changes in the ranks of

some genotypes (TDB, TDE, TDF) across environments. TDF (*Vrn4* line) appears to be most environmentally sensitive with prominent changes in rank between the growth chamber and greenhouse environments. Otherwise, the ranking is in general agreement with that of previous researchers (Goncharov and Rigin, 1991; Gotoh, 1976; Kato et al., 1988) who generally rank the vernalization genes according to their strength on promotion of heading dates: *Vrn1* > *Vrn3* > *Vrn4* > *Vrn2*.

There was a significant genotype by environment interaction when data from the growth chamber and the greenhouse were evaluated together. This may have been due to differential response of genotypes resulting from photoperiod settings in the greenhouse (14 h) and the growth chamber (12 h), or to the somewhat variable temperature, light intensity, moisture, fertility, and other environmental factors, which were probably greater in the greenhouse than in the growth chamber. Variations in environmental factors may also have obscured subtle differences in days to spike emergence.

There were significant effects due to genotype when an analysis of variance (AOV) was performed after sorting by environment. For both the growth chamber and the greenhouse, effects due to genotype were significant and effects due to replications in time were not. However, when the critical values for LSD were applied to the means, it was not possible to distinguish all genotypes by mean number of days to spike emergence. The means of genotypes in the growth chamber encompassed a wider range than those in the greenhouse, with a smaller calculated LSD value, allowing classes to be separated more distinctly. The greenhouse data were less discriminatory, and multiple comparisons on means of plants grown in the greenhouse failed to distinguish between any lines with a dominant vernalization gene.

Freeze Analysis. There were significant differences between the LT_{50}s for some of the lines studied. This suggests the vernalization genes or their associated linkage groups appear to be affecting the cold hardiness in the respective genotypes studied. The differences in cold hardiness in the freeze test do not appear to be due to differences in maturity, since all plants tested were at the same vegetative stage of development. TD-, TDD and TDF all appear to have the same approximate levels of hardiness, and differ from the other isolines in this regard. TDE (*Vrn3* line) was significantly more hardy than those three lines. TDB (*Vrn2* line) was significantly more hardy than TDE. TDC, the true winter line, was significantly hardier than all other lines.

If truly isogenic, then all lines should have identical cold hardiness alleles at all loci. The differential response across NILs implicates the vernalization genes in affecting cold hardiness. However, there appear to be differences across the lines other than the vernalization genes (Zeven et al., 1986). There is a possibility of a cold hardiness gene(s), closely linked to a vernalization gene(s), whose linkage was not broken in developing the isolines. This comment is of particular importance when considering the close linkage between the *Fr1* and *Vrn1* alleles recently identified by Galiba et al. (1995).

When considering the pedigrees of the different lines the contributing parent for the different vernalization alleles can often be determined. For TDD (*Vrn1* line), the contributor of the *Vrn1* allele was TD-, but the contributor of the v*rn2* allele is unknown (Zeven et al., 1986). Presumably, *vrn3* and *vrn4* were also contributed by Triple Dirk. TDD (*Vrn1* line) is not hardy and is not significantly different in hardiness from TD- (recurrent parent). TDC (winter line) differs for at least two loci with TD- and has a significant increase in hardiness. This increase in cold hardiness could be due to the

effect of a closely linked cold hardiness allele, such as *Fr1* described by Galiba et al. (1995).

TDB (*Vrn2* line) is somewhat more intriguing. The pedigree confirms that 'Winter Minflor' was the contributor of *vrn1* for both TDB and TDC (Zeven et al., 1986), with *vrn3* and *vrn4* presumably coming from Triple Dirk. One might expect that TDB would be similar in hardiness to either TD- (which has the identical *Vrn2* allele) or to TDC (which presumably has all identical vernalization alleles except for *Vrn2*). However, TDB is intermediate in hardiness, being significantly hardier than TDD or TD-, but not as hardy as TDC. The difference appears to be due to the *Vrn2* locus, or genes closely linked to it, although there is a possibility that the difference is due to effects of *vrn3* or *vrn4*, or genes closely linked to either, if the contributor of these alleles was actually Winter Minflor rather than TD-.

For TDE, the *Vrn3* allele was contributed by 'Loro.' (Zeven et al., 1986) as were the *vrn1* and *vrn2* alleles. The *vrn4* allele presumably came from Triple Dirk. TDE is more hardy than the recurrent parent but less so than the winter type. Once again the *Vrn3* locus, or genes closely linked to it, appear to contribute to increased hardiness. Alternatively, the differences may be partially due to the effects of the recessive *vrn1* and *vrn2* alleles, or genes closely linked to them, that were contributed by Loro. No conclusion can be reached as TDE was significantly different from TD- and TDC.

The same inferences can be made to TDF (*Vrn4* line) except that this line is not hardy. It is interesting to note that the LT_{50} of TDF is not significantly different from TD- or TDD. 'Gabo' contributed the *Vrn4*, *vrn1* and *vrn2* alleles (Pugsley, 1973), and the *vrn3* allele presumably was contributed by Triple Dirk. Only the *vrn3* allele is

identical when comparing TDF and TD-, suggesting that this allele does not independently contribute to increased cold hardiness. This differs from the results with TDE (*Vrn3* line) which suggest cold hardiness associated with the dominant (*Vrn3)* allele, or genes closes linked to it.

Does the presence of a dominant vernalization gene make a plant more susceptible to freeze damage? It appears that in all lines studied the presence of a dominant vernalization allele increased susceptibility. In all lines with *Vrn1*, the plants had low levels of cold hardiness, suggesting that the vernalization gene does not confer hardiness; rather, it may even cause susceptibility. Based on the work of Galiba et al. (1995), the *Vrn1* allele may be closely linked to a *fr* gene, which in the TD background fails to confer hardiness. However, the mere removal of the *Vrn1* allele, and any corresponding closely linked genes, does not have a uniform effect on hardiness. TDF remains sensitive, whereas TDC becomes hardy. TDB and TDE are intermediate and significantly different from other lines. This suggests that the differential level of hardiness may be associated with *Vrn2*, *Vrn3*, or the effects of closely linked cold hardiness genes.

CONCLUSIONS

An accurate assessment of number of days to spike emergence can only be obtained from plants grown under strict environmental conditions, preferably in a growth chamber rather than a greenhouse environment. Spike emergence data used to characterize lines with different vernalization requirements were variable, probably due to subtle environmental differences, making it difficult to compare lines. In the lines studied, the *Vrn* alleles contributed differentially to number of days to spike emergence in the absence of vernalization, with *Vrn1* having the strongest response, followed by *Vrn3*, *Vrn2* and *Vrn4*, although statistical differences were difficult to detect. The true winter type (*vrn1vrn2vrn3vrn4*) was significantly later than all other lines.

There were significant differences in cold hardiness as measured by LT_{50} between some, but not all, of the NILs. Since the NILs should differ at relatively few loci, namely one of the four vernalization genes studied, and all are in a common Triple Dirk genetic background, the implication is that the vernalization genes have an effect on cold hardiness. However, the possibility of effects due to closely linked genes can not be excluded. The true winter type (TDC) was the most hardy in the group, suggesting that the absence of a dominant vernalization allele makes the plant more resistant to cold. There also appears to be an uneven contribution to hardiness by the different dominant vernalization genes, as evidenced by the LT_{50}s of TDB (*Vrn2* line) and TDE (*Vrn3* line), which were intermediate in hardiness but less hardy than TDC (winter line). This suggests that cultivars with the *Vrn2* and *Vrn3* alleles should be used in spring types where increased winter hardiness is desired. These alleles may have particular utility for facultative wheat cultivars.

REFERENCES

Aksel, R. 1994. Quantitative-genetic analysis of reciprocal crosses between a winter and a spring cultivar of common wheat (*Triticum aestivum* L.). Theor. Appl. Genet. 89:1032-1038.

Anderson, J.A., C.M. Taliaferro, and D.L. Martin. 1993. Evaluating freeze tolerance of bermudagrass in a controlled environment. HortScience. 28:955.

Andrews, C.J., M.K. Pomeroy and I.A. de la Roche. 1974. Changes in cold hardiness of overwintering winter wheat. Can. J. Plant Sci. 54:9-15.

Ashworth, E.N. 1992. Formation and spread of ice in plant tissues. *In* J. Janick (ed.) Hort. Reviews. Vol. 13:215-255. John Wiley & Sons, New York.

Brule-Babel, A.L. and D.B. Fowler. 1988. Genetic control of cold hardiness and vernalization requirement in winter wheat. Crop Sci. 28:879-884.

Burke, M.J. and L.V. Gusta, H.A. Quamme, C.J. Weiser, and P.H. Li. 1976. Freezing and injury in plants. Ann. Rev. Plant Physiol. 27:508-528.

Cahalen, C. and C.N. Law. 1979. The genetic control of cold resistance and vernalisation requirement in wheat. Heredity 42: 125-132.

Chen, P and L.V. Gusta. 1978. The role of water in cold hardiness of winter cereals. p 165-174. *In* P.H. Li and A. Sakai (ed.) Plant cold hardiness and freezing stress: Mechanisms and crop implications. Vol. 1 Academic Press, New York.

Cloutier, Y. and D. Siminovitch. 1982. Correlation between cold- and drought-induced frost hardiness in winter wheat and rye varieties. Plant Physiol. 69:256-258.

Davies, W. J. and T. A. Mansfield. 1983. The role of abscisic acid in drought avoidance. p. 237-268. *In* F. T. Addicott (ed.) Abscisic acid. Praeger, New York.

De la Roche, A.I. 1979. Increase in linolenic acid is not a prerequisite for development of freezing tolerance in wheat. Plant Phys. 63:5-11.

De Silva, N.S., P. Weinberger, M. Kates, and A.I. de la Roche. 1975. Comparative changes in hardiness and lipid composition in two near-isogenic lines of wheat (spring and winter) grown at 2°C and 24°C. Can. J. Bot. 53: 1899-1905.

Dexter, S.T. 1956. The evaluation of crop plants for winter hardiness. Adv. Agron. 8:203-239.

Flood, R.G. and G.M. Halloran. 1983. Developmental responses in tetraploid wheat (*Triticum turgidum*). Can. J. Bot. 61:2539-2545.

Flood, R.G. and G.M. Halloran. 1984. Temperature as a component of the expression of developmental responses in wheat. Euphytica 33:91-98.

Flood, R.G. and G.M. Halloran. 1986. Genetics and physiology of vernalization response in wheat. Adv. Agron. 39:87-125.

Freyman, S. and M.S. Kaldy. 1979. Relationship of soil fertility to cold hardiness of winter wheat crowns. Can. J. Plant Sci. 59:853-855.

Fowler, D.B., J. Dvorak, and L.V. Gusta. 1977. Comparative cold hardiness of several *Triticum* species and *Secale cereale* L. Crop Sci. 17:941-943.

Fowler, D.B., D. Siminovitch, and M.K. Pomeroy. 1973. Evaluation of an artificial test for frost hardiness in wheat. Can. J. Plant Sci. 53:53-59.

Galiba, G., S.A. Quarrie, J. Sutka, A. Morgounov, and J.W. Snape. 1995. RFLP mapping of the vernalization (*Vrn1*) and frost resistance (*Fr1*) genes on chromosme 5A of wheat. Theor. Appl. Genet. 90:1174-1179.

Galiba, G., R. Tuberosa, G. Kocsy and J. Sutka. 1993. Involvement of chromosomes 5A and 5D in cold-induced abscisic acid accumulation in and frost tolerance of wheat calli. Plant Breeding 110:237-242.

Goncharov, N.P. and B.V. Rigin. 1991. Number of *Vrn* genes controlling spring habit in bread wheat. Wheat, Barley and Triticale Abstracts 8:2241.

Gordon-Kamm, W.J. and P.L. Steponkus. 1984. Lamellar-to-hexagonal$_{II}$ phase transitions in the plasma membrane of isolated protoplasts after freeze-induced dehydration. Proc. Natl. Acad. Sci. USA 81:6373-6377.

Gotoh, T. 1976. Studies on varietal differences in vernalization requirement in wheat. Japan J. Breed. 26:307-327. (in Japanese).

Gullord, M., Olien, C.R., Everson, E.H. 1975. Evaluation of freezing hardiness in winter wheat. Crop Sci. 15:153-157.

Gusta, L.V., M. Boyachek, and D.B. Fowler. 1978. A system for freezing biological materials. HortScience 13:171-172.

Gusta, L.V. and D.B. Fowler. 1976. Effects of temperature on dehardening and rehardening of winter cereals. Can. J. Plant Sci. 56:673-678.

Gusta, L.V. and D.B. Fowler. 1977. Factors affecting the cold survival of winter cereals. Can. J. Plant Sci. 57:213-219.

Gusta, L.V. and D.B. Fowler. 1979. Cold resistance and injury in winter cereals. p. 159-178. *In* H. Mussell and R.C. Staples (ed.) Stress physiology in crop plants. Wiley. New York.

Gusta, L.V., D.B. Fowler, and N.J. Tyler. 1982. Factors influencing hardening and survival in winter wheat. p. 23-40. *In* P.H. Li and A. Sakai (ed.) Plant cold hardiness and freezing stress: Mechanisms and crop implications. Vol. 2 Academic Press, New York.

Guy, C.L. 1990. Cold acclimation and freezing stress tolerance: Role of protein metabolism. Ann. Rev. Plant Physiol. Plant Mol. Biol. 41:187-223.

Hoogendoorn, J. 1985. The basis of variation in dates of ear emergence under field conditions among the progeny of a cross between two winter wheat varieties. J. Agric. Sci. 104:493-500.

Houde, M., J. Danyluk, J. Laliberte, E. Rassart, R.S. Dhindsa and F. Sarhan. 1992. Cloning, characterization, and expression of a cDNA encoding a 50-kilodalton protein specifically induced by cold acclimation in wheat. Plant Physiol. 99:1381-1387.

Kato, K., H. Ikoma and K. Hayashi. 1988. Geographical distribution of the genes for vernalization response and its implication for the adaptability of wheat. p. 533-539. *In* T.E. Miller and R.M.D. Koebner (ed.) Proc. 7th Int. wheat genet. symp. Cambridge, England, 19 July, 1988, Institute of Plant Science Research, Cambridge, England.

Kenrick, J.R. and D.G. Bishop. 1986. The fatty acid composition of phosphatidylglycerol and sulfoquinovosyldiacylglycerol of higher plants in relation to chilling sensitivity. Plant Physiol. 81:946-949.

Kirby, E.J.M., and M. Appleyard. 1981. Cereal Development Guide. *In* A. Barron (ed.), Cereal Unit, Stoneleigh, England.

Lalk, I. and K. Dorffling. 1985. Hardening, abscisic acid, proline and freezing resistance in two winter wheat varieties. Physiol. Plant. 63:287-292.

Laroche, A., X.M. Geng and J. Singh. 1992. Differentiation of freezing tolerance and vernalization responses in Cruciferae exposed to a low temperature. Plant Cell and Environ. 15:439-445.

Law, C.N., and G. Jenkins. 1970. A genetic study of cold resistance in wheat. Genet. Res. 15:197-208.

Lee, S.P. and T.H.H. Chen. 1993. Molecular biology of plant cold hardiness development. p. 1-29 *In* P.H. Li and L. Christersson (ed.) Advances in plant cold hardiness. CRC Press, Boca Raton.

Levitt, J. 1980. Responses of plants to environmental stress. Section II. p. 67-344. Chilling, freezing, and high temperature stresses. Vol. 1, 2nd Ed. Academic Press, New York, 1980.

Lynch, D.V. and P.L. Steponkus. 1986. Plasma membrane lipid alterations associated with cold acclimation of winter rye seedlings (*Secale cereale* L. cv *Puma*). Plant Physiol. 83:761-767.

Lyons, J.M., J.K. Raison, and P.L. Steponkus. 1979. The plant membrane in response to low temperature: An overview. p. 1-24. *In* J. M. Lyons, D. Graham, and J.K. Raison (ed.) Low temperature stress in crop plants: The role of the membrane. Academic Press, New York.

Metcalf, E.L., C.E. Cress, C.R. Olien, and E.H. Everson. 1970. Relationship between crown moisture and killing temperature for three wheat and three barley cultivars. Crop Sci. 10:362-365.

Millard, M.M., O.B. Veisz, D.T. Krizek and M. Line. 1995. Magnetic resonance imaging (MRI) of water during cold acclimation and freezing in winter wheat. Plant Cell Environ. 18:535-544.

Nordin, K., V. Lang, E. Mantyla, P. Heino, B. Welin, M. Baudo, K. Holmstrom, and E. T. Palva. 1993. Role of ABA in regulation of low temperature-induced genes in *Arabidopsis thaliana*. p. 45-56. *In* P.H. Li and Christersson (ed.) Advances in plant cold hardiness. CRC Press, Boca Raton.

Pomeroy, M.K. and C.J. Andrews. 1989. Low temperature injury in winter cereals. p. 107-122. *In* P.H. Li (ed.) Low temperature stress physiology in crops. CRC Press, Inc., Boca Raton.

Pomeroy, M.K., C.J. Andrews and G. Fedak. 1975. Cold hardiness and dehardening responses in winter wheat and winter barley. Can. J. Plant Sci. 55:529-535.

Pomeroy, M.K. and D.B. Fowler. 1973. Use of lethal dose temperature estimates as indices of frost tolerance for wheat cold acclimation under natural and controlled environments. Can. J. Plant Sci. 53:489-494.

Pugsley, A.T. 1971. A genetic analysis of spring-winter habit of growth in wheat. Aust. J. Agric. Res. 22:21-31.

Pugsley, A.T. 1972. Additional genes inhibiting winter habit in wheat. Euphytica 21:547-552.

Pugsley, A.T. 1973. Control of developmental patterns in wheat through breeding. p.857-859. *In* Proc. 4th Int. wheat genet. symp. 6-11 Aug., Columbia, MO. Agric. Exp. Stn. Univ. of Missouri, Columbia.

Rajashekar, C., L.V. Gusta, and M.J. Burke. 1979. Membrane structural transitions: Probable relation to frost damage in hardy herbaceous species. p. 255-274. *In* J. M. Lyons, D. Graham, and J.K. Raison (ed.) Low temperature stress in crop plants: The role of the membrane. Academic Press, New York.

Roberts, D.W.A. 1986. Chromosomes in 'Cadet' and 'Rescue' wheats carrying loci for cold hardiness and vernalization response. Can. J. Genet. Cytol. 28:991-997.

Roberts, D.W.A. 1989. Identification of loci on chromosome 5A of wheat involved in control of cold hardiness, vernalization, leaf length, rosette growth habit, and height of hardened plants. Genome 33:247.

Salisbury, P.A., G.J. Berry, and G.M. Halloran. 1979. Expression of vernalization genes in near-isogenic wheat lines: Methods of vernalization. Can. J. Genet. Cytol. 21:429-434.

SAS institute. 1991. SAS user's guide: Statistics. SAS Inst., Cary, N.C.

Steponkus, P.L. 1984. Role of the plasma membrane in freezing injury and cold acclimation. Annu. Rev. Plant Physiol. 35:543-584.

Steponkus, P.L. and S.C. Weist. 1979. Freeze-thaw induced lesions in the plasma membrane. p. 231-254. *In* J.M. Lyons, D. Graham, and J.K. Raison (ed.) Low temperature stress in crop plants: The role of the membrane. Academic Press, New York.

Steponkus, P.L. and S.C. Weist. 1978. Plasma membrane alterations following cold acclimation and freezing. p. 75-91. *In* P.H. Li and A. Sakai (ed.). Plant cold hardiness and freezing stress: Mechanisms and crop implications. Academic Press, New York.

Sutka, J. 1981. Genetic studies of frost resistance in wheat. Theor. Appl. Genet. 59:145-152.

Sutka, J. and G. Kovacs. 1985. Reciprocal monosomic analysis of frost resistance on chromosome 5A in wheat. Euphytica 34:367-370.

Sutka, J. and J.W. Snape. 1989. Location of a gene for frost resistance on chromosome 5A of wheat. Euphytica 42:41-44.

Thomashow, M.F. 1990. Molecular genetics of cold acclimation in higher plants. Adv. Gen. 28:99-131.

Thompson, G.A. 1979. Molecular control of membrane fluidity. p. 347-363. *In* J.M. Lyons, D. Graham, and J.K. Raison (ed.) In low temperature stress in crop plants: The role of the membrane. Academic Press, New York.

Trunova, T.I. 1987. Winter wheat frost hardiness and protein synthesis at chilling temperatures. p. 43-57. *In* P.H. Li. (ed.) Plant cold hardiness. Alan R. Liss, Inc., New York.

Trunova, T. I. 1982. Mechanisms of winter wheat hardening at low temperatures. p. 41-54. *In* P.H. Li and A. Sakai (ed.) Plant cold hardiness and freezing stress: Membranes and crop implications. Vol. 2 Academic Press, New York.

Wightman, F. 1979. Modern chromatographic methods for the identification and quantification of plant growth regulators and their application to studies of the changes in hormonal substances in winter wheat during acclimation to cold stress conditions. *In* T.K. Scott (ed.) Plant regulation and world agriculture. Plenum Press, New York.

Zeven, A.C., J. Waninge and L.T. Colon. 1986. The extent of similarity between near-isogenic lines of the Australian spring wheat variety Triple Dirk with their recurrent parent. Euphytica 35:381-393.

Table 1. Triple Dirk near-isogenic lines.

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*

(Pugsley 1971, 1972, 1973; Salisbury et al., 1979; Zeven et al., 1986).

Table 2. Mean days to spike emergence for Triple Dirk near-isogenic lines grown in growth chamber and greenhouse environments.

		Environment	
Line	**Haploid Genotype**	**Growth Chamber**	**Greenhouse**
		-----Mean Days to Spike Emergence----	
TD-	***Vrn1 Vrn2*** *vrn3 vrn4*	59.9	55.7
TDD	***Vrn1*** *vrn2 vrn3 vrn4*	62.7	58.1
TDE	*vrn1 vrn2* ***Vrn3*** *vrn4*	72.8	63.4
TDB	*vrn1* ***Vrn2*** *vrn3 vrn4*	75.5	68.7
TDF	*vrn1 vrn2 vrn3* ***Vrn4***	86.6	62.5
TDC	*vrn1 vrn2 vrn3 vrn4*	165.7	128.9
LSD	(P<0.05)	9.5	14.1

Table 3. Mean lethal temperatures (LT50s) for Triple Dirk near-isogenic lines.

Line	Haploid Genotype	LT_{50} (°C)
TDC	*vrn1 vrn2 vrn3 vrn4*	-11.7
TDB	*vrn1* ***Vrn2*** *vrn3 vrn4*	-9.4
TDE	*vrn1 vrn2* ***Vrn3*** *vrn4*	-8.1
TD-	***Vrn1 Vrn2*** *vrn3 vrn4*	-6.7
TDF	*vrn1 vrn2 vrn3* ***Vrn4***	-6.6
TDD	***Vrn1*** *vrn2 vrn3 vrn4*	-6.6
LSD	(P<0.05)	1.1

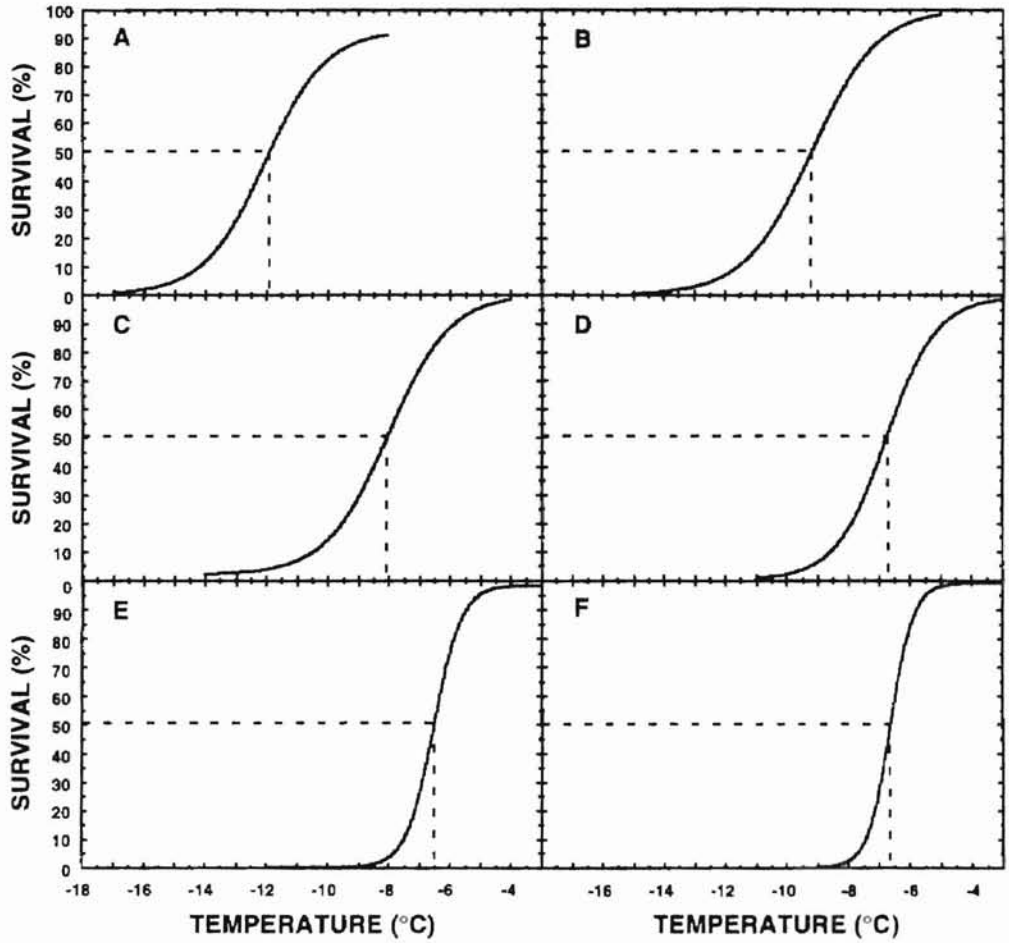

Fig. 1. Sigmoidal response curves for survival of the Triple Dirk near-isogenic lines used to estimate LT50s for (A) genotype: *vrn1vrn2vrn3vrn4*, (B) *vrn1Vrn2vrn3vrn4*, (C) *vrn1vrn2Vrn3vrn4*, (D) *Vrn1Vrn2vrn3vrn4*, (E) *Vrn1vrn2vrn3vrn4*, and (F) *vrn1vrn2vrn3Vrn4*.

CHAPTER 2

NON-RADIOLABELED DETECTION OF RESTRICTION FRAGMENT LENGTH POLYMORPHISMS IN WHEAT

ABSTRACT

A non-radiolabeled detection system to identify restriction fragment length polymorphisms (RFLPs) was developed using digoxigenin-labeled probes. The system was optimized while initiating a molecular mapping study to identify molecular markers linked to *Vrn3* on 5DL in hexaploid wheat. Two near-isogenic lines differing at the *Vrn3* locus were used to optimize systems of DNA extraction, digestion, electrophoresis, hybridization and detection. Additionally, parameters were optimized for probe labeling reactions. The probe Xbcd450, which has known homology to 5DL, was used to identify polymorphisms between the two lines. Both chemiluminescent and colorimetric detection methods were evaluated. Although the non-radiolabeled detection system using digoxigenin identified discernible bands, inconsistencies occurred in labeling reactions and in hybridization reactions, most frequently resulting in a high level of background.

INTRODUCTION

The use of molecular markers offers increasingly important opportunities in plant breeding programs, particularly in identifying traits with low heritability, or those which are difficult to discriminate phenotypically. Marker assisted selection has been identified as an important technique to enhance breeding efforts, particularly for quantitative traits (Patterson et al., 1991), in using exotic germplasm (Tanksley et al., 1989), and in early selection methodologies (Tauer et al., 1992). However, this is not to suggest that the advent of molecular techniques will dispense with the need for classical plant breeding (Patterson et al., 1991). The objective of this research was to establish a procedure for non-radiolabeled detection of restriction fragment length polymorphisms (RFLPs) in hexaploid wheat. This research marks the inception of the use of non-radiolabeled detection of RFLPs in molecular mapping of wheat in the United States.

LITERATURE REVIEW

RFLP Analysis. RFLPs are genetic markers which exist due to insertions, deletions, inversions, translocations, and minor base pair differences throughout the genome. RFLPs result due to differences in the length of DNA fragments following digestion of genomic DNA with sequence specific restriction endonucleases (Botstein et al., 1980; Beckmann and Soller, 1983). Hexaploid wheat has a relatively low level of polymorphisms (Devos and Gale, 1992). By digestion with restriction endonucleases, the DNA can be cut into many shorter fragments usually between a few hundred and a few thousand base pairs in length (Young, 1992). This distinctive set of fragments then defines an organism's RFLP genotype.

In eukaryotes there is difficulty in analyzing these fragments due to the large number generated following digestion. In wheat, the DNA composition has been estimated at 16 billion base pairs (May and Appels, 1987). As a result, following digestion, it is impossible to identify individual fragments. Therefore, labeled probes are used. Probes are short nucleotide sequences which correspond to a complementary sequence in the genomic DNA. Many probes are single or low copy. That is, the probe only hybridizes to one or few sequences in the genomic DNA. For wheat, numerous probes have been identified for use in RFLP analysis (Xie et al., 1993; Gill, 1993; Gill et al., 1993; Gill et al., 1992).

Although similar organisms tend to have similar base sequences, there are many changes, in general, throughout the entire DNA sequence. RFLPs can exploit these differences. When a labeled probe binds to its complementary sequence, now digested and separated by size (molecular weight) using gel electrophoresis, the digested fragment

of interest can be identified. Different size fragments between two individuals evidence a polymorphism. If the polymorphism is linked to a particular gene or quantitative trait loci (QTL), then the different size fragments can be identified and used as molecular markers. These identified RFLPs form a distinctive set of DNA fragments identifying a particular gene or QTL that can then be used in a breeding program. The gene of interest can be identified using RFLPs rather than screening for the physical trait itself.

Molecular and RFLP Analysis of Wheat. Much progress has been made recently in molecular mapping of wheat. The homoeologous group chromosomes have been divided among research facilities as part of the International Triticeae Mapping Initiative. Concerted efforts at molecular mapping are now underway for the homoeologous chromosomes for group 1 (Appels, 1993), group 2 (Sharp, 1993; Nelson et al., 1995b), group 3 (Devos et al., 1992; Sorrells, 1993; Nelson et al., 1995c), group 4 (Dvorak and Dubcovsky, 1993; Nelson et al., 1995a), group 5 (Gill, 1993; Xie et al., 1993; Nelson et al., 1995a), group 6 (Hart, 1993) and group 7 (Gale, 1993; Hohmann et al., 1994; Nelson et al., 1995a, Chao et al., 1989). Genome mapping has also been launched in Canada (Kim and Townley-Smith, 1993), France (Leroy et al, 1993), Japan (Tsunewaki, 1992), Australia (Sharp, 1992), and Russia (Badaev, 1992). Significant efforts have also been made to map the D-genome donor of hexaploid wheat (Kam-Morgan et al., 1989; Gill et al., 1990, 1992; Lagudah et al., 1991; Gill et al., 1993). Recent research also suggests the possibility of using microsatellite sequences as genetic markers in hexaploid wheat (Roder et al., 1995). Additionally, RFLP mapping of rye continues (Wang et al., 1991; Plaschke et al., 1993) providing insight into wheat genome mapping due to the chromosome homoeology between wheat and rye chromosomes (Naranjo et al., 1987).

DNA Extraction, Digestion and Blotting Techniques for Wheat. Leaf tissue should be harvested prior to the expansion of tillers and the onset of flower initiation (Osborn et al., 1986) to allow for easier extraction of DNA. Cool temperatures are necessary to prevent the degradation of the DNA by nucleases, and the fresh tissue should be immediately chilled at harvest by freezing in liquid nitrogen (Nelson, 1994).

There are two major published methods for extracting wheat DNA. DNA may be extracted by a method using hexadecyltrimethylammonium bromide (CTAB) (Gill et al., 1990; Hoisington et al., 1994), or by a sodium dodecyl sulfate (SDS) extraction method (Nelson, 1994; Leroy et al., 1995). Under the CTAB method, the nucleic acids are preferentially bound. Proteins, debris and contaminants are separated based on their lack of affinity. In the SDS method, a high affinity between the detergent and protein allows the proteins to be removed, leaving the nucleic acids in solution, which can then be precipitated with ethanol. The DNA can be further purified by a phenol extraction, with recovery by ethanol precipitation (Gill et al., 1990), which may be necessary if there is difficulty in obtaining a complete digestion.

Five restriction enzymes are routinely used to digest wheat DNA (Nelson, 1994). The size of the resulting fragments depends on the restriction enzyme added, its specific cutting site, and the number of times that the particular sequence occurs in the sample (Beckmann and Soller, 1986). Restriction endonucleases *Eco*RI, *Eco*RV, *Hind*III, *Xba*I and *Dra*I are commonly used for genomic wheat digests (Nelson, 1994; Reiter, 1992; Gill et al., 1990) and spermidine is added to enhance digestion (Gill et al., 1990).

The different size fragments are then separated by gel electrophoresis, generally in a 0.8% agarose gel at low voltages (Nelson, 1994; Hoisington et al., 1994; Leroy et al.,

1995). The fragments can be visualized by UV illumination following the intercalation of ethidium bromide. Some procedures suggest adding ethidium bromide directly to the gel, or to the running buffer, or to both so that ethidium bromide from the runnning buffer replaces that which migrates out of the gel during electrophoresis (Sambrook et al., 1989). Others suggest using a staining and destaining technique with ethidium bromide after the DNA has been run on the gel (Nelson, 1994).

There are numerous modifications of Southern blotting. All basically involve the transfer of DNA fragments from an agarose gel onto a membrane which can be used in hybridization. The original procedure described by Southern (1975) utilized a wicking blot built on a sponge soaked in a transfer solution which allowed movement of the DNA from the gel to the membrane by capillary action. Some procedures (Nelson, 1994) suggest soaking the gel in a weak acid such as 0.1 M HCl to depurinate large DNA fragments to facilitate transfer.

Probes and Labeling. Probes must be labeled in some way to identify the bands to which they hybridize. The most common method has been with ^{32}P (Gill et al., 1993; Nelson, 1994). However, environmental and safety issues suggest the use of non-radioisotopes. Non-radiolabeled probes such as those labeled with digoxigenin (DIG) have been estimated to have comparable sensitivities (Leroy et al., 1995) as radiolabeled probes. The use of a non-radiolabeled system may even be cost effective over the traditionally used radioactive method for RFLP analysis, given the ability to reuse probes, substrate and blots (Leroy et al., 1995). Additionally, non-radiolabeled probes are presumed much safer to use with shelf life advantages since there is no radioactive decay, and are also available in countries with restricted availability of radioisotopes.

The digoxigenin in DIG labeled probes is coupled to deoxyuracine 5′ triphosphoric acids (dUTPs) via an alkali-labile ester-bond. A mixture of deoxyribonucleosides containing DIG-11-dUTP in the reaction mixture results in the incorporation of labeled nucleotides in the newly synthesized DNA. The intensity of labeling is determined by the ratio of DIG labeled deoxyuracine 5′ triphosphoric acids (DIG-11-dUTPs) to deoxythymidine 5′ triphosphoric acids (dTTPs). However, there appear to be large differences in the intensity of labeling required, as well as the quantity of labeled probe necessary in the hybridization solution for acceptable detection.

Two methods are commonly used to generate DIG labeled probes: the polymerase chain reaction (PCR) and random primer labeling. PCR labeling has been cited as an efficient method of incorporating Dig-11-dUTPs into a probe (Lu et al., 1993). Introduction of non-radiolabeled substituted nucleotides in the reaction mixture permits the synthesis of large quantities of labeled probe (Lu et al., 1993). However, since the digoxigenin labeled DNA is synthesized approximately half as efficiently as unlabeled DNA, longer extension times for each thermal cycle are required for the labeling reaction than in comparable reactions without digoxigenin (Leroy et al., 1995; Lu et al., 1993). PCR uses Taq DNA polymerase to amplify the target sequence flanked by primers. Specific primer pairs are added to the mixture and serve as the starting point for the initiation of DNA synthesis. T3 and T7 are synthetic oligodeoxyribonucleotides that are homologous to plasmid DNA which flanks the insert DNA. T3 and T7 have been used successfully as primers to label probes in pBluescript vectors. such as Xbcd450 (Nelson, 1994). M13 forward and reverse are similar but more specifically designed to amplify inserts in pUC vectors.

Random primer labeling is similar, but this technique utilizes the Klenow enzyme and random oligonucleotide primers to synthesize the complementary DNA strand. The resulting labeled probe is a mixture of many sized fragments. Random primer labeling (Boehringer Mannheim, 1995) requires the probe to serve as a DNA template. The fragment used as the template in the labeling reaction is excised from a purified plasmid fraction following a restriction enzyme digestion. The random primer method does not make as efficient use of the DIG-11-dUTPs as the PCR reaction, and it has been estimated that as much as 70% of the labeled fraction remains in the nucleotide mixture following a labeling reaction (Boehringer Mannheim, 1995). It is not uncommon to bulk several reactions to obtain the quantity of probe desired.

Prehybridization and Hybridization. There are many variations of the prehybridization procedure. In general, 2-5 hours are required for prehybridization in a solution containing 0.2% (Hoisington et al., 1994) to 1% blocking reagent (Boehringer Mannheim, 1995). Hybridization of the labeled probe to the DNA bound on the membrane depends on many variables, including hybridization temperature, length of hybridization, salt concentration, guanine-cytosine content of the probe, length of the probe, volume of hybridization solution, and the degree of homology between the probe and the target site. Generally a temperature of 65° C is used, which is about 20° C below the actual melting temperature of double-stranded DNA in a 0.6 M NaCl solution (Nelson, 1994). However, shorter probes with high homology to the target site hybridize with the greatest efficiency in the shortest time. More cross hybridization occurs by lowering the hybridization temperature, increasing the salt concentration, or lengthening the hybridization time (Nelson, 1994). For DIG labeled probes, hybridizations are generally performed

overnight (16-18 hours) in a hybridization solution with 1% blocking reagent. Typically, the hybridization solution also has a 5X SSC (75 mM sodium citrate, pH 7.0) concentration, and low concentrations of the detergents N-lauroylsarcosine and SDS. although the concentrations of these detergents vary greatly across protocols. In general, the hybridization volume should be kept to a minimum to ensure maximum encounter rates between the probe and the bound DNA; however, too small of a volume will result in insufficient probe movement (Nelson, 1994).

Visualization. There are two primary methods of visualization: chemiluminescent detection and colorimetric detection. The chemiluminescent detection system uses Lumigen™ PPD which is an alkaline phosphatase substrate, that forms an unstable intermediate upon dephosphorylation, which decays emitting light (477 nm). Cetyltrimethylammonium bromide in the substrate solution also forms micelles containing a fluorescein surfactant which emits light (525 nm), further enhancing the signal. A luminograph can then be made by exposure to X-ray film.

Detection can also be through a colorimetric system that allows visualization of a blue precipitate in the presence of the substrates nitroblue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt (X-phosphate). The precipitates form directly on the membrane and have a tendency to degrade over time so the results are best preserved by scanning the image into an electronic data base.

There are three basic procedures describing technical procedures for DIG-labeled probes and detection of nucleic acids. The Boehringer Mannheim Genius System User's Guide (1995) describes the basic steps (Fig. 1) for using non-radioactive nucleic acid labeling and detection, although it is not specifically optimized for wheat. The other two

systems are designed for RFLP mapping in wheat. Hoisington et al. (1994) have described the optimized system using digoxigenin in the wheat biotechnology program for the International Maize and Wheat Improvement Center in Mexico (CIMMYT). Leroy et al. (1995) have described the optimized system using digoxigenin in the French National Institution of Agronomic Research (INRA) wheat mapping program.

Overview of the Boehringer Mannheim Procedure. Boehringer Mannheim (1995) does not detail a DNA extraction procedure. Digested DNA fragments are electrophoresced on a nucleic-acid grade agarose gel in TAE (40 mM Tris-HCl, 1 mM EDTA, pH 8.0 with glatial acetic acid), or TBE buffer (90 mM Tris, 90 mM Boric acid, 1 mM EDTA, pH 8.0). The gel is denatured for 30-60 minutes, and then neutralized for 30-60 minutes. The DNA is transferred to a nylon membrane compatible with the DIG system, such as MSI Magnagraph. The DNA can be blotted by capillary transfer using 10X or 20X SSC (300 mM sodium citrate, pH 7.0), or by vacuum-blotting. The DNA is bound to the membrane by UV crosslinking. The membrane is then rinsed in 5X SSC and is ready to be hybridized. Prehybridizations are for 2 hours in a prehybridization solution containing 1% blocking reagent. Prehybridizations are performed in sealable bags with at least 20 ml of prehybridization solution for each 100 cm^2 of membrane surface area. Hybridizations are in a standard buffer (5X SSC, 0.1% N-lauroylsarcosine, 0.2% SDS, 1% blocking reagent) with 5-25 ng ml^{-1} of labeled probe in at least 20 ml of hybridization solution for each 100 cm^2 of blot, and hybridizations are overnight at 50-68°C. Detection can be either by a chemiluminescent system or a colorimetric system.

Overview of the CIMMYT Procedure. CIMMYT uses a CTAB method of DNA extraction (Hoisington et al., 1994). Gel electrophoresis is overnight (18 h) at 20 volts

for a large gel (20 by 25 cm), with 15 µg of DNA loaded in each lane. Blotting proceeds overnight to Magnagraph MSI membranes using a phosphate capillary transfer method, followed by UV cross linking and baking at 80°C for 2 hours. Probes are labeled by nick translation, random primer, or PCR from a purified plasmid template. The prehybridization and hybridization solutions (5X SSC, 0.5% SDS, 0.1% N-lauroylsarcosine, and 0.2% blocking reagent) contain less blocking reagent than the other protocols. Prehybridizations are in siliconized glass bottles for 2 hours at 65°C with rotation. Hybridization are performed similarly for 16-18 hours with 2-3 ml for each 100 cm^2 of membrane. Wash conditions are variable and depend on the stringency required for the particular probe. Typically, the membranes are washed twice in 0.10X SSC at room temperature for 15 minutes each time, followed by three washings at the hybridization temperature (65°C) for 15 minutes each time. Membranes are incubated in a 0.2% blocking solution for 30 minutes and incubated with an anti-DIG alkaline phosphatase (AP) conjugate (Boehringer Mannheim) for 30 minutes. Excess antibody is removed by washing 3 times in a Tris buffer (0.01 M Tris-HCl, pH 7.5, 0.15 M NaCl), followed by washing three times in a Tris buffer containing blocking reagent (0.01 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.1% blocking reagent) for 15 minutes for each wash. Membranes are equilibrated for 5 minutes in a detection buffer (0.01 M Tris-HCl, pH 9.5, 0.15 M NaCl, 0.05 M $MgCl_2$). Membranes are incubated in a 1,2-dioxetane substrate solution (AMPPD®, Tropix) for 20 minutes and then exposed to X ray film overnight.

Overview of the INRA Procedure. The INRA suggests an SDS DNA extraction solution (100 mM Tris-HCl, 50 mM Na_2EDTA, 0.5 M NaCl, 1.25% SDS, 8.3 mM NaOH and 3.8 mg ml^{-1} Na bisulfite, pH 8.0) (Leroy et al., 1995). Ten µg of digested wheat

DNA are loaded per lane on a 20 by 25 ml 0.8% agarose gel and electrophoresced in TAE buffer at 30 volts overnight. DNA fragments are transferred to Hybond N+ nylon membrane with an alkaline buffer (0.4 NaOH) using a 1% agarose gel at the bottom and a sponge at the top. No UV cross linking or baking is necessary with this type of membrane. When possible, probes are labeled using PCR on bacterial lysate containing the plasmid vector. The addition of 10% glycerol in the reaction mix improves efficiency and specificity of the PCR reaction. The prehybridization and hybridization solutions contain 5X SSC, 0.5% SDS, 0.1% N-lauroylsarcosine, and 1% blocking reagent. Prehybridizations are for 5 hours at 65°C with multiple filters in a flat bottom plastic box. Hybridizations are in a heat sealable plastic bag in 40 ml of solution (for large filters) overnight at 65°C. After hybridization, filters are washed twice with a high stringency wash solution (0.1X SSC, 0.1% SDS) at room temperature for 5 minutes each time, followed by three washings at 65°C for 15 minutes each time. Filters are incubated at room temperature in a maleic acid buffer (0.1 M maleate, 0.15 M Nacl, pH 7.5) containing 1% blocking reagent, for one hour, and then incubated at room temperature for 30 minutes with anti-DIG AP conjugate (Boehringer Mannheim). After incubation, filters are rinsed in a Tris Buffer (100 mM Tris-HCl pH 8.0, 150 mM NaCl) three times for 30 minutes each. The membranes are then equilibrated in a detection buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) for five minutes. Membranes are incubated in an Lumigen-PPD substrate (Boehringer Mannheim) solution for five minutes and allowed to activate overnight. Detection is made the following day by exposing X-ray film for 4-6 hours.

MATERIALS AND METHODS

A chemiluminescent detection procedure was developed while trying to identify RFLPs for near-isogenic-lines differing at the *Vrn3* locus. Optimization of the procedure was primarily through hybridizations with the probe Xbcd450, which is a barley cDNA of 1.2 kb cloned in a pBluescript vector and available from M.E. Sorrells of Cornell University (Nelson, 1995a).

Genetic Stocks. Triple Dirk C (TDC) and Triple Dirk E (TDE) are two of the near-isogenic lines (NILs) developed by Pugsley (1971, 1972, 1973) and summarized in Table 1. TDC is of true winter habit and is homozygous recessive for the *vrn3* allele on chromosome arm 5DL. TDE is of spring habit and is homozygous dominant for the *Vrn3* allele. Both of these NILs are photoperiod insensitive (*Ppd1*) (Zeven et al., 1986).

DNA Extraction, Digestion, and Blotting. Plants of the parental types were grown and plant tissue was harvested when the plants were approximately one to two months old. This was early enough that the tissue was small and easy to grind, and with limited oils, polysaccharides, lignins or other compounds which could complicate DNA extraction. Multiple plants were grown in the same pot to save space and tissue was harvested twice, following regrowth. Plant tissue was harvest by cutting tissue about 10 cm above the crown. The harvested tissue was inserted into a coin envelope and frozen immediately in liquid nitrogen to avoid degradation, and stored at -80°C, if not processed immediately. Tissue was ground by pulverizing to a fine powder with mortar and pestle kept cold by the continual addition of liquid nitrogen (Nelson 1994; Leroy et al., 1995).

Both the CTAB method used by Gill et al (1990), and the SDS extraction technique outlined as part of the International Triticeae Mapping Initiative (ITMI)

(Nelson, 1994) were evaluated in this study. Since the SDS extraction procedure by Nelson (1994) proved the easiest and most reliable, the DNA used in the RFLP analysis was extracted by this procedure. After initial extraction, the DNA was washed with ethanol to remove salts and contaminants. The DNA was then resuspended in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM Na_2EDTA). RNAases were eliminated by digesting with RNAaseA (Gill et al., 1990).

DNA was quantified after determining the optical density at 260 nm with a spectrophotometer (UV 160U, Shimadzu). Five restriction endonucleases *Eco*RI, *Eco*RV, *Hind*III, *Xba*I and *Dra*I were used for the genomic digestions. DNA was digested in a mixture with final concentrations of 4.0 mM spermidine, 1.0 mM DTT, 1X buffer as recommended by the manufacturer, and 2.5 units of restriction enzyme per μg of DNA. The digestions were allowed to proceed overnight and arrested by adding a gel loading buffer (4.6 mM Tris, 20 mM EDTA, 0.125% bromophenol blue, and 50% glycerol).

Due to the large genome size of wheat, 12 μg of digested DNA were loaded per lane. Digested parental DNA was always loaded on the gels in the same order by enzyme to avoid errors and for easier comparisons. Digested DNA was electrophoresced on an 0.8% agarose gel using a horizontal gel electrophoresis apparatus (Biometra Horizon® 11*14, Life Technologies, Gibco BRL) for 7 to 7.5 hours, which was the time required for the bromophenol blue marker dye to migrate 3/4 the way across the gel. Gels were electrophoresced in TAE running buffer at low voltage (20-25 volts) to prevent the bands from bending. Longer runs at this voltage would result in fragments running off the end of the gel. Ethidium bromide (5 μg 100 ml^{-1} gel) was added to the liquid gel before

pouring and solidification. The DNA fragments were visualized following the intercalation of the ethidium bromide into the DNA fragments, and photographs were taken of each gel while fragments were fluorescing under illumination with UV light (302 nm).

Prior to transfer of DNA fragments to nylon membranes, the gels were soaked for 30 minutes in denaturing solution (0.4 M NaOH, 0.6 M NaCl) and then transfered to neutralizing solution (0.5 M Tris, pH 7.5, 1.5 M NaCl) (Hoisington et al., 1994) for 30 minutes. The DNA was transferred to Magnagraph nylon (MSI) membranes using a modified Southern (1975) blotting procedure. The blot was constructed with two sheets of filter paper wetted with 20X SSC, rather than on a sponge in a reservoir of transfer buffer. DNA was allowed to tranfer overnight.

Labeling of Probes. Both the PCR and random primer methods were used to label probes. The procedure described by Lu et al. (1993) was used to label probes using PCR. Three probes of known homology to chromosome arm 5DL, Xbcd450, Xrz395, and Xpsr426, were initially targeted to optimize the labeling reaction. Xbcd450 (1.2 kb) and Xrz395 (1.4 kb) are both barley cDNAs cloned into pBluescript vectors (Nelson, 1995a), and Xpsr426 (0.5 kb) is a wheat genomic DNA cloned into a pUC18 vector (Plaschke et al., 1993). Probes were labeled at the higher labeling intensity recommended by Leroy et al. (1995) with a 1:5.25 ratio of dTTP:DIG-11-dUTP. In general, bacteria containing the plasmid vector were grown up for 6-8 hours on a LB broth with selection (ampicillin) at 37°C with vigorous shaking. 800 µL of the culture were taken and added to a 1.5 ml microcentrifuge tube. The bacteria were pelleted by centrifuging at 12,000*x*g for 30 seconds. The supernatant was discarded and 400 µl of 1% Triton X-100 was used to

resuspend the bacterial pellet. The bacterial suspension was then boiled for 5 minutes and then placed on ice for 5 minutes. This bacterial lysate was stored at 4°C, and was used as the DNA template in the PCR reaction mixture.

A 25 µl reaction mixture was used to label probes with ingredients in the following concentrations: 10% glycerol, 1X buffer (Perkin Elmer), 0.2 mM $MgCl_2$ (Perkin Elmer), 0.2 µM each of appropriate primer pair, 50 µM of deoxyadenosine 5′ triphosphoric acid (dATPs), 50 µM of deoxyguanosine 5′ triphosphoric acid (dGTPs50 µM of deoxycytidine 5′ triphophoric acid (dCTPs), 42 µM of dTTPs, 8 µM of DIG-11-dUTPs (Boehringer Mannheim), and 0.025 units μl^{-1} of Taq DNA polymerase (AmpliTaq®, Perkin Elmer). For probes in pBluescript vectors such as Xbcd450, primer pairs were T3 (ATT AAC CCT CAC TAA AG) and T7 (AAT ACG ACT CAC TAT AG). For probes in pUC vectors such as Xpsr426, M13 forward (GTA AAA CGA CGG CCA GT) and reverse (AAC AGC TAT GAC CAT GA) were added as primers. The reaction mix was run in a GeneAmp PCR system 9600 (Perkin Elmer Cetus) to amplify and label the probe using the thermocycler profile by Leroy et al. (1995).

Despite several modifications of the PCR reaction mix and the thermocycler program, Xpsr426 did not label well through PCR. Xpsr426 was labeled using a random primer labeling procedure (Boehringer Mannheim, 1995), and the labeled product was quantified. For random primer labeling, plasmid was purified using a plasmid purification kit (Qiagen) and the probe excised with an excision enzyme (*Pst*1) by overnight digestion. The probe was separated by gel electrophoresis, excised, and purified using a gel extraction kit (Qiagen). Three different concentrations of probe were

used as DNA template, 0.3, 0.6, and 0.9 µg, to determine optimum template concentration in a 20 µl labeling reaction.

For labeling, the probe was denatured by heating in boiling water for 10 minutes, and quickly chilled on ice for 5 minutes. While on ice, the random primer hexanucleotides, nucleotide mix, and Klenow enzyme were added according to the protocol by Boehringer Mannheim (1995). Since the Klenow fragment lacks the exonuclease 5'-3' activity, the fragments accumulate. The reaction was incubated overnight (18 hours) at 37°C and stopped by adding 2 µl of 0.2 M EDTA, pH 8.0. The labeled product was precipitated, washed of salts, and resuspended in 50 µl of TE buffer (Boehringer Mannheim, 1995).

Hybridization and Detection. Membranes were probed with digoxigenin labeled Xbcd450 to optimize the hybridization and detection procedures. Initially the CIMMYT procedure was followed, but modification by increasing the percentage of blocking reagent in solutions was necessary due to the high background. Many parameters from the INRA and Boehringer Mannheim procedures were then adopted until acceptable results were obtained. In general, prehybridizations were at 65°C for 2 hours in 20 ml of solution with 1% blocking reagent (Boehringer Mannheim) in Rubbermaid™ containers. Hybridizations were at 65°C for 12 to 18 hours in 20 ml of hybridization solution with 1% blocking reagent (Boehringer Mannheim) and probe concentrations of 2.5 to 50 ng ml^{-1} in Seal-A-Meal® (Dazey) bags.

Membranes were rinsed in a high stringency wash (0.1X SSC, 0.1% SDS) for two times, 5 minutes each, at room temperature, and then three times, 15 minutes each, at 65°C. Membranes were incubated in 50 ml of 1X blocking solution for one hour at room

temperature, and then incubated in 50 ml of Anti-DIG-AP solution with 1% blocking reagent, for 30 minutes. Excess antibody was removed by rinsing four times, 10 minutes each, at room temperature in a Tris buffer (10 mM Tris, pH 7.5, 100 mM NaCl).

Filters were activated by incubating for 5 minutes in equilibration buffer (10 mM Tris, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$). Both the Boehringer-Mannheim digoxigenin-based chemiluminescent detection system (Boehringer Mannheim, 1995; Hoisington 1992; Hoisington et al., 1994; Khairallah et al., 1993), and the colorimetric methods of detection (Boehringer Mannheim, 1995) were evaluated using DIG-labeled Xbcd450 as the probe while the system was being optimized. For the chemiluminescent system, Lumiphos was added to the DNA side of a membrane, and the membrane placed between two acetate sheets and exposed to X-ray film. For the colorimetric detection system, the color precipitate solution (Boehringer Mannheim, 1995) was added directly to the DNA side of the membrane, covered with an acetate sheet, and the color precipitate allowed to develop overnight (16 hours) in the dark without movement.

RESULTS AND DISCUSSION

Comparison of the Methods of DNA Extraction. Although Gill et al. (1990) and Hoisington et al. (1994) use a CTAB solution for extraction of DNA in wheat, inconsistent yields were obtained using this method. Additionally, degradation was more frequently experienced with the CTAB method, probably the result of more pipetting steps. The CTAB method also took more time to complete, especially with the necessary phenol: chloroform step. The SDS method of DNA extraction was adopted with consistent results. DNA extracted using the SDS method was digestible without a phenol: chloroform purification step. The ITMI procedure, with modification, using a SDS extraction buffer was found to be the easiest and most reliable. Modifications included digestion with RNAaseA and ethanol precipitation with washes for increased quality and a more definite calculation of quantity of DNA.

Southern Blotting. Two methods of blotting were evaluated. The alkaline transfer method (Nelson, 1994; Leroy et al., 1995) using Hybond N+ offers the quickest method of transfer, and omits the need for UV crosslinking. However, this gave a noticeably higher background than the SSC blot transfer method to MSI Magnagraph membrane. Due to the ease and effective transfer using the SSC blot transfer, other methods of wick blotting and transfer buffers were not analyzed. A depurinating acid wash (0.5 HCl) is often suggested to enhance transfer of particularly large DNA fragments (Nelson, 1994), but was found not to be necessary for the digested samples blotted.

Labeling Probes. The PCR reaction using bacterial lysate gave positive results for two (Xbcd450 and Xrz395) of the three probes labeled. Better yields were obtained when using 25 µl reaction volume in each tube rather than a 100 µl bulk reaction. Minor

adjustments in the amount of primer and $MgCl_2$ were necessary to optimize the reaction for each probe. T3 and T7 synthetic oligodeoxyribonucleotides successfully primed labeling reactions for probes in pBluescript vectors, such as Xbcd450. M13 forward and reverse failed to successfully amplify Xpsr426, which is in a pUC18 vector. Indeed, this program or various modifications of it typically produced multiple bands, the faintest of which was thought to be labeled probe, even when purified plasmid was used as the DNA template.

The Boehringer Mannheim (1995) random primer labeling procedure was used to label Xpsr426. Although Boehringer Mannheim recommends adding 1-3 μg of DNA template to the labeling mix (20 μl), no decrease in total labeled probe (500 pg ml^{-1}) was observed for template starting quantities as low as 0.6 μg, suggesting that this amount of template is sufficient in the labeling reaction. Best results were by allowing the reaction to proceed overnight for maximum quantities of labeled probe.

Hybridization with Labeled Probe. Different prehybridizations times were evaluated without any improvement in level of background. However, at least four other factors have a large effect on decreasing the level of background. These include increasing the percentage of blocking reagent, increasing the volume of prehybridization and hybridization solution, omitting air pockets or bubbles in the solution, and using the optimum probe concentration in the hybridization solution.

The recommended blocking reagent concentration of 0.2% by CIMMYT in the prehybridization and hybridization solutions was too low. Unacceptably high levels of background were experienced with this concentration, particularly following long detection periods. Higher concentrations of blocking reagent in the prehybridization and

in the hybridization solutions decreased background. A 1% concentration of blocking reagent is recommended by Boehringer Mannheim and is also used by INRA. This concentration significantly improved detection while minimizing background.

The volume of hybridization solution is critical to prevent the membrane from drying, and to allow for uniform exposure to reagents in the solution. A minimum volume of 20 ml is necessary for prehybridization/hybridization for 11 by 14 cm membranes. Larger volumes are more desirable but will require additional labeled probe to maintain the same concentration.

Exposure of the membrane to direct contact with air bubbles, particularly during the hybridization, will result in high background, often evidenced as small rings or dots after detection which obscure banding patterns. Air bubbles are particularly prevalent in prehybridizations and hybridizations performed in Seal-A-Meal or plastic bags. When sealing a bag, the inclusion of some air should be anticipated. These bubbles should be isolated and heat sealed in a corner away from the membrane.

The use of optimal probe concentration is also critical in minimizing the level of background. The amount of probe should be quantified and the correct concentration added to the hybridization solution. However, there can be a larger order of error associated with quantification, particularly if the dot blot dilution method is used to quantify labeled probe (Boehringer Mannheim, 1995). Too much probe in the hybridization solution wastes reagent and causes excessive background. Too little probe will fail to identify bands with sufficient intensity. Best hybridization results using Xbcd450 were obtained at probe concentrations of 2.5 ng ml^{-1}, which is much lower than the 50-100 ng ml^{-1} range suggested by Hoisington et al. (1994), or the 20 ng ml^{-1}

suggested by Leroy et al. (1995). However, it should be noted that the probes by Hoisington et al. (1994) were labeled at a 1:20 (dTTP:DIG-11-dUTP) ratio, rather than 1:5.25.

Probes which have hybridized to nonspecific target sites must be removed to avoid excess background. There are numerous variations in the wash procedure, but a high stringency wash was most successful. A high stringency wash removed the largest portion of probe bound to nonspecific sites yet left the probe bound to the target sites relatively undisturbed. Interpretations of luminographs suggest an advantage in using lower concentrations in the hybridization producing slightly weaker signals but with less background, than the use of higher probe concentrations producing stronger signals with higher background. However, the wash as well as the probe concentration must be optimized for each probe.

The effect of using plastic storage containers (Rubbermaid^{TM}) rather than plastic bags (Seal-A-Meal®) was analyzed. For long incubations at high temperatures such as 65°C, the effect of evaporation is obvious, changing salt and probe concentration. Plastic containers are advantageous in that they minimize the exposure to air bubbles since air pockets are no longer trapped inside the container. Additionally, they offer advantages in that it is easier to manipulate the membrane in a plastic container than in the plastic bags. However, the plastic containers are deficient in that they allow for increased levels of evaporation causing changes in the concentration of reagents in the hybridization solution, and increasing background. Most plastic containers are also deficient in that they lack a perfectly flat bottom, thus requiring additional hybridization solution for full

coverage of the membranes. Plastic bags can be placed in plastic containers during hybridization for containment in the event a heat seal breaks on the plastic bag.

In some cases a pH gradient was apparent on the membranes following hybridization. This problem has been attributed to using TAE running buffer without circulation. TBE has a higher buffering capacity and should be substituted to avoid this problem.

Additionally, significant levels of background were attributed to alkaline phosphatase activity resulting from the side of the membrane without DNA. Luminographs using the chemiluminescent detection system were incomprehensible due to the high level of background. This problem was eliminated by using a higher quality filter paper (Whatman 3MM Western Chromatography) in constructing the Southern blot.

Visualization. Chemiluminescent detection and the production of a luminograph (X ray film exposed to light emitted by the alkaline phosphatase reaction) was the easiest method of visualization. A typical example of a luminograph for the parental survey using Xbcd450 is shown in Fig. 2. This system also allowed for the easiest method of stripping the membranes for reuse. Multiple exposures could be made until the optimal intensity of signal for interpretation had been reached. Typically a long (12 h) exposure was made initially. If significant background intensity resulted, obscuring banding patterns, then a shorter exposure (5-6 h) was made. However, shorter exposures frequently resulted in the bands being too faint for interpretation.

The colorimetric system was also evaluated. A typical image formed following precipitation is shown in Fig. 3. The precipitate giving the image in Fig. 3 was developed on the same membrane as that used for the chemiluminescent detection. Following the

development of the Luminograph in Fig. 2, excess Lumiphos was removed and colorimetric detection made without further hybridization. Bands are discernible using both systems.

CONCLUSIONS

The potential of molecular markers to be utilized in a traditional breeding program represents a tremendous savings in time and labor, particularly for quantitative trait loci, and traits with low heritability. Techniques for identifying molecular markers using non-radiolabeled methods have developed quickly in the past few years. A system identifying discernible banding patterns in hexaploid wheat has been developed for hybridizations using DIG labeled Xbcd450.

Although it is anticipated that labeling procedures and hybridization should be similar for other probes, minor adjustments in solutions and technique may be necessary to optimize hybridization for each probe tested. Inconsistencies have already been detected in working with the three probes initially evaluated, particularly in the labeling methodology. Further work is needed to optimize labeling and hybridization conditions using other probes such as Xrz395 and Xpsr426.

A final analysis of working with chemiluminescent systems reveals extreme sensitivity to individual technique and nontolerance of minor changes in solution concentrations, stringency washes, or hybridization conditions. Additionally, the system is sensitive to conditions peculiar to the individual probe being tested. Numerous steps to refine the techniques of Hoisington et al (1994) and Leroy et al. (1995) were necessary to optimize the system using Xbcd450, primarily to eliminate a high level of background.

REFERENCES

Appels, R. 1993. Chromosome group reports: Group 1. p. 85-87. *In* D. Hoisington and A. McNab, (ed.) Progress in genome mapping of wheat and related species: Proceedings of the 3rd public workshop of the international Triticeae mapping initiative. Mexico, D.F.: CIMMYT.

Badaev, N. 1992. Russian Triticeae mapping initiative. p. 79. *In* D. Hoisington and A. McNab, (ed.) Progress in genome mapping of wheat and related species: Proceedings of the 3rd public workshop of the international Triticeae mapping initiative. Mexico, D.F.: CIMMYT.

Beckmann, J.S. and M. Soller. 1983. Restriction fragment length polymorphisms in plant improvement: Methodologies, mapping and costs. Theor. Appl. Genet. 67:35-43.

Beckmann, J.S. and M. Soller. 1986. Restriction fragment length polymorphisms and genetic improvement of agricultural species. Euphytica 35:111-124.

Boehringer Mannheim. 1995. Genius™ system user's guide for membrane hybridization. Version 3.0. Indianapolis, IN.

Botstein, D., R.L. White, M. Skolnick, and R.W. Davis. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am. J. Hum. Genet. 32:314-331.

Chao, S., P.J. Sharp, A.J. Worland, E.J. Warham, R.M.D. Koebner, and M.D. Gale. 1989. RFLP-based genetic maps of wheat homoeologous group 7 chromosomes. Theor. Appl. Genet. 78:495-504.

Devos, K.M., M.D. Atkinson, C.N. Chinoy, C.J. Lui, and M.D. Gale. 1992. RFLP-based genetic map of the homoeologous group 3 chromosomes of wheat and rye. Theor. Appl. Genet. 83:931-939.

Devos, K.M. and M.D. Gale. 1992. The use of random amplified polymorphic DNA markers in wheat. Theor. Appl. Genet. 84:567-572.

Dvorak, J. and J. Dubcovsky. 1993. Chromosome group reports: Group 4. p. 92-95. *In* D. Hoisington and A. McNab, (ed.) Progress in genome mapping of wheat and related species: Proceedings of the 3rd public workshop of the international Triticeae mapping initiative. Mexico, D.F.: CIMMYT.

Gale, M.D. 1993. Chromome group reports: Group 7. p. 103-107. *In* D. Hoisington and A. McNab, (ed.) Progress in genome mapping of wheat and related species: Proceedings of the 3rd public workshop of the international Triticeae mapping initiative. Mexico, D.F.: CIMMYT.

Gill, B.S. 1993. Chromosome group reports: Group 5. p. 98. *In* D. Hoisington and A. McNab, (ed.) Progress in genome mapping of wheat and related species: Proceedings of the 3rd public workshop of the international Triticeae mapping initiative. Mexico, D.F.: CIMMYT.

Gill, B.S., K.S. Gill, W.J. Raupp, D. Delaney, R.S. Kota, L. Mickelson, D. Hassawi, A. K. Fritz, T.S. Cox, S.H. Hulbert, R.G. Sears, T.R. Endo, D. Namuth, and N.L.V. Lapitan. 1993. Genetic and physical mapping in *Triticum tauschii* and *Triticum aestivum*. p. 10-17. *In* D. Hoisington and A. McNab. (ed.) Progress in genome mapping of wheat and related species: Proceedings of the 3rd public workshop of the international Triticeae mapping initiative. Mexico, D.F.: CIMMYT.

Gill, K.S., D. Hassauri, W.J. Raupp, A.K. Fritz, B.S. Gill, T.S. Cox and R.G. Sears. 1992. An updated genetic linkage map of *Triticum tauschii*, the D-genome progenitor of wheat. In B.S. Gill, W. J. Raupp, and H. Corke (ed.) Progress in genome mapping of wheat and related species: Proceedings of the 2nd public workshop of the international Triticeae mapping initiative. Manhattan, Kansas, 1991, 27-29 Sept., 1991. Report No. 10, University of California Genetic Resources Conservation Program, Davis, CA.

Gill, K.S., E.L. Lubbers, B.S. Gill, W.J. Raupp, and T.S. Cox. 1990. A genetic linkage map of *Triticum tauschii* (DD) and it relationship to the D-genome of bread wheat (AABBDD). Genome 34:362-376.

Hart, G.E. 1993. Chromosome group reports: Group 6. p. 99-102. *In* D. Hoisington and A. McNab, (ed.) Progress in genome mapping of wheat and related species: Proceedings of the 3rd public workshop of the international Triticeae mapping initiative. Mexico, D.F.: CIMMYT.

Hohmann, U., T.R. Endo, K.S. Gill, and B.S. Gill. Comparison of genetic and physical maps of group 7 chromosomes from *Triticum aestivum* L. Mol. Gene Genet. 245: 644-653.

Hoisington, D. 1992. Chemiluminescent methods for RFLP analysis in the Triticeae. p. 39-40. *In* Gill, B.S., Raupp, W.J. and Corke, H. (ed.). Progress in genome mapping of wheat and related species: Proceedings of the 2nd public workshop of the international Triticeae mapping initiative. Report No. 10, University of California Genetics Resources Conservation Program, Davis, CA.

Hoisington, D., M. Khairallah and D. Gonzalez-de-Leon. 1994. Laboratory protocols: CIMMYT applied molecular genetics laboratory. 2nd Ed., Mexico, D.F.: CIMMYT.

Kam-Morgan, L.N.W., B.S. Gill, and S. Muthukrishan. 1989. DNA restriction fragment length polymorphism: A strategy for genetic mapping of D genome of wheat. Genome 32:724-732.

Khairallah, M., F. Acevedo, F. Dorregaray, S. Feingold, P. Lacaze, D. Gonzalez de Leon, and D. Hoisington. 1993. RFLP analysis in the Triticeae: Scale-up of operations and the use of chemiluminescent techniques. p. 56-58. *In* D. Hoisington and A. McNab, (ed.). Progress in genome mapping of wheat and related species: Proceedings of the 3rd public workshop of the international Triticeae mapping initiative. Mexico, D.F.: CIMMYT.

Kim, W.K. and F. Townley-Smith. 1993. Canada Wheat Genome Mapping Group. 1993. p. 82-84. *In* D. Hoisington and A. McNab, (ed.) Progress in genome mapping of wheat and related species: Proceedings of the 3rd public workshop of the international Triticeae mapping initiative. Mexico, D.F.: CIMMYT.

Lagudah, E.S., R. Appels, and D. McNeil. 1991. The Nor-D3 locus of *Triticum tauschii*: natural variation and genetic linkage to markers in chromosomes 5. Genome 34:387-395.

Leroy, P., M. Merlino, S. Negre, J.C. Caissard, M. Atkinson, Y.H. Lu and M. Bernard. 1995. Non-radioactive methods of detection on southern blots. *In* Plant molecular biology manual. 2nd Ed. Kluwer Academic Publishers, Dordrecht.

Leroy, P., M. Bernard, S. Bernard, G. Branlard, L. Jestin, M. Rousset, P. Nicolas, G. Doussinault, J. Jahier, J. Trottet, P. Joudrier, M.F. Gautier, F. Quetier, C. Hartmann, and I. Sissaef. 1993. French Triticeae mapping initiative. p. 80-81. *In* D. Hoisington and A. McNab, (ed.) Progress in genome mapping of wheat and related species: Proceedings of the 3rd public workshop of the international Triticeae mapping initiative. Mexico, D.F.: CIMMYT.

Lu, Y.H., S. Negre, P. Leroy, and M. Bernard. 1993. PCR-mediated screening and labeling of DNA from clones. Plant Mol. Biol. Reptr. 11(4): 345-349.

May, C.E. and R. Appels. 1987. The molecular genetics of wheat: toward an understanding of 16 billion base pairs of DNA. p. 165-198. *In* E.G. Heyne (ed.) Wheat and wheat improvement. Agron Monogr. 13. ASA, Madison, Wisconsin.

Naranjo, T., A. Roca, P.G. Goicoechea, and R. Giraldez. 1987. Arm homoeology of wheat and rye chromosomes. Genome 29:873-882.

Nelson, J.C. 1994. 1994 ITMI wheat mapping workshop laboratory manual. International Triticeae mapping initiative (ITMI). Cornell Univ. Aug., 1994. Ithaca, NY.

Nelson, J.C., M.E. Sorrells, A.E. Van Deynze, Y.H. Lu, M. Atkinson, M. Bernard, P. Leroy, J.D. Faris, and J.A. Anderson. 1995. Molecular mapping of wheat: major genes and rearrangements in homoeologous groups 4, 5, and 7. Genetics 141: 721-731.

Nelson, J.C., A.E. Van Deynze, E. Autrique, M.E. Sorrells, Y.H. Lu, M. Merlino, M. Atkinson, P. Leroy. 1995. Molecular mapping of wheat: Homoeologous group 2. Genome 38: 516-524.

Nelson, J.C., A.E. Van Deynze, E. Autrique, M.E. Sorrells, Y.H. Lu, S. Negre, M. Bernard, and P. Leroy. 1995. Molecular mapping of wheat: Homoeologous group 3. Genome 38:525-533.

Osborn, T.C., D.C. Alexander, and J.F. Fobes. 1986. Identification of restriction fragment length polymorphisms linked to genes controlling soluble solids content in tomato fruit. Theor. Appl. Genet. 73:350-356.

Patterson, A.H., S.D. Tanksley, and M.E. Sorrells. 1991. DNA markers in plant improvement. Adv. in Agron. 46:39-90.

Plaschke, J., A. Borner, D.X. Xie, R.M.D. Koebner, R. Schlegel, and M.D. Gale. 1993. RFLP mapping of genes affecting plant height and growth habit in rye. Theor. Appl. Genet. 85:1049-1054.

Pugsley, A.T. 1971. A genetic analysis of the spring-winter habit of growth in wheat. Aust. J. Agric. Res. 22:21-31.

Pugsley, A.T. 1972. Additional genes inhibiting winter habit in wheat. Euphytica 21:547-552.

Pugsley, A.T. 1973. Control of developmental patterns in wheat through breeding. p. 857-859. *In* Proc. 4th Int. wheat genet. symp. 6-11 Aug., Columbia, MO. Agric. Exp. Stn., Univ. of Missouri, Columbia.

Reiter, R. S. 1992. Genetic linkage of the *Arabidopsis* genome: Methods for mapping with recombinant inbreds and random amplified polymorphic DNAs (RAPDs). p. 170-190. *In* C. Koncz, N. Chua and J. Schell (ed.) Methods in Arabidopsis Research. 1992. World Scientific, River Edge, NJ.

Roder, M.S., J. Plaschke, S.U. Konig, A. Borner, M.E. Sorrells, S.D. Tanksley, and M.W. Ganal. 1995. Abundance, variability and chromosomal location of microsatellites in wheat. Mol. Gene Genet. 246:327-333.

Salisbury, P.A., G.J. Berry, G.M. Halloran. 1979. Expression of vernalization genes in near-isogenic wheat lines: Methods of vernalization. Can J. Genet. Cytol. 21:429-434.

Sambrook, J., E.F. Fritsch, T. Maniatis. 1989. Molecular cloning, a laboratory manual. 2nd Ed. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, New York.

Sharp, P.J. 1992. ATMI-The Australian Triticeae mapping initiative. p. 49. *In* Gill, B.S., Raupp, W.J. and Corke, H. (ed.). Progress in genome mapping of wheat and related species: Proceedings of the 2nd public workshop of the international Triticeae mapping initiative. Report No. 10, University of California Genetics Resources Conservation Program, Davis, CA.

Sharp. P.J. 1993. Chromosome group reports: Group 2. p. 88-90. *In* D. Hoisington and A. McNab, (ed.) Progress in genome mapping of wheat and related species: Proceedings of the 3rd public workshop of the international Triticeae mapping initiative. Mexico, D.F.: CIMMYT.

Sorrells, M.E. 1993. Chromosome group reports: Group 3. p. 91-97. *In* D. Hoisington and A. McNab, (ed.) Progress in genome mapping of wheat and related species: Proceedings of the 3rd public workshop of the international Triticeae mapping initiative. Mexico, D.F.: CIMMYT.

Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

Tanksley, S.D., N.D. Young, A.H. Paterson, and M.W. Bonierbale. 1989. RFLP mapping in plant breeding: New tools for an old science. Bio/Tech. 7:257-264.

Tauer, C.G., S.W. Hallgren and B. Martin. 1992. Using marker-aided selection to improve tree growth response to abiotic stress. Can. J. For. Res. 22:1018-1030.

Tsunewaki, K. 1992. Japan wheat genome mapping initiative. p.54-57. *In* Gill, B.S., Raupp, W.J. and Corke, H. (ed.). Progress in genome mapping of wheat and related species: Proceedings of the 2nd public workshop of the international Triticeae mapping initiative. Report No. 10, University of California Genetics Resources Conservation Program, Davis, CA.

Wang, M.L., M.D. Atkinson, C.N. Chinoy, K.M. Devos, R.L. Harcourt, C.J. Liu, W.J. Rogers and M.D. Gale. 1991. RFLP-based genetic map of rye (*Secale cereale* L.) chromosome 1R. Theor. Appl. Genet. 82:174-178.

Xie, D.X., K.M. Devos, G. Moore, and M.D. Gale. 1993. RFLP-based genetic maps of the homoeologous group 5 chromosomes of bread wheat (*Triticum aestivum* L.). Theor. Appl. Genet. 87:70-74.

Young, N.D. 1992. Restriction fragment length polymorphisms (RFLPs) and crop improvement. Expl. Agric. 28:385-397.

Zeven, A.C., J. Waninge and L.T. Colon. 1986. The extent of the similarity between near-isogenic lines of the Australian spring wheat variety Triple Dirk with their recurrent parent. Euphytica 35:381-393.

Table 1. Near-isogenic lines differing at the *Vrn3* allele.

Experimental Line	Genotype (haploid)
Triple Dirk C	*vrn1 vrn2 vrn3 vrn4 Ppd1*
Triple Dirk E	*vrn1 vrn2 Vrn3 vrn4 Ppd1*

(Pugsley 1971, 1972, 1973; Salisbury et al., 1979; Zeven et al., 1986)

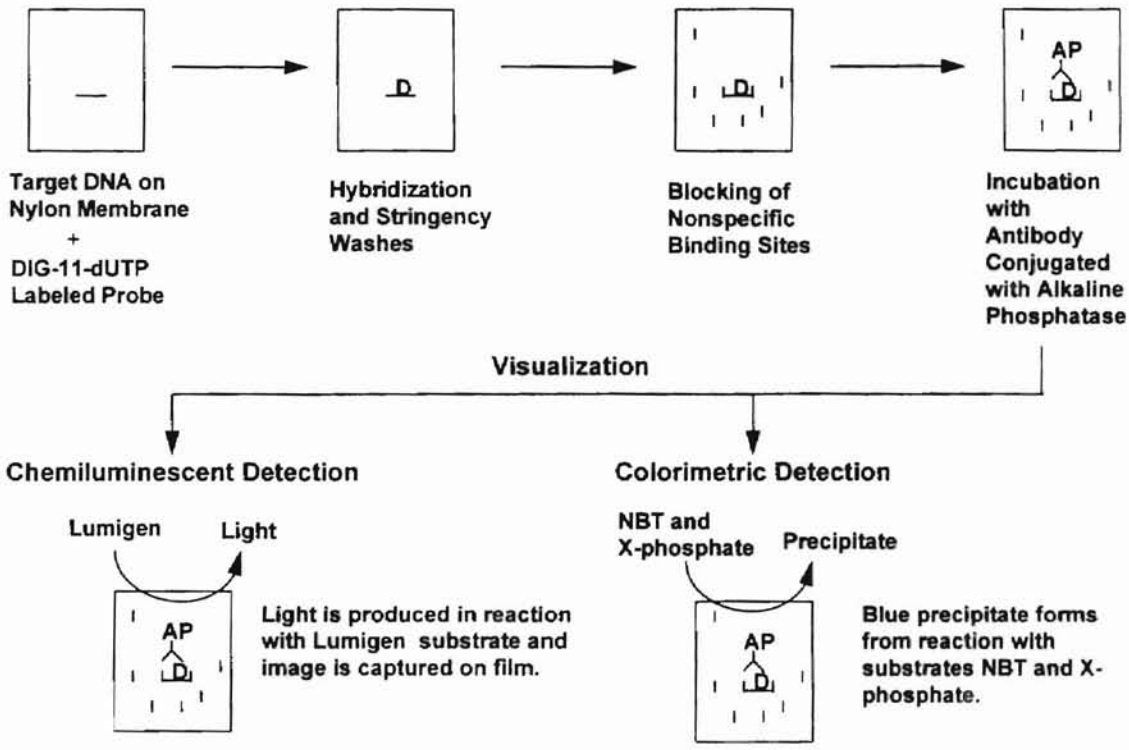

Fig. 1. Overview of a non-radiolabeled detection procedure (Boehringer Mannheim, 1995).

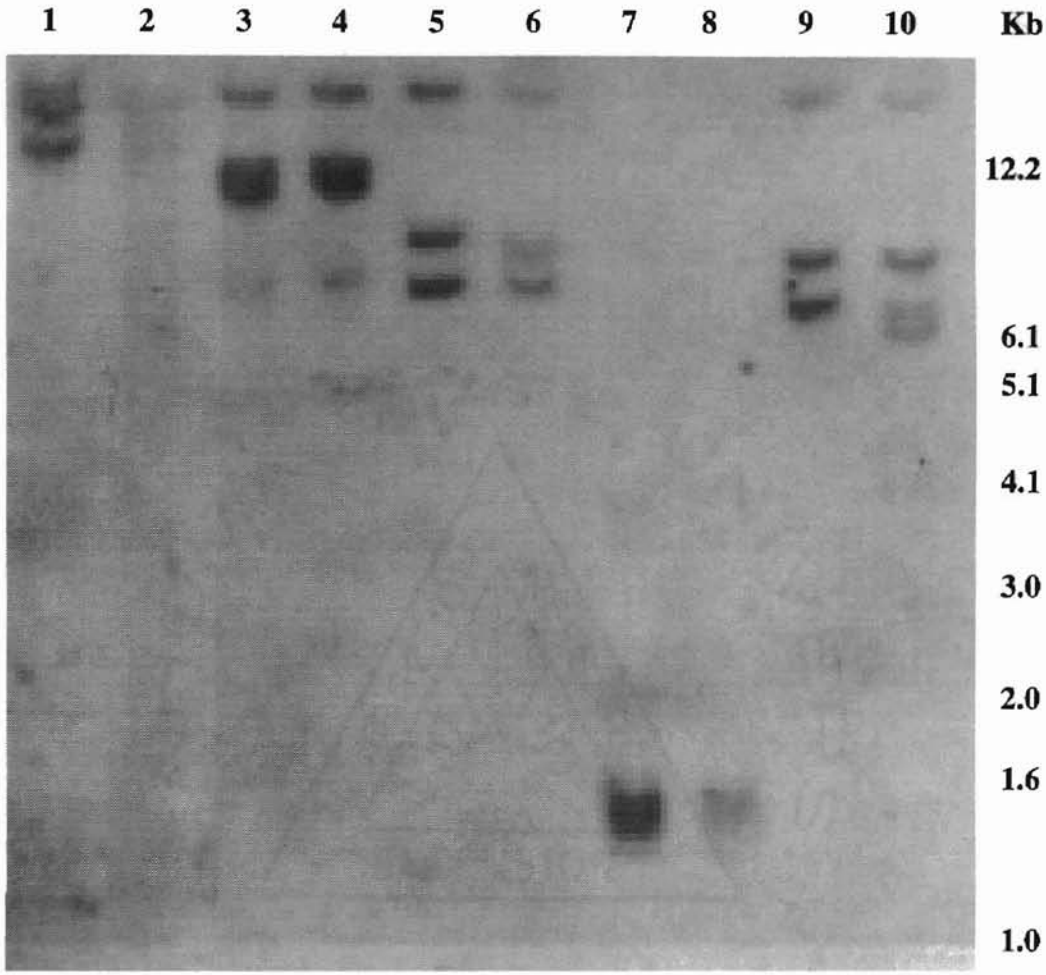

Fig. 2. Luminograph using chemiluminescent detection following hybridization with digoxigenin labeled Xbcd450 as a probe. By lanes: (1) *Eco*RI digest of 12 µg of Triple Dirk C (*vrn3vrn3*), (2) *Eco*RI digest of 12 µg of Triple Dirk E (*Vrn3Vrn3*), (3) *Eco*RV digest of 12 µg of Triple Dirk C, (4) *Eco*RV digest of 12 µg of Triple Dirk E, (5) *Hind*III digest of 12 µg of Triple Dirk C, (6) *Hind*III Digest of 12 µg of Triple Dirk E, (7) *Dra*I digest of 12 µg of Triple Dirk C, (8) *Dra*I digest of 12 µg of Triple Dirk E, (9) *Xba*I digest of 12 µg of Triple Dirk C, (10) *Xba*I digest of 12 µg of Triple Dirk E, and nucleic acid molecular size standards (Kb).

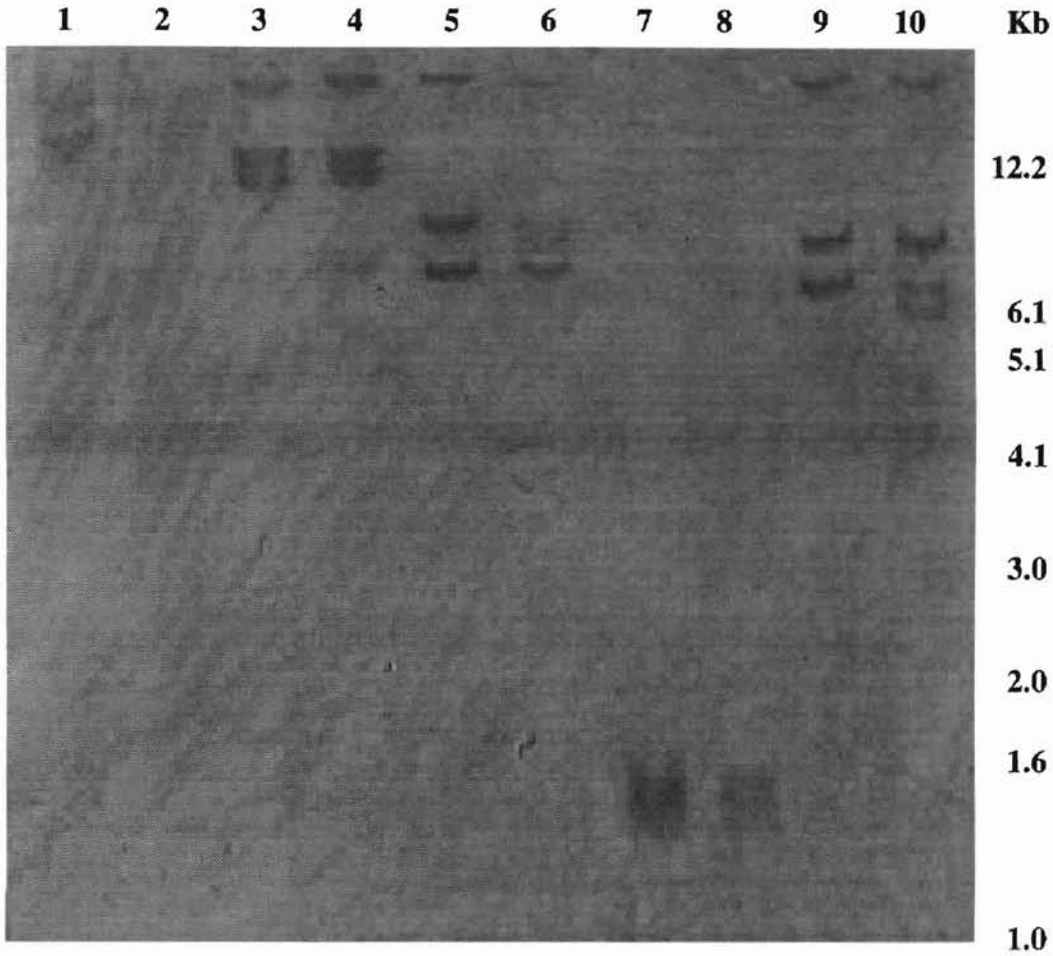

Fig. 3. Image of precipitates formed using colorimetric detection following hybridization with digoxigenin labeled Xbcd450 as a probe. By lanes: (1) *Eco*RI digest of 12 µg of Triple Dirk C (*vrn3vrn3*), (2) *Eco*RI digest of 12 µg of Triple Dirk E (*Vrn3Vrn3*), (3) *Eco*RV digest of 12 µg of Triple Dirk C, (4) *Eco*RV digest of 12 µg of Triple Dirk E, (5) *Hind*III digest of 12 µg of Triple Dirk C, (6) *Hind*III Digest of 12 µg of Triple Dirk E, (7) *Dra*I digest of 12 µg of Triple Dirk C, (8) *Dra*I digest of 12 µg of Triple Dirk E, (9) *Xba*I digest of 12 µg of Triple Dirk C, (10) *Xba*I digest of 12 µg of Triple Dirk E, and nucleic acid molecular size standards (Kb).

APPENDIXES

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. Preheat the extraction buffer to 65°C in a water bath.

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 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 containing upper phase. Pour through a Miracloth™ filter into a 50 ml polypropylene tube.

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

7. 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.

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

9. 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.

10. Remove contaminants by centrifuging for 10 minutes at 10,000 rpm to pellet material that did not go into solution. Enhance the purity of the DNA 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 but the improvement in quality is more important.

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

12. 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. Centrifuge at 10,000 *x*g for 15 minutes to pellet the DNA.

13. Wash the pellet with 1 ml of 95% ethanol, then with 1 ml of 70% ethanol. Resuspend the pellet in 200 µl of TE overnight at 4°C.

14. 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

15. 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. 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.

2. 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).

3. 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.

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

5. Pour an 0.8% agarose gel (0.8 grams 100 cm^{-1}) and allow to solidify for at least one hour prior to loading samples.

6. Add running buffer (TBE) until the top of the gel is barely submerged.

7. 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.

8. Pull the samples into the gel by running at 100 volts for 15 minutes. Then electrophoresce at 25 volts until the bromophenol blue marker dye has migrated at least 3/4 the way across the gel (7 to 7.5 hours for 0.8% on a 11*14 BRL Horizon).

9. Denature for 40 minutes in denaturing solution (0.4 M NaOH, 0.5 M NaCl).

10. Neutralize for 40 minutes (0.5 M Tris pH 7.5, 1.5 M NaCl).

11. Transfer the DNA fragments to a nylon membrane (Magnagraph MSI) by Southern blotting. Prepare the blot by laying a foundation of two sheets of filter paper soaked in 20X SSC (300 mM sodium citrate, pH 7.0). Lay the gel (upside down) on the filter paper. Lay a piece of nylon membrane (prewet with H_2O and immersed in 20X SSC 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 20X SSC. Add 2 sheets of filter paper soaked in 20X SSC. 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 overnight. For optimal results, ensure that the absorbent material does not contact the gel or foundational filter paper.

12. Fix the DNA to the membrane in a UV Crosslinker (UV Stratalinker™ 1800) by applying 120,000 μjoules. Crosslink the DNA side only (upper right side nicked), and do not cross link the back side as this may increase background. Rinse the membrane in 2X SSC (30 mM sodium citrate, pH 7.0), for 15 minutes to remove any agar debris.

side for orientation. Add 1 sheet 3MM Western blot chromatography paper (Whatman) soaked in 20X SSC. Add 2 sheets of filter paper soaked in 20X SSC. 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 overnight. For optimal results, ensure that the absorbent material does not contact the gel or foundational filter paper.

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PROCEDURE FOR PREHYBRIDIZATIONS AND HYBRIDIZATIONS

1. Calculate and prepare the amount of prehybridization and hybridization solution needed. Estimate a minimum of 20 ml for prehybridization solution and 20 ml for hybridization for each probe. For large membranes use 0.1 ml for each cm^2 of membrane. Increased amounts of solution have been referenced as giving decreased background (Leroy et al., 1995).

2. Ensure that there are adequate quantities of 10% blocking stock solution for all prehybridization/hybridization steps, and all blocking and incubation steps. Prepare the 10% blocking stock solution by dissolving dry blocking reagent (Boehringer Mannheim) in maleic acid buffer at 65°C with gentle stirring. The solution should be autoclaved, and then refrigerated as soon as possible to avoid a brown discoloration which occurs with prolonged exposure to heat.

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

4. Prehybridize membranes in a 1X prehybridization solution for 2 hours at 65°C in Rubbermaid™ or plastic trays.

5. Denature the probe by placing in boiling water for 10 minutes and chilling quickly on ice for 5 minutes. It is best to boil the probe in a minimum volume of 500 µl to minimize reannealing. Add the denatured probe (50 ng) to 20 ml 1X hybridization solution for a labeled probe concentration of 2.5 ng ml^{-1}. If using probe that has previously been diluted in hybridization solution then boil for 20 minutes and chill on ice for 8 minutes.

6. Hybridize one or two membranes in a Seal-A-Meal® (Dazey) bag in a minimum of 20 ml of hybridization solution. If using two membranes, then place the DNA side of one face down and the DNA side of the other face up. Cut three sides of the Seal-A-Meal bag and placing the membrane inside. Heat seal all sides. Cut one corner and add the hybridization solution. Eliminate air, and bubbles, by rolling to the outside with a glass pipette. Heat seal the remaining corner. Check for any remaining air pockets, or bubbles, and isolate these from the membrane by heat sealing in a corner.

7. Incubate at 65°C overnight (12-18 hours). Use very gentle shaking to avoid whipping up bubbles. It is best to use a shaker that rocks back and forth rather than a circular orientation. Face the DNA side of the bottom membrane down and the DNA side of the top membrane up. Use size 2 Rubbermaid trays for 11 by 14 membranes.

8. Seal-A-Meal bags may be place inside Rubbermaid containers for containment in the event of heat seal failure.

9. After hybridization the membranes should be washed to remove excess probe.

PROCEDURE FOR WASHING AND DETECTION

1. Leave the hybridization oven on following hybridization steps. If using low stringency wash, then lower the oven temperature to 50°C instead of 65°C.

2. Recover the probe solution by cutting one corner of the hybridization bag and draining the solution into a 50 ml polypropylene tube. Store the recovered probe at -20°C in a 50 ml polypropylene tube.

3. Cut the remaining sides of the hybridization bag and lift out the membrane with clean (preferable sterilized) filter forceps and immediately rinse (2 minutes) in a high stringency wash solution (0.10X SSC, 0.10% SDS) at room temperature.

4. Wash two times for 5 minutes each in 100 ml of a high stringency wash solution at room temperature with shaking. Wash three times for 15 minutes each in 100 ml of a high stringency wash solution at 65°C with shaking. (Note: For a lower stringency wash, wash 2 times for 15 minutes each at room temperature, then wash 2 times for 15 minutes each at 65°C or hybridization temperature. For a lower stringency wash solution use 0.15X SSC, 0.1% SDS.)

5. Rinse the membrane for 5 minutes in 100 ml Tris wash buffer (10 mM Tris, pH 7.5; 100 mM NaCl) in a Rubbermaid container.

6. Incubate the membrane in 50 ml of 1X Blocking Solution (45 ml Maleic acid buffer, 5 ml 10X Blocking Stock Solution) in a Rubbermaid container for 30 minute to 1 hour to bind any nonspecific sites that may now be open.

7. Incubate the membrane in the Anti-Dig-AP solution (3 µl anti-Dig conjugate and 45 ml 1X blocking solution) in a Rubbermaid container for 30 minutes. The antibody binds to the probe at this time and is conjugated to alkaline phosphatase which can be used in the detection reaction. During the incubation steps, the Lumiphos substrate is taken from storage at 4°C and allowed to warm to room temperature. The acetate sheets used in the detection should also be cleaned by spraying with 95% ethanol and wiped dry with a kimwipe tissue.

8. Rinse excess antibody from the membrane. Rinse four times for 10 minutes each at room temperature in 100 ml of Tris wash buffer (10 mM Tris, pH 7.5; 150 mM NaCl) in a Rubbermaid container with gentle shaking. Use clean forceps, preferably autoclaved, and a clean container for each rinse.

9. Equilibrate the membrane for 5 minutes in 50 ml of Tris equilibration buffer (10 mM Tris, pH 9.5; 100 mM NaCl; 50 mM $MgCl_2$).

10. Position the membrane with the DNA side up (nick in the upper left side) on a clean acetate sheet. Do not attempt to drain any excess Tris equilibration buffer since

drying will result in high background. Residual Tris equilibration buffer should not affect the detection process.

11. Apply the chemiluminescent substrate. Dot 500 µl of Lumiphos directly on the DNA side of the membrane. Place the second acetate sheet on top of the membrane and allow surface adhesion to spread the Lumiphos. Separate the two sheet and drain excess substrate by tilting the acetate sheets 90° and allow the excess to drain from the edges. Replace the sheets and smooth out any air bubbles. Wipe away any excess form the outside of the sheets. Protect the Lumiphos from light by covering with a lid or paper.

12. Incubate the substrate at room temperature for 4 hours protected from light, such as in a cassette holder (Kodak X-Omatic®).

13. Expose X-ray film to the membrane for a period of 4-5 hours, though exposure times will vary for each hybridization and for each probe tested. Wipe any excess substrate from the outside of the acetate sheets, or the inside of the cassette holder as this can emit light in reaction catalyzed by contaminant alkaline phosphatase.

14. Develop film and reexpose for a longer or shorter time, if necessary.

15. If not proceeding with colorimetric detection, then immediately remove the probe from the membrane by immersing the membranes in a stripping solution (0.2 M NaOH, 0.1% SDS) twice, for 15 minutes each, at 37°C with gentle shaking. Rinse in TE and air dry. Store in a cool dry place.

16. If proceeding with colorimetric detection, rinse membranes briefly in Milli-Q H_2O for three minutes to remove residual Lumiphos. Note: It may be best to reincubate with 1X blocking solution and antibody, followed by rinse steps, but these steps may be omitted. Rinse twice in 2X SSC for 15 minutes each. Equilibrate in detection buffer for 4 minutes. Add 10 ml color-substrate solution (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5; 45 µl nitroblue tetrazolium salt, 75 mg ml^{-1} in dimethyl formamide; 35 µl 5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt, 50 mg ml^{-1}, in dimethylformamide) directly to the DNA side of the membrane. Place an acetate sheet over the top, and allow the color to develop fully, usually overnight.

PROCEDURE FOR LABELING PROBES BY PCR.

1. Optimize the concentration of nucleotides, primers, $MgCl_2$ and template for each plasmid/probe combination. Prepare a PCR worksheet calculating the ingredients to optimize the thermocycler reactions (see Polymerase Chain Reaction Worksheet for Labeling Probes with Digoxigenin, this Appendixes). Initially, perform a nine point optimization test by manipulating the concentration of $MgCl_2$ at three levels (1 mM, 1.5 mM, and 2.0 mM) and concentration of primer pairs at three levels (0.2 µM, 0.4 µM, and 0.8 µM). For most reliable results prepare a bulk reaction to divide into aliquots.

2. Prepare a reaction mix of 25 µl and overlay with 50 µl of mineral oil. Use thin walled PCR microtubes for best heat dispersion.

3. Generate DIG-labeled probes using a thermocycler program such as that optimized by Leroy et al. (1995) (see Thermocycler Program for Labeling and Amplifying DIG-labeled Probes, this Appendixes).

4. Pipet the reaction product only (approximately 25 µl), leaving the mineral oil behind, and place in a new tube. Add gel loading dye to a 1X concentration (6 µl). Load each sample on a 1% agarose gel and electrophoresce for 2 hours at 50 volts. Ensure that the bands are in the expected molecular weight range for the probe amplified. After excising, purify with a gel extraction kit (Qiagen).

5. Each 25 µl labeling reaction should produce approximately 5-50 ng of labeled probe. Labeled probes may be stored at -20°C for up to a year.

Restriction enzyme digestion worksheet.

DNA Sample and Concentration			Reaction Volume		Ingredients (10X buffer, 40 mM spermidine, and 10 mM DTT)				Enzyme (2.5 units μg^{-1} DNA digested)			Digestion Volume	5X Loading Buffer	Gel Loading Volume
#	Name	$\mu g\ \mu l^{-1}$	μg DNA	μl Sample	μl H_20	μl Buffer	μl Sperm	μl DTT	Name	units μl^{-1}	μl	μl	μl	μl
1														
2														
3														
4														
5														
6														
7														
8														
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10														
11														
12														
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17														
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Date:			Optimization for 1:5.25 DIG-labeled probe from bacterial lysate											
Probe:			Primer Set 1-- 5 uM each of T3 and T7											
Program:			Primer Set 2-- 5 uM each of M13 Forward and Reverse											
Tube #	PCR H_2O	50% Glycerol	10X PCR Buffer	$MgCl_2$ 25 mM	dATPs 2.5 mM	dCTPs 2.5 mM	dGTPs 2.5 mM	dTTPs 2.5 mM	DIG-dUTP 1.0 mM	Primer Set 1	Primer Set 2	TAQ 5U ul-1	Template (Lysate)	Total Volume
1	10.76	5.00	2.5	1	0.5	0.5	0.5	0.42	0.20	1	0	0.125	2.5	25
2	9.76	5.00	2.5	1	0.5	0.5	0.5	0.42	0.20	2	0	0.125	2.5	25
3	7.76	5.00	2.5	1	0.5	0.5	0.5	0.42	0.20	4	0	0.125	2.5	25
4	10.26	5.00	2.5	1.5	0.5	0.5	0.5	0.42	0.20	1	0	0.125	2.5	25
5	9.26	5.00	2.5	1.5	0.5	0.5	0.5	0.42	0.20	2	0	0.125	2.5	25
6	7.26	5.00	2.5	1.5	0.5	0.5	0.5	0.42	0.20	4	0	0.125	2.5	25
7	9.76	5.00	2.5	2	0.5	0.5	0.5	0.42	0.20	1	0	0.125	2.5	25
8	8.76	5.00	2.5	2	0.5	0.5	0.5	0.42	0.20	2	0	0.125	2.5	25
9	6.76	5.00	2.5	2	0.5	0.5	0.5	0.42	0.20	4	0	0.125	2.5	25
NC	12.76	5.00	2.5	1.5	0.5	0.5	0.5	0.42	0.20	1	0	0.125	0	25
Master Mix	H_2O	Glycerol	10X PCR Buffer	$MgCl_2$	dATPs	dCTPs	dGTPs	dTTPs	DIG-dUTP	Primer 1	Primer 2	TAQ	Template	Total
1X	-7.745	5	2.5	1	0.5	0.5	0.5	0.42	0.2	1	0	0.125	0	4
10	-77.45	50	25	10	5	5	5	4.2	2	10	0	1.25	*	40
Add after aliquotting master mix to each tube														
Tube #	PCR H_2O	50% Glycerol	10X PCR Buffer	$MgCl_2$ 25 mM	dATPs 2.5 mM	dCTPs 2.5 mM	dGTPs 2.5 mM	dTTPs 2.5 mM	DIG-dUTP 0.25 mM	Primer Set 1	Primer Set 2	TAQ 5U ul-1	Template (Lysate)	Total Volume
1	4.00	0.00	0	0	0	0	0	0.00	0.00	0	0	0	2.5	25
2	3.00	0.00	0	0	0	0	0	0.00	0.00	1	0	0	2.5	25
3	1.00	0.00	0	0	0	0	0	0.00	0.00	3	0	0	2.5	25
4	3.50	0.00	0	0.5	0	0	0	0.00	0.00	0	0	0	2.5	25
5	2.50	0.00	0	0.5	0	0	0	0.00	0.00	1	0	0	2.5	25
6	0.50	0.00	0	0.5	0	0	0	0.00	0.00	3	0	0	2.5	25
7	3.00	0.00	0	1	0	0	0	0.00	0.00	0	0	0	2.5	25
8	2.00	0.00	0	1	0	0	0	0.00	0.00	1	0	0	2.5	25
9	0.00	0.00	0	1	0	0	0	0.00	0.00	3	0	0	2.5	25
NC	6.50	0.00	0	0	0	0	0	0.00	0.00	0	0	0	0	25

Polymerase Chain Reaction worksheet for labeling probes with digoxigenin.

REAGENTS

SDS (Sodium Dodecyl Sulfate) extraction buffer.

Ingredient/Stock Solution	Quantity for 1 Liter	Final Concentration
5 M NaCl	100 ml	500 mM
0.25 M EDTA	100 ml	100 mM
20% SDS	62.5 ml	1.25 %
Milli Q H_2O	to volume	--
Sodium bisulfite	0.19g $100ml^{-1}$	

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 immediately before use and adjust pH to 7.8-8.0.

(Nelson, 1994)

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 solution*.

Ingredient	Amount to Add for 100 ml	Final Concentration
Milli-Q H_2O	62 ml	--
20X SSC, pH 7.0 (Sodium Citrate)	25 ml	5X
5% Laurylsarcosine	2 ml	0.1%
20% SDS (Sodium Dodecyl Sulfate)	1 ml	0.2%
10% Blocking Reagent Stock Solution	10 ml	1%

*Older protocols recommend adding the blocking reagent in its dry powdered form to the solution with the other ingredients premixed. The pH of the solution (without blocking reagent) prior to heating should be adjusted to 7.3 to 7.4 using weak (0.25M) HCl. Back titrating should be avoided as an increase in salt content will affect hybridization. The blocking reagent is then added and stirred into solution with constant heating for 30 minutes to 1 hour at 65°C. This tedious technique requires care and attention to maintaining constant temperature without overheating, with additional concerns of effects due to evaporation.

Tris wash buffer.

Ingredient	Amount to Add for 1 Liter	Final Concentration
Milli-Q H_2O	960 ml	--
1 M Tris buffer, pH 7.9	10 ml	10 mM Tris
5 M NaCl	30 ml	100 mM NaCl

(Hoisington et al., 1994)

Tris equilibration buffer.

Ingredient	Amount to Add for 500 ml	Final Concentration
Milli Q H_2O	460 ml	--
1 M Tris buffer, pH 9.5	5 ml	10 mM Tris
5 M NaCl	10 ml	100 mM NaCl
1 M $MgCl_2$	25 ml	50 mM $MgCl_2$

(Hoisington et al., 1994)

Maleic acid buffer.

Ingredient	Amount to Add for 1 Liter	Final Concentration
Maleate	11.61 g	0.1 M
5 M NaCl	30 ml	0.15 M
NaOH (pellets)	approximately 7.2 g	pH 7.5 at 20°C*
Milli Q H_2O	to volume	--

*In adjusting the pH above 7.0, add individual NaOH pellets. Autoclave and store at room temperature.

(Boehringer Mannheim, 1995)

VITA

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Candidate for the Degree of

Master of Science

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