DISSECTION OF KERNEL HARDNESS AND BREAD MAKING QUALITY IN WHEAT USING MOLECULAR MARKERS

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CERTIFICATE

Certified that the work incorporated in this thesis entitled, “Dissection of kernel hardness and bread making quality in wheat using molecular markers” submitted by Ms. Aditi Galande was carried out by the candidate under my supervision. The material obtained from other sources has been duly acknowledged in the thesis.

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Research Guide

DECLARATION

I hereby declare that the thesis entitled “Dissection of kernel hardness and bread making quality in wheat using molecular markers” submitted for Ph.D. degree to the University of Pune has not been submitted by me to any other university for a degree or diploma.

Date:

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…. to my mother

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Aditi Galande

LIST OF ABBREVIATIONS

AACC : American Association of Cereal Chemists

AFLP	:	Amplified Fragment Length Polymorphism
APS	:	Ammonium per sulphate
AS-PCR	:	Allele-specific PCR
BAC	:	Bacterial Artificial Chromosome
BMQ	:	Bread making quality
bp	:	base pair
cm	:	centimeter
cM	:	centiMorgan
CTAB	:	Hexadecyltrimethyl Ammonium Bromide
cv	:	cultivar
DH	:	Doubled Haploid
DNA	:	Deoxy Ribonucleic Acid
dNTPs	:	Deoxy Ribonucleotide Tri Phosphate
DT	:	ditelosomic
DWR	:	Directorate of Wheat Research
EDTA	:	Ethylene Diamine Tetra Acetic acid
EST	:	Expressed Sequence Tag
g/gm	:	gram
GPC	:	Grain Protein Content
h	:	hour
hec	:	hectare
HMW	:	High- Molecular Weight
HRS	:	Hard Red Spring wheat
HRW	:	Hard Red Winter wheat
HW	:	Hectolitre weight
IAA	:	Iso-Amyl Alcohol
ISSR	:	Inter Simple Sequence Repeat
ITMI	:	International Triticeae Mapping Initiative
K_2O	:	Potassium oxide
kb	:	kilobase pair
KCl	:	Potassium chloride
kg	:	kilogram
Kg/hec	:	Kilogram per hectare
KH	:	Kernel hardness

LMW	:	Low- Molecular Weight
LOD	:	Log of the Odd (Base 10 logarithm of the likelihood ratio)
min	:	minute
M	:	Molar
MAS	:	Marker Assisted Selection
Mb	:	Megabase pair
MC	:	Moisture content
$MgCl_2$	:	Magnesium chloride
mm	:	millimeter
mM	:	millimolar
MT	:	million tons
MMT	:	million metric tons
MW	:	Molecular Weight
N_2	:	Nitrogen
NaCl	:	Sodium Chloride
ng	:	nanogram
NIL	:	Near Isogenic Line
NIR	:	Near Infrared Reflectance
nM	:	nanomoles
NT	:	nulli-tetrasomic
P	:	Phosphorus
PAGE	:	Poly-Acrylamide Gel Electrophoresis
PC	:	Protein Content
PCR	:	Polymerase Chain Reaction
pmoles	:	picomoles
PVP	:	Polyvinyl Pyrrolidone
QTL	:	Quantitative Trait Loci
RAPD	:	Random Amplified Polymorphic DNA
RFLP	:	Restriction Fragment Length Polymorphism
RIL	:	Recombinant Inbred Line
rpm	:	revolution Per Minute
s	:	second
SCAR	:	Sequence Characterized Amplified Region
SDS	:	Sodium Dodecyl Sulphate

SRW	:	Soft Red Winter wheat
SSR	:	Simple Sequence Repeat
STMS	:	Sequence Tagged Microsatellite Site
STS	:	Sequence Tagged Site
TAE	:	Tris-acetate EDTA
TE	:	Tris-EDTA
TEMED	:	Tetramethylethylenediamine
TKW	:	Thousand-Kernel Weight
Tm	:	Melting temperature
Tris	:	Tris-hydroxymethyl amino methane
var	:	variety
°C	:	Degree centigrade
µg	:	microgram
µl	:	microlitre
µM	:	micromolar

CHAPTER 1: Review of Literature

Harnessing Biotechnology in Wheat Quality Improvement

1.1 Wheat: the leading crop of the world

Of all the cereal crops, wheat is grown in some 100 countries around the world and has the widest adaptation. Though it is cultivated as far north as Finland and as far south as NewZeland, the heaviest concentration of wheat production is in the temperate zone of the Northern hemisphere, which includes the major grain growing areas of Canada, North America, Europe, Asia and North Africa. In the Southern hemisphere, the main wheat producing countries include Australia, Argentina, Brazil and South Africa. The international importance of wheat is evident from the 1996-97 worldwide production estimates of 579 MMT of wheat compared to 573, 558 and 132 MMT for corn, rice, and soybean, respectively. Besides, the world wheat production has exhibited a steady growth from 1960 to 1990, ranging from a low of 225 MMT in 1961 to a record 593 MMT in 1990 (Oleson 1994). Today, wheat is the leading grain produced, averaging 600 MMT annually and representing almost one-third of all the cereal production.

In India, with the advent of green revolution, which included introduction of high yielding, photo-incentive, and dwarf wheat genotypes, the scenario of wheat production has undergone a major change as is evident from the yield and production data shown in Table 1.1. Figure 1.1 depicts how wheat cultivation has spread from Punjab, Uttar Pradesh and Haryana to Himachal Pradesh, most of the

Table1.1: All India production and yield of wheat

Year	Production (MT)	Yield (Kg/hec)
50-51	6.4	663
60-61	11.0	851
70-71	23.8	1307
80-81	36.3	1630
90-91	55.1	2281
95-96	62.1	2483
99-2000	68.7	2621

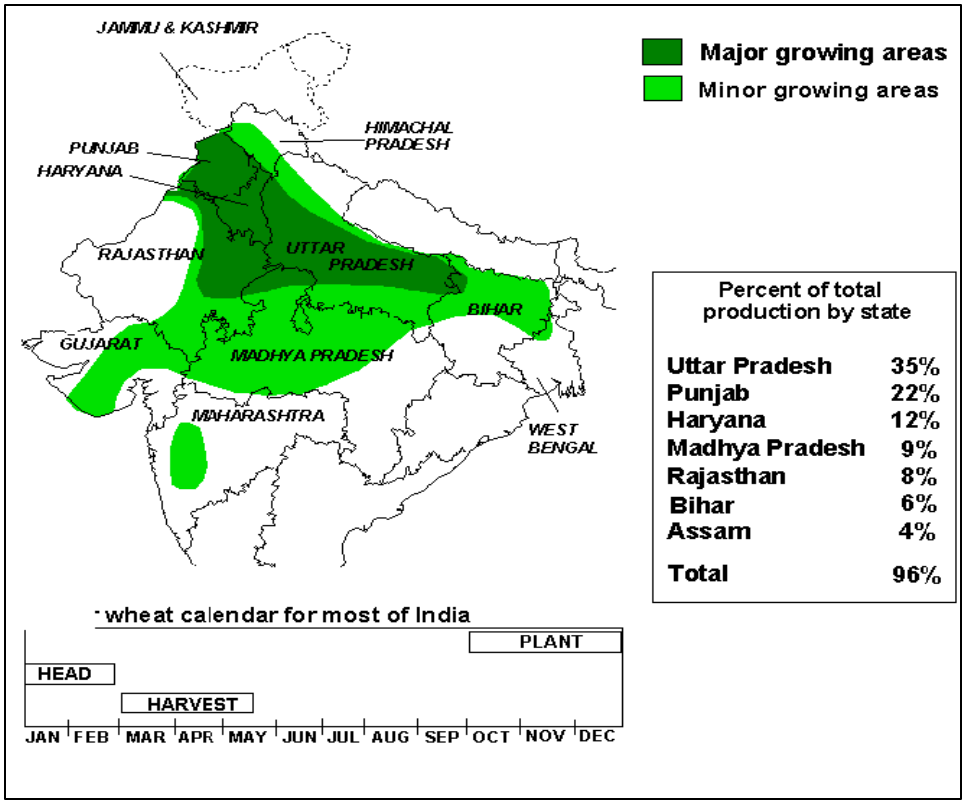

Fig 1.1 Wheat production in India

Madhya Pradesh, Gujarat, Rajasthan, Bihar and Maharashtra. India is now the second largest producer of wheat next to China in the world. With the increase in the area under wheat cultivation and food grain production, there has been greater availability of wheat/person/day in India (Nagarajan 1997).

1.2 Wheat utilization: More demand for quality and value-added products

Though utilization of wheat can be divided into four categories namely food, feed, pharmacological and industrial (Fig 1.2), food is the major use of wheat where close to 75% of world wheat production is consumed for this purpose. Being unique among all the food grains, wheat alone has the visco-elastic dough properties that are essential in making many products depending on the geographical location and culture of the consumer. In western countries, leavened pan bread is the obvious wheat-based food, but now this form is common worldwide. Wheat is also the basis of many breakfast cereals, cookies, and cakes, and it is an important ingredient of many processed items. Pasta is another common form of wheat-based food and many forms of noodles are most evident in Asia, together with steamed breads. More common in the Middle East is the wide range of flat and pocket breads (Fig 1.3).

In India, wheat is mainly used to make rotis, parathas, chapattis and naans. Among these, chapatti is the most popular traditional Indian homemade product and around 85% of the total wheat produced in the country is utilized for this purpose. As a result, until recently Indian wheat has been selected for good roti/chapatti making qualities. However, the change in the life style of Indians involving preference for various kinds of bread and bakery products in the diet demands for the improvement of wheat for domestic consumption. Moreover, with the availability of surplus wheat for export purposes, there is a tremendous need for the improvement of Indian wheat keeping the buyer's requirements in mind leading to breeding of wheat more for quality than quantity (Pena 1997). Understanding the genetic control of specific grain components, the relationship of these components with processing qualities; and the rapid identification and manipulation of quality-related traits based on the use of reliable, fast and low-scale quality testing methodology have, therefore, become the main objectives of recent wheat research (Pena 1997).

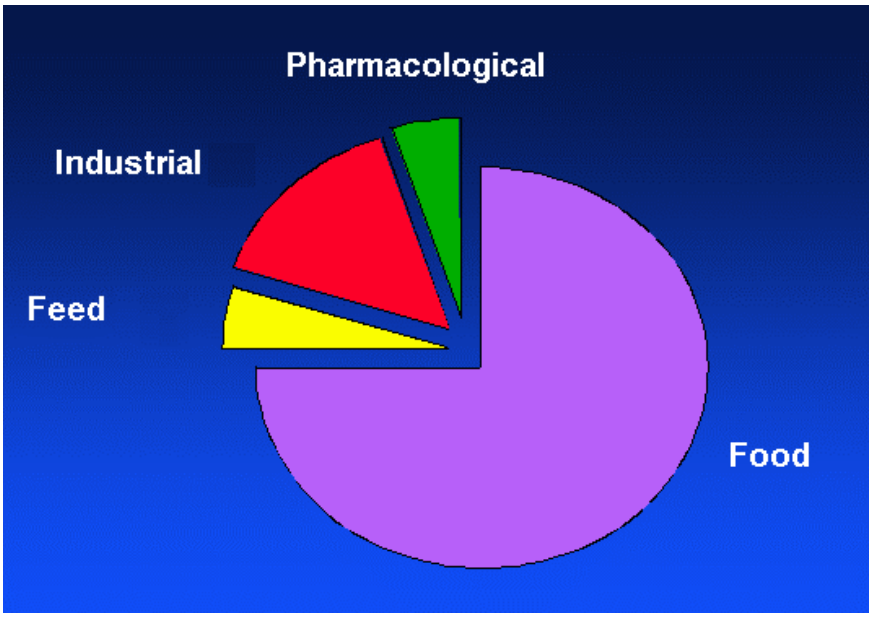

Fig 1.2 Wheat utilization

Fig1.3 Different end-uses of wheat

1.3 Important traits involved in wheat quality breeding

Wheat is unique among all the cereal grains as its flour makes a cohesive mass of dough when mixed with water, which can be molded to make innumerable products. There are large differences between grain quality requirements for the major baked food types such as bread, pastries and cookies and also within each of the types. The overall bread making quality of wheat depends on several factors such as water absorption, loaf volume, internal and external loaf characteristics; and tolerance to mixing and fermentation. All these quality factors are correlated to the physical and chemical properties of flour and dough (Misra and Gupta 1995). The physical characters include grain appearance score, kernel hardness, vitreousness of kernel, 1000-kernel weight, hectolitre weight, kernel size and shape while the chemical characters are protein content, protein quality (gluten), sedimentation test etc as summarized in Table 1.2.

Several other tests such as farinograph, extensograph, mixograph and alveograph can estimate the dough mixing or visco-elastic properties (Faridi 1985, Bushuk, 1985, Menjivar 1990). Other bread making quality tests like the Pelshenke dough ball test; the Zeleny sedimentation test and the SDS (sodium dodecyl sulphate) sedimentation test can give valuable information about the baking quality of wheat. Pasta making quality generally depends upon cookability, stickiness, firmness, elasticity, cooking tolerance and water absorption. A number of workers have developed successful methods for estimating cooked spaghetti firmness and resilence (Dexter *et al.* 1985) and have associated cooking quality with protein content, gluten composition and solubility, farinograph mixing characteristics, SDS-sedimentation volume; and mixograph characteristics (Matsuo *et al.* 1982).

In order to match suitability of end product with wheat quality, three basic factors are taken into consideration as shown in Table 1.3 (Pena 1997; Tipples *et al.* 1994). Among these, kernel hardness and gluten proteins are the two major parameters, which determine the quality of wheat and these are now discussed in greater details in the following paragraphs.

Table 1.2: Various quality criteria used in baking industries

Quality criterion	Refers to	Effect on
Physical characters		
Grain appearance score	Grain size, shape and colour	Price of grain in market
Kernel hardness	Texture of grain	Grading of wheat and milling and end-use qualities
Vitreousness of kernel	Hardness of grain	Flour and semolina recovery
Impurities	Bran pieces, black particles	Aesthetic value
Chemical characters		
Protein content	% of total protein	End use quality and nutritional quality
Moisture content	Presence of moisture in grain	Keeping quality of grains
Gluten content	Dry and wet gluten	Visco-elastic properties
Sedimentation test	Volume of sediment after treating gluten with lactic acid and SDS	Loaf volume of bread
1000-kernel weight	Weight of 1000 grains in grams	Kernel size and density
Hectolitre weight (test weight)	Weight per unit volume	Plumpness of grain and flour yield
Damaged kernel	Infections	Quality and aesthetic value of the end product

Table 1.3 Wheat quality characteristics for diverse end-uses

End-use	Grain hardness	Grain protein (%)	Gluten (dough) strength type
Leavened breads			
Pan type, buns	Hard	>13	Strong-extensible
Hearth, French	Hard/medium	11-14	Medium-extensible
Steamed	Hard/soft	11-13	Medium/weak
Unleavened (flat) breads			
Arabic	Hard/medium	12-14	Medium-extensible
Chapatti, tortilla	Medium	11-13	Medium-extensible
Crackers	Medium/soft	11-13	Medium
Noodles			
Yellow alkaline	Medium	11-13	Medium/strong
White	Medium/soft	10-12	Medium
Cookies, cakes, pastries	Soft/very soft	8-10	Weak or Weak-extensible

1.4 Kernel hardness: An important parameter of end product quality

Kernel hardness (KH) is an important parameter, which is used as grading factor to determine wheat type (Pomeranz and Williams 1990) and also to define end-product quality (Jolly *et al.* 1996). It has a profound effect on milling, baking as well as end-use qualities of bread wheat with greater significance in both the wheat and flour industry and in domestic and world trade (Bettge *et al.* 1995).

KH refers to the texture of the kernel, that is, whether the endosperm is physically hard or soft (Giroux and Morris 1998). Soft wheat kernels fracture more easily and produce fine textured amorphous powder-like flour with less starch damage. Whereas hard grains are difficult to crush and produce coarser-textured

flour with higher levels of starch damage composed of many angular aggregates of starch and protein (Jolly *et al.* 1993). Higher starch damage leads to more water absorption, which also influences crumb softness and increases the shelf life of bread (Tipples *et al.* 1994).

1.4.1 Methods of kernel texture/ hardness measurement

The "biting" or "chewing" test is the oldest form of apparatus to be employed in the evaluation of wheat kernel texture. Several later reports, including those of Harper and Peter (1904), Roberts (1910), Jelinek (1927), Newton *et al.* (1927), Pence (1935) and others have employed versions of a crushing, biting, or princer-type implement to enumerate wheat hardness. Shollenberger and Coleman (1926) and even some later studies such as that of Phillips and Niernberger (1976) have relied on visual evaluation of "Dark, hard, vitreous" or "yellow berry" kernels to define kernel texture.

KH is currently measured by methods such as particle size index (PSI) of flour, time and resistance required to grind grain and near infrared reflectance spectroscopy (NIR) of whole grain meal. Among these, NIR is most favoured due to its non-destructive nature. Although these methods are useful in determining the texture of bulked grain lots, they have limitations in determining the composition of mixtures of hard and soft wheat and in assessing the genotypic hardness. Further, phenotypic hardness varies considerably due to environment affecting reliable determination of genotypic hardness (Morris 1992, 1998).

1.4.2 Important factors affecting kernel texture

Various factors influencing kernel texture in wheat have been reviewed by Pomeranz and Williams (1990) in great details. Among these, three important factors namely protein content, kernel size and moisture content are discussed below.

I Protein content

The relation of wheat hardness and protein content has been studied by many investigators with various confounding results and these are summarized in Table 1.4. Based on these reports, it can be seen that different methods of expressing texture may also lead to different confusions. Various investigators

further feel that strong varietal, seasonal and location interactions play an important role in explaining the relationship between these two factors.

Table 1.4 Relationship of wheat protein and hardness

Investigator(s)	Method(s)	Results
Newton *et al.* (1927)	Cracking	No relation
Worzella (1942)	Particle size	No relation
Berg (1947)	Particle size	Varietal character, uninfluenced by protein
Fajerson (1950)	Particle size	Varietal character, influenced by protein
Symes (1961)	Particle size	Protein effect varies among varieties
Williams (1967)	PSI, starch damage	No relation
Symes (1969)	Particle size	No relation
Greenaway (1969)	Wheat hardness index	Relation with protein per square meter of flour and protein
Seckinger and Wolf (1970)	Microscopy of endosperm particles	Protein particles for hard (unlike soft) wheat compact and hard to disrupt
Barlow *et al.* (1973)	Penetrometer	No varietal differences
Moss *et al.* (1973)	Pearling resistance, PSI	Negative relation for single cultivar
Trupp (1976)	PSI (protein by dye binding)	Very low relation affected by variety & environment
Stenvert and Kingswood (1977)	Time to produce a fixed volume of ground wheat	Positive relation with protein content, cultivar- dependent
Moss (1978)	Starch damage, particle size	Optimum hardness and starch damage related to minimum protein
Obuchowski and	Miscellaneous	No relation

Investigator(s)	Method(s)	Results
Bushuk (1980a, 1980b)		
Miller *et al.* (1981b)	Work required to grind	No relation
Miller *et al.* (1982)	Time to grind, work to grind, particle size, NIR	No relation
Miller *et al.* (1984)	Grinding time and NIR	Significant correlation
Sourdille *et al.* (1996)	NIR	Positive relation
Bushuk (1998)	Miscellaneous	Positive relation
Giroux *et al.* (2000)	NIR	Significant correlation

PSI= particle size index
NIR= near-infrared spectroscopy

Using three different methods of texture measurement, Miller *et al.* (1984) have found significant correlations between KH and PC for grinding time and NIR values. In the same year, 1984, Williams and Sobering have reported that soft wheats become harder as protein content is increased while Pomeranz *et al.* (1985), in a study of wheats grown in widely differing locations, have found very weak correlations between protein and kernel texture. Wheats of high protein content tend to be hard, have strong gluten and produce good quality bread whereas wheats of low protein content tend to be soft, have weak gluten and produce small loaves of inferior crumb structure (Bushuk 1998; Tipples *et al.* 1994).

II Kernel size:

Shaw and Gaumnitz (1911) have found for the first time that large kernels required more pressure to break them than did small kernels. Newton *et al.* (1927) have shown that hardness, as defined by cracking strain, increased with the size of kernel. On the other hand, Worzella (1942) has found no correlation between kernel size and granulation, using a pioneer version of PSI test. Chesterfield and Lind (1971) have also found no consistent relationship between pearling resistance and kernel size. In further studies, Chung (1972) has considered kernel size as an important variable while Fowler and De la Roche (1975) have reported a non-significant correlation between hardness (grinding time) and kernel weight. Kosmolak (1978) has also observed no significant relationship between these two factors. Miller *et al.* (1981a) have reported that the energy required to grind a 50-g sample of HRW wheat was directly related to kernel size whereas Williams and Sobering (1984) have reported that the coefficient of correlation between PSI percent and kernel weight was strongly positive for the hardest cultivars, changing to negative for soft wheats. Using Brabender microhardness tester, Pomeranz and Afework (1984) have found that grinding time and PSI increased as kernel size decreased, whereas PSI and NIR reflectance were directly related to kernel size. Williams *et al.* (1987) have found kernel size to exert a small effect on hardness while, Pomeranz *et al.* (1988) have reported that kernel size had a direct effect on the measurement of hardness in wheat. Thus the studies of correlation between kernel size and hardness have also shown varying results.

III Moisture content

All the methods of measuring wheat kernel texture are affected by moisture content of the sample (Newton *et al.* 1927). Tarutin and Orloff (1935) have reported that resistance to crushing decreased as the moisture content of the grain increased. McCluggage (1943) has found no conclusive effect of moisture on pearling test of wheat grains. Katz *et al.* (1961) have studied the influence of moisture on the hardness of HRW, SWW and durum wheats using a penetrometer. They have found that all wheats became progressively softer as moisture content was increased. Symes (1961) has observed that a range in moisture from 6 to over 18% had a much more pronounced effect on the PSI of the hard wheats, than on that of the soft wheat. Stenvert (1974) has reported that

a moisture range of 11-19% had relatively little effect on the hardness. Obuchowski and Bushuk (1980a) have surveyed 12 methods of hardness measurement and found that a moisture range of 9.5-15.5% affected all the methods whereas Miller *et al.* (1981b) have shown that the influence of moisture was very marked on the grinding time. Williams and Sobering (1988) have confirmed that moisture had an extremely strong effect on grinding time, particularly with soft wheats. According to Pomeranz and Williams (1990) it is clear that moisture content is extremely important in any measurement of wheat kernel texture.

1.4.3 Genetic analysis of kernel hardness

A better understanding of the physico-chemical mechanism and the genetic control of endosperm texture would aid in breeding, marketing and proper utilization of wheat grains (Greenblatt *et al.* 1994). During the attempt of studying genetic control of KH, Symes (1969) and Baker (1977) have demonstrated that the difference in hardness between hard and soft wheat is due to one major gene, designated as *Hardness* (*Ha*), located on the short arm of chromosome 5D (Mattern *et al.*, 1973 and Law *et al.*, 1978). According to Morrison *et al.* (1989), chromosome 5A may also carry a minor locus for hardness while Baker (1977) and Pomeranz and Williams (1990) have reported presence of two major and three minor genes controlling the expression of kernel hardness. Simmonds *et al.* (1973) have suggested that hardness is due to a cementing agent between starch and proteins and it has been found to be involved with the continuity of protein matrix and the strength with which it physically entraps starch granules (Stenvert and Kingswood 1977). A 15-kDa protein is shown to be present in the extracts of water washed starch granules from soft wheat and is absent in those of hard grains (Greenwell and Schofield 1986; Schofield and Greenwell 1987; Morrison *et al.* 1992; Jolly *et al*.1993). Thus this protein is a marker for grain softness and has been called as “friabilin” (Greenwell and Schofield 1989) or “Grain softness protein” (GSP) by Jolly *et al.* (1990). Jolly (1991) has also shown its correlation with qualitative level of kernel hardness. Friabilin is present on the surface of water-washed starch; and is abundant on soft wheat, scarce on hard wheat and absent on durum wheat starch (Oda *et al.*, 1992; Morris *et al.*, 1994; Bettge *et al.*, 1995; Greenblatt *et al.*, 1994). It is primarily composed of two subpolypeptides,

puroindoline-a and puroindoline-b (Blochet *et al.*, 1993, Gautier *et al.*, 1994; Giroux and Morris 1997, 1998). Experimental evidences have indicated that friabilin is associated with kernel softness, which is a dominant trait. However, this polypeptide does not appear to be specific for soft wheat suggesting that it is also present in the endosperm of hard wheat (Jolly *et al.* 1993). Friabilin alone thus is not useful as a marker of grain hardness or softness (Wrigley 1994). Moreover, environment has also been shown to modulate the penetrance of the *Ha* locus (Jolly *et al.* 1993).

Using RFLP markers, Sourdille *et al.* (1996) have reported presence of a major locus *Xmta9*, which is linked closely to the gene, *ha* (hard) on 5DS and seven other minor loci distributed on different chromosomes. The genes for puroindoline a (*pin-a*), puroindoline b (*pin-b*) and grain softness protein (*Gsp-1*), closely linked to the *Ha* (soft) locus have been shown to be associated with the expression of grain softness (Tranquilli *et al.*,1999 and Turner *et al.*, 1999). Giroux and Morris (1998) have found that presence of a single mutation in either protein, a null in *pin-a* or glycine to serine sequence change at position 46 in *pin-b*, is associated with hard grain texture. Recently, Lillemo and Morris (2000) have also reported a leucine to proline mutation at position 60 in pin b in hard wheats from Northern Europe. Giroux and Morris (1998) have further designed PCR primers for *pin-a* and *pin-b* sequences using the single nucleotide change in *pin-b* (Giroux and Morris 1997). Preliminary results have indicated that bread wheat varieties with the null *pin-a* deletion had harder grains than varieties with the glycine to serine mutation (Giroux *et al.* 1998, Morris *et al.* 1999). Tranquilli *et al.* (1999) have further developed a more reliable, codominant CAPS (cleavage amplified polymorphic sequence) marker based on the same point mutation in *pin-b*, which can be used to determine kernel texture. However, as reported by Morris (1998), till now, the emphasis of all the molecular studies has been restricted to the *Ha* locus and there is a need to further understand other genetic factors contributing to the variation for KH beyond that explained by *Ha*.

1.5 The gluten proteins of wheat: Another parameter responsible for wheat quality

Wheat protein also known as gluten comprises roughly 78-85% of total wheat endosperm protein. Gluten is a very large complex protein composed

mainly of polymeric glutenins and monomeric gliadins (MacRitchie, 1994). They are deposited and stored in protein bodies during endosperm development. Glutenins confer elasticity while gliadins confer, mainly viscosity and extensibility to the gluten complex (Pogna *et al.* 1987). Thus, gluten is responsible for most of the viscoelastic properties of wheat flour doughs and is commonly known as flour or dough strength of wheat (Pena 1997). Work done on wheats with similar hardness has shown that the variation in bread making quality depends almost on the variation in glutenin components of flour protein (Orth and Bushuk, 1972). Fu and Sapristein (1986) have confirmed that most variation in dough strength parameters was explained by the amounts of soluble and insoluble glutenin. To obtain a semi-quantitative estimation of the amount of glutenin, the Zeleny and SDS-sedimentation tests (Axford *et al.*, 1979) are currently and most widely used as rapid tests to screen early generation wheat lines for bread making quality (Weegels *et al.* 1996). These tests are based on the expansion of mainly glutenins in lactic acid or SDS/lactic acid solution.

1.5.1 Gliadins

Gliadins constitute about 40% of the total endosperm protein and are a heterogenous mixture of single-chain polypeptides of molecular weight ranging from 28-70kDa (Payne *et al.* 1982). They are further divided into four groups, α-, β-, γ- and ω-gliadins based on their electrophoretic mobility at low pH. Some of them such as α, β- and γ-gliadins show interchain disulfide bond, which is lacking in case of ω-gliadins. Most of the genes coding for ω- and γ-gliadins are tightly clustered at three homologous loci such as *Gli-A1*, *Gli-B1* and *Gli-D1* and as a result, the gliadin polypeptides coded by each locus are inherited strictly as a block, which is referred to as gliadin allele (Pogna *et al.* 1994). Some gliadins have been found to be strongly associated with glutenins (Beitz and Wall 1975). Significant correlations between gliadin components and gluten quality have been observed by several researchers. A consistent relationship was found between the presence of γ-gliadin designated band 45 and gluten strength, and between the presence of γ-gliadin band 42 and gluten weakness in durum wheats (Damidaux *et al.* 1978). Boggini and Pogna (1989) have confirmed that γ-gliadin 45 has strongly favorable influence on the bread making quality of durum wheat

as well. Recently, gliadins 42 and 45 have been found to be the only genetic markers of quality (Pogna *et al.* 1990). Metakovsky (1991) have shown significant correlations between gliadin alleles (*Gli-B1b*, *Gli-B2c* and *Gli-A2b*) and gluten strength in Italian bread wheat cultivars.

1.5.2 Glutenins

Glutenins are classified into two subunits, the high molecular weight (HMW) and the low molecular weight (LMW) glutenins (Huebner *et al.* 1976). Both classes of subunits are present in the flour as cross-linked proteins resulting from interpolypeptide disulfide linkages. The HMW subunits are minor components in terms of quantity but are major determinants of elasticity of gluten (Payne *et al.*, 1980).

I HMW glutenins and their allelic variation

HMW glutenin subunits are controlled by genes located in complex loci in group 1 and group 6 chromosomes. The *Glu-A1*, *Glu-B1*, and *Glu-D1* loci possess genes coding for high molecular weight glutenin (HMWG) subunit components. Allelic variation has been studied in the greatest detail for the HMWG subunits; in particular, the *Glu-D1* locus has been shown to have a stronger influence on bread making quality than variation at the other *Glu-1* loci (Branlard and Dardevet, 1985, Primard *et al.* 1991, Dong *et al.*, 1992). These variations result because of differences in molecular weight and have arisen in evolution by unequal crossing over (Payne 1987). Allelic variation in glutenin composition is exploited by wheat breeders in the development of new varieties with improved bread-making qualities.

Several HMW subunits of glutenin are found to be associated with bread making quality. Payne and co-workers (1987) have analysed numerous unselected progeny of crosses between common wheat cultivars for both SDS-sedimentation volume (which is correlated with loaf volume) and subunit composition and have shown that certain allelic subunits impart differential effects on gluten quality. Payne (1987) has given quality scores for several HMWG subunits. There are also differences in quality effects among glutenins of the same locus; for example, subunit 5+10 is more closely associated with better bread making quality and 2+12 with poor bread making (Kolster *et al.* 1991,

Payne *et al.*, 1981). Since the x- and y-type of HMW subunits encoded by the *Glu-B1* and *Glu-D1* loci are always inherited as pairs, it is not possible to determine the effects of individual subunits on gluten quality. However, analysis of the segregating progeny from crosses involving the recombinant cultivar, Fiorello, which contained the unusual combination of subunits 1Dx5 and 1Dy12, suggests that the y-type subunit 10 is responsible for the 'good quality' of subunit pair 5+10 (Pogna *et al.*, 1987). Later, Lafiandra *et al.* (1993) have demonstrated that the polypeptide designated as subunit 5 (called as 5*) in the allelic combination 5+12 by Pogna *et al.* (1987) is different from the polypeptide also designated as subunit 5 in the 5+10 combination. This 5* polypeptide from 5*+12 lacks one sulphydryl group in the N-terminal domain like subunit 2, which is the cause to have an effect by enabling larger glutenin polymers to be formed. Further, using NILs, Rogers *et al.* (1991) have shown that both types of subunits (5 & 10) are equally important for dough properties, SDS-sedimentation and loaf volume. Branlard and Dardevet (1985) have shown that the alveograph parameters *W* (gluten strength) and *P* (tenacity); and the Zeleny sedimentation value are correlated positively with subunits 7+9 and 5+10, and negatively with 2+12, whereas subunit 1 is correlated with *W*, and subunit 2* and 17+18 with *G* (swelling). Obukhova *et al.* (1997) have indicated that the high quality of dough is mainly determined by the *Glu-A1*a allele (subunit 1), whereas the low quality of dough was related to the *Glu-A1*c allele (null allele).

D'Ovidio and Anderson (1994) have designed locus specific primers to detect the presence or absence of 1Dx5 gene and to select for the entire locus 1Dx5-1Dy10. The oligonucleotide primers have further been developed for all six HMWG genes by D'Ovidio *et al.* (1995). Recently Ahmad (2000) has reported the utility of specific PCR primers to identify wheat genotypes carrying glutenin allelic combinations, which are related to good or poor BMQ. Bustos *et al.* (2000) have developed a set of AS-PCR markers for HMW-glutenin of wheat and have also designed primers specific for X-null or 2* alleles which would facilitate marker assisted selection.

II LMW glutenins

LMW glutenin subunits represent a specific class of wheat storage proteins encoded at the *Glu-3* loci designated as *Glu-A3*, *Glu-B3*, and *Glu-D3* (D'Ovidio *et*

al., 1997). Sreeramulu and Singh (1997) have further identified new LMWG subunits called as *Glu-4* (*GluD-4* and *GluD-5*) subunits. In contrast to gliadins and HMW glutenin subunits, which are easily resolved by Acid-PAGE or SDS-PAGE, the LMW glutenin subunits have proved much more difficult to analyse by SDS-PAGE because of overlap with gliadins (Pogna *et al.* 1994). Also, when the genes on chromosome 1D that code for the D group of LMWG subunits have been mapped, they are shown to be unbreakably linked to *Gli-D1* and are not found at a separate locus (Payne 1987). Genetic correlations between dough quality and LMW glutenin abundance have been found to be very difficult to establish in hexaploid wheat (Gupta *et al.* 1989). Some gliadins and LMW glutenin alleles have been found to be significantly correlated with dough extensibility (Branlard and Dardevet, 1985, Cerny *et al.* 1992,and Gupta *et al.* 1989, 1991, Manifesto *et al.*, 1998; Obukhava *et al.*, 1997). Alleleic variation for LMWG subunits encoded at *Glu-B3* locus has been shown to be responsible for differences in gluten visco-elastic properties (Pogna *et al.* 1990). Redaelli *et al.* (1997) have shown strong positive effects on dough extensibility by *Gli-D1* (gliadins)/*Glu-D3* (LMWGs) alleles.

Contrary to the current belief that wheat bread making quality is determined primarily by variation at the *Glu-1* locus, Rousset and Dvorak (1997) have shown that this trait is under a complex control and the Glu-1 loci (HMWGs) are only a component of the genetic control of the trait. Gupta *et al.* (1989) and Neito-Taladriz *et al.* (1994) have proposed that the effective predictive methods of dough properties should be based on the composition of both, HMWG and LMWG subunits. Moreover, LMWGs are in large excess in doughs than HMWGs making their purification and characterization more difficult (Masci *et al.* 1998). Thus, gluten quality seems to be a complex, multigenically controlled trait that cannot be accounted for by simple additive effects of individual alleles at loci coding for storage proteins (Metakovsky *et al.* 1997). According to Pena (1997), there is much still to be known about the genetic control of gluten proteins and its impact on bread making quality, particularly, about the role of the LMWG subunits which do play a role in BMQ as important as that of HMWG (Gupta *et al.*, 1990; Masci *et al.* 1998 and Manifesto *et al.*, 1998).

1.6 New biotechnological strategies for wheat quality enhancement

As detailed in the previous text, wheat quality traits and their parameters are very complex, often polygenic and are ill-defined, making it difficult for a breeder to combine them in a genotype through successive crossing. The conventional approach involves selecting two better quality parental genotypes and crossing them to produce superior varieties with the quality traits of interest. These methods of breeding are time-consuming, laborious and environment dependent. The number of genes and their interaction effects controlling the expression of these QTLs are poorly understood. It becomes further more difficult if the traits show very low heredity and possess environmental effects.

Various biotechnological approaches like *in vitro* tissue culture, gene transfer and use of DNA markers have emerged as powerful tools to complement conventional methods of breeding by generating genetic variability necessary for creating novel cultivars with desirable characters and reducing the time taken to produce cultivars with improved characteristics.

1.6.1 Gene transfer in wheat

Among various methods of wheat transformation, particle bombardment has been used widely to develop highly efficient transformation systems for wheat (Vasil *et al.* 1992, 1993, Weeks *et al.* 1993). Gene dosage studies have indicated that bread making quality of wheat could be improved by integration and expression of specific HMW-GS genes (Flavell *et al*. 1989; Shewry *et al*. 1995). Since bread making quality is the major thrust of wheat quality improvement studies, efforts have been made to manipulate the proportion of HMW-GS that are known to be associated with good bread making quality.

Using biolistic bombardment of cultures of immature embryos, Altpeter *et al.* (1996) have introduced the HMW-GS 1Ax1 gene, known to be associated with good bread making quality, into the Bobwhite cv. of wheat. They have found that the accumulation of HMW-GS 1Ax1 is consistent and the transgenic plants are normal, fertile and show Mendelian segregation thus suggesting the possibility of manipulating both the quantity and quality of HMW-GS. Blechl and Anderson (1996) have introduced a gene encoding novel hybrid HMW-glutenin subunit (Dx5: Dy10), under the control of its native endosperm specific promoter in wheat cv. Bobwhite. The hybrid HMW-GS accumulated in the endosperm at levels similar to that of native subunits and could be identified from the latter by its distinct mobility

on SDS-PAGE. Barro *et al.* (1997) have transformed two near-isogenic lines of wheat, which have been derived from crossing mutants of the cultivars Olympic and Gabo with null alleles at the *Glu-1* (HMW-GS) loci. Blechl *et al.* (1998) have successfully transformed bread wheat in order to increase the proportions of HMW subunits and improve the functional properties of flour. Vasil and Anderson (1997) have also been successful in transforming wheat with the HMW-GS, including its integration and expression and have reported the possibilities of engineering wheat gluten in order to improve bread making qualities. Expression of HMW subunits has also been reported in tetraploid pasta wheat (He *et al.* 1999) and Tritordeum, a novel amphidiploid cereal produced by crossing tetraploid wheat with *Hordeum chilense* (Rooke et al 1999). The ability to change the levels and composition of the HMW-GS of wheat by genetic engineering thus reaches the possibility of tailoring flour properties to end-user specifications of dough strength (Blechl *et al.* 1998).

1.6.2 Use of DNA markers in wheat quality improvement

With the introduction of several DNA markers for genome mapping and tagging qualitative traits, there is a tremendous success in crop improvement programmes. Breeding laboratories all over the world are poised to develop and use the technologies such as microsatellite markers, large insert DNA libraries and EST etc. There are currently a number of ongoing discussions and plans for various plant genome programs. A broadly planned and executed “Crop Plant Genome Program” is considered to be a logical format to advance all aspects of crop development. Such attempts are being made in various crops such as rice, maize and arabidopsis.

Wheat genome is characterized by a large genome size (16 X 10^9 bp/1C) (Bennett and Smith, 1976), almost 10 to 20 times larger than crops like cotton and rice. Development of markers in wheat has been very challenging because of its polyploid nature and presence of high proportion of repetitive DNA. Efforts on wheat genome mapping using DNA markers have been initiated with the formation of the International Triticeae Mapping Initiative (ITMI) in 1990. RFLPs have been used as the preliminary system of markers to map the wheat genome by various workers like Chao *et al.* (1989), Liu *et al*. (1991), Anderson *et al.* (1992), Devos *et al.* (1992), Devos and Gale (1992) and Boyko *et al.* (1999). With

further advancement of technology, new types of markers, namely RAPD, ISSR, STMS, SSR and AFLP were employed in wheat genome mapping, which has led to the establishment of genetic linkage maps. Kojima *et al.* (1998) and Peng *et al.* (2000) have used RAPD markers for the construction of linkage maps while Cadalen *et al.* (1997), Roder *et al.* (1998), Stephenson *et al.* (1998), Penner *et al.* (1998), Messmer *et al.* (1999), Nachit *et al.* (2001) have extensively used SSR, STMS and AFLP primers for the same.

In recent years, the genetic improvement of wheat using DNA markers has received considerable attention from plant breeders with the purpose of increasing the grain yield, to minimize crop loss due to unfavourable environmental conditions, and attack by various pests and pathogens. Efforts have been made to tag various complex quality traits such as grain protein content (Blanco *et al.*, 1996, Mesfin *et al.*, 1999, Prasad *et al.* 1999 and Dholakia *et al.*, 2001), grain size (Varshney *et al.* 2000, Ammiraju *et al.* 2000), preharvest sprout tolerance (Anderson *et al.*, 1993, Roy *et al.*, 1999 and Zanetti *et al.*, 2000), vernalization response (Galiba *et al.* 1995, Nelson *et al.* 1995), red grain colour (Nelson *et al.* 1995) and amylose content (Araki *et al.* 1999). D'Ovidio and Anderson (1994) have developed discriminating primers for the well-known gene HMW-G 5+10 subunit pair, which will enhance the selection for good bread making genotypes whereas Perretant *et al*. (2000) and Zanetti *et al*. (2001) have found QTLs linked to bread making quality. Bread making quality being the major aspect of wheat quality, various related parameters like flour colour, flour yield, flour viscosity, kernel hardness, kernel morphology and texture have been studied by many researchers like Parker *et al.* (1998), Parker and Langridge (2000), Parker *et al*. (1999) Udall *et al.*, (1999), Sourdille *et al.* (1996) and Campbell *et al.* (1999). However, these quality traits being complex and polygenic, a lot more information needs to be unraveled in order to exploit these technologies meaningfully for wheat quality enhancement.

Genesis of my thesis

In recent years, India is producing surplus wheat due to increase in the area under wheat cultivation and introduction of high yielding varieties. However, Indian wheat has been selected and improved for chapatti/roti making characters and is not highly suitable for making products such as bread, pasta, noodles and

cookies. To increase the export potential of Indian wheat and to meet the international standards it is essential to enhance the quality of Indian wheat. Wheat varieties producing good quality bread with smooth crust, uniform grain size and high loaf volume will be preferred not only in the export market but also within the country due to changed life-style of majority of Indian population. Therefore, research work on tagging of wheat quality traits was initiated at Plant Molecular Biology group of NCL in collaboration with various other wheat breeding research organizations. Since it was my ambition to carry out research in crop biotechnology, which will be eventually useful to Indian farmers, I decided to join this programme for my Ph.D. work. I focused my work on two quality traits, namely kernel hardness and bread making quality and made an attempt to identify molecular markers that are associated with them.

Organization of my thesis

I have organized the thesis in the following order:

Chapter 1: Introduction - Review of literature

Chapter 2: Materials and Methods

Chapter 3: Results

Chapter 4: Discussion

Chapter 5: Thesis summary

Bibliography

Curriculum vitae

CHAPTER 2: Materials and Methods

2.1 Selection of plant material for KH and BMQ traits

For the identification of markers associated with the two traits namely kernel hardness (KH) and bread making quality (BMQ), a study was undertaken to analyze different wheat genotypes from various wheat breeding centers in India for the quality parameters, responsible for deciding bread quality. Parents for KH were identified based on traditionally used criterion and then got tested for hardness from CFTRI, Mysore, India. Crosses were attempted among suitable bread wheat varieties and the following two different populations were developed at the Directorate of Wheat Research, Karnal, India (Table 2.1).

Table 2.1 Crosses attempted for the two quality traits

Quality trait	Parental cross	No. of RILs	Generation
Kernel Hardness	NP4 (hard) X HB208 (soft)	100	F_7
Bread Making Quality	CPAN3004 (good) X HD2329 (poor)	120	F_8

A set of 100 RILs at F_7 generation was developed from a cross between the hexaploid Indian wheat landrace NP4 (hard grain) and HB208 (soft grain) following single seed descent method. The KH value was about five times higher in NP4 as compared to HB208 (Table 2.2A). A second set of 120 RILs at F_8 generation was also developed from a cross between CPAN3004, a wheat cultivar with good BMQ and HD2329, a wheat cultivar with poor BMQ (Table 2.2B).

These two parental pairs were analysed for different traits influencing KH and BMQ and the values are detailed in Table 2.2 A & 2.2 B.

All the four parental genotypes along with their respective RIL populations were grown at Agharkar Research Institute's field-station, Hol, near Pune in 11x11 lattice design with two replicates for two successive seasons i.e.1997-98 and 1998-99 for NP4 X HB208 cross while 1998-99 and 1999-2000 for CPAN3004 X HD2329 cross, respectively. The RILs were orderly planted in doubled rows, with

Table 2.2A Quality parameters influencing KH in the selected parents

Traits	NP4	HB208
Kernel hardness per se (NIR)	5.8	1.1
Protein Content (%)	13.84	12.04
Moisture Content (%)	8.24	8.48
Hectolitre Weight (gm)	78.1	77.8
1000-Kernel Weight (gm)	42	34

Table 2.2B Quality parameters influencing BMQ traits in the selected parents

Traits	CPAN3004	HD2329
Sedimentation Value (cc)	8.02	7.55
Protein content (%)	11.47	11.17
Kernel hardness	3.07	3.85
Glu-1 Protein score	9	7
Hectolitre weight (kg/hl)	77.9	79.7
1000-Kernel weight (g)	38	45

2m length having distance of 23cm in between them (Fig 2.1). The fertilizer applied was in a ratio of 120kg N2: 60kg P: 40kg K_2O in field experiments. Phenotypic data was collected in the successive years from all the RILs in a row to avoid biased selection.

2.2 Measurements of KH and BMQ related traits

Hardness values of the kernels of parents as well as RILs was determined by NIR (Near Infrared Reflectance) spectroscopy with a Technicon 400 Infra-analyzer (Technicon Corp, NY) according to the AACC method (AACC, 1989). The reliability of the NIR kernel texture method lies on the calibration of the instrument and grinder variability. Protein content (PC) was determined by Kjeldahl method (AACC, 1983) on gel-tech autoanalyser (Tecator, Sweden) calibrated for the estimation of Nitrogen content. The PC was calculated using the

formula: PC (%)= Nitrogen content x 5.7. Moisture content (MC) was measured using hot air oven (AACC, 46-12, 1983). Hectolitre weight (HW) was measured by

Fig 2.1: Representative picture of RILs of NP4 X HB208 sown in rows in the field

the hectolitre weight-measuring funnel of the SINARFP Auto 6080 moisture analyzer (Tecator, Sweden) while 1000-kernel weight (TKW) in grams was determined by obtaining the weight of 1000 grains using electronic counter (Misra and Gupta 1995). Sedimentation volume (SV) was determined by sedimentation test, based on the fact that gluten protein absorbs water and swells considerably when treated with lactic acid in the presence of SDS (Misra and Gupta, 1995). *Glu -1* protein score (PS) was determined according to Payne *et al.* (1987) after extracting HMW glutenin as described by Singh *et al*. (1988) and running the extracted proteins on 12% SDS-PAGE.

2.3 Isolation of genomic DNA

2.3.1 Using CTAB method

Total genomic DNA of the parents as well as RILs of cross NP4 X HB208 was extracted from 15 days old seedlings grown in the glass house by modified Hexacetyltrimethylammonium bromide (CTAB) method of Rogers and Bendich (1988). Young leaf tissue (10g) was ground to a fine powder in liquid nitrogen using mortar and pestle. To this, 2X CTAB buffer (2% CTAB, 100mM Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl, 1% PVP and 2% β-mercaptoethanol) was added and the contents were mixed to form an emulsion, which was incubated at 60° C for 20 min. After incubation, the emulsion was allowed to cool down to room temperature and then was equally distributed in SS34 centrifuge tubes. In each tube, an equal volume of Chloroform:IAA (24:1) mixture was then added; the tubes were capped and gently swirled to mix the contents. The tubes were centrifuged at 10,000 rpm for 10 min at room temperature in a Sorvall RC-5B centrifuge (Du Pont, USA). The aqueous layer formed after centrifugation was recovered and distributed in fresh SS34 tubes. To this, an equal volume of CTAB precipitation buffer (1% CTAB, 500 mM Tris-HCl pH 8.0, 10 mM EDTA) was added, the contents were thoroughly mixed and kept at room temperature for 15 min. Subsequently, the pellet of the precipitated DNA obtained by centrifuging the tubes at 10,000 rpm for 10 min at 15° C, was dried and dissolved in high salt TE buffer (1M NaCl, 10 mM Tris-HCl pH 8.0, 1mM EDTA). The dissolved DNA was reprecipitated by adding two volumes of chilled ethanol and the precipitated DNA

was either spooled out or pelleted by centrifugation at 10,000 rpm for 10 min at 4° C. The DNA pellet was washed with 70% ethanol, dried and dissolved in an appropriate volume of TE buffer (10mM Tris-HCL pH 8.0, 1mM EDTA pH 8.0). To remove RNA from the samples, 100 µg/ml RNase A (DNase free) was added and incubated at 37° C for 1h. Subsequently, an equal volume of Chloroform:IAA (24:1) mixture was added, the contents were mixed and centrifuged at 10,000 rpm for 10 min at 15° C. Following centrifugation, the aqueous layer containing DNA was recovered and stored at -20° C until further use.

2.3.2 DNA isolation by Anderson *et al.* (1992)

The genomic DNA of the parents as well as RILs of cross CPAN3004 X HD2329 was extracted from 15 days old seedlings grown in the glass house according to the procedure as described by Anderson *et al.* (1992). Young leaf tissue (10 gm) was ground to a fine powder in liquid nitrogen using mortar and pestle. To this, 15-20 ml of extraction buffer (500mm NaCl, 100 mM Tris-HCl pH8.0, 50 mM EDTA and 0.84% SDS; a pinch of Sodium Bisulphite was added just before use and pH adjusted with NaOH to 7.8-8.0) was added and the solution was pre-heated at 65^0C. The contents were mixed to form an emulsion, which was incubated at 65^0C for 20-30 min, inverting tubes every 5-10 min. After incubation, the emulsion was allowed to cool down to room temperature, and was then equally distributed into SS34 centrifuge tubes. In each tube, equal volume of Chloroform: IAA (24:1) was added, the tubes were capped and gently swirled to mix the contents. The tubes were centrifuged at 10,000 rpm for 10 min at 4^0C in a Sorvall RC-5B centrifuge (Du Pont, USA). The aqueous layer formed after centrifugation was recovered and distributed into fresh SS34 tubes. To this, again second wash of Chloroform: IAA (24:1) was given as described above. After the supernatent was recovered in fresh tubes, either two volumes of chilled ethanol or one volume of isopropanol was added and the precipitated DNA was either spooled out or pelleted with centrifugation at 10,000 rpm for 10 min at 4^0C. The DNA pellet was washed with 70% ethanol, dried and dissolved in an appropriate volume of TE buffer (10mM Tris-HCl pH8.0, 1mM EDTA pH 8.0). To remove RNA from the samples, RNase A (DNase free, 10 mg/ml stock solution) equivalent to

0.3% of the total volume of sample was added to the dissolved DNA and incubated at 37^0C for 1 h. Then it was stored at -20^0C until further use.

2.4 DNA quantification

Extracted genomic DNA (1µl) was loaded on a 0.8% agarose gel in 0.5X TAE buffer contaning ethidium bromide (10 µg/ml) along with known dilutions of λ bacteriophage DNA (50 ng, 100 ng, 200ng, 500ng etc). The DNA concentration of the sample was estimated by visual comparison of the band with known dilutions of bacteriophage DNA. Purity and concentration of the extracted DNA for each sample was also checked spectrophotometrically at 230nm, 260nm, 280nm and 300nm.

2.5 PCR amplification using various DNA primers

1) RAPD analysis

Five hundred and eighty decamer random primers of series A to Z and of further series AA, AB, AC, AX, AY and AZ from Operon Technologies, USA, were used to amplify the DNAs of all the four parents and their respective two RIL populations. Primers, which produced clear and reproducible amplification pattern upon repetitive trials, were used in further investigations. PCR amplifications were performed in 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM dNTPs, 0.4 mM spermidine, 5 picomoles of primer, 1 unit of *Taq* DNA polymerase (Perkin Elmer, USA) and 15 ng of genomic DNA per 25µl reaction in PTC-200 thermocycler (MJ Research, USA) according to the protocol by Eastwood *et al.* (1994) with some modifications.

The PCR conditions included

Initial denaturation: 94^oC for 4 min

5 cycles : 92^oC for 30 s
35^oC for 2 min
72^oC for 1.5 min

35 cycles : 92^oC for 5 s
40^oC for 20 s
72^oC for 1.5 min

1 cycle :72^oC for 5 min

Amplified products were electrophoresed on 2% agarose gels in 0.5X TAE buffer and visualized under UV light after staining with ethidium bromide. The agarose gels were then photographed using PENTAX K1000 and gel documented using the gel-documentation system by Pharmacia Biotech imagemaster VDS.
Each set of amplification was accompanied by a control reaction that contained all the other reaction components except template DNA to ensure the reproducibility of amplification products.

2) ISSR analysis

A set of one hundred inter simple sequence repeat primers (UBC 801-900) procured from Department of Biotechnology, University of British Columbia, Vancouver, Canada were used for the analysis using four parents and both the populations. The primers, which gave clear and repetitive polymorphic patterns, were used for further analysis.
A 25µl reaction contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM dNTPs, 2.0 % formamide, 0.4 mM spermidine, 0.2 µM of primer, 1 unit of *Taq* DNA polymerase (Perkin Elmer, USA) and 20 ng of genomic DNA. The thermal cycling protocol was as described by Nagaoka and Ogihara (1997) and contained following steps:

Initial denaturation: 94^{o}C for 5 min
45 cycles : 94 ^{o}C for 30 s
50 ^{o}C for 45 s
72 ^{o}C for 2 min
1 cycle : 72 ^{o}C for 5 min

All ISSR-PCR amplifications were performed in PTC-200 thermocycler (MJ Research, USA). The amplified products were resolved in 2% agarose gels in 0.5X TAE buffer, and visualized and photographed as detailed earlier.

3) SSR analysis

One hundred seventy three microsatellite primer pairs were used for the analysis. Out of these, 128 primer pairs (designated as "*gwm*") were selected from

all chromosomal regions of wheat, from the previously published microsatellite map of wheat by Roder et al. (1998), while remaining 45 primer pairs (designated as "*psp*") were kindly provided by Dr.M.D.Gale, John Innes Research Center, UK.

The PCR amplifications of microsatellite primers selected from wheat microsatellite linkage map were performed in 25µl reaction volumes following the protocol by Roder et al. (1998) with minor modifications in annealing temperature and reaction conditions. The reaction mixture contained 100nM of each primer, 200µM of each dNTP, 0.4 mM spermidine, 2% formamide, 1.5mM $MgCl_2$, 0.8 units of *Taq* DNA polymerase (Perkin Elmer, USA) and 25 ng of template DNA. The PCR thermal cycling programme was as follows:

Initial denaturation : 95°C for 5 min
35 cycles : 94°C for 1 min
: 50°C/ 55°C/ 60°C or 63°C for 1 min
(Depending on GC content of individual microsatellite)
: 72°C for 2 min
1 cycle : 72°C for 5 min

The PCR amplifications using microsatellite primers obtained from Dr. Gale were done in 30µl reaction volume. Each reaction contained 100 ng of DNA, 10X reaction buffer, 2mM dNTPs, 2µM of primers and 1.5 units of *Taq* DNA polymerase (Perkin Elmer, USA) and was performed according to the protocol described by Stephenson et al. (1998) involving

Initial denaturation : 94°C for 5 min
30 cycles : 94°C for 1 min
-0.5°C/min (RAMP)
: 55°C/ 58°C /61°C /63°C or 65°C
+ 0.5°C/min (RAMP)
: 72°C for 1 min
1 cycle : 72°C for 5 min

All PCR amplifications were performed in PTC-200 thermocycler (MJ Research, USA). The amplified products were resolved on 2.5% metaphor agarose (FMC Bio Products,USA) gels in 1xTBE buffer, followed by ethidium bromide staining and photography as detailed earlier.

4) Locus-specific amplification using "puroindoline-a" and "puroindoline-b" - primers

These oligonucleotide primers were synthesized as reported by Gautier *et al.* (1994), as follows.

i) *pin-a* specific primers

5'- ATGAAGGCCCTCTTCCTCA-3': sense strand

5'- TCACCAGTAATAGCCAATAGTG-3': anti-sense strand

ii) *pin-b* specific primers

5' -ATGAAGACCTTATTCCTCCTA -3': sense strand

5'-TCACCAGTAATAGCCACTAGGGAA-3': anti-sense strand

Each reaction of 25µl contained 50 ng of DNA, 20 picomoles of primer, 0.4 mM spermidine, 0.1 mM dNTPs, 10X buffer and 0.7 units of *Taq* DNA polymerase (Perkin Elmer, USA). The PCR protocol was also according to Gautier *et al.* (1994) with some modifications in annealing temperatures.

Initial denaturation : 93^{o}C for 4 min

35 cycles : 94^{o}C for 1 min

: 62^{o}C for 1 min

: 72^{o}C for 2 min

1 cycle : 72^{o}C for10 min

The amplified products were resolved on 10% polyacrylamide gel in 1X TBE buffer, followed by ethidium bromide staining and photography as detailed earlier.

5) 1Dx5-1Dy10 STS marker (HMW glutenin subunit specific primer)

The oligonucleotide primers were synthesized on the basis of the HMW-glutenin gene (1DX5) from *Triticum aestivum* cv Cheyenne as reported by Anderson *et al*. (1989) and D'Ovidio and Anderson (1994). They had the following sequences:

(F): GCCTAGCAACCTTCACAATC

(R): GAAACCTCCTGCCGACAAG

This primer pair was used only with CPAN3004X HD2329 population. The 25µl PCR mix contained 1X reaction buffer, 300µM of each dNTP, 250nM of each primer, 2.5 units of *Taq* DNA polymerase (Perkin Elmer, USA) and 50 ng of DNA. Thermal cycling conditions were according to D'Ovidio and Anderson (1994) as detailed below.

Initial denaturation: 94^{o}C for 2 min

35 cycles : 94 ^{o}C for 1min

60 ^{o}C for 2min

72 ^{o}C for 2.3 min

1 cycle : 72 ^{o}C for 5 min

The amplified products were resolved on 1.5% agarose gels in 1xTAE buffer, followed by ethidium bromide staining and photography as detailed earlier.

6) LMW and gliadin SSR primer analysis

Two wheat SSRs located within the LMW glutenin subunits in *Glu-A3* (*Xpsp1*) and the γ-gliadins in *Gli-B1* (*Xpsp2*) and one SSR located on chromosome arm 1AL (*Xcnl5 1A*) were analyzed. The primers and internal repeat sequences are indicated in Table 2.3 and reported by Manifesto et al. (1998). These primer pairs were also used with CPAN3004X HD2329 population. The PCR reaction mix and the thermal cycling conditions were also as described by Manifesto et al. (1998) with slight modifications in annealing temperature. Fifteen microlitre of rection mix contained 25 ng of DNA, 100nM of each primer, 200µM of each dNTP, 1X PCR buffer and 0.5 units of *Taq* DNA polymerase (Perkin Elmer, USA). Thermal cycling conditions were as follows:

Initial denaturation : 94^{o}C for 2 min

35 cycles : 94^{o}C for 1min

55^{o}C (*Glu-A3* and *Gli-B1*) for 1min

60^{o}C (*Xcnl5*) for 1min

72^{o}C for 2 min

1 cycle :72^{o}C for 5 min

The amplified products were resolved on 2.5% metaphor agarose (FMC Bio Products, USA) gels in 1xTBE buffer, followed by ethidium bromide staining and photography as detailed earlier.

Table2.3 SSR primer sequences and repeats

SSR loci	Repeat	Primers
Xpsp2 (Gli1)	$(CAA)_n$	F: GCA GAC CTG TGT CAT TGG TC
		R: GAT ATA GTG GCA GCA GGA TAC G
Xpsp1 (*Glu3*)	$(CAG)_n$ $(CAA)_n$	F: TCC CGC CAT GAG TCA ATC
		R: TTG GGA GAC ACA TTG GCC
Xcnl5	$(CT)_n$	F: GGT GAT GAG TGG CAC AGG
		R: CCC AAC AGT TGC AGA AAA TTA G

2.6 Analysis of HMW and LMW glutenins in the RILs of cross NP4 X HB208

2.6.1 Extraction of HMW and LMW glutenins

HMW and LMW glutenins were extracted according to Singh *et al*. (1991). A single grain of wheat was crushed gently in one eppendorf (~ 20mg flour). After crushing, 50% propanol was added. The mixture was incubated at 65^{o}C for 30 min with vortexing twice intermittently and then spinned at 10,000rpm at RT for 1 min. The supernatant was discarded and the same step was repeated twice. After the final spin, supernatant, which contained gliadins, was discarded and the precipitate was subjected to drying. Glutenin extraction buffer (50% propan-1-ol, 0.08M Tris-HCl pH8.0) was then added to the residue containing 1% freshly

prepared dithiothreitol. The mixture was vortexed thoroughly and kept at 65^{0}C for 30 min and then spinned at 10,000 rpm for 5 min. Glutenin extraction buffer (pH - 8.0) containing 1.4 % freshly mixed 4-vinylpyridine was added to each tube and incubated for 15 min for protein alkylation. The sample was then centrifuged at 10,000 rpm for 2 min. An aliquot of the supernatant was transferred to a new tube containing the dilution sample buffer, (2% SDS, 40% glycerol, 0.02% Bromophenol blue and 0.08M Tris-HCl pH8.0). After brief vortexing, the tubes were kept at 65^{0}C for 15 min and then centrifuged at 10,000rpm for 2 min, followed by storing at -20^{0}C.

2.6.2 Poly -Acrylamide Gel Electrophoresis (PAGE)

A 1.5 mm thick slab gel was casted by preparing 10% separating gel acrylamide (0.8% cross linker) and 3% stacking gel acrylamide as follows:

Separating gel- total volume : 60 ml
2X separating gel buffer pH 8.9 : 30ml
30% acrylamide : 20ml
Distilled water : 10ml
TEMED : 200µl
10% APS : 120µl

Stacking gel- total volume : 9ml
2X Stacking gel buffer pH 6.8 : 4.5ml
Stock acrylamide (30%) : 1.0ml
Distilled water : 3.5ml
TEMED : 20µl
10%APS : 60µl

2.6.3 Preparation of discontinuous polyacrylamide gel

The glass plates were cleaned thoroughly and were sandwiched together to form a gel of 1 mm thickness. The separating gel solution was prepared by combining all reagents except APS and TEMED. The solution was degassed for 15 min under vacuum. APS and TEMED were added to the degassed separating

gel solution and poured immediately into the gel assembly upto the mark. The gel solution was overlaid with distilled water to get a clear line gel formation. The gel was allowed to polymerise for about 45 min and then the overlayed distilled water was completely rinsed off. The stacking gel solution was prepared by combining all reagents except APS and TEMED. The solution was degassed for 15 min under vacuum. A suitable comb was then placed in the gel sandwich and tilted slightly to prevent air from being trapped. APS and TEMED were added to the stacking gel solution and poured into the gel sandwich. The comb was properly aligned and the gel was allowed to polymerise for about 30 min. The comb was removed gently and the wells were rinsed completely with distilled water and finally with running buffer.

The sample was electrophoresed at 80 mA for about 5 h (at least 45 min after the blue dye ran out). After electrophoresis, the gel was removed from the glass plate and placed in freshly prepared Coomassie Brilliant Blue staining solution for overnight with gentle agitation. The staining solution was drained out and the gel was rinsed with water three or four times for destaining. After destaining, the gel was photographed using PENTAX K1000 and gel documented using the gel-documentation system by Pharmacia Biotech imagemaster VDS.

2.7 Analysis of the amplification data

The genotype of each sample in case of RAPD and ISSR analysis was scored as presence or absence of amplified DNA locus. In case of SSR primers and other locus specific markers scoring was done based on the size variation of the alleles in parents. Segregation ratio of each marker in the RIL population was tested for goodness of fit to 1:1 using Chi-square test. Molecular marker linkages were determined with MAPMAKER v3.0 (Lander *et al.* 1987) at LOD 3.0. Few heterozygotes identified in case of microsatellite markers were coded as missing data in the MAPMAKER analysis. Single point analysis to determine molecular marker-putative QTL associations was done using QGENE (Nelson 1997) based on simple linear regression (R^2). Markers with significant P values (<0.05) were interpreted as putative QTLs (pQTL). Significant marker loci were then combined in a multiple linear regression model to determine the cumulative effect.

2.8 Chromosomal assignment of the RAPD and ISSR markers linked to the traits

Assignment of significant markers to specific chromosomes was done through PCR amplification using template DNA from each of the 21 NT (nulli-tetrasomic) lines derived from Chinese Spring. Identification of the arm location was done in the same way using chromosome specific DT (ditelosomic) lines of Chinese Spring (Sears 1966, Sears and Sears 1978).

CHAPTER 3: Results

3.1 Genetic analysis of kernel hardness in bread wheat using PCR based markers

3.1.1 Distribution of kernel hardness in RIL population

Genetic analysis of kernel hardness was carried out using hard and soft wheat varieties NP4 and HB208, respectively and their RIL population (100 in number). The phenotypic values of all the five traits under study were collected from the average of all the RI lines as well as parents grown in two replications as detailed in Materials and Methods.

Table 3.1 Phenotypic trait values in parents and their range in RILs

Traits	NP4	HB208	Range in RILs
NIR	5.8	1.1	7.6- 0.2
PC (%)	13.84	12.04	17.8- 10.0
MC (%)	8.24	8.48	6.3-8.8
HW (gm)	78.1	77.8	81.1- 61.8
TKW (gm)	42	34	45.6- 15.8

Frequency distribution of NIR value, PC, MC, HW and TKW in the form of histograms is depicted in Fig.3.1. As summarized in Table 3.1, the two parents differed significantly in their NIR values (5.8 for NP4 and 1.1 for HB 208) while the progeny showed hardness scores ranging from 7.6 to 0.2. The difference in PC between the parents, NP4 and HB208 was not very high however, in the progeny it ranged from 17.8% to 10.0%. Also for MC, eventhough the parents differed little in their values, 8.24% for NP4 and 8.48% for HB208, the range of MC values in the progeny was 6.3% -8.8%. In case of HW, the parental values were 78.1g for NP4 and 77.8g for HB208 while progeny range was 81.1g to 61.8g. TKW values were 42 g for NP4 and 34 g for HB208 and the values in the progeny ranged from 45.6g to 15.8g. All the traits showed continuous variation with transgressive segregants. In case of NIR value and PC, the progeny showed transgressive segregants in both the directions, for HW and TKW, it was more towards softness, whereas for MC, it was towards hardness. The recombinant inbred population

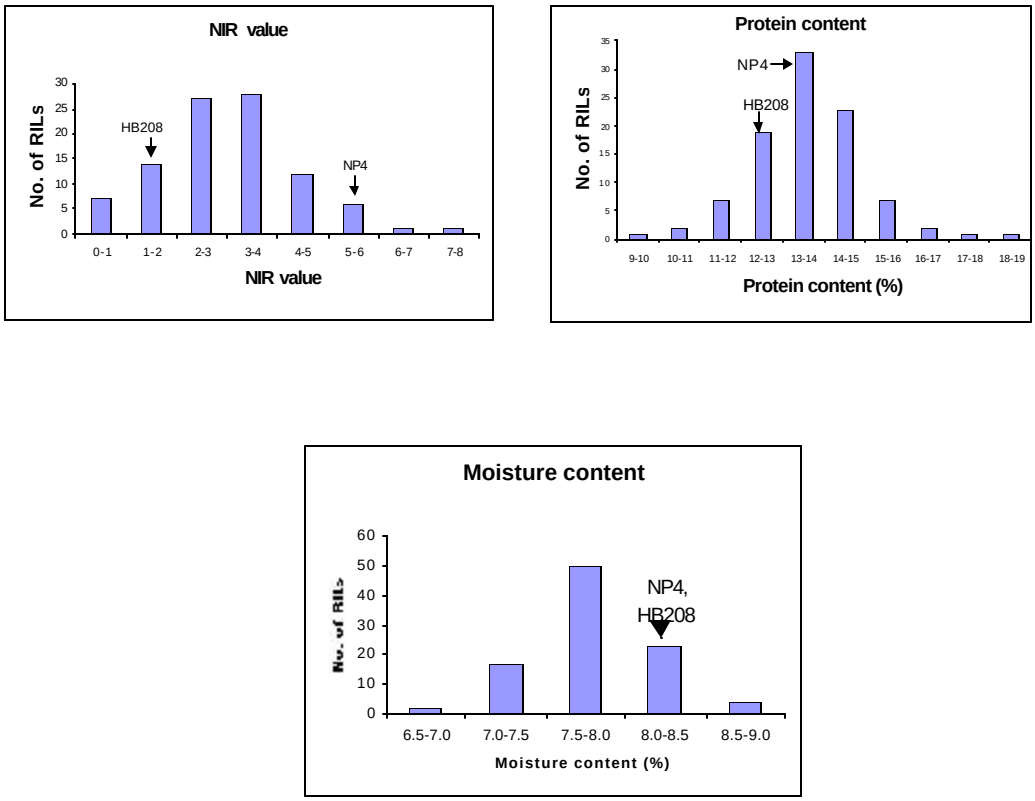

Fig 3.1: Frequency distribution of NIR value, PC, MC, HW and TKW in the RIL population of a cross NP4 x HB208

generated from the cross NP4 x HB 208, thus segregated for all the five traits with transgressive segregants.

3.1.2 Identification of putative markers associated with KH and related traits

I Using RAPD, ISSR and STMS primers

All the ISSR, RAPD and STMS primers were initially used for parental survey followed by selective genotyping (Lander and Botstein 1989) and putative markers were then attempted with the entire RIL population.

Out of 580 RAPD primers used to detect polymorphism, 300 primers could amplify the parental DNAs; among these 42 primers equivalent to 14% of the RAPD primers could reveal polymorphism between the two parental genotypes. These polymorphic primers were used for selective genotyping using extreme RILs, such as ten soft grained and ten hard-grained RILs. Seven primers showed phenotype specific segregation for 8 loci indicating their putative association with kernel hardness. These markers were further used to screen the entire population (100 in number) of RILs (Table 3.2). Figure 3.2 represents a segregation pattern of a RAPD marker $OPA04_{750}$ in both the parents (NP4 and HB208) as well as in the representative RILs.

In ISSR analysis, out of 100 primers attempted with the parents, 32 primers showed amplification of which 20 primers were found to be polymorphic, (62.5% polymorphism). Twelve primers out of 20 showed phenotype specific segregation for 21 different loci and were then used to genotype the total RIL population (Table 3.3). As shown in Figure 3.3, two bands of 1200bp and 1000bp are found to be segregating in the parents and RILs using the ISSR primer UBC880.

Twelve ISSR and seven RAPD primers revealing 29 loci segregating in phenotype specific manner were further tested for goodness of fit to 1:1 ratio using chi square analysis. Twenty-five markers segregated in accordance with expected ratio of 1:1 with probabilities ranging from 0.001- 0.70. Four markers, $UBC818_{1078,}$ $UBC827_{1350,}$ $UBC836_{872}$ and $OPC11_{650}$ deviated from the expected ratio showing skewed segregation towards one of the parents (Tables 3.2 & 3.3).

When 173 STMS primer pairs covering all the chromosomes were used for parental screening, 120 primers were successful in amplifying loci from parental

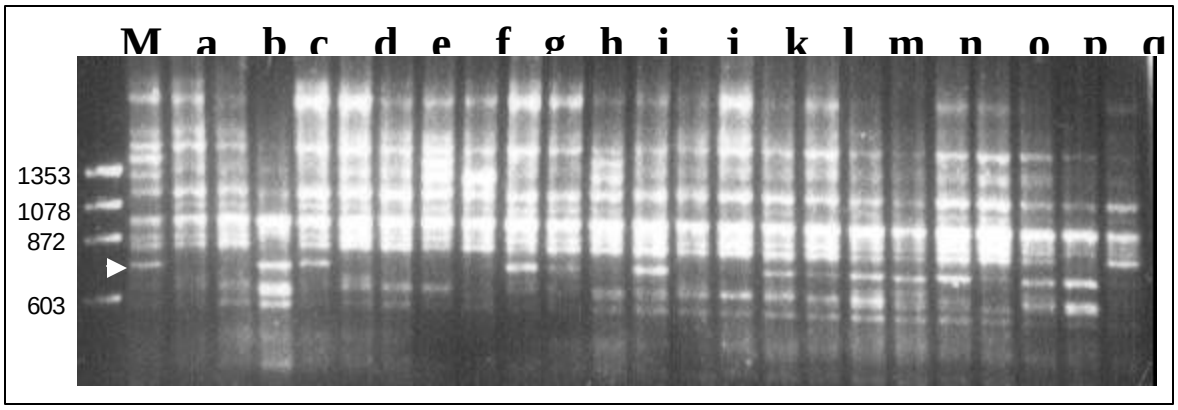

Fig 3.2 Amplification profile of parents and RILs of KH using OPA04

M: ØX174/*HaeIII* digest, Lane a: HB208, Lane b: NP4, Lanes c-x: representative RILs of KH segregating for the marker $OPA04_{750}$ indicated with an arrow

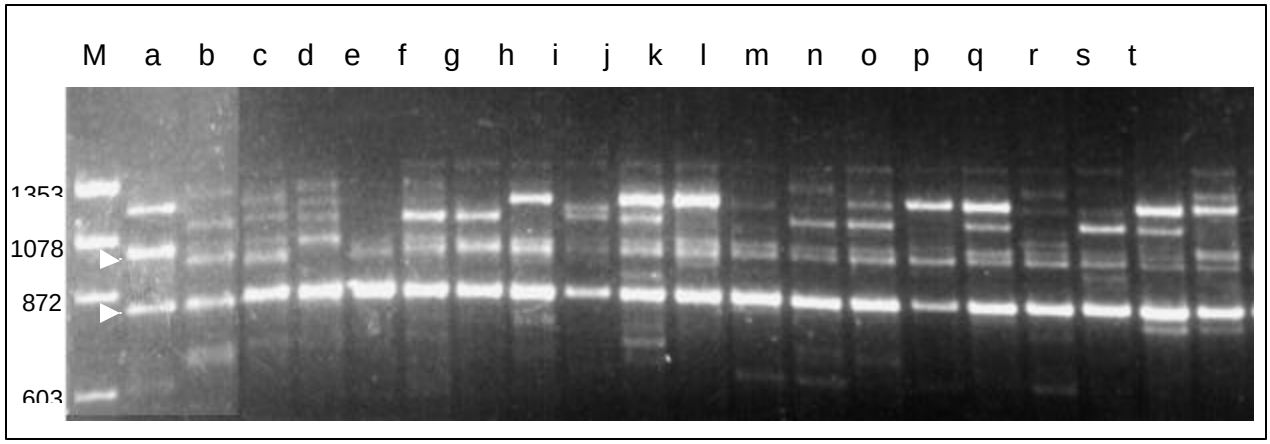

Figure 3.3 Amplification profile of parents and RILs of KH using UBC880

M: ØX174/*HaeIII* digest, Lane a: HB208, Lane b: NP4, Lane c-t: representative RILs of KH segregating for the marker $UBC880_{1200}$ and $UBC880_{1000}$ indicated with the arrows

DNAs and out of these, 45 primer pairs gave polymorphism (37.5% polymorphism). Out of 45 polymorphic primers, only 14 were further used for population screening after selective genotyping. Eleven markers were found to be segregating in 1:1 ratio when analyzed with chi square test of goodness of fit (Table 3.4) while three markers namely, *Xgwm*174, *Xgwm*630 and *Xpsp*3119 showed skewed segregation. Figure 3.4 shows presence of a band of 190bp from primer *Xpsp*3000 present in HB208 (lane a), its allele of 270bp in NP4 (lane b) and out of 17 representative RILs, 8 showed presence of 190bp band while 9 showed presence of 270bp band, lane 'j' represents a heterozygote.

Table 3.2: Segregation data of polymorphic RAPD primers

Primer	Sequence	Fragment size (bp)	a:b segregation	Chi-square	Probability
OPA 04	AATCGGGCTG	750	63:34	8.5	0.001-0.01
OPB 15	GGAGGGTGTT	880	38:48	2.96	0.05-0.10
OPC 11	AAAGCTGCGG	1000	39:59	4.04	0.02-0.05
OPC 11	AAAGCTGCGG	650	74:24	25.04	–
OPC 16	CACACTCCAG	860	48:42	1.36	0.10-0.20
OPH 12	ACGCGCATGT	900	44:52	0.8	0.30-0.50
OPI 20	AAAGTGCGGG	690	37:56	4.1	0.02-0.05
OPJ 01	CCCGGCATAA	700	53:32	7.4	0.001-0.01

Table 3.3: Segregation data of polymorphic ISSR primers

Primer	Sequence	Fragment size (bp)	a:b segregation	Chi-square	Probability
UBC807	AGA GAG AGA GAG AGA GT	650	36:55	4.42	0.02-0.05
UBC811	AGA GAG AGA GAG AGA GGA	870	42:58	2.56	0.10-0.20
UBC812	GAG AGA GAG AGA GAG AA	1000	36:56	4.64	0.02-0.05
UBC812	GAG AGA GAG AGA GAG AA	800	35:57	5.48	0.02
UBC815	CTC TCT CTC TCT CTC TG	1000	61:34	7.54	0.001-0.01
UBC815	CTC TCT CTC TCT CTC TG	900	56:39	3.14	0.05-0.10
UBC815	CTC TCT CTC TCT CTC TG	800	42:53	1.46	0.20-0.30
UBC815	CTC TCT CTC TCT CTC TG	700	41:54	1.94	0.10-0.20
UBC818	CAC ACA CAC ACA CAC AG	1078	64:29	12.74	--
UBC827	ACA CAC ACA CAC ACA CG	1350	17:75	34.28	--

UBC827	ACA CAC ACA CAC ACA CG	1300	60:32	8.48	0.001-0.01
UBC835	AGA GAG AGA GAG AGA GY*T	700	35:48	4.58	0.02-0.05
UBC836	AGA GAG AGA GAG AGA GYA	872	67:30	13.78	--
UBC848	CAC ACA CAC ACA CAC AR*G	890	48:52	0.16	0.50-0.70
UBC848	CAC ACA CAC ACA CAC ARG	400	44:56	1.44	0.20-0.30
UBC856	ACA CAC ACA CAC ACA CYA	700	39:60	4.42	0.02-0.05
UBC856	ACA CAC ACA CAC ACA CYA	610	42:57	2.26	0.10-0.20
UBC856	ACA CAC ACA CAC ACA CYA	590	53:46	0.5	0.30-0.50
UBC873	GAC AGA CAG ACA GAC A	880	54:44	1.04	0.30
UBC880	GGA GAG GAG AGG AGA	1200	45:53	0.68	0.30-0.50
UBC880	GGA GAG GAG AGG AGA	1000	42:56	2.0	0.10-0.20

Y = C, T R = A, G

Table 3.4: Segregation data of polymorphic STMS primers

Primer	Repeat Sequence	Fragment size (bp)	a:b segregation	Chi-square	Probability
Xgwm261	(CT) 21		40:50	2.0	0.10-0.20
Xgwm497	(GT) 29 imp		45:52	0.58	0.30-0.50
Xgwm368	(AT) 25		32:52	7.2	0.001-0.01
Xgwm174	(CT) 22		29:69	16.04	-
Xgwm499	(GA) 32		49:47	0.2	0.5-0.7
Xgwm156	(GT) 14		52:42	1.36	0.2-0.3
Xgwm264	(CA) 9A (CA) 24		48:38	2.96	0.05-0.10
Xgwm630	(GT) 16		32:64	10.4	-
Xpsp3000	(CAA) 15		36:55	4.42	0.02-0.05
Xpsp3050	(CT)16(TA)14		53:41	1.8	0.1-0.2
Xpsp3152	(GT)10(GA)21		31:59	8.84	0.001-0.01
Xpsp3119	(TG)18		30:36	11.92	-
Xpsp3027	(GA)17		45:46	0.82	0.001-0.01
Xpsp3131	(GAA)13imp		54:46	1.04	0.3

imp: imperfect repeat

II Using glutenin markers and locus specific primers

Initial screening of both the parents for the HMW and LMW glutenin subunits showed good and distinct polymorphism for HMWG- 2* and LMWG -B & D subunits. Hence the glutenin analysis of all 100 RILs was further carried out to

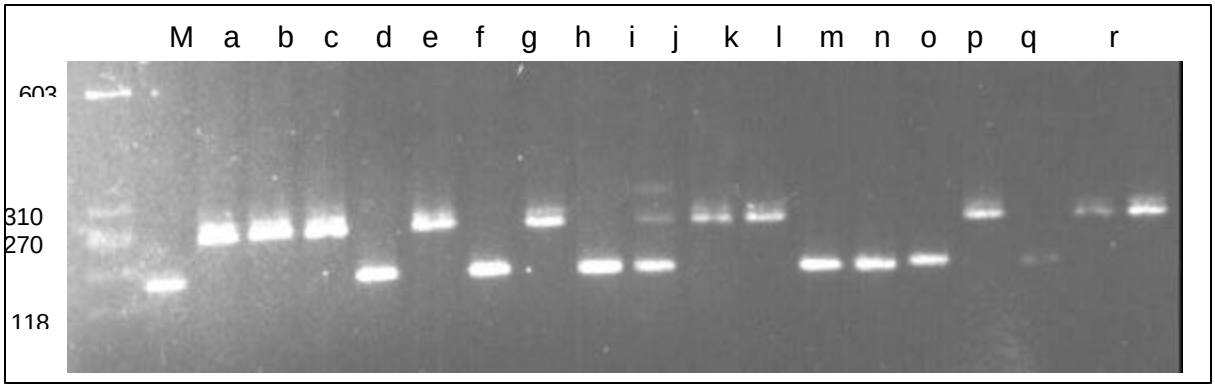

Fig 3.4 Amplification profile of parents and RILs of KH using *Xpsp*3000
M: ØX174/*HaeIII* digest, Lane a: HB208, Lane b: NP4,
Lane c-s: representative RILs of KH segregating for marker *Xpsp*3000 with 190bp allele as seen in HB208 and 280bp allele present in NP4

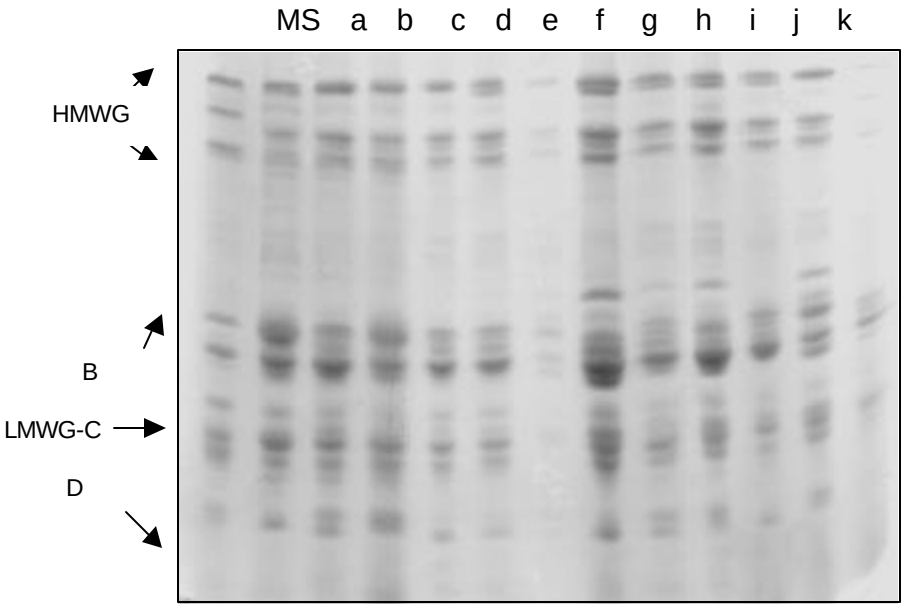

Fig 3.5 Analysis of HMW and LMW glutenin subunits in the RIL population of NP4 X HB208 showing the polymorphism for HMWG and LMWG-B&D subunits.
CS: Chinese Spring, MS: MACS 2496, j: NP4, k: HB208, a-i : representative RILs

detect polymorphism with these three putative markers after selective genotyping. Fig. 3.5 explains the polymorphic pattern of HMWG (2*) and LMWG (B & D) among the parents, NP4 and HB208 as well as few representative RILs.

Using the protocol as given in section 2.5, both the parents were screened with the '*pin-a*' and '*pin-b*' primers. However, no polymorphism was observed inspite of many variations in the reaction conditions and thermal cycling protocol.

3.1.3 MAPMAKER analysis

The genetic linkage map was generated with all the segregating markers using MAPMAKER version 3.0 at LOD 3.0. Twenty-nine markers were assigned to eight linkage groups while twenty-four markers remained unlinked. All the linked markers covered 426.2cM distance, which is quite small as compared to the total map distance of wheat genome. Linkage group 3 consisted of six markers (UBC815$_{900}$, UBC815$_{1000}$, UBC827$_{1300}$, UBC02$_{860}$, UBC818$_{1078}$ and OPJ01$_{700}$) and it covered maximum map distance of 119.4cM while linkage group 1 comprised 5 markers (UBC856$_{610}$, *Xpsp*3000, *Xgwm*630, UBC812$_{800}$ and *Xpsp* 3131) covering 101.3cM map distance. Linkage groups 5 and 6 consisted of four markers each (Linkage Group 5: *Xpsp*3119, OPH12$_{900}$, UBC856$_{700}$ and UBC880$_{1200}$; Linkage Group 6: *Xgwm*499, OPA04$_{750}$, UBC848$_{400}$ and UBC880$_{1000}$) covering a maximum map distance of 63.4cM and 51.1cM, respectively. Three markers constituted linkage group 4 namely, *Xpsp*3027, UBC88$_{700}$ and UBC815$_{700}$ and linkage group 2 (*Xgwm*397, OPI20$_{690}$ and UBC827$_{1000}$) whereas linkage group 7 and 8 consisted of two markers each (Linkage group 7: OPB15$_{284}$ and UBC812$_{1000}$; Linkage group 8: OPC11$_{650}$ and UBC856$_{590}$).

3.1.4 Association of all markers with the traits

Contribution of all the linked and unlinked markers to the individual phenotype was estimated by single and multiple marker analysis using a software QGENE (Table 3.5) and its details for all the five traits are given below:

i) Kernel hardness by NIR

Three ISSR markers ($UBC807_{650}$, $UBC873_{880}$ and $UBC880_{1000}$), four RAPD markers ($OPB15_{880}$, $OPJ01_{700}$, $OPC16_{860}$ and $OPA04_{750}$) and three STMS

Table 3.5: Percentage of phenotypic variation in the marker-trait associations

Trait	Markers	R^2 with single marker effect X100	Probability	R^2 with multiple marker model x100
NIR value	OPB15$_{880}$	8.61	0.007	18.9
	Xgwm 264	7.97	0.009	
	OPJ01$_{700}$	5.97	0.026	
	*Xpsp*3000	5.83	0.019	
	OPC16$_{860}$	5.36	0.031	
	UBC807$_{650}$	4.79	0.040	
	UBC873$_{880}$	4.29	0.045	
	Xgwm 397	3.93	0.062	
	UBC880$_{1000}$	3.62	0.066	
	OPA04$_{750}$	3.26	0.083	
Protein content	OPB15$_{880}$	10.46	0.003	18.7
	UBC815$_{1000}$	8.87	0.003	
	*Xpsp*3000	8.71	0.003	
	OPI 20$_{690}$	8.48	0.005	
	UBC856$_{610}$	6.4	0.013	
	UBC827$_{1300}$	5.85	0.022	
	UBC807$_{650}$	5.69	0.025	
	Xgwm 264	5.21	0.037	
	OPJ01$_{700}$	5.07	0.042	
	Xgwm 397	4.61	0.043	
	UBC818$_{1078}$	4.46	0.047	
	UBC812$_{800}$	4.39	0.050	
	UBC812$_{1000}$	4.28	0.053	
	UBC880$_{1200}$	3.09	0.090	
Moisture content	UBC815$_{1000}$	7.99	0.006	16.3
	UBC856$_{610}$	5.57	0.021	
	OPI20$_{690}$	4.52	0.045	
	OPC16$_{860}$	4.38	0.053	
	UBC812$_{800}$	4.21	0.055	
	OPC11$_{650}$	4.06	0.051	
	Xpsp3152	3.97	0.056	
	UBC827$_{1300}$	3.35	0.086	
	UBC856$_{700}$	3.32	0.077	
Hectolitre weight	*Xgwm* 497	8.51	0.009	21.5
	UBC880$_{1000}$	7.1	0.016	
	UBC873$_{880}$	6.41	0.023	
	*Xpsp*3119	5.67	0.032	
	Xgwm 174	4.52	0.056	
	UBC880$_{1200}$	3.9	0.077	
Thousand kernel weight	UBC873$_{880}$	6.41	0.016	12.1
	UBC880$_{1200}$	5.83	0.023	
	UBC856$_{590}$	4.97	0.035	
	Xgwm 174	4.02	0.061	

markers, (*Xgwm*264, *Xgwm*397 and *Xpsp*3000) were found to be associated with NIR values with multiple linear regression of 18.9%. The R^2 values of these markers by single marker analysis ranged from 8.61% to 3.26%.

i) Protein content

A total of 14 markers (8 ISSR, 3 RAPD and 3 STMS) showed association with PC with a total phenotypic contribution of 18.7%. R^2 values by single marker analysis ranged between 10.46% to 3.09%. Markers like $OPB15_{880}$, $UBC815_{1000}$, *Xpsp*3000 and $OPI20_{690}$ showed significant association with phenotypic contribution of 10.46% (P=0.003), 8.87% (P=0.003), 8.71% (P=0.003) and 8.48% (P=0.005), respectively.

iii) Moisture content

Five ISSR ($UBC815_{1000}$, $UBC856_{610}$, $UBC812_{800}$, $UBC827_{1300}$ and $UBC856_{700}$), three RAPD ($OPI20_{690}$, $OPC16_{860}$ and $OPC11_{650}$) and one STMS, *Xpsp*3152 were found to be associated with MC contributing 16.3% to the total phenotypic variation.

iv) Hectolitre weight

Three ISSR markers, $UBC880_{1000}$, $UBC880_{1200}$ and $UBC873_{880}$ (with phenotypic contribution of 7.11%, 6.41% and 3.95%, respectively) and three STMS markers (*Xgwm*497, *Xgwm*174 and *Xpsp*3119 with phenotypic contribution of 8.51%, 4.52% and 5.67%, respectively) were found to be associated with HW with 21.5% of total phenotypic variation. Thus HW showed the highest R^2 value explained by multiple marker model.

v) 1000-kernel weight

$UBC873_{880}$, $UBC880_{1200}$ and $UBC856_{590}$ (with phenotypic contribution of 6.41%, 5.82% and 4.97%, respectively) and only one STMS marker, *Xgwm*174 contributing 4.02% to the phenotypic variation in TKW were found to be associated with 12.1% contribution to total phenotypic variation.

3.1.4 Molecular markers shared by different traits

In the QGENE analysis of all the five traits, many markers were found to be shared among the traits (Table 3.6).

- Markers associated with KH like OPB15$_{880}$, OPJ01$_{700}$, UBC807$_{650}$, *Xgwm*264, *Xgwm*397 and *Xpsp*3000 were associated with PC, UBC873$_{880}$ and UBC880$_{1000}$ with HW whereas UBC 873$_{880}$ with TKW. Only two markers namely OPA04$_{750}$ and OPC16$_{860}$ were unique to KH.
- UBC880$_{1200}$ with low contribution to PC was shared by both HW and TKW while; OPI20$_{690}$, UBC815$_{1000,}$ UBC856$_{610}$, UBC827$_{1300}$, UBC818$_{1078}$, UBC812$_{800}$ and UBC812$_{1000}$ were unique to PC.
- Four markers namely UBC815$_{1000}$, UBC827$_{1300}$, UBC812$_{800}$ and OPI20$_{690}$ were shared between PC and MC whereas one marker OPC16$_{860}$ was shared between KH and MC. None of the markers were found to be shared between MC and HW /TKW.
- Markers like UBC873$_{880}$, UBC880$_{1200}$ and *Xgwm*174 contributed to both, HW and TKW. UBC856$_{590}$ was observed to be a unique marker associated with TKW only and not to any other trait in this analysis.

Apart from individual molecular markers shared by more than one phenotypic trait, the linkage groups as detailed earlier were also shared by various phenotypic traits. Linkage group 3 was shared by three traits namely KH, PC and MC, whereas linkage group 5 was shared by four traits namely PC, MC, HW and TKW. Linkage group 2 consisting of three markers was shared by KH, PC and MC while linkage group 1 was also shared by KH, PC and MC. Linkage groups 6, 7 and 8 were shared by two traits each, Linkage group6 by KH and HW, Linkage group 7 by KH and PC and Linkage group 8 by MC and TKW. Linkage group 4 was not shared by any of these traits.

3.1.5 Chromosomal localization of RAPD and ISSR markers

Chromosome assignment for all RAPD and ISSR markers associated with these traits was done using nulli-tetrasomic (NT) and ditelosomic (DT) lines. For example, as shown in Figure 3.6a, a marker band (UBC812$_{800}$) is observed to be absent in the NT lines for chromosome 2B, but is present in the NT lines for remaining chromosomes. Thus the marker was assigned to chromosome 2B. Further, the location of this marker was confirmed to chromosome arm using

Table 3.6 Molecular markers shared by different traits

Traits	NIR	PC	MC	HW	TKW
NIR	*OPA04$_{750}$	OPB15$_{880}$ OPJ01$_{700}$ Xgwm264 Xpsp3000 Xgwm397 UBC807$_{650}$	OPC16$_{860}$	UBC873$_{880}$ UBC880$_{1000}$	UBC873$_{880}$
PC	Same as NIR and PC	* UBC812$_{1000}$	UBC815$_{1000}$ OPI 20$_{690}$ UBC856$_{610}$ UBC827$_{1300}$ UBC812$_{800}$	UBC880$_{1200}$	UBC880$_{1200}$
MC	Same as NIR and MC	Same as PC and MC	*OPC11$_{650}$ *Xpsp3152 *UBC856$_{700}$	NIL	NIL
HW	UBC880$_{1000}$ UBC873$_{880}$	Same as PC and HW	NIL	*Xgwm497 *Xpsp3119	UBC880$_{1200}$ UBC873$_{880}$ Xgwm174
TKW	Same as NIR and TKW	Same as PC and TKW	NIL	Same as HW and TKW	*UBC856$_{590}$

* : Markers unique to each trait

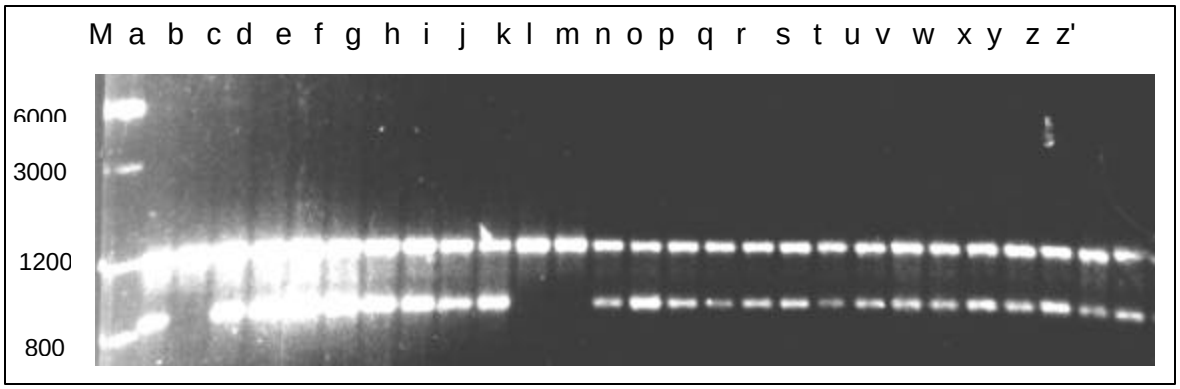

Fig 3.6a: Chromosomal localization of marker UBC812$_{800}$ showing absence of the 800bp band in the lanes k and l, which are nullisomic for chromosome 2B. M: 1KB ladder, a: HB208 b: NP4 lanes c-z': nullisomic lines in the background of Chinese Spring

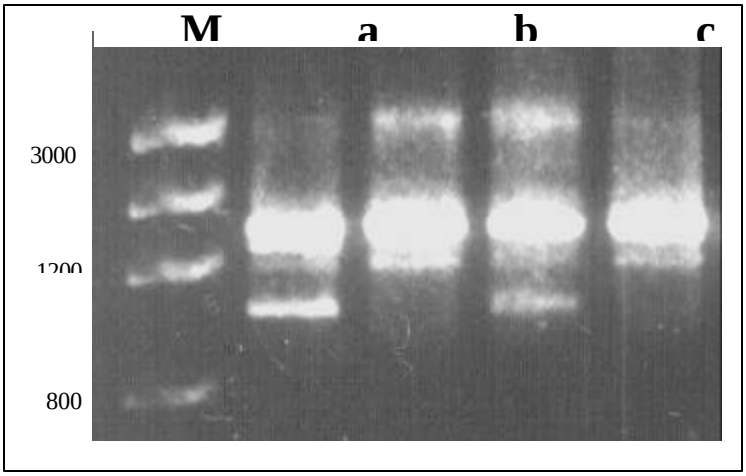

Fig 3.6b: Chromosome arm localization showing presence of marker UBC812$_{800}$ on long arm of chromosome 2B
M: 1Kb ladder, a: HB208, b: NP4, c: ditelosomic for long arm of 2B, d: ditelosomic for short arm of 2B

ditelosomic lines from 2B, i.e. 2BL and 2BS (Fig. 3.6b). The marker band is absent in 2BS and present in 2BL; hence it is assigned to long arm of chromosome 2B. This analysis indicated that three ISSR markers $UBC807_{650}$ contributing to KH and PC and $UBC815_{1000}$ contributing to PC and MC, were present on the short arm of chromosome 6B whereas $UBC812_{800}$ contributing to PC alone was localized on the long arm of 2B. Other ISSR and RAPD markers could not be assigned to chromosomes, as they did not show any amplification in Chinese Spring in which background NT and DT lines were established.

3.1.6 Correlations among all the traits

It was observed that different traits influencing the kernel texture shared some of the molecular markers; I further studied the correlations among all these traits (Table 3.7). Interestingly some of the traits showed significant correlations. For example, NIR and PC were found to be significantly and positively correlated (r= +0.8) in this population. HW and TKW also showed significant (+0.6) positive correlation whereas NIR and HW and PC and HW showed negative correlations. Similarly PC and MC, NIR and TKW and PC and TKW were negatively correlated.

Table 3.7 Correlation data between the phenotypic traits

Traits	NIR	PC	MC	HW	TKW
NIR	0.00				
PC	0.835*	0.00			
MC	0.127***	-0.136***	0.00		
HW	-0.517*	-0.540*	-0.015	0.00	
TKW	-0.434**	-0.443**	0.143***	0.699*	0.00

*P= 0.00, **P< 0.01 ***P<0.05

- *Part of the content of section 3.1 involving analysis of KH using RAPD and ISSR markers has been accepted as a full-length paper in* ***Theoretical and Applied Genetics (2001) 103: 601-606***

- *Part of the content of section 3.1 involving analysis of KH using STMS markers has been communicated as a full length paper to* ***Euphytica (2001)***

3.2 Identification of DNA markers associated with bread making quality

3.2.1 Genetic analysis of BMQ related traits

The parents CPAN3004 and HD2329 differ in PC, SV, TKW, HW, PS and KH with values of 11.47%, 8.02 cc, 38 g, 77.9 kg/hl, 9; and 3.07 for CPAN3004 and 11.17%, 7.55 cc, 45g, 79.7 kg/hl, 7 and 3.85 for HD2329, respectively (Table 3.8).

Table 3.8: Phenotypic values of the parents and their range in RILs

Traits	CPAN3004	HD2329	Range in RILs
SV (cc)	8.02	7.55	4-13
PC (%)	11.47	11.17	10-17
KH	3.07	3.85	0.6-8.5
PS	9	7	4-10
HW (kg/hl)	77.9	79.7	50-85
TKW (g)	38	45	10-45

Figure 3.7 depicts phenotypic distribution of various traits in 120 RILs developed by crossing CPAN3004 and HD2329. Traits like PC, SV and PS shown continuous variation in the RIL population, indicating polygenic nature of these traits whereas TKW shows bimodal distribution indicating that there are two major genes involved in the phenotypic expression in this cross. HW and KH show discontinuous distribution indicating dominant nature of some of the alleles. Significant transgressive segregants towards both the parental values were observed for PC, SV, PS and KH, whereas, transgressive segregants towards lower parental values were observed for TKW and HW.

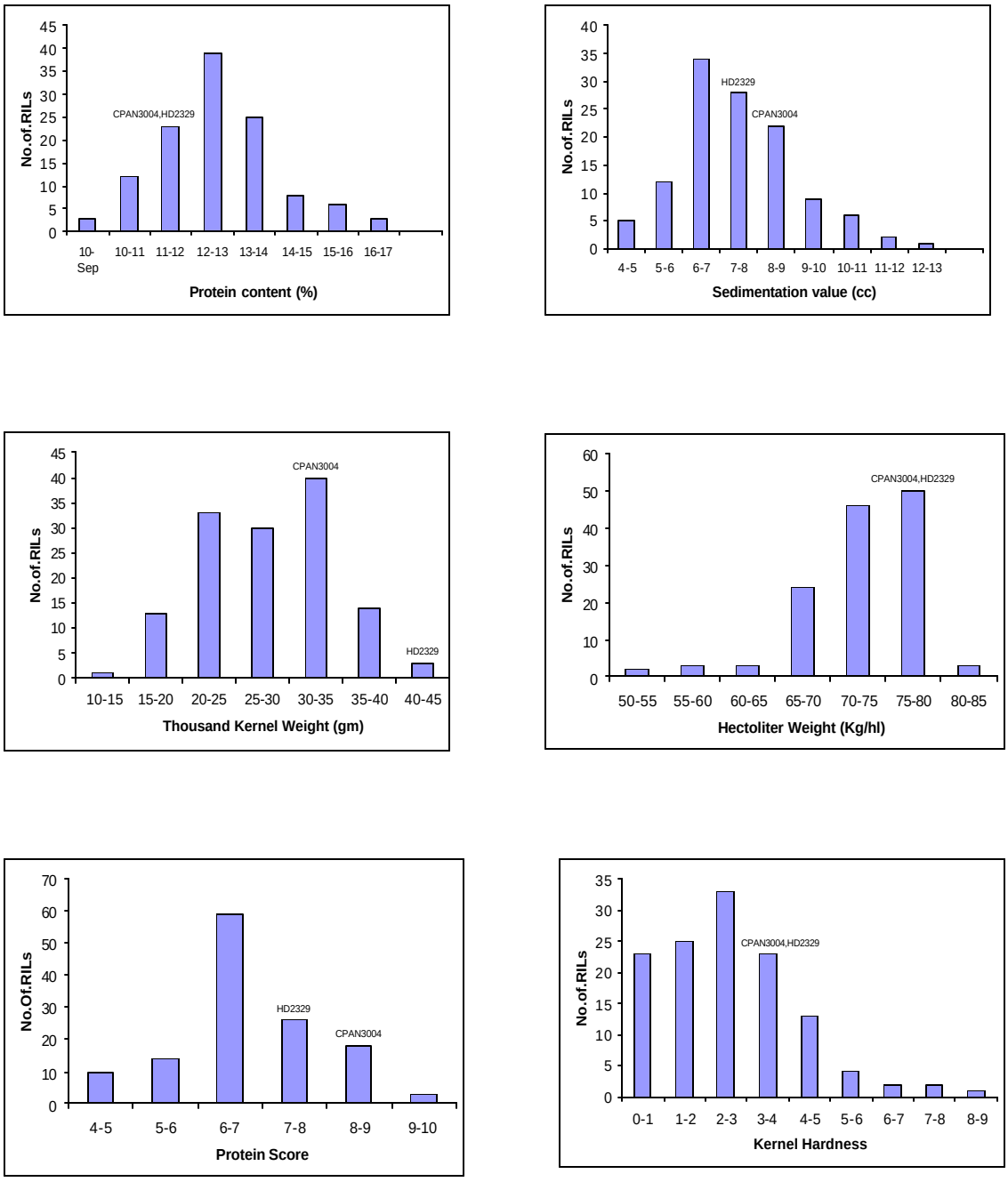

Fig 3.7: Frequency distribution of bread making quality related traits in the RIL population of CPAN3004 X HD2329

3.2.2 Identification of putative markers associated with BMQ related traits

A total of 173 microsatellite primer pairs covering all the wheat chromosomes were used for parental screening. Out of these, 40 primer pairs gave polymorphism among the parents. Figure 3.8 depicts the segregation pattern of marker *Xgwm* 205 showing presence of a band of 194bp in CPAN3004 and its allele of 225bp in HD2329. Nine RILs show presence of CPAN3004 allele, 194bp whereas 10 RILs show presence of HD2329 allele.

Out of 100 ISSR primers used, 32 primers were successful in amplifying parental loci and 14 primers gave polymorphism among the parents. Only 26 among the 580 RAPD primers attempted showed polymorphism in parental screening. Selective genotyping was then performed with all the polymorphic primers identified from parental survey using 20 RILs showing extreme phenotypes for all the traits together as far as possible and for individual traits which were not included in the combined trait RILs.

Using the protocols as given in section 2.5, both the parents were screened with the locus specific primers for 1DX5-1DY10 subunit pair (D'ovidio and Anderson 1994) and for LMW and gliadin genes (Manifesto *et al*. 1998). 1DX5-1DY10 primers showed distinct polymorphism between both the parents as well as in selective RILs and hence were further used to genotype entire population. The LMW glutenin and gliadin primers failed to give reproducible polymorphic patterns.

All markers that showed phenotype-specific segregation were further used for analyzing the whole population. All the markers showed goodness of fit to 1:1 ratio when analyzed using Chi-square test. Single point regression ($p<0.05$) using QGENE software served as the primary method of detecting associations between markers and QTLs. The DNA marker data obtained for each BMQ related trait is described below and summarized in Table 3.9.

i) Sedimentation value (SV):

The SDS sedimentation test was selected as an indirect method to evaluate BMQ because of its positive and significant correlation with loaf volume (Manifesto *et al* 1998). Three microsatellite markers namely *Xgwm*205, *Xpsp*3100

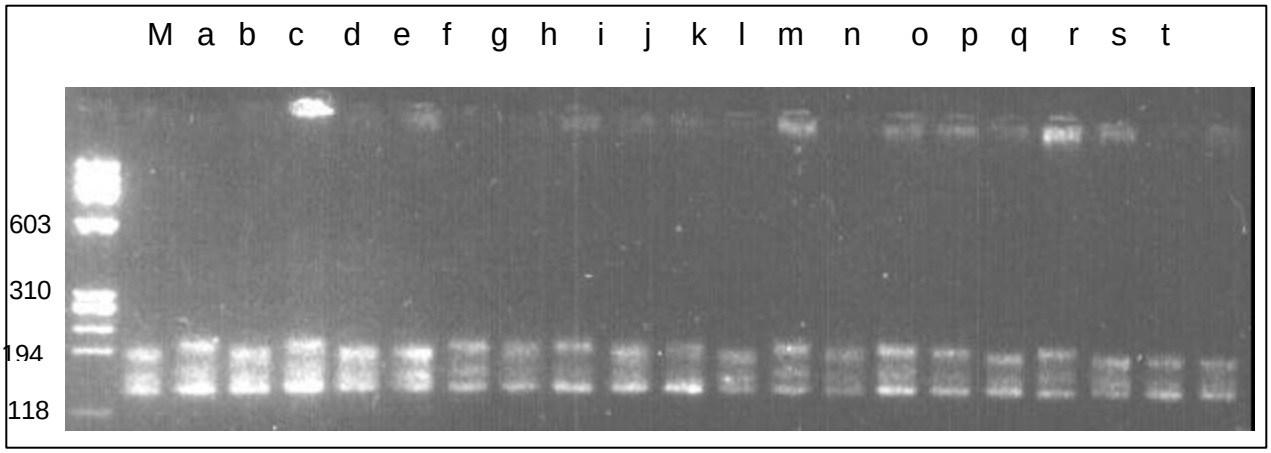

Fig 3.8 Amplification profile of parents and RILs of BMQ using *Xgwm*205
M: ØX174/*HaeIII* digest, a: CPAN3004, b: HD2329,
c-u: representative RILs of BMQ segregating for marker *Xgwm*205 with 194bp allele as seen in CPAN3004 and 225bp allele present in HD2329

and *Xpsp*3152 were found to be significantly associated with sedimentation value, with regression coefficient values (R^2 X100 values) of 10.13% (P=0), 7.36% (P<0.03), and 6.79% (P<0.01), respectively (Table3.9). These markers were previously mapped on chromosome 5A & 5B, 6A and 1B, respectively (Roder *et al* 1998; Stephenson *et al* 1998). 1Dx5-1Dy10 STS marker amplified a 450bp-fragment specific to that locus in CPAN3004, which was absent in HD2329. This marker was used to genotype the whole population and it contributed only 6.75% (P<0.01) to the total phenotypic variation. The best multiple regression model explained 18.75 % of the total phenotypic variance.

ii) Protein content

One microsatellite marker *Xpsp*3200 previously mapped on chromosome 6D (Stephenson *et al* 1998) was identified to be associated with PC significantly (P=0) with R^2 value of 13.75% while the marker *Xgwm*44 mapped on 7D was found to be associated with a contribution of 3.39% only.

iii) Kernel hardness

One RAPD $OPH12_{900}$ and one microsatellite marker *Xgwm*644 were found to be associated with KH with phenotypic contribution of 6.05% (P= 0.01) and 4.21% (p=0.04), respectively. In previous section, I have shown $OPH12_{900}$ to be polymorphic but with an insignificant contribution to phenotype, in a cross HB208 X NP4 studied for KH, whereas the same primer gave a good R^2 value in the present population. Interestingly, the marker *Xgwm*644 that was previously reported to be associated with PC by Khan and his co-workers (Khan *et al*. 2000) did not show any contribution to PC but was found to be associated with KH in our analysis.

iv) Protein score

Only one ISSR marker $UBC848_{610}$ was associated with the Glu-1 score with a very little phenotypic contribution of 3.46% (P<0.05).

Table 3.9: Association of DNA markers with BMQ related traits

Trait	Marker	R^2 X100 value	Probability	Chromosomal locations
Sedimentation value	Xgwm205	10.13	0.0	5A, 5B
	Xpsp3100	7.36	0.03	6A
	Xpsp3152	6.79	0.01	1B
	1Dx5-1Dy10 STS	6.75	0.01	1D
Protein Content	Xpsp3200	13.75	0.04	6D
	Xgwm 44	3.39	0.05	7D
Kernel Hardness	$OPH12_{900}$	6.05	0.01	-
	Xgwm 644	4.21	0.04	6B, 7B
Protein score	$UBC848_{610}$	3.46	0.05	-
1000-Kernel Weight	Xgwm205	4.62	0.04	5A, 5B

- : markers unable to localize

v) 1000-kernel weight

One microsatellite marker *Xgwm*205 was associated with TKW (P<0.04) with a contribution of 4.62%. This marker was also found to control SV with contribution of 10.13%. The ISSR markers contributing to TKW in the population (developed from crossing Rye Selection 111X Chinese Spring) studied by Ammiraju *et al*. (2000) failed to give any significant contribution in this study though most of them did show polymorphism in parental survey.

No DNA marker was found to be significantly associated with hectolitre weight (HW) in this population.

3.2.3 Genetic correlations among the BMQ related traits

BMQ is a complex trait governed by many other traits. In order to develop wheat cultivars with good BMQ, it is necessary to pyramid these traits with positive effect on BMQ. This prompted me to study correlation among various BMQ related traits.

As shown in Table 3.10, a positive correlation was observed between PC and KH, PC and SV, PC and PS, KH and SV and HW and TKW, while negative correlation was observed between PC and HW and TKW. Similarly negative correlation was observed between PC and HW, PC and TKW, KH and HW, KH and TKW, HW and SV and TKW and SV (Table 3.10). Even though the markers identified in this study were associated with different traits, none of them were shared among these interrelated traits, indicating that these markers/QTLs are specific to respective traits except for the marker *Xgwm*205, which was shared by SV and TKW wherein TKW and SV were negatively correlated.

Contents of section 3.2 have been communicated as a full length paper to *Biochemical Genetics* ***(2001)***

Table 3.10: Summary of genetic correlations among bread making quality related traits in the RIL population CPAN3004 X HD2329

Quality measurement	PC	KH	HW	TKW	SV	PS
Protein content (PC)	1.000					
Kernel hardness (KH)	0.875*	1.00				
Hectoliter weight (HW)	-0.64*	-0.614*	1.00			
1000-kernel weight (TKW)	-0.59*	-0.502	0.808*	1.00		
Sedimentation value (SV)	0.21**	0.192**	-0.27**	-0.29**	1.00	
Protein score (PS)	0.16***	0.00	0.00	0.00	0.00	1.00

* P=0.00, ** P < 0.01, *** P < O.O5

CHAPTER 4: Discussion

4.1 Success parameters of tagging quality traits

The success of mapping and tagging of agroeconomically important traits depends upon various parameters such as diversity in parents, size of population generated and marker system adopted. I have discussed these aspects with respect to the results that were obtained in the present studies.

4.1.1 Features of the two populations used for marker analysis

In my work on finding DNA markers associated with kernel hardness and bread making quality traits, two parents each were selected for crossing to develop the RIL populations at the Directorate of Wheat Research, Karnal, India. Usually the parents that provide assurance of sufficient genetic variation within populations for the traits of interest are selected for crossing (reviewed by Lee, 1995). In the present study, two parents namely, NP4 and HB208 were selected for identification of QTLs for kernel hardness. The difference for hardness between the two parents is high enough leading to significant contribution and more number of markers contributing to the component traits (Table 3.5). On the contrary, in case of CPAN3004 and HD2329, which are good and poor bread making genotypes, respectively the component traits analysed by us contributing to BMQ did not show significant variation thereby identifying only a few major and a few minor loci affecting the phenotype. Such a population is good to actually make the bread to identify markers linked to BMQ rather than the component traits. Efforts in this direction are in progress in my laboratory.

Apart from differences in the parents, size of the population is also important in case of QTL studies. Many previous studies have indicated that usually larger populations can give more number of QTLs identified and a higher proportion of genetic variance (Anderson *et al.* 1993; Parker *et al.* 1998). For example, Zanetti *et al.* (2000) have used a population of 226 RILs for genetic analysis of preharvest sprout tolerance in wheat and have detected 183 loci associated with the trait. Zanetti *et al.* (2001) have also identified 187 loci linked to various bread making quality parameters such as Zeleny sedimentation values, protein content, kernel hardness and 1000-kernel weight of 226 RILs

from a cross between wheat and spelt wheat. In my analysis, the population size was 100 in case of KH and 120 in BMQ cross. There have been reports of QTL tagging using a population of 78 RILs for preharvest sprout tolerance in wheat (Anderson *et al.* 1993), for tagging flour viscosity in wheat (Udall *et al*. 1999) and even 65 RILs for QTL analysis of grain protein content (Blanco *et al*. 1996). However, it is possible that higher contributions as well as more number of contributing markers could have been observed if the population size was larger than that used in my studies.

The frequency distribution of most of the phenotypic traits in the RIL population of both the crosses showed presence of polygenes controlling the phenotypes. Secondly, transgressive segregants were observed in case of all the traits involved in both the crosses, which could probably be due to the accumulation of complementary alleles from both the parents (Tanksley, 1993; Schon *et al.*, 1993; Veldboom *et al.*, 1994; Sourdille *et al.* 1996). Favorable transgressive segregants are preferred by the breeders for creating populations with adequate genetic variation in QTL studies (Lee 1995). The two populations that were used in the present work fulfilled this criterion and were thus found to be suitable for marker analysis.

4.1.2 ISSR and STMS as more useful markers for QTL analysis in wheat

In wheat, various quality traits such as grain protein content (Blanco *et al*. 1996, Joppa *et al.* 1997, Mesfin *et al.* 1999), kernel hardness (Sourdille *et al.*1996), preharvest sprout tolerance (Anderson *et al*. 1993) and seed size (Campbell *et al.* 1999) have been studied using RFLPs. In my initial attempt to use RFLPs, I attempted various wheat Southern hybridization protocols from many laboratories all over the world to obtain good results in wheat. I also transformed around 400 RFLP clones derived from wheat genomic (wg, mwg clones); and barley (bcd) and oat (cdo) cDNA libraries into suitable plasmids to use them as RFLP probes. Some of these clones like *Xwg*114 (*EcoRI*, *Bam HI*), *Xwg*241 (*BglI*, *BamHI*), *Xmwg*561 (*EcoRI*), *Xcdo*57 (*EcoRI*), *Xcdo*89 (*HindIII*), *Xcdo*388 (*EcoRV*), and *Xcdo*580 (*HindIII*) showed good RFLP profiles in the

parental survey as well as in the selective genotyping. The next obvious step was to attempt all these polymorphic RFLP probes with the entire populations. However, considering the cost, time and labour involved to carry out hundreds of Southern hybridization reactions, it became an unaffordable and difficult task for me and hence I switched to PCR-based markers.

During last decade, random and dominant markers such as RAPD (Randomly Amplified Polymorphic DNA; Williams *et al.*, 1990; Welsh and McClelland, 1990) and ISSRs (Inter-simple sequence repeats- Zietkiewicz *et al.*, 1994) have emerged as reliable and speedy PCR marker systems for detecting a higher percentage of polymorphism as well as more number of polymorphic loci due to their multilocus nature and technical simplicity (Devos and Gale 1992). In wheat, RAPDs have been used for mapping purposes by Kojima *et al*. (1998) and also for mapping rust resistance (Naik *et al.* 1998), yellow berry resistance (Ammiraju *et al.* 2001) and grain protein content (Dholakia *et al.* 2001). Use of ISSRs as markers in wheat has been reported by Nagaoka and Ogihara (1997). Later, ISSRs have been successfully used for tagging seed size (Ammiraju *et al.* 2000) and grain protein content (Dholakia *et al.* 2001). Codominant simple sequence repeat markers (SSRs) have also emerged as a powerful tool to analyse the complex genome of wheat. These microsatellites are locus specific, highly informative and show a much higher level of polymorphism in wheat than any other marker system (Plaschke *et al.* 1995; Roder *et al.* 1995, Ma *et al.* 1996; Bryan *et al.* 1997). STMS markers have been used successfully to tag kernel texture (Campbell *et al.*, 1999), vernalization response (Korzun *et al.* 1997), grain protein content (Prasad *et al.*, 1999) and 1000-kernel weight (Varshney *et al.* 2000).

Many new PCR-based marker technologies such as AFLP, SSCP, and EST have been developed in recent years (Saghai-Maroof *et al.* 1994, Vos *et al.* 1995) and some of these such as AFLP (Parker *et al.* 1998, 1999) and STS markers (Roy *et al.* 1999, Parker and Langridge 2000) have been used in wheat for the analysis of various quality traits. Taking into account the advantages of PCR based markers; I undertook the task of identification of RAPD, ISSR and

STMS markers associated with various component traits of kernel hardness and bread making quality such as SV, MC, PC, KH, PS, HW and TKW. Among the three marker systems, ISSR and STMS revealed high percentage of polymorphism (62.5% and 37.5% in case of cross NP4X HB208 and 32% and 23%, respectively in case of cross CPAN3004 X HD2329) and more number of markers in both the populations in the present study as against RAPD markers (14% for NP4X HB208 and 5% polymorphism in case of cross CPAN3004 X HD2329), which is in accordance with all the previous reports.

4.2 Less usefulness of previously reported loci associated with KH and BMQ in the present study

KH being an important end product quality trait, efforts have been made to study the genetic basis of KH by previous researchers (Symes, 1969; Mattern *et al.*, 1973; Law *et al.*, 1978; Baker, 1977; Pomeranz and Williams, 1990). More recently, a 15-kDa protein, friabilin has been identified and its correlation with kernel hardness has also been shown by Greenwell and Schofield (1989) and Jolly *et al.* (1993). Friabilin is primarily composed of two polypeptides, puroindoline-a and puroindoline-b (Giroux and Morris 1997). Giroux and Morris (1998) have shown presence of a single mutation in either protein, a null in *pin-a* or glycine to serine sequence change in *pin-b*, to be associated with hard grain texture. The single nucleotide change in *pin-b* has been further exploited to create discriminating PCR primers for *pin-a* and *pin-b* sequences (Giroux and Morris 1997). In order to analyse if the same locus is responsible for hardness in the population under present study, I used these primers to amplify the genomic DNA from both the parents, NP4 and HB208. However, no polymorphism was detected indicating that in this cross the hardness might be due to some other genes/loci

It is well known that the 1Dx5-1Dy10 allele pair of the HMW glutenin subunit gene has been used as diagnostic PCR approach to select the good bread making wheat cultivars. Accordingly, D'Ovidio and Anderson (1994) have designed locus specific primers to detect the presence or absence of 1Dx5 gene

and to select for the entire locus since the alleles 1Dx5 and 1Dy10 are strictly associated. When I used this primer to genotype the entire population of CPAN3004 X HD2329, as detailed in chapter 3, it contributed only 6.75% ($P<0.01$) to the total phenotypic variation indicating that in this cross BMQ was controlled by genetic variation not explained by these alleles alone and there might be some additional genes/loci influencing overall expression of BMQ. According to Manifesto *et al.* (1998) the percentage of inter cultivar variation in bread-making potential that can be explained by variation in HMW subunit composition covers a range of 15% to over 60%, depending on the group of cultivars.

Apart from the HMW glutenins, LMW glutenin subunits and gliadins also play an important role in determining the bread making quality of wheat as suggested by Manifesto *et al.* (1998). They have developed SSR primers to detect these LMW glutenin and gliadin loci. When I used these primers to amplify the DNA of parents CPAN3004 and HD2329 with many variations in the PCR conditions I failed to obtain reproducible polymorphism.

As discussed in Chapter 1, KH is an important parameter of BMQ and HMWG and LMWG are major proteins governing the BMQ of wheat. There has been no previous report of studying these proteins as markers for a population developed for kernel texture. With this purpose, I extracted the HMW and LMW glutenins from the parents as well as RILs of KH and analysed them to study their effect on KH. However, none of the protein subunits showed significant contribution to any of the component traits of KH, though they detected distinct polymorphism among RILs.

With the less success of previously reported loci in both the populations under study, finding out additional loci using molecular markers thus became very essential to analyze such genetically complex traits.

4.3 Identification of new loci associated with KH and related traits: KH, PC, MC, HW and TKW (NP4 X HB208)

Although *Ha* locus has long been known to be important for kernel hardness, Sourdille *et al.* (1996) have shown that this gene alone can explain around 60% of the variation to KH, suggesting involvement of some other loci in its phenotypic expression (Morris 1992). In my attempt to study such other loci as well as other parameters influencing the KH, I have identified 21 PCR-based markers associated with all the five traits contributing to kernel hardness (Table 3.5). Thus, presence of a number of markers with minor effects on phenotypic variation and their localization on different chromosomal arms have indicated the complexity of this trait and strongly support involvement of additional chromosomal regions apart from *Ha* on 5DS. However, only two ISSR markers got localized using NT and DT wheat lines whereas the chromosomal locations of STMS markers were already known (Roder *et al.*, 1998 and Stephenson *et al.*, 1998). The localization of RAPD and remaining ISSR markers could not be done as explained in chapter 3, however, they might get localized to previously reported chromosomal locations or even to new locations for the presence of QTLs to KH, PC and other traits.

Sourdille *et al.* (1996) have reported a major RFLP locus, *Xmta*9 on 5D and four additional regions on chromosomes 2A, 2D, 5B and 6D contributing to hardness and three loci having interaction effects located on chromosomes 5A, 6D and 7A. Perretant *et al.* (2000) have also shown the presence of a major locus for KH on 5D. In my studies I have identified new loci 1B, 1D, 3B and 6B contributing to KH *per se* in my studies. Genes controlling PC have been found previously on chromosomes 1A, 1B and 7A by Stein *et al.* (1992) while Joppa *et al.* (1997) have shown presence of a major locus on 6BS for high grain protein content (GPC) in durum wheat. Blanco *et al.* (1996) and Prasad *et al.* (1999) have also shown 4B contributing to GPC in bread wheat. Recently Dholakia *et al.* (2001) have observed loci 2B, 2D, 4B, 5B, 6B and 7A governing the expression of GPC. I have also found loci 2B and 6B contributing to GPC in this cross whereas loci namely 1B, 1D and 3B identified by me have not been reported

earlier. Loci 6B and 2B have been shown to carry QTLS for kernel texture and flour protein quantity, respectively by Campbell *et al.* (1999) whereas; Ammiraju *et al.* (2000) have shown presence of loci for TKW on chromosomes 2D and 6B. In my analysis, I have shown chromosomes 6A and 6B carrying loci controlling MC and loci 1A, 3D and 4A controlling expression of HW for the first time. To the best of my knowledge, till now there has been no report describing molecular studies of traits such as MC and HW.

4.4 Identification of new loci associated with BMQ related traits: SV, PC, KH, PS and TKW (CPAN3004 X HD 2329)

Previous studies in wheat by Manifesto *et al.* (1998) have revealed the existence of genes/QTLs controlling sedimentation value on chromosome 1BS and have shown that microsatellite marker derived from ϒ-gliadin gene located on chromosome 1BS could be used as a molecular marker for the *Gli-B1*/*Glu-B3* chromosomal region. Further, they have shown that this microsatellite marker is significantly associated (r=0.7) with differences in the SV. Various studies in the past decade have demonstrated that HMW subunits on homeologous group 1 chromosomes have significant influence on BMQ (Shewry *et al.*, 1992 and D'Ovidio *et al.*, 1995). Sourdille *et al.* (1996) and recently Perretant *et al.* (2000) have shown presence of a major locus for KH on chromosome 5D. As seen earlier, there are some common loci shared between PC and KH on chromosomes 2B and 6B in the cross NP4 X HB208. In the cross CPAN3004XHD2329, I have observed one microsatellite marker contributing to KH located on 6B while Joppa *et al.* (1997) have shown presence of a major locus on 6B for high grain protein content (GPC). I have further found chromosomal regions 6D and 7D to be associated with PC, which were not reported previously. Ammiraju *et al.* (2000) have shown presence of loci for TKW on chromosomes 6B and 2D in the population Chinese Spring X Rye Selection111. These loci did not contribute to TKW in my study, which indicated population specificity of markers as reported by Melchinger *et al.* (1998). Further, I identified the chromosomal regions 5A and 5B contributing to TKW, which was

previously reported by Campbell *et al.* (1999), Guira and Saluescu (1996) and Petrovic and Worland (1988). Other chromosomal regions identified in this study such as 5A, 5B and 6A for SV and 7B for KH were not reported previously. Such new and unique markers to each trait can be exploited in marker assisted selection (MAS) programmes.

4.5 Interrelations of different traits observed in both the crosses

Both the traits under present study namely kernel hardness and bread making quality are influenced by many other related traits (Morris, 1992; Tipples *et al*., 1994; Lee, 1995; Rousset and Dvorak, 1997; Pena, 1997; Bushuk, 1998). It is, therefore, advantageous to study the interrelations among them in order to design a suitable strategy for breeding purposes.

4.5.1 Trait interrelations in cross NP4x HB208

In my studies on the cross NP4 X HB208, an interesting observation was made wherein most of the markers were associated with more than one trait. Moreover, the linkage groups as detailed in section 3.1 were also shared by all these traits indicating probable sharing of loci on different chromosomes. The correlation studies also supported interrelation in these traits with high and significant correlation (r= 0.8) between the traits KH and PC. HW and TKW exhibited strong positive correlation between them (r=0.6) suggesting that the test weight of grain and its size could be related as confirmed by sharing of most of the markers associated with them. In both the crosses, KH was significantly negatively correlated to HW and TKW, indicating that grain size and test weight do affect hardness of the grain. The effect of MC on KH has been studied by many researchers like Obuchowski and Bushuk (1980a), Williams *et al.* (1987) and Pomeranz and Williams (1990) who have shown significant effect of MC on the texture of the grain. Interestingly, I also found positive correlations between KH and MC (Table 3.7). Campbell *et al.* (1999) and Ammiraju *et al.* (2000) have found grain size negatively affecting the protein content of grains which was

confirmed in my results wherein PC consistently showed negative correlations with MC, HW and TKW.

4.5.2 Evidence suggesting interrelation of KH and PC based on molecular data

The relation of KH and PC has been studied by many investigators using different approaches. For example, Symes (1961) has found genotypic interaction with the relationship between kernel texture and PC. Baker and Dyck (1975) have shown a highly significant positive correlation between PC and KH whereas Miller *et al.* (1984) have observed low correlations between PC and KH. In the same year, Williams and Sobering (1984) have reported that soft wheats become harder as PC is increased, in a replicated series of trials grown over a wide range of agroclimatic conditions. Later, Pomeranz *et al.* (1985) have also found very weak correlations between PC and kernel texture.

According to Tipples *et al.* (1994) and Bushuk (1998), hard wheats are generally high and soft wheats are low in PC. Sourdille *et al.* (1996) have reported that some marker loci involved in KH are mapped in the same regions as storage proteins. Recently, Giroux *et al.* (2000) have found that selection for increased PC results in a linear increase in KH. They have reported significant positive correlations for KH and PC and have also suggested that these traits may share some genetic factors. However, to date, no clear molecular evidence has been produced in support of this. When I studied the correlations between KH and PC in the cross NP4 X HB208 and the population therein, I found that KH and PC were significantly correlated (+0.8). Interestingly, I identified three markers (one RAPD and two ISSRs) and two microsatellite markers *Xpsp*3000 and *Xgwm*264 contributing to PC as well as to KH, indicating that few loci contributing to KH and PC were linked to each other in the present population. This was also supported by identification of chromosomes 2B and 6B carrying marker loci for both KH and PC.

Sourdille *et al.* (1996) have discussed the biochemical association of gliadin loci and hardness; however, could not find any clear genetic linkage

supporting this interaction. On the other hand, in my study, the microsatellite marker X*psp*3000, showed association with KH, which has been mapped on the Gli-1 locus on chromosome 1DS by Stephenson *et al.* (1998), providing clear evidence of correlation between KH and storage proteins, namely the gliadin loci.

4.5.3 SV, PC, KH, PS, TKW and HW interrelations in cross CPAN3004X HD2329

In case of the cross CPAN3004XHD2329 also, KH and PC were significantly correlated (r=0.8), but they did not share any markers between them. PC was negatively correlated with HW and TKW even in this cross while HW and TKW showed positive correlation (r=0.8) with no marker being common between them. In fact none of the traits involved in the cross CPAN3004XHD2329 shared any markers, which might be due to the less number of markers analyzed in this population. Also, except for one RAPD marker ($OPH12_{900}$) none of the markers attempted for the cross NP4X HB208 was common to those screened with CPAN3004XHD2329 population. This confirms the population specificity of markers as reported by Melchinger *et al.* (1997).

In my studies, SV was found to exhibit positive correlations with PC and KH, which was in accordance with all the previous reports indicating that high PC and harder grains produce good quality breads (Tipples *et al.* 1994, Bushuk 1998). PS showed a positive correlation with PC, indicating that high protein content flour will give good protein quality supported by the previous report of Tipples *et al.* (1994). It also explains that wheats of higher PC tend to have strong gluten and produce good quality bread whereas wheats with low PC tend to have weak gluten and produce small loaves of inferior crumb structure.

Sharing of markers as well as linkage groups by such different traits suggests the possibility of these traits sharing the QTL intervals, which could not be detected with such a small number of markers. Interval mapping using more markers spanning the entire genome thus would be the next immediate step in my work.

4.6 Prospective strategies of breeding based on the molecular data

In a process of understanding the importance of kernel hardness and related traits towards the bread making quality of wheat, I reported 16 RAPD and ISSR markers and 8 STMS markers that were associated with various traits, related to kernel texture. The identification of microsatellite markers, which are locus-specific, would be of great help to breeders in order to accelerate marker-assisted selection (MAS). For example, the microsatellite markers like *Xpsp*3000 and *Xgwm*264, having relatively good effects on the phenotypic variance of PC and KH both could be specifically used as an indirect selection tool in MAS. Identification of many such markers would be a promising approach to tackle with the complex traits/QTLs in breeding. Moreover, detecting additional QTLs/gene(s) using more microsatellite markers for interval mapping would also be useful for mapping purposes. Further analysis of these populations in different environments and use of larger RIL population would facilitate to localize kernel QTLs with more accuracy.

Understanding the genetic basis of BMQ in wheat would help breeders in devising their breeding strategies and selection methods for improved grain quality (Manifesto *et al.* 1998). Research in the past two decades has focused on the role of HMW subunits in determining BMQ of wheat and has resulted in the development of cultivars with these alleles (Payne 1987 and Dong *et al.* 1991) However, significant variations in the BMQ are still shown among cultivars with maximum *Glu-1* index according to Manifesto *et al.* (1998). They have selected a segregating population derived from two parents, which have similar HMW glutenin subunit composition but are different in their BMQ and have shown that even LMW glutenin and gliadin subunits contribute to differences in BMQ. There are some additional reports on the effect of LMW subunits and the gliadins on BMQ (Branlard and Dardevet 1985, Gupta *et al.* 1989,1991). However, rather difficult analysis of different LMW glutenin subunits and gliadin alleles makes them not very suitable as selection criteria in practical breeding programmes

(Groger *et al.* 1997). When I screened the BMQ population with the primers for LMW glutenins and gliadins as given by Manifesto *et al.* (1998), no significant contribution was observed to any of the component traits suggesting involvement of some other loci. In my study, using 1Dx5-1Dy10 primers in the same population, I have also demonstrated that HMW subunits contributed to only a small extent and a significant proportion of variation in BMQ could be explained by loci other than HMW subunits.

Direct evaluation of BMQ is expensive and it requires special facilities (Lee, 1995). According to Dong *et al.* (1991) and Groger *et al.* (1997) a selection using HMW glutenin subunits seems to be profitable for the first approximate distinction between good and poor BMQ. My results showing DNA markers associated with various traits of BMQ can be of further use to breeders for predicting BMQ of the preselected material. Microsatellite markers, namely, *Xpsp*3200 and *Xgwm*205, having relatively large effects and explaining more than 10% of the phenotypic variance, will have potential for use in MAS programmes.

The low variances explained in these crosses by the putative markers of SV, PC, KH and TKW suggest the possible involvement of additional smaller or larger effect loci, which were not identified in my study either due to inadequate genome coverage or due to loose linkage between QTLs and markers as given by Anderson *et al.* (1993). More number of primers, preferably STMS and AFLP, have to be used to study a complex trait like BMQ. According to Anderson *et al.* (1993), epistasis, environmental effects and error associated with the measurement of the phenotypic traits could also be the reasons for less number of QTLs identified. My studies with both the populations suggest that these quality traits are, in most of the cases, controlled by many minor loci and only a few major loci. This makes breeder's job more difficult since minor effect of such low contributing QTLs cannot be detected and further pyramided without the tools of molecular markers.

Thus my thesis work represents an initial step to the vast area of deciphering quantitative traits like kernel hardness and bread making quality. It

gives some insight about the complexity of these traits and how such traits should be studied from different dimensions in order to understand them thoroughly. In my analysis, I could not study the environmental effects on these traits and hence these traits need to be studied across different populations and environmental conditions for their complete analysis and will form the future direction of my work.

CHAPTER 5: Summary

5.1 Introduction

Wheat has been a food crop for mankind since the beginning of agriculture and has been used over 8000 years. It is grown worldwide and is one of the most widely adapted cereals. World acreage of wheat is estimated to be 500 million acres, with over 560 million tonnes of grain yield. With a total production of 68.7 million tonnes, India today is the second largest wheat producing country in the world.

Most of the wheat produced in the world is traded as the main raw material for the manufacture of hundreds of diverse food products such as all kinds of bread, pasta, macaroni, spaghetti, noodles, cakes, biscuits, cookies, roti, paratha, chapatti and naan. Many of these wheat products consumed around the world require specific grain quality attributes for their manufacture. Grain quality thus becomes a breeding issue as important as grain yield especially when a country reaches to a surplus level of production.

5.2 Why the emphasis on grain quality studies in India?

- The increased production of wheat in recent years demands for export of wheat.
- The changed scenario of over production of wheat has led to attempts of improving the quality of grain with buyer's requirement in mind. Bread with high loaf volume, smooth crust, uniform and smaller grain in the crumb is always preferred.
- Until now, Indian wheat has been selected for best chapatti or roti-making qualities and hence attempts need to be made for improvement of Indian wheat for good bread making quality for export as well as for domestic consumption.

5.3 Rationale in using DNA markers

All the quality traits are generally polygenic in nature and their expression is controlled by quantitative trait loci (QTLs). In order to study such complex traits at molecular level, one needs to find out markers associated with these QTLs.

With the advent of molecular marker technology, many PCR-based markers like RAPD (Randomly Amplified Polymorphic DNA), ISSR (Inter Simple Sequence Repeat), microsatellites and AFLP (Amplified Fragment Length Polymorphism) have gained popularity as tools to dissect and study the genetic basis of various agronomically important quality traits in crops like rice, maize and barley.

The genetics of hexaploid wheat is complicated due to polyploidy, complex segregational patterns and epistatic effects. Most of the genes that have been identified and located until now are the ones affecting qualitative variation, rust resistance and those determining most of the proteins that have been characterized through gel electrophoresis (SDS-PAGE). Very little work has been done as regards the wheat grain quality characteristics and hence molecular efforts will be very useful to explore the vast area of quality attributes specific to a particular end use.

With these perspectives, two important quality traits viz. kernel hardness and bread making quality were identified for molecular studies in this thesis.

5.4 Important research findings

5.4.1 Genetic analysis of kernel hardness using PCR-based markers and study of trait-interrelations in a cross NP4 X HB208

In wheat, kernel hardness (KH) is one of the most important quality parameters having a profound effect on milling, baking as well as end-use qualities. It refers to the texture of the kernel, in terms of physically hard or soft endosperm. Hard wheat flour is generally used for bread, whereas soft wheat flour is used for pastries, cakes and confectioneries. Also the primary basis of discriminating different end uses of wheat is not protein content but is grain texture, which also influences the economic value of wheat grain. Assessment of endosperm texture is, therefore, necessary in characterizing the end-use quality of wheat.

Kernel hardness is a complex genetic trait involving various directly and indirectly contributing components such as kernel hardness *per se*, protein content, moisture content, hectolitre weight and 1000-kernel weight. In an

attempt to identify DNA markers associated with this trait, I screened 100 recombinant inbred lines (RILs) derived from a cross between a hard grain land-race, NP4 and a soft grain variety, HB 208 with using 100 ISSR, 580 RAPD and 173 STMS primers.

Twenty-nine markers were assigned to 8 linkage groups covering 426.2cM whereas 24 markers remained unlinked. A multiple marker model could explain percentage of phenotypic variation for kernel hardness as 18.9%, whereas that for protein content, moisture content, hectolitre weight and 1000-kernel weight was 18.7%, 16.3%, 21.5% and 12.1%, respectively. It is for the first time that I have shown association of 26 PCR-based (RAPD, ISSR and STMS) markers with all the four traits contributing to kernel hardness.

Molecular marker analysis using dominant ISSR and codominant microsatellite markers revealed presence of QTLs for the above traits on chromosomes 1A, 1D, 2A, 2B, 3D, 4A, 5D, 6A and 6B. Thus, presence of a number of markers with minor effects to phenotypic variation indicates the complexity of this trait and strongly supports involvement of additional chromosomal regions apart from *Ha* (hardness) on 5DS. Most of the markers are associated with more than one trait and the linkage groups have also been shared by all these traits indicating probable sharing of loci on different chromosomes. My results thus indicate that phenotypic expression of kernel hardness is controlled by many QTLs and is interdependent on various related traits.

I further attempted to study interrelation among traits like kernel hardness and protein content, which play a major role in determining the kernel texture. Two microsatellite markers in my study, *Xpsp*3000 and *Xgwm*264 contributed to phenotypic expression of both KH and PC. *Xpsp*3000, which has been mapped on the Gli-1 locus on 1DS by Stephenson et al (1998), provided a strong evidence for a locus, which was involved in kernel hardness expression and was mapped in the same region as storage proteins, namely the gliadin loci.

All the above analysis will enhance our knowledge about genetic basis of kernel hardness and is a step to increase the breeding efficiency to develop cultivars with better grain quality.

5.4.2 Identification of DNA markers associated with bread making quality related traits

Breeding for end use quality has become an important objective after fulfilling considerable high yield requirements of wheat during last one decade. Wheat cultivars with better processing characteristics and good bread making quality (BMQ) are the major end use requirements for the baking industry. However, BMQ is a unique and complex feature, which depends on many interrelated parameters and not much is known about its genetic basis, except for the role of high molecular weight glutenin sub units at Glu-1/ Glu-3 loci.

In order to decipher the chromosomal regions associated with BMQ related traits, a segregating recombinant inbred line (RIL) population of 120 individuals was developed by making a cross between CPAN3004, a wheat cultivar with good BMQ and HD2329, a wheat cultivar with poor BMQ, at the Directorate of Wheat research, Karnal, India. I studied the inheritance and genetic correlations of six traits, which influence the BMQ, namely SDS-sedimentation value, protein content, kernel hardness, Glu-1 protein score, 1000-kernel weight and hectoliter weight in this population using 580 RAPD primers, 100 ISSR and 173 microsatellite primer pairs. Single marker analysis revealed the presence of QTLs for sedimentation value on chromosomes 1B, 5A, 5B and 6A; for protein content on 6D and 7D; for kernel hardness on 6B and 7B; and for 1000-kernel weight on 5A and 5B.

My results show that DNA markers for various traits related to BMQ can be of further use to breeders for predicting BMQ of the preselected material. More significantly, the two-microsatellite markers, *Xpsp*3200 and *Xgwm*205, having relatively large effects explaining more than 10% of the phenotypic variance, will have potential for use in MAS programmes.

In summary, my work with two quality traits namely kernel hardness and bread making quality has shown the complexity of wheat quality traits and strongly suggests their interdependence.

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