MOLECULAR APPROACHES TO DECIPHER QUANTITATIVE TRAITS GOVERNING GRAIN QUALITY IN WHEAT

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CERTIFICATE

Certified that the work incorporated in this thesis entitled, "**Molecular Approaches to Decipher Quantitative Traits Governing Grain Quality in Wheat**" submitted by Mr. Bhushan B. Dholakia 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 "**Molecular Approaches to Decipher Quantitative Traits Governing Grain Quality in Wheat**" 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.

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TO MY PARENTS

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Bhushan Dholakia *Date:*

LIST OF ABBREVIATIONS

AACC	:	American Association of Cereal Chemists
AFLP	:	Amplified Fragment Length Polymorphism
BAC	:	Bacterial Artificial Chromosome
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
DWR	:	Directorate of Wheat Research
EDTA	:	Ethylene Diamine Tetra Acetic acid
EST	:	Expressed Sequence Tag
FFD	:	Factor Form Density
g/gm	:	gram
GPC	:	Grain Protein Content
h	:	Hour
ha	:	Hectare
HMW	:	High- Molecular Weight
HRS	:	Hard Red Spring wheat
HRW	:	Hard Red Winter wheat
IAA	:	Iso-Amyl Alcohol
IFPRI	:	International Food Policy Research Institute
ISSR	:	Inter Simple Sequence Repeat
ITMI	:	International Triticeae Mapping Initiative
K_2O	:	Potassium Oxide
kb	:	kilobase pair
KCl	:	Potassium Chloride
kg	:	kilogram
LMW	:	Low- Molecular Weight
LOD	:	Log of the Odd (Base 10 logarithm of the likelihood ratio)
Lr	:	Leaf Rust
m	:	Minute
M	:	Molar
MAS	:	Marker Assisted Selection
Mb	:	Megabase pair
$MgCl_2$	:	Magnesium Chloride
mm	:	milimeter
mM	:	milimolar
mt	:	milliontones
MW	:	Molecular Weight
N_2	:	Nitrogen
NaCl	:	Sodium Chloride
ng	:	nanogram
NIL	:	Near Isogenic Line
NIR	:	Near Infrared

nM	:	nanomoles
P	:	Phosphorus
PAGE	:	Poly-Acrylamide Gel Electrophoresis
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
TKW	:	Thousand-Kernel Weight
Tm	:	melting temperature
Tris	:	Tris-hydroxymethyl amino methane
var	:	Variety
YB	:	Yellow Berry
Yr	:	Yellow Rust
°C	:	degree centigrade
µg	:	microgram
µl	:	microlitre
µM	:	micromolar

Chapter 1

Review of Literature

Enhancing the quality of Indian wheat through integration of biotechnological approaches

1.1 Taxonomy, origin and phylogeny of wheat:

Wheat belongs to the sub-tribe Triticinae of tribe Triticeae in the grass family Poaceae. The sub-tribe triticinae is of recent origin, and contains about 35 genera including *Triticum*, *Aegilops*, *Thinopyrum*, *Dasypyrum*, *Lophopyrum* and *Secale*. The various species of these genera easily hybridize with each other resulting in either a direct exchange of genetic material or polyploidy. The cytogenetic studies by Sakamura (1918) and Kihara (1924) have shown that the various species of *Triticum* and *Aegilops* form a polyploid series based on a basic haploid set of 7 chromosomes, and consist of three ploidy levels: diploids (2n= 14), tetraploids (4n= 28) and hexaploids (6n= 42). Emmer wheat is generally regarded as one of the ancestors of the wheats. The site of origin of wild emmer, *T.dicoccoides* is considered to be the upper area of the Fertile Crescent of the Near East (Tigris-Euphrates region) (Nevo 1988). Percival (1921) concluded that bread wheat was originated by hybridization from emmer type and wild species of grass. Kihara (1924) gave genomic formula AA to *T. monococcum*, AABB to *T. turgidum* and AABBDD to *T. aestivum*. So, the genomic phylogeny of bread wheat can be schematically represented in two crosses- (a) AA x BB = AABB and (b) AABB x DD = AABBDD. By finding the origin of A, B and D genomes the origin of bread wheat was deduced. Molecular studies (Dvorak *et al.* 1993; Jiang and Gill, 1994) identified *T.urartu*, a biological species of the former, as the A genome ancestor of emmer and durum wheat. Morris and Sears (1967) identified *Aegilops squarrosa* as donor of D genome to bread wheat. The identity of the B genome donor is elusive till date, though morphological, geographical, cytological and molecular evidences have been used to implicate *Aegilops speltoides* (SS) or a closely related species as the B genome donor (Riley *et al.* 1958, Dvorak and Appels 1982, Dvorak and Zhang, 1990, 1992). However, studies on chromosome banding, in-situ hybridization, meiotic pairing and isozymes by Waines and Barnhart (1992) suggested that the genome of *Aegilops speltoides* is not identical to the genome of present day tetraploid and hexaploid wheat. The present scenario about B genome donor of bread wheat includes three possibilities: (i) the

original donor may now be extinct (ii) it may yet be undiscovered and (iii) more than one species may have contributed to the genome, which in turn has undergone rearrangement after incorporation into tetraploid wheat.

1.2 Types of wheat, their cultivation and consumer preferences:

Wheat (*Triticum spp*.) is one of the most important winter cereals in India contributing around 32% of total foodgrain basket of the country. Area under wheat cultivation is around 24.5 mha and production is of the order of 68.7 mt in 1997 (Anonymous 1997). India now is the second largest wheat producing nation in the world and contributes approximately 12% (1995-96) to the world's wheat basket with an area of 11% under wheat cultivation in the world. China is the largest producer of wheat with 13% area and its contribution of 19% of the total wheat production worldwide (Anonymous 1997). In other words, China and India together produce nearly one-third of the global wheat.

Wheat is cultivated in many different countries representing all the continents and interestingly, is harvested throughout the year taking into account all the wheat producing regions (Table-1.1). In India, three types of wheat are cultivated: (i) *Triticum aestivum* (bread wheat), (ii*) Triticum durum* (durum wheat) and (iii) *Triticum dicoccum* (dicoccum wheat). Common wheat or bread wheat is mainly grown in the alluvial soils of the Indo-Gangetic plains comprising Punjab, Uttar Pradesh, Bihar and parts of Rajasthan and accounts for a major share of wheat produced in the country. It is used to prepare different home-made foods and *Chapati*, which is a major food component in most parts of India. Durum wheat is considered to be the best suited for drought conditions or restricted irrigated conditions of Punjab, Madhya Pradesh, Karnataka, Tamil Nadu, Gujarat, West Bengal and Himachal Pradesh and is used for semolina (*suji*) preparation. Dicoccum or khapli wheat is grown in a few states of India viz. Maharashtra, Karnataka and Tamil Nadu and is used in some pockets of Karnataka and Maharashtra to make special local preparations like vermicelli (*Sevai*).

Table-1.1: Harvesting time of wheat during a year in different parts of the world.

Adopted from Percival (1921)

Month	Parts of the world
January	Argentina, Australia, Chile & NewZealand
February	India
March	India & northern Egypt
April	Asia minor, India, Iran, Cuba, Mexico & southern Egypt
May	Algeria, central Asia, China, Japan, Morocco & Tunisia
June	Italy, Greece, Japan, southern France, southern USA, Spain & Turkey
July	Austria, Bulgaria, Canada, France, Germany, Hungary, northern USA, Romania & southern part of former Soviet Union
August	Belgium, Canada, central part of former Soviet Union, England, Holland, northern France & USA
September	Canada, Norway, Scotland & Sweden
October	Finland & northern part of former Soviet Union
November	Argentina, southern Africa & Peru
December	Argentina, Australia & Burma

1.3 Challenges in meeting the projected demand of wheat and its quality improvement:

According to census, population of India has grown at the rate of 2.1% per anum in 1985, 1.8% in 2000 and by 2020 it will be around 1.3 billion. From the finite land resources, adequate food grain is to be produced for the deeming population and so the country would require approximately 105 to 109 mt of wheat by the year 2020 (Nagarajan *et al.* 1997). This means that wheat production has to increase by another 40 mt and unless production increases annually at the rate of 1.8%, the projected demand can not be achieved. As there is no scope of area expansion, this also means that the current national average of wheat yield is to be increased from 2,700kg/ha to 4,200kg/ha in less than 25 years (Nagarajan *et al.* 1997). Viewed in this light, the challenge for

increasing wheat supplies in developing world is as great today as it was three decades ago at the start of Green Revolution.

As far as global scenario is concerned, by 2020, two-third of the world's wheat consumption will occur in developing countries. Wheat demand worldwide is calculated to rise by 40% from 552 mt in 1993 to 775 mt in 2020 Rosegrant *et al.* 1997). The expected increase in demand is partly motivated by population growth, but also due to substitution of rice and coarse grain cereals with increase in income and population, which is increasingly based in urban areas. Across Asia, rapid economic growth and urbanization are creating dramatic changes in dietary patterns. As income rises, households tend to increase their consumption of bread and high value food such as meat, poultry products, fruits and vegetables. The IFPRI (International Food Policy Research Institute) IMPACT model (Rosegrant *et al.* 1997) projects that Asia will account for 42% of global wheat demand in 2020 as opposed to 37% in 1993 and for India, the anticipated demand with this model is around 96 mt by 2020. The increased demand for wheat will have to be met through a combination of increased imports and enhanced domestic production with improved grain quality using different approaches that are technologically feasible.

India can produce surplus grain of bread wheat, durum and dicoccum with the advantage of diverse climate and these different wheats can be supplied for the product development and to the consumers. Over the last several years, wheat has been exported to some of the countries as goodwill gesture in emergency time. Due to surplus wheat production from last few years, Govt. of India has liberalized the wheat export. However, getting access to the export market demands high grain quality standards, competitive prices and reliable supply in quantity and quality (Anonymous 1997). Therefore, a coordinated effort is required from research, development and marketing agencies to produce better quality wheat and to have well-organized overseas promotion and marketing system to facilitate export of Indian wheat. As we grow more than 60 different varieties in different agroclimatic and

agroecological zones, it will be possible to supply any kind of wheat grain required by the importer nation.

1.4 Commercial classification of wheat based on quality and its utilization:

The common wheats must be classified by properties other than the botanical features for commercial purpose. World wheat trade has become increasingly complex, with more emphasis being placed on technical specifications as per the specific requirements of the buyers. Individual varieties may vary in their breadmaking quality, dough characteristics and milling properties. However, broad classes of wheats are recognized and used on the basis of world trade (Orth and Shellenberger, 1988). This has led to classification based on physical and chemical properties of wheat grain related to their processing performance. Major factors used to distinguish wheats are hardness or softness of the grain, spring or winter habit, white or red bran and more recently, the protein content and such different classes of wheat grains are shown in Fig 1.1. Within these, wheats can be further described by bushel weight or test weight (a measure of bulk density), cleanliness, the degree of soundness, moisture content and measures of the properties of their doughs and processing quality. Wheat quality can be fairly defined by reference to these parameters. Some of these are influenced primarily by genetic factors while others are governed by environmental factors. Wheats can be closely matched to many different end products according to their hardness and protein content. Durum wheats are preferred for pasta; soft bread wheats are for cakes, biscuits and pastries while hard bread wheats for noodles, pan breads, flat breads and other products. Hard grains with higher protein content give flour, which is more suited for products such as pan breads, some specific noodles like Cantonese noodles etc. On the other hand, low protein and soft grains are well suited for cakes, biscuits and pastries (Orth and Shellenberger, 1988). Fig. 1.2 shows representative examples of different end products of bread wheat. The combination of quality characteristics genetically present in varieties and those influenced by environment should be carefully balanced to produce

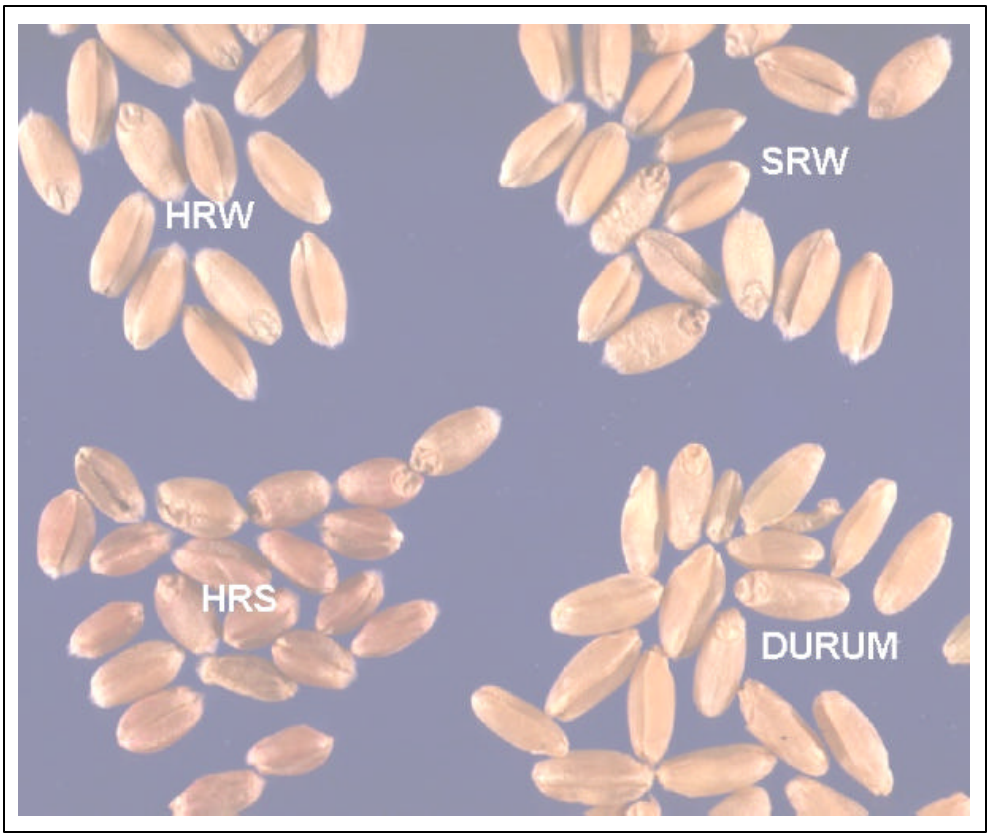

Fig 1.1: Different types of wheat grains.

(Source: http://www.psu.missouri.edu/plsci274/images)

Fig 1.2: Various end use products of common wheat.

(Source: http://www.wheatfoods.org)

wheats with particular end use in mind. Information about the different food products, which can be prepared, based on hardness and protein content of wheat grain is presented in Table-1.2.

Table-1.2: Requirements of grain protein content and hardness for different wheat based products.

Food products	% GPC	Hardness of wheat
Cakes, Biscuits & Pastries	8.0-9.5%	Soft bread wheat
Thickeners & Puddings	9.5-11.0%	Soft – less hard bread wheat
White Noodles (Japanese)	9.5- 10.0%	Mixed type bread wheat
Yellow Noodles (Chinese)	11.0-12.5%	Mixed type bread wheat
Middle/ Eastern flat breads (*Chapaties*)	10.5-11.5%	Mixed – hard bread wheat
Pan breads	12.0-13.5%	Hard bread wheat
High protein flour	13.5-15.0%	Hard bread wheat
Pasta & Macaroni	14.0-15.0%	Durum wheat

1.5 Various criteria contributing to wheat grain quality:

The criteria of wheat quality are as varied as their different uses. Wheat, which is suitable for a particular use/product, may have certain characters that make it entirely unsatisfactory for other purposes. Moreover, different parts of wheat grain/kernel have different uses; for example, endosperm is used for flour, germ is for diet supplements and bran for feed. Though individual variety is an important factor that influences the grain quality, generally it is marketed according to the class wherein each class consists of group of varieties with similar characteristics and is suited for similar purpose/ end use (Halverson and

Zeleny, 1988). Based on this, factors that influence the wheat grain quality have been broadly classified in two groups- physical characteristics and chemical characteristics. Following subsection describes each parameter in its respective group, its utility and limitation.

1.5.1 Physical characteristics:

Wheat quality has different meaning or definition as per required end use. Generally, for marketing purpose wheat is often assigned a numerical grade based on the results of certain tests such as test weight, percent of damaged kernels and presence of impurities.

Vitreousness: It is often correlated with hardness of grains, which indirectly is an index of protein and gluten content. Kernel vitreousness is now a grading factor only in grading of spring wheat. For durum wheat, it is of primary interest as it is associated with kernel hardness and semolina yield. It is visually scored with X-ray film viewer. Video densitometer, a device commonly used for scanning of the gels, can also be used for kernel vitreousness.

Colour: Generally wheat is classified as red or white based on the colour of the bran. These two basic colours as well as certain variations within each of them are commonly used for wheat grading purpose. Red wheats are predominant varieties grown in Europe, north and south America and parts of Asia and they are usually preferred over white wheats for bread flour production or mixing with other wheats for that purpose. White wheats are produced predominantly in Australia, India and Pakistan, in the Pacific coast region of the United States as well in the states of New York and Michigan. They are generally used for cake, cookies, pastries and crackers flour in United States whereas in India, Pakistan, some parts of Asia and Africa it is preferred for making *Chapati* as principal wheat food.

Yellow berry: It is an important quality parameter and is a physiological disorder affecting the grains of durum wheat, bread wheat and triticale. Yellow berry arises due to deficiency of nitrogen concentration in soil and is recognized by its softer, light coloured and starchy endosperm, which lacks the vitreous texture of normal grains (Sharp 1927). Grains affected with yellow berry have higher moisture content, higher level of starch and lower amount of protein content compared to normal grains, thereby affecting bread making quality in bread wheat and pasta making quality in durum wheat.

Test weight (weight per unit volume): The weight of wheat per unit volume is one of the widely used and simplest criteria of wheat quality. In most countries, it is expressed in metric systems as kilogram per hectoliter and in the United States, test weight is expressed as pounds per bushel. It usually determines the plumpness of the grain. Test weight is affected by uniformity of kernel size and shape, as these factors determine in which manner kernels orient themselves in a container. Other factor which influences the test weight is density of the grain, which in turn, is determined by structure of the grain and chemical composition. Test weight is a rough index for the flour yield and several studies have shown positive correlations between them. However, as the differences in test weights caused by inherent variety characteristics often do not reflect the differences in milling yield, this test can not be used as accurate predictor of flour yield.

Grain weight: This is a function of grain size and density and generally expressed in grams per 1000 grains. This is determined by electronic seed counter and balances which are considered to be more reliable guide of flour yield than test weight. Grain weight generally varies from 20- 45 grams per 1000 grains, based on the type of wheat i.e. hard or soft grains.

Grain size and shape: These are more closely related to grain weight and affect the flour yield. Shuey (1960) developed a procedure of sizing the wheat grains according to average cross-section area and reported a correlation of

0.957 between predicted and actual commercial milling yield. However, separating large grains from smaller ones and milling them separately may improve efficiency of milling (Shuey and Gilles 1969).

Grain hardness: This has been considered as a major factor for grain quality and is used in differentiating hard and soft classes of wheat. When hard grains are used for making flour, their endosperm is cracked along the line of aleurone layer giving more flour yield. On the other hand, most part of aleurone layer in soft wheat remains with endosperm, which causes inconsistency in flour during milling operation. Water absorption has a direct effect on the amount of bread produced and is considered to be an important quality factor. When hard grains are reduced to flour, large number of starch granules get damaged which in turn absorb more water. While in softer wheat, as the number of damaged granules of starch is low, water absorption is less. Various methods for measuring hardness like particle size index, pearling resistance, grinding resistance (time to grind), sound of grinding, starch damage, near-infrared (NIR) analysis and crushing or slicing of individual kernels have been proposed and among these, NIR is used widely for testing of grain hardness.

Damaged grains: Wheat may be damaged in the field before harvest, during harvest or artificial drying operations, and during subsequent storage or handling. These types of damages decrease the storage life and processing values of the grain and affect the grade. Infections caused by pathogens before harvest may affect the grain quality. Immaturity at harvest can cause shriveled grains, which reduces the yield and quality of flour. Wet weather before harvest, when wheat has matured, may result in sprouting of the kernels. Sprouted wheat reduces flour yield with resultant flour having high α-amylase and protease activity, which affects bread-making quality. In the field, heavy frost while wheat is still maturing can cause blistering of the bran, which results in discoloration of the kernels. Such frost-damaged wheat grains produce low flour yields with high ash content in the flour. While threshing, improperly adjusted

equipment may break the grains leading to decrease in the flour yield. Excessive heating from artificial drying or spontaneous heating during storage may affect the gluten quality of the wheat, causing discoloration of the grains. High level of moisture in wheat during storage leads to development of infection by fungi and other pathogens, which may also result in discoloration of grains. Wheat damaged by insect infestations during storage also decreases the flour yield with presence of insect's fragments in flour. Damaged and infected grains have no market value except it can be used as a feed.

Impurities: Amount and type of impurities or other matter in wheat are important quality criteria. Most of such materials can be removed from wheat in the mills during the screenings, which have value in animal feeds but considerably less value than the wheat on weight basis.

1.5.2 Chemical characteristics:

Moisture content: It is one of the most important factors for determination of wheat grain quality. Moisture content is inversely related to the dry matter of grain and has more effect on keeping quality of wheat as dry and sound wheat that can be kept for years when it is stored properly but wet wheat with high moisture may deteriorate faster in few days. Generally, moisture content is measured using hot-air oven (Misra and Gupta, 1995). Electric moisture meters are also used to measure moisture content of most of wheat when large samples are needed to check quickly, but these instruments must be calibrated against reference method like hot-air oven method.

Protein content: Protein content of wheat is usually determined by Kjeldahl method (AACC, 1983 Method 46-10) or its different modifications (AACC methods 46-11A, 46-12 and 46-13). This method is quite precise and reproducible among various laboratories. Another faster method based on NIR analysis is also used which is calibrated against the Kjeldahl method. Protein content varies from 6-20% depending on variety and class of wheat with

environmental conditions during the growth. Wheats having high protein content get high premium price in the market as they are useful in blending with low protein wheat flours for bread production. Flours of low protein content wheats are useful in making other different products like cakes, cookies and biscuits.

Protein quality: The approach to wheat protein quality is based on considering potential end product. Gluten quality varies and is based on varietal characteristics. Wheats having same amount of total protein produces flours giving different outcome during baking which many times may be due to qualitative variation in gluten proteins. Excessive heat and low humidity during maturation of wheat in field have deleterious effect on gluten quality leading to poor quality of end product. Bread making potential can be estimated by two widely used tests. The wheat- meal fermentation time test which is also known as the Pelshenke test or dough-ball test (AACC, 1983 Method 56-50) is used in many countries. The sedimentation test (AACC, 1983 Method 56-61A) or its adaptations (AACC, 1983 Method 56-62, 56-63 and 56-70) developed by Zeleny is another useful technique to measure the bread making strength of wheat. The sedimentation test requires less time than the former technique.

Amylase activity: Wet weather before the harvest when wheat crop is matured can cause sprouting (pre-harvest sprouting) of the grains and such sprouted grains have high α-amylase activity. In some cases though sprouting of kernels is not visible, the level of α-amylase activity remains high. Increased amylase activity with high level of other proteolytic activities have negative effect on bread making quality (Fox and Mulvihill, 1982) The falling number test (AACC, 1983 Method 56-81B) is a more practical method for measuring α-amylase activity than the amylograph method (AACC, 1983 Method 22-12).

Crude fiber and ash content: These two factors are inversely related to flour yield and are related to amount of bran in wheat grain. On percentage basis, small or shriveled kernels have more bran that gives more crude fiber and ash

resulting in lower flour yield in comparison to normal plump wheat grains. Wheat generally contains 2.0-2.7% crude fiber and 1.4-2.0% ash considering 14% moisture content in grain.

Fat acidity: Under ideal storage conditions, wheat and other grains can be kept for longer time as the chemical changes in the grain are very slow. However, in unfavorable conditions, these changes occur rapidly resulting in deterioration of the grain. At earlier stage of deterioration, high lipase activity breaks down fats resulting in increased free fatty acids. Therefore, to measure the degree of soundness of grains a fat acidity test (AACC, 1983 Methods 02-01A, 02-02A, 02-03A and 02-04A) has been developed, which is defined as the number of milligrams of potassium hydroxide required to neutralize the free fatty acids from 100 gm of grain and is calculated on moisture-free basis. Normal wheat grains have fat acidity value around 20 and deteriorated wheats have high values reaching up to 100 in extreme cases.

1.6 Role of glutenin and gliadin in wheat grain quality:

Common wheats cover a wide range of technological characteristics and hence end uses. The main products from these wheats can be classified into relatively distinct groups based on grain hardness and protein content. The term 'quality' means the performance of a cultivar at a specific protein level, in a test that reflects a specific end product. When the cultivars are selected on similar protein quality, their breadmaking potential is directly related to the protein content. In such cases, protein content becomes an accurate measure of breadmaking potential and thus, it is used to market wheat by several wheat exporting countries. However, functional properties of flour, such as ability of hard wheat flour to make bread, depends on other constituents of the flour, the ingredient and process used. Work done on wheats with similar hardness have shown that the variation in protein quality for breadmaking depends almost on variation in glutenin components of flour protein (Orth and Bushuk, 1972).

Wheat storage proteins namely, gliadin and glutenin are the main component of gluten, which is the major contributor to the rheological and bread making properties of wheat flour. Glutenin is a high molecular weight protein (MW > 1 million) made up of about 20 polypeptide subunits linked by disulfide bonds. It forms about 40% of the flour protein and contributes significantly to viscoelastic properties of the doughs. Early studies on glutenin structure in relation to functionality focussed on composition of its HMW subunits. These subunits are controlled by three loci, located on long arm of chromosome 1A, 1B and 1D and denoted as *Glu-1A*, *Glu-1B* and *Glu-1D,* respectively (Payne *et al.* 1987). To correlate the breadmaking potential with HMW subunit composition, two statistical approaches have been used. Based on the SDS-sedimentation volume, Payne (1987) assigned quality score to each subunit and then individual values were summed to calculate the total quality score for a wheat cultivar, with maximum score of 10. In another approach, Ng and Bushuk (1988) developed an equation for predicting the unit loaf volume, based on HMW subunit composition of a bread wheat cultivar. Further work in this area has suggested the role of specific HMW subunit in contribution of glutenin to breadmaking quality. Payne *et al.* (1979) observed a direct relationship between breadmaking and proportion of subunit *Glu-1Ax1.* Several studies have shown that amount and relative proportion of HMW subunit is an important factor in functional performance for breadmaking quality (Kolster *et al.* 1992; Kolster and Vereijken, 1993; Marchylo *et al.* 1992). Khelifi and Branlard (1992) and Menifesto *et al.* (1998) have shown that allelic variations in composition of LMW glutenin subunits also contribute to breadmaking quality, however, determination of the best combination for LMW subunits is in progress. It is generally accepted that glutenins are mainly responsible for viscoelastic properties, but gliadins are important in conferring extensibility to dough. Three loci, *Gli-A1*, *Gli-B1* and *Gli-D1,* that are located on distal end of chromosome arms 1AS, 1BS and 1DS respectively, encode the ω-gliadins, most of γ-gliadins and some β-gliadins. Separate complex loci on group-6 chromosome (*Gli-A2*, *Gli-B2* and *Gli-D2*) encode other gliadins (Skerritt, 1998). Dal Belin Peruffo *et al.*

(1985) have identified individual gliadins encoded by *Gli-B1* as markers of dough strength in bread wheat. Some gliadin alleles have also been shown to be positively associated to dough extensibility and dough strength (Branlard and Felix 1994, Metakovsky *et al.* 1997)

Many tests are developed for end use quality characteristics of bread wheat, which can be screened with small amount (~50 g) of wheat grains. These are grain hardness (PSI), protein content, which can be measured by NIR spectroscopy and protein quality (gluten strength) which can be predicted by different tests like SDS-sedimentation, micromixograph, SDS-PAGE of HMW glutenins and SDS-gel protein test (Bushuk, 1998). To assess the dough strength for breadmaking performance of a wheat variety, several tests based on dough rheology (farinograph, extensigraph, mixograph and alveograph) are available. However, ultimate and best screening test for breadmaking potential is the bake test, which reflects the product that will eventually be made from a wheat variety. For the soft common wheats, which are generally used for other types of bread like French bread, Arabic flat bread and other noodles, both starch and protein quality are important factors. Wheats that are very soft, with lowest protein content and weakest dough strength are more suitable for cookies, cakes and some type of noodles. The early generation test for such wheat grains include grain hardness (PSI), protein content and Pelshenke test or SDS- sedimentation test. At later stages as more grains are available, quality testing can be performed with Falling number value (for prediction of sprouting), ash content, flour colour etc, while the rheological tests include amylograph, farinograph and alveograph for this class of softer wheat.

1.7 Development of quality wheat through conventional plant breeding efforts:

Main objectives of plant breeding are to obtain increased yield, improved quality in terms of nutritional and technological aspects; and resistance to biotic and abiotic stresses. Conventional plant breeding usually involves production of variability by making sexual crosses among selected genotypes having

characters to be combined to develop population of plants with superior quality. Using conventional plant breeding methodologies, breeders have developed some of the varieties having good quality characters in last few years and there has been a continued increase in productivity of wheat in India. Earlier, the variety such as Kalyansona yielded better compared to Lerma Rojo 64 and WL 711 was more productive than Kalyansona. Varieties developed later, such as HD 2329, WH542 and PBW 343 registered better yield over one another. Recently, Singh *et al.* (2000) have reported few wheat varieties having better quality characters. For example, PBW 373 possesses the boldest grains with high protein content and produces best bread loaves, while best cookies are made from PBW 396.

One important limitation of conventional plant breeding is that the agronomically desirable characters are often ill-defined and many of them are polygenic. Another limitation is that the range of accessible genes is limited to related species that can be crossed sexually. Also, in conventional plant breeding efforts, breeders select desirable individuals from a large breeding material through the expression of a trait of interest, which is a time consuming, laborious and environment dependent process (Jones and Lindsey, 1999). Though breeders have been able to transfer many traits governed by one or few dominant genes, it becomes difficult to monitor the transfer of recessive genes. In case of complex quantitative traits such as yield, quality and tolerance to biotic/abiotic stresses, it is often very difficult and also requires multilocation evaluations with replications to identify individuals carrying such traits. This becomes even more difficult when the trait has low heritability, complex epistatic interactions and large environmental effects.

1.8 Quality improvement using genetic transformation and transgenic approach:

Rapid developments in genetic engineering and molecular biology have provided major advancement in modification of agronomically useful traits in crop plants. Useful genes for such important traits present in sexually

incompatible wild species can now be introduced either by cloning and transformation or by protoplast fusion (somatic hybridization) into desirable plants. It is now technically possible to identify, isolate, modify, transfer and obtain expression of a range of specific genes controlling novel traits to facilitate crop improvement for upgrading existing varieties as well as creating superior genotypes. In addition to this, modification of plants by various types of genetic manipulation, modern technologies of tissue culture and molecular biology can be useful for plant breeders in aspects of handling and multiplication of plants and accelerating the screening and selection procedures.

Breadmaking quality of wheat genotypes is determined by specific alleles of high-molecular weight glutenin subunits (HMW-GS). Gene dosage studies have indicated that breadmaking quality of wheat could be improved by integration and expression of specific HMW-GS genes (Flavell *et al.* 1989; Shewry *et al.* 1995). The manipulation of the proportion of HMW-GS that are known to be associated with good breadmaking quality and of the crosslinking by altering cystine residues, are the key targets for improvement of wheat grain quality. Blechl and Anderson (1996) introduced a gene encoding novel hybrid HMW-glutenin subunit (Dy10: Dx5), 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 analysis. Altpeter *et al.* (1996) used the gene of a chromosome 1A-encoded subunit (HMW-GS 1Ax1) known to be associated with good breadmaking quality, to develop transgenic wheat in cv. Bobwhite that lacks HMW-GS 1Ax1 by nature. They used biolistic bombardment method on cultured immature embryos and showed that the amount of HMW-GS 1Ax1 protein produced in different transgenic lines varied from 0.6% to 2.3% of total protein, resulting in an increase up to 71% in total HMW-GS proteins. In both of these studies, the total level of HMW-GS was more in transgenic seeds, indicating that the accumulation of this transgenic HMW-GS was not at the cost of their native counterpart and high level of expression of HMW-GS was maintained for several generations without any

structural instability. However, as cv. Bobwhite contains the chromosome 1BL/1RS translocation associated with the sticky dough, it's not an ideal background to explore the functionality of HMW-GS (Barro *et al.* 1997). To study the effects of HMW subunit transgene expression on dough properties in a suitable background, Barro *et al.* (1997) transformed two near-isogenic lines of wheat, which were derived from crossing mutants of the cultivars Olympic and Gabo with null alleles at the *Glu-1* (HMW subunit) loci. The effect of this transformation with one or two HMW subunit genes was determined using a small-scale mixograph, which showed stepwise increase in dough elasticity. Similarly, 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 pasta wheat with *Hordeum chilense* (Rooke *et al.* 1999a). These examples have demonstrated that genetic engineering can be used to improve the functional properties of wheat grain. However, high level expression can also lead to dramatic changes, which can be detrimental to bread making quality (Rooke *et al.* 1999b). Transformation with both the existing and novel, modified genes will be useful in bioengineering of wheat and resulting loci are expected to be useful in modifying and improving wheat grain quality characteristics.

1.9 DNA marker technology and wheat quality improvement:

Wheat research is benefited from rich tools for genetic analysis in the form of several aneuploid stocks in the variety Chinese Spring developed by Sears (1966). By 1980, around 100 genes had been placed on the classical wheat map with cytogenetic analysis. Hart (1987) placed numerous isozyme loci on the wheat chromosomes using the Chinese Spring stocks. Efforts on wheat genome mapping using DNA markers started after formation of the International Triticeae Mapping Initiative (ITMI) in 1990. However, the progress in developing saturated map was slow because of large genome size of wheat ($16x10^9$ bp/1C) (Bennett and Smith, 1976), polyploid nature, high proportion of repetitive DNA and lack of sufficient polymorphism. RFLPs were the first marker

systems, which were employed for genome mapping for humans (Botstein *et al.* 1980) and later they were utilized in plant genome mapping (Helentjaris *et al.* 1986, Weber and Helentjaris, 1989) including wheat (Chao *et al.* 1989, Liu and Tsunewaki 1991, Anderson *et al.* 1992, Devos *et al.* 1992, Devos and Gale 1993a, b, Xie *et al.* 1993, Nelson *et al.* 1995a, b, c, Marino *et al.* 1996). As low level of polymorphism was detected with D-genome of bread wheat, *Aegilops tauschii* was used to prepare RFLP maps of D-genome (Lagudah *et al.* 1991, Gill *et al.* 1991, Gill *et al.* 1993). With further technological advancement, new type of DNA markers were employed in wheat genome mapping leading to its well-established genetic linkage maps as summarized in Table-1.3.

With the development of highly informative and technically simple marker systems, marker based analysis of agronomically important traits in wheat has gained momentum. Molecular markers associated with a number of genes/QTLs for disease resistance, physiological responses and yield-related traits have been identified in wheat as well as in other crops. In wheat, several molecular marker approaches have facilitated the identification of chromosomal regions associated with agronomically important traits like dwarfing and vernalization response (Korzun *et al,* 1997), leaf rust resistance (Feuillet *et al.* 1995, 1997; Naik *et al.* 1998), cadmium uptake (Penner *et al.* 1995), pre-harvest sprouting tolerance (Roy *et al.* 1999), resistance to common bunt (Demeke *et al.* 1996) and powdery mildew resistance (Qi *et al.* 1996). Many researchers across the globe are engaged in overall improvement of wheat quality. For example, molecular markers have been utilized to identify a number of QTLs such as kernel traits, kernel hardness and flour viscosity in wheat (Campbell *et al.* 1999, Sourdille *et al.* 1996, Udall *et al.* 1999) as well as in other crops like in maize for grain yield, protein and starch concentration (Stuber *et al.* 1987, Goldman *et al.* 1993, Austin and Lee, 1998), in rice for cooking and eating quality (Tan *et al.* 1999) and in soybean for seed oil and protein content (Lee *et al.* 1996). These efforts for wheat are summarized in Table-1.4. Similarly, DNA marker technology has been used extensively for tagging/

Table-1.3: Molecular maps of wheat (*Triticum* spp.).

No.	Genome	Type of marker	No. of mapped loci	Mapping population	Reference
1	Bread wheat	RFLP, SSR	264, 2	'Chinese Spring' x 'Courtot' DH population	Cadalen *et al.* (1997)
2	Bread wheat	STMS	279	'Opata85' x 'W7984'	Roder *et al.* (1998)
3	Bread wheat	SSR	53	'Chinese Spring' x 'Synthetic'	Stephenson *et al.* (1998)
4	Bread wheat	AFLP, SSR	620, 42	'Garnet x 'Saunders' DH population	Penner *et al.* (1998)
5	Bread wheat	RFLP	197	*T. aestivum* cv. 'Chinese Spring' x *T. spelta* var *dutamellanum*	Liu and Tsunewaki (1991)
6	Durum wheat	RFLP, PCR, Biochemical, Morphological	259	*T. turgidum* (Messapia x MG4343)	Blanco *et al.* (1998)
7	Durum wheat	AFLP, RFLP	88	*T. turgidum* (Messapia x MG4343)	Lotti *et al.* (2000)
8	Einkorn wheat	RFLP, RAPD, ISSR	81	*T. monococcum* x *T. boeoticum* spp. *boeoticum*	Kojima *et al.* (1998)
9	Wild emmer wheat	AFLP, RAPD, STMS	543	*T. dicoccoides* var. *Hermon 52* x *T. durum* var. *Langdon*	Peng *et al.* (2000)
10	D-genome progenitor *Aegilops tauschii*	RFLP	546	*T. tauschii* (TA 1691 x TA 1704)	Boyko *et al.* (1999)
11	D-genome	RFLP	127	*T. tauschii* (TA 1691 x TA 1704)	Gill *et al.* (1991)
12	D-genome	STMS	55	'Opata85' x 'W7984'	Pestsova *et al.* (2000)
13	Durum wheat	RFLP, AFLP, SSR, Storage Proteins	306	'Jennah' x 'Khetifa'	Nachit *et al.* (2001)
14	Bread wheat	RFLP, Microsatellites	230	*T. aestivum* var 'Forno' x *T. spelta* 'Oberkulmer'	Messmer *et al.* (1999)

Table-1.4: Tagging/mapping of gene/ QTL governing traits for wheat quality improvement.

Trait	Gene/QTL	Type of marker	Population	Reference
Grain protein content	QTL	RFLP	65 RILs (F_7)	Blanco *et al.* (1996)
	Major locus	RFLP	3 RIL populations	Mesfin *et al.* (1999)
	QTL	STMS	100 RILs (F_8)	Prasad *et al.* (1999)
Preharvest sprouting tolerance	QTL	RFLP	2 RIL (F_5) populations	Anderson *et al.* (1993)
	Major gene	STMS, STS	100 RILs (F_5)	Roy *et al.* (1999)
	QTL	RFLP, STMS	204 RILs (F_5)	Zanetti *et al.* (2000)
Vernalization response	*Vrn1*	RFLP	-	Galiba *et al.* (1995)
		RFLP	Molecular mapping	Nelson *et al.* (1995a)
		RFLP, STMS	114 F_2 lines	Korzun *et al.* (1997)
	Vrn-A^{m}1, Vrn-A^{m}2	RFLP	Molecular mapping	Dubcovsky *et al.* (1998)
Bread making quality	*Glu-D1*	PCR	-	D'Ovidio and Anderson (1994)
	QTL	RFLP, STMS	204 RILs (F_5)	Zanetti *et al.* (2001)
	QTL	Biochemical, RFLP	187 DH lines	Perretant *et al.* (2000)
Grain/kernel hardness	*Ha*	RFLP	-	Nelson *et al.* (1995a)
		RFLP	114 RILs (F_7)	Sourdille *et al.* (1996)
	QTL	RAPD, ISSR	100 RILs (F_7)	Galande *et al.*(2001)
Red grain colour	*R1*, *R3*	RFLP	Molecular mapping	Nelson *et al.* (1995a)
Flour colour	QTL	RFLP, AFLP	150 F_4 lines	Parker *et al.* (1998)
	Major locus	STS	150 F_4 lines	Parker and Langridge (2000)
Milling yield	QTL	RFLP, AFLP	150 F_4 lines	Parker *et al.* (1999)
Amylose content	*Wx-B1*	RFLP	98 Single chromosome substitution lines	Araki *et al.* (1999)
Noodle quality	*GBSS*	PCR	-	Briney *et al.* (1998)
Flour viscosity	QTL	RFLP	78 RILs	Udall *et al.*(1999)
Kernel morphology and texture	QTL	RFLP, STMS, PCR	78 RILs	Campbell *et al.* (1999)
Grain yield and 50-grain weight	QTL	RFLP	3 RIL (F_5) Populations	Kato *et al.* (2000)
1000-Grain weight	QTL	STMS	100 RILs	Varshney *et al.* (2000)

mapping of different agronomically important traits (Table -1.5) as well as for disease resistance gene(s) (Table-1.6)

1.10 Genesis of thesis:

Among the cereals, wheat is a unique gift of nature to the mankind as once it is converted into dough, it can be molded into innumerable products of the choice. Wheat in India is largely consumed in the form of *chapati*. However, due to improvement in living standards of the population, there is an increased demand for convenience foods that has led to rise in consumption of bakery products. As in past few years, there has been surplus production of wheat, increasing proportion of wheat is now being milled into flour to be used in production of bread, biscuits and other products. In this context, quality of Indian wheat needs to be improved not only for domestic usage, but also for achieving the international standards for export purpose. The quality requirements of these various products are different and they determine suitability of a wheat variety for particular end use. Most of the characters/ traits, which govern grain quality for different end products, are polygenic in nature and hence it is difficult to identify and isolate genes governing these traits by conventional methods of plant breeding. Recent advancement in different biotechnological approaches offers better options to improve the wheat grain quality. One such approach of molecular marker technology has already shown a greater improvisation in plant breeding research by way of marker assisted selection both for qualitative and quantitative traits. When I joined Plant Molecular Biology Unit at NCL, work on wheat genome analysis was already in progress. I found this study fascinating and hence, undertook the work involving identification of molecular markers associated with the quantitative traits governing grain quality in hexaploid wheat. The work described in this thesis includes grain quality parameters namely grain protein content, grain size and grain shape traits and yellow berry tolerance in wheat.

Table-1.5: Tagging/mapping of agronomically important traits in wheat

Trait	Gene/ QTL	Type of marker	Reference
Cadmium uptake	*Cdu1*	RAPD	Penner *et al.* (1995)
Dwarfing	*Rht12*	RFLP, STMS	Korzun *et al.* (1997)
	Rht-1B, Rht-1D	RFLP	Sourdille *et al.* (1998)
Fertility restoration	*Rf3*, *Rf4*	RFLP	Ma and Sorrells (1995)
	Rf3	RFLP	Kojima *et al.* (1997)
Haploid formation	QTL	RFLP	Torp *et al.* (1998)
Heading time and Photoperiod response	QTL	RFLP	Sourdille *et al.* (2000a)
Ear compactness, Spike length and No. of spikelets	QTL	RFLP	Sourdille *et al.* (2000b)
Free-threshing habit	QTL	RFLP	Simonetti *et al.* (1999)
Aluminum tolerance	*Alt2*	RFLP	Luo and Dvorak (1996)
Boron toxicity	QTL	RFLP, AFLP	Jefferies *et al.* (2000)
Ear emergence time	QTL	RFLP	Kato *et al.* (1999)
Plant height	QTL	RFLP, PCR	Cadalen *et al.* (1998)
	QTL	RFLP	Kato *et al.* (1999)

Table-1.6: List of disease resistance gene(s) tagged/ mapped in wheat

Trait	No. of genes tagged/mapped	Technique
Powdery mildew resistance	12	RFLP, AFLP, RAPD, STS
Cereal cyst nematode resistance	3	RFLP, STMS
Wheat streak mosaic virus resistance	1	RAPD, STS
Hessain fly resistance	14	RFLP, RAPD
Common bunt resistance	2	RAPD
Karnal bunt resistance	1	RFLP
Stem rust resistance	7	RFLP, STS, SCAR, RGA clone
Leaf rust resistance	20	RFLP, RAPD, STS, STMS, SCAR, RGA clone
Stripe rust resistance	3	RFLP, RAPD, STMS
Loose smut resistance	2	RFLP, RAPD
Fusarium head blight (FHB) resistance (scab resistance)	1	RFLP, AFLP
Russain wheat aphid resistance	1	RAPD, SCAR
Eye spot disease	1	RFLP
Tan spot disease	1	RFLP
Septoria nodorum blotch resistance	1	RAPD
Septoria tritici blotch resistance	1	AFLP

I have organized my thesis in the following order:

Chapter-1: Review of literature

Chapter-2: Protein content in wheat: A trait analyzed at two agroclimatic zones in India using molecular markers

Chapter-3: Molecular marker analysis of wheat grain size, a determinant of consumer preference

Chapter-4: Dissection of grain shape and size traits in wheat using PCR-based DNA markers

Chapter-5: Yellow berry tolerance in wheat: Inheritance and molecular marker studies

Thesis summary and future directions

Bibliography

Curriculum vitae

Chapter 2

Protein content in wheat: A trait analyzed at two agroclimatic zones in India using molecular markers

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Part of the contents of this chapter has been accepted as a full-length paper in Biochemical Genetics (2001) (In Press)

ABSTRACT:

Grain protein content (GPC) of hexaploid wheat is one of the important factors that determines the end product quality as well as plays a pivotal role in human nutrition. In an attempt to identify PCR-based DNA markers linked to GPC, 106 recombinant inbred lines (RILs) were developed by single seed descent method from a cross between two wheat cultivars PH132 and WL711 which differ significantly in GPC. The RILs were phenotyped for GPC at two diverse agroclimatic locations, namely, Pune and Ludhiana as this trait is influenced by environment. Screening of parents with different PCR-based markers, the selective genotyping and whole population analysis revealed fifteen markers associated with the trait. Two markers ($UBC844_{1100}$ and $OPA4_{800}$) were observed to be associated with the trait in both the locations, whereas six and seven markers were found to be specific to Pune and Ludhiana, respectively. Finally, the markers associated with GPC at Pune and Ludhiana together accounted for 18.8% and 24.0% of phenotypic variation, respectively.

2.1 INTRODUCTION:

Hexaploid wheat (*Triticum aestivum* L.), the second most important cereal crop in the world, is used in preparation of many food products like various types of breads, biscuits, cookies, cakes, noodles etc. The quality of these end products depends on specific characters like grain size, grain hardness, amount of protein and starch in wheat seeds. Among these, grain protein content (GPC) as well as its specific subunit composition are very important determinants of grain quality. For example, presence of HMW glutenin subunit 5 + 10 imparts good bread making quality (Dong *et al.* 1991) while 2 + 10' is associated with poor bread making quality (Rodriguez-Quijiano and Carrillo, 1996). Wheats need to have certain minimum levels of protein content and quality for good bread making (Bhatt and Derera, 1975). Although the knowledge about genetic control of specific subunits of seed storage proteins like gliadin and glutenin of wheat and their regulation is advancing (Muller *et al.* 1995), the physiology and genetic control of total GPC in wheat grain is poorly understood (Barneix *et al.* 1998). The increase in GPC, which has been achieved with the use of nitrogenous fertilizers, it is restricted due to negative correlation between productivity and GPC (Cox *et al.* 1985; Day *et al.* 1985). On the contrary, the possibility of increasing GPC without affecting the productivity has been reported in previous studies (Johnson and Mattern, 1987; Day *et al.* 1985). Recently, analysis of 12 high and 12 low GPC RILs (F_7) developed from two crosses namely, ND683/Bergen and Glupro/Bergen has shown the overlapping distribution of grain yield between the two groups, indicating that the lines with high GPC and acceptable yield level can be obtained and selected in these populations (Mesfin *et al.* 2000).

GPC is governed by a complex genetic system and is influenced by environment. Halloran (1975) and Konzak (1977) have shown that a few major genes with many other genes of small effect govern the trait. Morris *et al.* (1978) have used intravarietal chromosome substitution lines to show a high GPC gene in variety 'Atlas 66' on chromosome 5D. However, reports by Kuspira and Unrau (1957) and Diehl *et al.* (1978) have suggested quantitative nature of

GPC trait with presence of several genes distributed throughout the wheat genome. Kuspira and Unrau (1957) have evaluated intervarietal chromosome substitution lines of Chinese Spring and have reported that chromosomes 3D, 4D, 5A, 5B and 7B affect GPC. Studies on wild emmer wheat (*T.turgidum* L. var. *dicoccoides*) have shown presence of genes for high GPC on group 1, 5 and 7 chromosomes (Leavy *et al.*1988). Stein *et al.* (1992) have reported that 'Plainsman V' may have genes for increased GPC on chromosome 1A, 1B and 7A and 'Wichita' may have on chromosome 5B. Molecular studies carried out on tetraploid wheat (Blanco *et al.* 1996, Joppa and Cantrell 1990; Joppa *et al.* 1997) and hexaploid wheat (Prasad *et al.* 1999) have also suggested polygenic nature of this trait with presence of several QTLs on different chromosomes. This makes it difficult to follow inheritance of GPC and its manipulations with conventional breeding programs.

Molecular marker-assisted breeding has received a considerable attention in recent years. To overcome the difficulties associated with RFLP markers to apply them cost-effectively in plant breeding programs, PCR based DNA markers have emerged as a powerful tool for genetic analysis due to their simplicity, easy handling and ability to generate higher level of polymorphism. Randomly amplified polymorphic DNA (RAPD) markers have become popular in short time because of their simple technical protocol and speed. RAPD markers have been used for tagging and mapping genes in wheat (Naik *et al.* 1998; Penner *et al.* 1995) as well as in other crops like tomato (Martin *et al.* 1991). Recently, RAPD markers have been utilized for mapping the shell thickness locus in oil palm (Moretzson *et al.* 2000).

Microsatellites or Simple Sequence Repeat (SSR) markers with tandem repeats of a basic motif of <6 bp are the most polymorphic and hence are highly useful markers in wheat (Roder *et al*. 1998) for applications such as tagging of stripe rust resistance gene (Peng *et al.* 1999). Inter Simple Sequence Repeat (ISSR) markers (Zeitkiewicz *et al.* 1994) have also emerged as an alternative system with reliability and advantages of microsatellites (SSR) along with broad taxonomic applicability of RAPDs. The technique

involves amplification of genomic segments flanked by inversely oriented, closely spaced microsatellite sequences by a single primer based on SSR anchored at 5' or 3' with 2-4 purine or pyramidine residues. Several studies have indicated that ISSR markers are potentially useful for cultivar identification and for phylogenetic studies (Blair *et al*. 1999, Charters *et al*. 1996, Fang and Roose, 1997, Gupta *et al*. 1994, Joshi *et al*. 2000, Kantety *et al.* 1995, Nagaoka and Ogihara, 1997, Parsons *et al*. 1997, Prevost and Wilkinson, 1999, Wu and Tanksley 1993, Zietkiewicz *et al*. 1994). Nagaoka and Ogihara (1997) have shown that ISSRs are more informative compared to RFLP and RAPD markers in wheat. The utility of ISSR markers for tagging agronomically important genes has been first demonstrated by Akagi *et al.* (1996) by identifying tight linkage with a nuclear restorer gene in rice. Similarly, Tsumura *et al.* (1996) have reported the Mendelian inheritance of ISSR markers in Douglas fir and sugi. Ratnaparkhe *et al.* (1998) have studied the inheritance of ISSR markers in chickpea and established linkage between an ISSR marker and Fusarium wilt resistance gene. Kojima *et al.* (1998) have shown the usefulness of ISSR and RAPD markers in genetic linkage map construction in Einkorn wheat. In the present chapter, I report identification of PCR-based markers associated with GPC in bread wheat and demonstrate their utility in studying the influence of varied agroclimatic conditions on such an important agronomic character.

2. 2 MATERIALS AND METHODS:

2. 2. 1 Plant material and phenotypic evaluation:

A set of 106 RILs (F_7) was developed from a cross between the hexaploid wheat cultivar, PH132 (high GPC) and WL711 (low GPC) following Single Seed Descent method at Punjab Agricultural University (P. A. U.), Ludhiana (Punjab, India). Parental genotypes alongwith RILs were grown in a field station at Hol, near Pune in 11 X 11 lattice design with two replicates for two successive seasons i.e.1996-97 and 1997-98. The RILs were orderly

planted in doubled rows, with 2m rows having distance of 23 cm and the fertilizer applied was in a ratio of 120kg N_2: 60kg P: 40 kg K_2O in field experiments. Phenotypic data of GPC was also collected from RILs grown for one season at P.A.U., Ludhiana to study the influence of another agroclimatic condition on this trait. These two locations represent two agroclimatic zones, which have a major share in annual wheat production. The GPC of parents as well as of the RILs was determined by Kjeldahl method (AACC method 46-10,1983) on gel-tech autoanalyser (Tecator, Sweden) calibrated for the estimation of Nitrogen content. The GPC was then calculated using the following formula:

GPC (%) = Nitrogen content x 5.7

Seeds of nullitetrasomic and ditelosomic lines in the background of Chinese Spring were obtained from Prof. B. S. Gill, Kansas State University, U.S.A. Genomic DNAs isolated from these lines were used in PCR to assign the markers found to be linked with GPC on specific wheat chromosomes (detailed in marker localization section 2.2.5).

2. 2. 2 DNA isolation:

Genomic DNAs were extracted from the leaves of 15 day old seedlings of parents, RILs and nullitetrasomic as well as ditelosomic lines, grown in glasshouse, by modified CTAB (Hexacetyltrimethyl ammonium bromide) protocol of Rogers and Bendich (1988). Young leaf tissue (10g) was ground to fine powder in liquid nitrogen using a mortar and pestle. To this, 2X CTAB buffer (2% CTAB, 100mM Tris-HCl pH 8.0, 20mM EDTA, 1.4 M NaCl, 1% PVP and 1% 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, following which it was equally distributed in SS34 centrifuge tubes. In each tube, an equal volume of Chloroform: IAA (24:1) mixture 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 room temperature in a Sorvall RC-5B centrifuge (Du Pont, U.S.A.). 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 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, 1 mM 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 (10 mM Tris-HCl pH 8.0, 1 mM 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 1 h. 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. The DNA concentration was estimated spectrophotometrically as well as by agarose gel (0.8%) electrophoresis by comparing with known concentration of λ DNA.

2. 2. 3 PCR amplification:

(A) ISSR analysis:

From a set of 100 ISSR primers obtained from Biotechnology Laboratory, University of British Columbia, Canada (UBC set no. 9), 85 primers were used in PCR analysis. Amplifications were carried out in 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1mM dNTPs, 2.0% formamide, 0.4 mM spermidine, 0.2 µM primer, 0.8 unit of Taq DNA polymerase (Perkin Elmer, U.S.A.) and 15ng of genomic DNA per 25 µl reaction. The PCR conditions included initial denaturation for 5 min at 94° C, followed by 45 cycles of 30 s at 94° C, 45 s at 50° C, 2 min at 72° C and final 5 min extension at 72° C.

(B) RAPD analysis:

Three hundred and fifty decamer random primers of series A to P, R and V from Operon Technologies, U.S.A., were used in this study. 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 $MgCl_2$, 0.1mM dNTPs, 0.4 mM spermidine, 5 picomoles of primer, 0.8 unit of Taq DNA polymerase (Perkin Elmer, U.S.A.) and 20ng of genomic DNA per 25 µl reaction. The PCR conditions include, initial denaturation of 4 min at 94° C, followed by 5 cycles of 30 s at 92° C, 2 min at 35° C, 1.5 min at 72° C; 35 cycles of denaturation at 92^0 C for 5 s, annealing at 40^0 C for 20 s, extension at 72^0 C for 1.5 min with final extension at 72^0 C for 5 min. .

Polymerase chain reactions of ISSR and RAPD were performed in PTC-200 thermal cycler (M. J. Research Co., USA.) and the amplified products were electrophoresed on 1.5% agarose gel in 0.5X TAE (0.02M Tris-acetate, 0.0005M EDTA pH 8.0) buffer for further detection with ethidium bromide staining.

(C) Microsatellite analysis:

Seventy-eight microsatellite primer pairs selected from previously published wheat linkage map (Roder *et al.* 1998) were used for the analysis. The PCR mixture contained 100nM of each primer, 200µM of each dNTP, 1.5mM $MgCl_2$, 0.8 unit of Taq DNA polymerase (Perkin Elmer, U.S.A.) and 50 ng of template DNA in 25 µl reaction volume. The PCR program included 95^oC of initial denaturation for 5 min, followed by 35 cycles of 1 min at 94^oC, 1min at either 50^oC, 55^oC, 60^oC or 63^oC (depending on GC content of individual microsatellite), 2 min at 72^oC and final extension step of 5 min at 72^oC. All PCR amplifications using STMS primers were performed in Gene-Amp 9700 thermal cycler (Perkin Elmer, U.S.A.). The amplified products were resolved on 3% metaphor agarose (FMC Bio Products, U.S.A.) gels in 1xTAE (0.04M Tris-acetate, 0.001M EDTA pH 8.0) buffer, followed by ethidium bromide staining.

2. 2. 4 Data analysis:

The markers, which were found to be polymorphic in parental screening, were used for selective genotype analysis (Lander and Botstein, 1989; Darvasi and Soller, 1992) using 10 RILs from each extreme representing the highest/lowest GPC in population. The markers, which were found to be co-segregating with phenotypic trait values in selective genotyping, were further analyzed on the entire population of 106 RILs along with parents. The genotype of each RIL with ISSR and RAPD was scored as presence or absence of amplified DNA marker as these are dominant markers. STMS being co-dominant marker, genotype of each RIL was scored based on the size variation of the alleles in the parents. Linkage relationships among the markers were determined by maximum likelihood analysis of the segregation pattern using Mapmaker (ver 3.0) (Lander *et al.* 1987). Simple linear regression analysis was performed using QGENE software (Nelson, 1997) to determine the significant association between markers and phenotype of each RIL. Only significant LOD scores (> 2.0) were interpreted to indicate cosegregation of putative QTLs for GPC and markers. Multiple regression model of QGENE was used to determine total phenotypic contribution by the different markers associated with GPC. Analysis of variance for lattice design was performed using AGROBASE software (Mulitze 1998). Genotypic and phenotypic variances were computed by the expectations of mean squares from analysis of variance and these were in turn used to estimate heritability (h^2). Genotypic variance was calculated using formula $R_g^2 = R_p^2 / h^2$, wherein R_g^2 = genotypic variance and R_p^2 = total phenotypic variance.

2. 2. 5 Marker localization:

The genomic DNAs of nullitetrasomic and ditelosomic lines of Chinese Spring (Sears 1966; Sears and Sears, 1978) were used to localize markers linked to the trait on specific wheat chromosome arms based on presence or absence of marker fragment in these lines. For example, if a specific marker

band was observed to be absent in nullitetrasomic lines N1A T1B and N1A T1D, was present in rest of the lines in equal intensity and present in lines N1B T1A and N1D T1A in double intensity (as both these lines have double set of 1A chromosome), then the marker was assigned to chromosome 1A. Further, the location of this marker band was confirmed to chromosome arm using ditelosomic lines from chromosome 1A, i.e. 1AS and 1AL. If the marker band was absent in 1AS and present in 1AL, it was assigned to long arm of chromosome 1A.

2. 3 RESULTS AND DISCUSSION:

2. 3. 1 Protein content loci interact with environment:

Two bread wheat genotypes, PH132 and WL711, which were used to generate RILs differed significantly in GPC (16.3% and 12.3%, respectively with mean values of two years' data at Pune location). One hundred and six RILs were grown at two different locations namely, Pune and Ludhiana, which come under two diverse agroclimatic zones (west plains zone and north zone, respectively) and contribute to a major share of annual wheat production in India. These RILs were phenotyped for GPC and mean of the two years GPC ranged from 12.3% to 19.0% at Pune whereas it ranged from 9.3% to 13.6% for one year at Ludhiana. Although Dhaliwal *et al.* (1994) showed the presence of two partially dominant genes in this cross, the frequency distribution curve (Fig. 2.1) in this analysis showed normal distribution in both the locations, indicating polygenic nature of the trait. This is supported by an earlier report (Prasad *et al.* 1999) confirming the role of several loci in controlling the trait in the same cross. The analysis of variance indicated that the GPC values of parental genotypes and RILs, grown at Pune location were significantly (P<0.05) higher (3-4%) compared to Ludhiana, which might be attributed to environmental influence on this trait. Transgressive segregation was observed towards high GPC at both the locations. Similar results were obtained for QTL mapping of GPC in *Triticum dicoccoides* by Gonzalez-Hernandez *et al*. (1998) using recombinant inbred

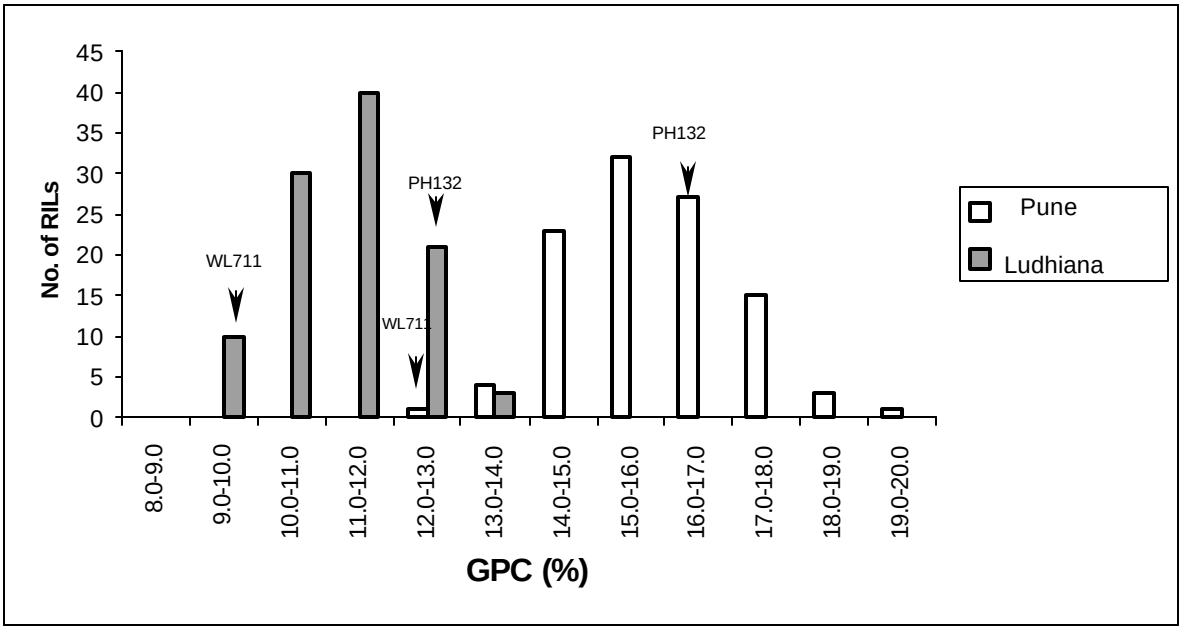

Fig 2.1: Frequency distribution of GPC in PH132 X WL711 RILs at two locations. Arrows indicate parental values at respective locations.

chromosomal lines (RICLs) for chromosome 5B. The GPC in these lines grown at three different locations showed significant difference as well as transgressive segregation indicating genotype and environmental interactions contributing to GPC.

2.3.2 DNA polymorphism in parental analysis:

The DNA polymorphism between parental genotypes was assessed using 85 ISSR, 350 RAPD and 78 STMS primers. In ISSR analysis, 50 primers generated scorable amplification among which 14 primers were polymorphic in the parents. This resulted in total of 65 amplified products, from which 18 were polymorphic between parental pair, depicting 28% polymorphism, while in case of RAPD analysis, 72 primers produced distinct polymorphism between the two parents. This resulted in total of 340 amplified marker loci, and 85 of them were polymorphic between parental combination with 24% polymorphism. This analysis showed that ISSR, which are based on anchored, repeat primers were probably a better choice as molecular markers compared to random primers in wheat. Similarly, Nagaoka and Ogihara (1997) also reported that ISSRs were more polymorphic in wheat in comparison to RFLP and RAPD. In case of STMS analysis with 78 primer pairs attempted, 65 pairs gave amplification and total 19 pairs produced scorable polymorphic pattern (30% polymorphism). Comparatively, microsatellite analysis showed more polymorphism than RAPD and ISSR markers and this was in accordance with the reports of Plaschke *et al*. (1995), Roder *et al.* (1995), Ma *et al.* (1996) and Bryan *et al*. (1997).

2.3.3 Identification of markers segregating with GPC trait:

In order to minimize the time and cost of analysis, all the polymorphic markers were analysed using selective genotyping approach (Darvasi and Soller 1992, Lander and Botstein 1989). For this purpose, 10 RILs of low GPC and 10 RILs of high GPC representing two extreme ends of the frequency distribution curve were subjected to PCR analysis using polymorphic DNA markers. Out of 122 polymorphic markers, 9 ISSR, 9 RAPD and 10 STMS

markers were found to be cosegregating with the phenotype of selected RILs. These markers were then attempted with the total RIL population containing 106 lines. Figures 2.2a and 2.2b show the segregation profiles of marker $UBC880_{1100}$ and Xgwm604 respectively, with parents and the representative RILs of GPC. As seen in Fig. 2.2a, the amplified band of 1100 bp is present in low GPC parent WL711 and it is absent in high GPC parent PH132. Out of 26 representative RILs, 12 RILs show presence of this marker whereas in remaining 14 RILs it is absent. Similarly, as shown in Fig. 2.2b a band of 125bp from marker Xgwm604 is present in PH132 (lane b), its allele of 115bp in WL711 (lane a) and out of 26 representative RILs, 19 show presence of the 125bp band, while remaining 7 RILs have an allele of 115bp. Majority of the 28 putative markers, identified with selective genotyping, showed expected 1:1 segregation at $P \leq 0.05$ by chi-square test when tried with whole population of 106 RILs.

In order to identify linkage among putative markers, Mapmaker (ver. 3.0) analysis was performed which revealed three linkage groups, one comprising 7 markers (Xgwm261, Xgwm219, Xgwm604, Xgwm644, Xgwm146, $UBC\ 813_{1300}$ and $OPC11_{700}$), second group with 6 markers (Xgwm515, $UBC810_{900}$, $UBC825_{1000}$, $OPM5_{870}$, $OPO10_{870}$ and $OPV14_{1200,}$) and third group comprising two markers ($OPA4_{800}$ and $UBC880_{900}$), whereas remaining 13 markers were unlinked. Single marker analysis based on simple linear regression using QGENE software revealed a total of 15 markers namely, Xgwm219, Xgwm368, Xgwm499, Xgwm515, Xgwm604, $UBC810_{900}$, $UBC844_{1100}$, $UBC873_{750,}$ $UBC880_{1100}$, $OPA4_{800}$, $OPD20_{1000}$, $OPH4_{1400}$, $OPM5_{870}$, $OPO10_{870}$ and $OPV14_{1200}$ associated with GPC trait. As the data shown in Table-2.1 suggested that the phenotypic variation explained by individual marker was low, it could be extrapolated to indicate the presence of minor genes governing GPC in this

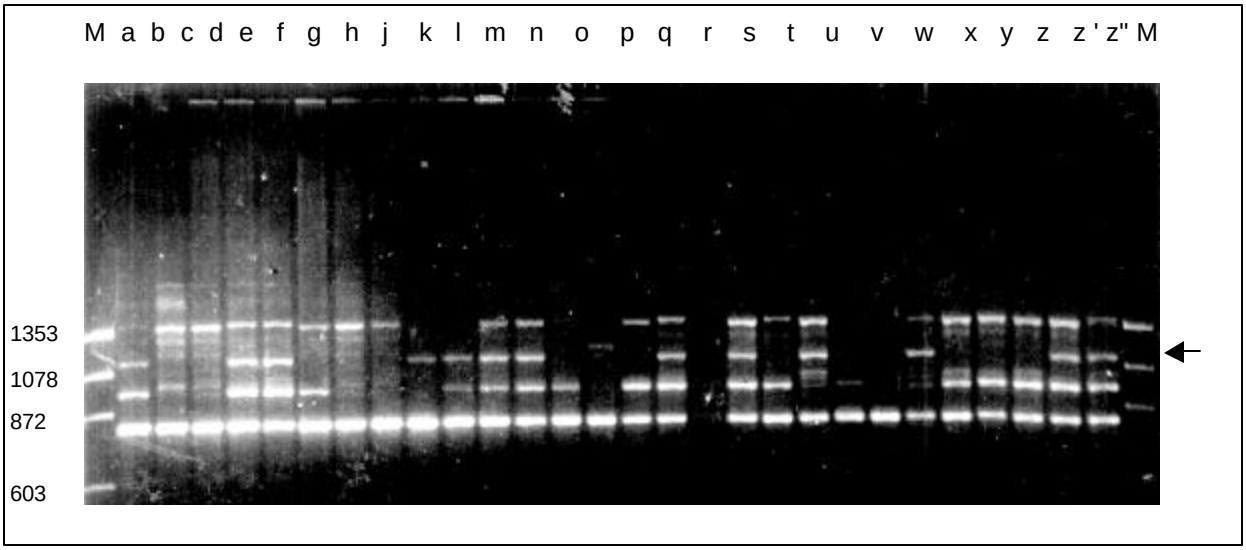

Fig 2.2a: Amplification profile of parents and RILs of GPC using $UBC880_{1100}$ marker.

M: f X 174/*Hae*III digest, Lane a: WL711, Lane b: PH132, Lane c-z": representative RILs of GPC segregating for the marker $UBC880_{1100}$, indicated with an arrow.

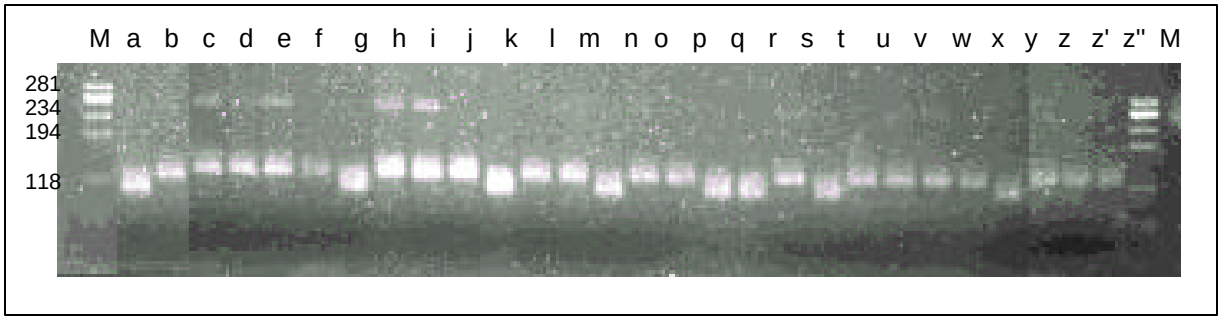

Fig 2.2b: Segregation of marker Xgwm604 in the RILs of GPC.

M= fX174/*Hae* III digest, Lane a: WL711, Lane b: PH132, Lanes c- z": RILs. A marker band of 125bp is present in PH132 and its allele of 115bp is present in WL711.

Table 2.1: Percent phenotypic variation of markers associated with GPC.

Marker	Chromosomal Location	R^2 X 100		P value	
		Pune	Ludhiana	Pune	Ludhiana
$UBC844_{1100}$	7AS	8.1	5.6	0.004	0.01
$OPA4_{800}$	+	3.4	6.7	0.06	0.01
Xgwm219	6BS	4.5	-	0.03	-
$UBC880_{1100}$	+	3.9	-	0.04	-
Xgwm604	5BL	3.6	-	0.05	-
$OPH4_{1400}$	+	3.5	-	0.06	-
Xgwm499	5BL	3.2	-	0.07	-
$UBC873_{750}$	+	3.0	-	0.08	-
$OPM5_{870}$	+	-	8.4	-	0.003
$OPV14_{1200}$	+	-	6.8	-	0.009
$OPO10_{870}$	2BL	-	6.5	-	0.009
Xgwm368	4BS	-	4.7	-	0.04
$UBC810_{900}$	+	-	4.4	-	0.04
Xgwm515	2DL	-	4.3	-	0.03
$OPD20_{1000}$	+	-	3.2	-	0.07

+ Chromosomal localization was not possible using nullitetrasomic analysis since markers were absent in Chinese Spring.

population. Multiple regression model using the linked markers at Pune and Ludhiana locations depicted a total of 18.8% and 24.0% of phenotypic variation, respectively. The heritability of GPC at Pune location was calculated to be 0.54, which indicated that the genetic variation accounted by 8 markers was of 34.8% at Pune location.

2. 3. 4 Chromosomal assignment of the markers:

After identifying the markers linked to GPC, next step was to localize these markers on the chromosome. The STMS markers (Xgwm219, Xgwm368, Xgwm499, Xgwm515 and Xgwm604) which were found to be associated with GPC were already mapped on wheat chromosomes by Roder *et al.* (1998). To localize the linked ISSR and RAPD markers, the nullitetrasomic lines in the background of Chinese Spring were used and PCR amplifications were performed on a full set of these lines alongwith the parents. This analysis revealed that $UBC844_{1100}$ was present on chromosome 7A, showing amplification of marker locus in all the lines except that of 7A. Similarly, marker $OPO10_{870}$ was found to be present on chromosome 2B. However, localization of other 5 markers ($OPA4_{800}$, $OPD20_{1000}$, $OPH4_{1400}$, $UBC873_{750}$ and $UBC880_{1100}$) was not possible with this approach as these marker loci were absent in the Chinese Spring itself, in the genetic background of which nullitetrasomic and ditelosomic lines were developed. Further, presence of $UBC844_{1100}$ marker on short arm of chromosome 7A was confirmed when it amplified only in ditelosomic line 7AS and not in 7AL. PCR analysis with ditelosomic lines of chromosome 2B revealed that marker $OPO10_{870}$ was present on chromosome arm 2BL. As other 3 markers ($OPM5_{870,}$ $OPV14_{1200,}$ and $UBC810_{900}$) were present on the same linkage group with less than 10 cM distance among each other (data not shown), these 3 markers were considered to be probably located on the same chromosome arm. This can only be confirmed using other mapped markers present on the chromosome arm 2BL. Previously, genes controlling GPC have been found on chromosome 1A, 1B and 7A by Stein *et al.* (1992). Leavy *et al.* (1988) have reported presence of the

genes for high GPC on chromosomes 1, 5 and 7 using F_2 population derived from *T. turgidum* L. var *dicoccoides*. This indicates that a QTL on long arm of chromosome 2B associated with GPC as identified by me, is an additional new locus contributing to GPC. Similarly, STMS markers Xgwm219, Xgwm368, Xgwm499, Xgwm515 and Xgwm604 linked to GPC were located on chromosome arms 6BS, 4BS, 5BL, 2DL and 5BL, respectively, indicating a new locus on the chromosome arm 4BS contributing to GPC in bread wheat, that has been previously reported to be linked to GPC in durum wheat (Blanco *et al.* 1996). Prasad *et al.* (1999) have also reported a microsatellite marker associated with a QTL for GPC on chromosome arm 2DL in the same cross.

2. 3. 5 Sensitivity of GPC trait to environment:

In order to identify environmentally neutral QTLs associated with the trait, marker genotype data were analyzed using the phenotypic data from two locations separately. As given in Table-2.1, 2 markers ($OPA4_{800,}$ and $UBC844_{1100}$) were found to be associated with GPC at both the locations. This indicated that these markers were linked with two QTLs, which were stable across the environment. Individually, these 2 markers showed difference in phenotypic contribution at both the locations (Table-2.1). However, multiple regression analysis with these 2 markers associated with GPC at both the locations revealed that phenotypic contributions were similar at Pune and Ludhiana locations (9.9% and 9.5%, respectively). This proved that these two loci were stable in contributing to phenotypic variation under both the agroclimatic conditions. Of the remaining 13 markers, 6 (Xgwm219, Xgwm499, Xgwm604, $UBC880_{1100}$, $OPH4_{1400}$ and $UBC873_{750}$) were found to be associated with at least 5 QTLs at only Pune location (Xgwm604 and Xgwm499 have been mapped on 5BL hence may suggest them to be one QTL) whereas other 7 markers (Xgwm368, Xgwm515, $OPD20_{1000}$, $OPM5_{870}$, $OPO10_{870}$, $OPV14_{1200}$ and $UBC810_{900}$) were associated with 7 QTLs specific to Ludhiana location. This could be due to environmental sensitivity of these loci. Thus, the data described here reveals identification of markers associated with 2

environmentally neutral QTLs, 5 QTLs specific to west plains zone represented by Pune and 7 specific to north zone represented by Ludhiana. Similar findings have also been reported in soybean for seed oil and protein content (Lee *et al.* 1996) as well as in rice for heading date and plant height (Lee *et al.* 1996) and in tomato for fruit size (Paterson *et al.* 1991). Lu *et al.* (1997) have reported two characters, namely, heading date and plant height in rice which are sensitive to environment in comparative mapping of QTLs for agronomic traits across environments using a doubled-haploid population. In QTL analysis of fruit size, soluble solids concentration and pH in tomato, Paterson *et al.* (1991) have reported 29 putative QTLs affecting these characters out of which only 4 have been detected in all the three environments, 10 in two environments whereas 15 are present in only single environment.

2. 3. 6 Application of markers linked to GPC in wheat breeding:

QTL analysis of agronomically important traits has been used to determine the number and locations of genes affecting a trait, which will help in indirect selection for a trait in marker assisted breeding programs. Quantitative trait that is influenced by many genes as well as by environment and governs the end use quality performance, is an ideal candidate for MAS (Lee and Penner, 1997). GPC is one such trait, which is widely studied due to its role in wheat end product quality as well as in human nutrition. My work has shown the presence of 15 markers associated with 14 genomic regions controlling GPC, out of which 2 are environmentally stable while the remaining 12 loci (5 from Pune and 7 from Ludhiana) are environment-specific. Lee *et al.* (1996) have reported five markers for seed protein content in soybean with phenotypic contribution of 8-13% in F_2 derived population of PI 97100 X COKER 237 and out of these, four markers are stable at two different locations. However, phenotypic variation explained by the markers in this study as well as in the above example is low indicating either the existence of other undetected loci in the population or the need to find closer markers to the detected QTLs. Lande and Thompson (1990) have concluded from their studies on marker assisted

selection (MAS) that the relative efficiency of MAS is the greatest for characters with lower heritabilities. The estimated heritability of GPC at Pune location as 0.54 shows that nearly half the proportion of variation of the trait is not genetically controlled. In this context, findings reported here with GPC are significant as they show presence of markers, which are stable as well as specific to environment. It is known that individual QTL appears to show a range of sensitivity to environment as some of them are detected in all the environments while many seem to be specific to a single environment. Quantitative traits, which are consistent in different environments, are obviously a better choice in breeding programs. Use of environment-specific QTLs may further improve the agricultural productivity at different agroclimatic zones. By combining several QTLs of a trait, with different environmental specificities into a single genotype, it may be possible to improve the phenotype, which is buffered against varied environmental conditions.

Chapter 3

Molecular marker analysis of wheat grain size, a determinant of consumer preference.

Part of the contents of this chapter has been published as a full length paper in Theoretical and Applied Genetics (2001) 102: 726-732

ABSTRACT:

To identify molecular markers associated with grain size, a complex character in hexaploid wheat, a recombinant inbred line (RILs) population was developed from a cross between Rye selection 111, having bold seed size and Chinese Spring, having small seed size by Single Seed Descent method. Different PCR based markers such as ISSR, RAPD and STMS were applied in parental screening and the polymorphic markers were used on selected RILs of both high and low grain size, representing extreme values of the trait in population. Markers co-segregating in phenotype-specific manner were further used for segregation analysis of whole RIL population. Single marker analysis with QGENE software revealed total 14 markers associated with grain size. While individually these markers contributed 2.6% to 14.8% of phenotypic variation, they showed 39% phenotypic contribution to grain size in multiple regression analysis.

3.1 INTRODUCTION:

The end use quality of wheat is greatly influenced by grain size (Campbell *et al*. 1999) and can be measured indirectly by thousand-kernel weight (TKW), which is positively correlated with agronomic yield (Baril, 1992) and flour yield (Chastain *et al.* 1995). Moreover, the high heritability values of grain weight in most of the cultivars studied so far have proved that this character is phenotypically the most stable yield component (Giura and Saulescu, 1996). Grain weight is also a superior parameter in predicting milling quality of the hard grain compared to test weight (Mishra and Gupta, 1995). Finally, large grains usually command consumer preference and thereby represent an important factor in controlling economic value of wheat. Development of improved grain size specific cultivars is thus an important breeding objective in wheat agriculture (Campbell *et al.* 1999).

Seed size in crop plants has been used as a model system for quantitative genetics since early 1900. The pioneering report of Johannsen (1903) on bean seeds established the first evidence of the interaction of genetic and environmental factors for quantitative variations. Also, seed weight that is one of the measure of seed size, was the first quantitative trait for which Sax (1923) reported linkage between seed color and seed weight in common bean (*Phaseolus vulgaris* L.). This was the first observation of linkage between a morphological character and a quantitative trait. Several researchers have attempted to identify the loci controlling seed weight in legumes using different molecular markers. Vallejos and Chase (1991) have identified the linkage between isozyme markers and seed weight in *Phaseolus vulgaris.* Fatokun *et al.* (1992) have identified 2 and 4 QTLs explaining 53% and 50% of the variation for seed weight in cowpea and mung bean, respectively. QTL analysis for seed weight has been further reported in other legumes including soybean (Mansur *et al.* 1993; Maughan *et al.* 1996; Mian *et al.* 1996), lentil (Abbo *et al.* 1992; Tahir *et al.* 1994), pea (Timmerman *et al.* 1996) and common bean (Park *et al.* 2000). Similarly, in cereals, several reports are available on identification of molecular markers associated with seed weight QTLs. Mather *et al.* (1997)

have reported QTLs for kernel weight on chromosome 1H and 7H of barley. In rice, Lin *et al.* (1996) have shown the presence of QTLs for grain weight on chromosomes 1, 2, 4 and 5 while Lu *et al.* (1997) have reported QTLs on chromosomes 1, 2, 3, 5, 6 and 8. Several QTLs for grain weight have been reported on different chromosomes of maize also (Edwards *et al.* 1992; Schon *et al.* 1994; Goldman *et al.* 1994; Ausin and Lee 1996).

Wheat grain size, like most of the traits of biological interest and agricultural importance, is a complex character and suggested to be of quantitative nature (Halloran 1976; Bannier 1979; Kharabova and Maistrenko 1980; Chojecki *et al.* 1983; Zheng *et al.* 1993; Giura and Saulescu, 1996; Campbell *et al.* 1999; Varshney *et al.* 2000). Due to polygenic nature of such traits, it's difficult to study the effect of individual locus controlling them in conventional approaches of plant breeding and therefore, any information on its genetic control is useful for increasing breeding efficiency. Identifying molecular markers linked to QTLs controlling seed size would facilitate selection in early generations and may contribute to improve end-use quality in wheat by accumulating such loci into elite backgrounds.

3.2 MATERIALS AND METHODS:

3.2.1 Plant material and phenotypic measurements:

A recombinant inbred population (RIL) of 113 individual lines was developed by making a cross between Rye Selection 111 (RS 111), an Indian genetic stock obtained through introgression of genes for bold seed size from rye, and Chinese Spring having small seed size. RS 111 was developed from crossing of a wheat line mono5B-C591 with Rye (*Secale cereale*) and then backcrossing with another wheat cultivar Sonalika. RILs were advanced by single seed descent method to the F_6 generation wherein panicles were bagged in each generation. The progenies in each generation were scored for seed size by recording 1000-kernel weight from first two tillers of five plants from each progeny.

3.2.2 DNA extraction:

DNA was extracted from 15 days old seedlings of parents and RILs grown in the glass house according to the procedure described by Anderson *et al.* (1992). Young leaf tissue (10gm) was ground to fine powder in liquid nitrogen using a mortar and pestle. To this, 15-20 ml of extraction buffer (500mM NaCl, 100mM Tris-HCl pH8.0, 50mM EDTA and 0.84% SDS; Sodium bisulphite was added just before use and pH adjusted with NaOH to 7.8-8.0) was added which was pre-heated at 65 °C. The contents were mixed to form an emulsion, which was incubated at 65 °C for 20-30 min, inverting tubes every 5-10 min. After incubation, the emulsion was allowed to cool down to room temperature, following which it was equally distributed into SS34 centrifuge tubes. In each tube, an 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°C in a Sorvall RC-5B centrifuge (Du Pont, U.S.A.). 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 supernatant 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°C. The DNA pellet was washed with 70% ethanol, dried and dissolved in an appropriate volume of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0). To remove RNA from the samples, 0.3 % of the total volume of sample, 10 mg/ml stock solution of RNase A (DNase free) was added to the dissolved DNA and incubated at 37°C for 1 hour. Then it was stored at -20°C until further use. The DNA concentration was estimated spectrophotometrically as well as by agarose gel (0.8%) electrophoresis by comparing with known concentration of λ DNA.

3.2.3 Screening of parents and RILs using PCR-based markers:

Using 100 inter simple sequence repeat primers (ISSR) (UBC set-9, University of British Columbia, Vancouver, Canada) amplifications were carried

out according to the protocol as described in Chaper-2 (Section 2.2.3A). The amplified products were resolved on 1.5% agarose gels followed by staining with ethidium bromide. In RAPD analysis, PCRs were performed using total of 480 RAPD primers (380 primers were from Operon Technologies, USA and 100 primers were from University of British Columbia, Canada) and the amplified products were checked as given in Chapter-2 (Section 2.2.3B). In case of microsatellite, out of 106 STMS primer pairs, 78 pairs were selected from the previously published map of wheat by Roder *et al.* (1998) and 25 primer pairs from all 21 chromosomes of wheat were kindly provided by Prof. M. D. Gale, John Innes Research Center, Norwich, UK. The PCR amplifications of microsatellite primers obtained from map published by Roder *et al.* (1998) were performed in 25µl reaction volume as described in Chapter-2 (section 2.2.3C). The PCR amplification of microsatellite primers obtained from Dr. Gale were done in 30µl reaction volumes with reaction mixture containing 200nM of each primer, 200µM of each dNTP, 1.5mM $MgCl_2$, 1.0 unit of Taq DNA polymerase (Perkin Elmer, U.S.A.) and 50 ng of template DNA. The PCR program consisted of 94^{o}C initial denaturation for 5 min, followed by 30 cycles at 94^{o}C for 1 min, ramping of 0.5^{o}C/min to annealing temperature of 50^{o}C, 55^{o}C, 60^{o}C and 63^{o}C (according to GC content of microsatellite primer), 72^{o}C extension for 1 min and final extension for 5 min. The amplified products were resolved on 3% metaphor agarose (FMC Bio Products, U.S.A.) gels, followed by ethidium bromide staining.

3.2.4 Data analysis:

The polymorphic markers identified in parental screening were used for selective genotype analysis (Lander and Botstein, 1989; Darvasi and Soller, 1992) using 13 RILs from each extreme representing the highest/lowest grain weight in population. The markers, which were found to be co-segregating with phenotypic trait values in selective genotyping, were further analyzed on the entire population of 113 RILs along with parents. The genotype of each RIL with ISSR, RAPD and STMS was scored and linkage analysis was performed

as described in Chapter-2 (section 2.2.4). Only significant LOD scores (>2.0) were interpreted to indicate co-segregation of putative QTLs for grain weight and genetic markers.

3.2.5 Chromosome assignment of markers:

Nullitetrasomic (NT) (Sears, 1966) and ditelosomic (Sears and Sears, 1978) lines of Chinese Spring wheat were used to localize ISSR and RAPD markers showing association with grain size on wheat chromosomes as given in Chapter-2 (section 2.2.5).

3.3 RESULTS AND DISCUSSION:

3.3.1 Frequency distribution of seed weight in RILs:

The parents used in this cross, Rye Selection 111 and Chinese spring differed significantly in their TKW (57.3 g and 29.16 g) value while the TKW in the RIL population ranged from 53.38 g to 16.99 g, indicating a continuous variation which is a characteristic of polygenic nature of the trait. A frequency distribution plot was prepared (Fig 3.1) using the TKW data of RILs which suggested a good fit to the normal distribution. Significant transgressive segregants occurred towards low TKW parental value whereas transgressive segregation was not significant towards high TKW parental value. The recovery of the progenies having grain weight as high/low as parents indicated that the population was useful for molecular tagging of the trait.

3.3.2 Parental screening and selective genotyping:

Three types of DNA markers namely, ISSR, RAPD and microsatellite were employed for parental screening. Out of 100 ISSR primers used to detect parental polymorphisms, 55 primers were successful in amplifying loci from parental DNAs. A total of 18 primers showed polymorphism (approximately 32.7%) out of the 55 primers that gave amplification. Out of 480 RAPD primers used, 350 were successful in giving amplification, among which a total of 73

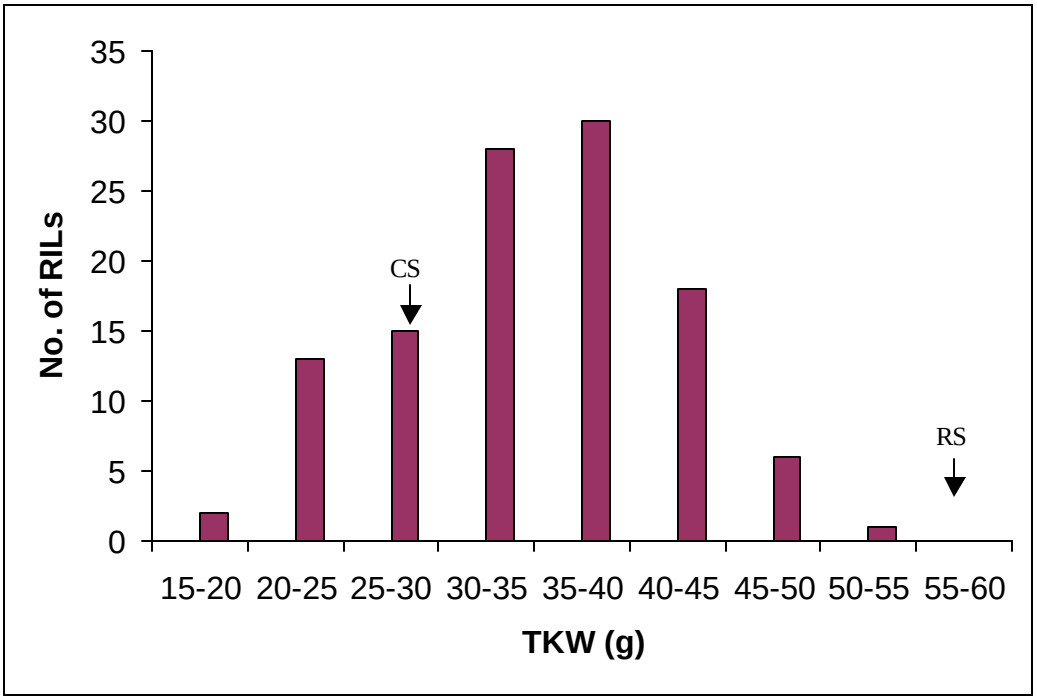

Fig. 3.1: Frequency distribution of TKW in Rye Selection 111 X Chinese Spring RILs. Arrows indicate parental values.

primers were polymorphic (approximately 23%). Eighty-two microsatellite primer pairs gave scorable amplification, out of 103 primer pairs attempted and 24 primer pairs showed polymorphism (31.2%). All these polymorphic loci were further used for selective genotyping with 13 each from both extreme tails of the population and were amplified using the identified polymorphic markers. Figures 3.2a and 3.2b give representative photographs of segregation analysis using one ISSR marker $UBC815_{850}$ and one RAPD marker $OPC5_{1400}$, respectively. It can be seen from the Fig. 3.2a that a band of molecular weight 850bp, indicated by an arrow, is present in Chinese Spring (lane b) and absent in Rye selection 111 (lane a). It is also present in 10 out of the 13 RILs with extreme low seed size (lanes p-z") and is absent in all the 13 extreme high seed size RILs (lanes c-o), suggesting its probability to be a linked marker. Similarly, as shown in Fig. 3.2b, marker $OPC5_{1400}$ is present in Chinese Spring (lane b), absent in Rye Selection 111 (lane a) and out of 24 representative RILs, it is present in 14 RILs while absent in remaining 10 RILs. Together 36 markers including 15 ISSR, 3 RAPD and 18 STMS were found to be useful during selective genotyping, which were then used to screen the 113 RILs and the data was recorded for linkage analysis. The genotypic data of all marker scores were analyzed according to Chi-square tests for goodness of fit. Majority of the markers in the analysis segregated in the expected ratio of 1:1 while few markers showed skewing towards either of the parental alleles.

3.3.3 Determination of markers associated with TKW:

In an attempt to study linkage of all the 36 markers, the marker segregation data was analyzed using MAPMAKER v.3.0 software. This analysis revealed four linkage groups with each having 6, 3, 2 and 2 markers, respectively, at LOD 3.0 with 23 markers remaining unlinked. Single marker analysis using simple linear regression was further done using QGENE software to determine, first the association between the markers and QTLs controlling the trait and secondly to explore the genetic basis of the TKW. Total of 14 markers including 7 ISSR ($UBC815_{850}$, $UBC815_{800}$, $UBC818_{1000}$, $UBC812_{750}$, $UBC842_{600}$,

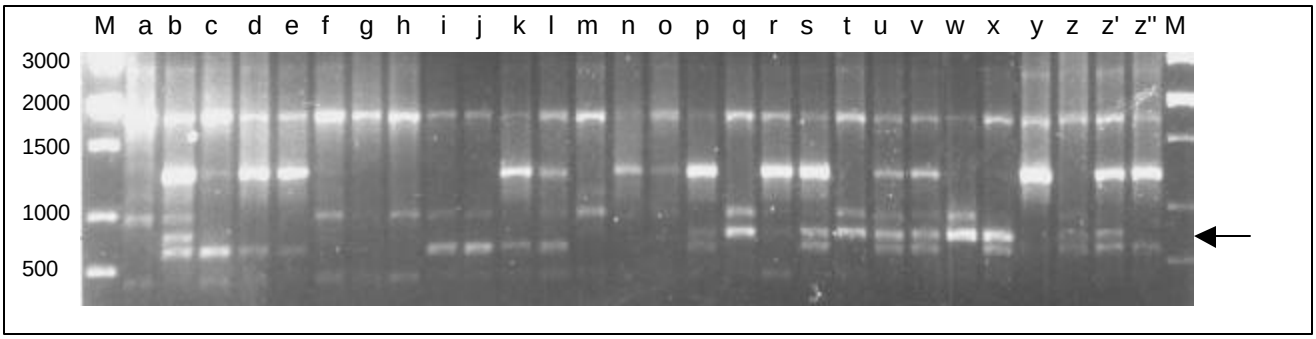

Fig 3.2a: Segregation of marker UBC815$_{850}$ in the RILs of Rye Selection 111 X Chinese Spring.

M= 1 kb Ladder; Lane a: Rye Selection 111, Lane b: Chinese Spring; Lanes c- z": RILs. An 850bp band, indicated with an arrow, is present in Chinese spring and absent in Rye Selection 111.

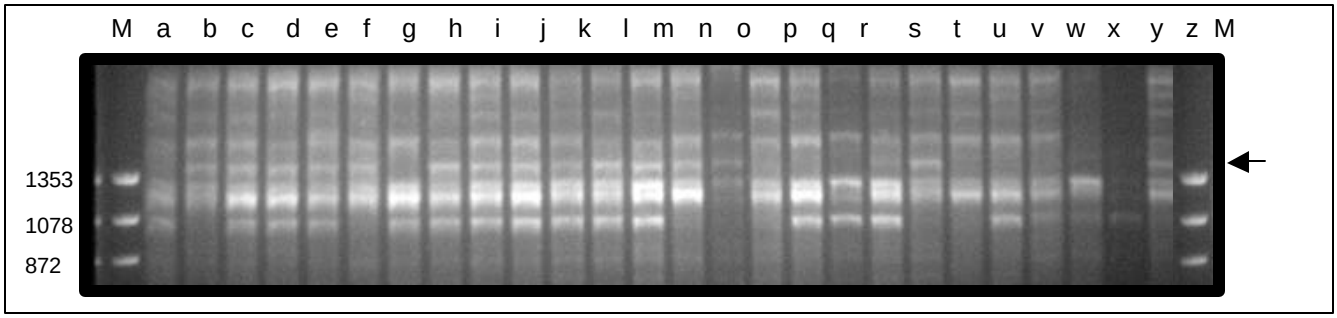

Fig 3.2b: Segregation of marker $OPC5_{1400}$ in the RILs of Rye Selection 111 X Chinese Spring.

M= f x174/*Hae*III digest; Lane a: Rye Selection 111, Lane b: Chinese Spring; Lanes c- z": RILs. A 1400bp band, indicated with an arrow, is present in Chinese spring and absent in Rye Selection 111.

UBC843$_{1200}$ and UBC814$_{750}$), 2 RAPD (OPC5$_{1400}$ and OPR8$_{1000}$) and 5 STMS (Xgwm644, Xgwm156, Xgwm499, Xpsp3151 and Xgwm251) were found to be associated with TKW (Table-3.1). The R^2 values for phenotypic contribution of these markers ranged from 0.148 to 0.026. Multiple linear regression model with these marker loci explained approximately 39% of the total phenotypic variance. This data is in line with results of Campbell *et al.* (1999) who identified markers associated with TKW on 1A, 1B, 3B and 7A chromosomes of wheat and together these markers explained 44% of the phenotypic variation in multiple regression model.

3.3.4 Identification of new loci governing TKW:

Among the markers identified associated with TKW in this analysis, the chromosomal positions of the 5 STMS markers were previously reported (Roder *et al.* 1998, Stephenson *et al.* 1998) as given in Table-3.1. To localize ISSR and RAPD markers linked to TKW, complete set of nullitetrasomic (NT) lines in the background of Chinese Spring was used. This analysis indicated that UBC815$_{850}$ was present on chromosome 2D, UBC815$_{800}$ on chromosome 6B and UBC812$_{700}$ on chromosome 1D. Marker analysis with ditelosomics further indicated that UBC815$_{850}$ was present on long arm of 2D, UBC815$_{800}$ on long arm of 6B and UBC812$_{750}$ on short arm of 1D. However, other ISSR and RAPD markers viz. UBC818$_{1000,}$ UBC842$_{600}$, UBC843$_{1200}$, UBC814$_{750}$, OPC5$_{1400}$ and OPR8$_{1000}$ could not be localized on to particular chromosomes, because of their absence in Chinese Spring in which background the nullitetrasomic lines were developed. It was therefore, attempted to study if any of these markers were present on the linkage groups established as detailed earlier, in order to localize them on specific chromosomal regions. Except linkage group one, markers on other groups did not represent any of the markers contributing to the trait. Linkage group one had 6 markers namely, UBC815$_{800}$, Xgwm644, OPC5$_{1400}$, UBC818$_{1000}$, OPR8$_{1000}$ and UBC842$_{600}$ linked to each other in the order described, with map distances of 10.6cM, 29.4cM,

Table 3.1: Percent phenotypic variation of the molecular markers with grain size (TKW)

Marker	Chromosomal location	R^2 X 100	P	Multiple regression R^2 X100
$UBC815_{850}$	2DL	14.8	0	39.0
Xgwm644	6BL	14.6	0.0002	
Xgwm156	5AL	10.6	0.001	
$OPC5_{1400}$	-	10.4	0.0009	
$UBC815_{800}$	6BL	9.5	0.001	
$UBC818_{1000}$	-	8.0	0.002	
Xgwm499	5BL	7.5	0.01	
$OPR8_{1000}$	-	6.8	0.006	
$UBC812_{750}$	1DS	6.4	0.007	
Xpsp3151	1AS	5.4	0.03	
$UBC842_{600}$	-	4.7	0.002	
Xgwm251	4BL	4.7	0.02	
$UBC843_{1200}$	-	2.9	0.07	
$UBC814_{750}$	-	2.6	0.08	

22.7cM, 32.7cM and 13.8cM respectively, between consecutive markers. The linkages among the markers indicated that all these markers might be located on chromosome 6B but probably associated with different QTLs as indicated by their distance.

In last two decades, several studies have identified different wheat chromosomal regions that are governing the variation in grain weight. According to the report of Halloran (1976) chromosome 4B is associated with seed size while that of Petrovic and Worland (1988) has identified that TKW is associated with chromosome 5D. Guira and Saulescu (1996) have reported that chromosomes 6D and 4A are associated with high TKW while 5B and 5D are associated with low TKW. Recently, Campbell *et al.* (1999) have demonstrated that chromosomes 1A, 1B, 3B, and 7A have the loci, which control TKW. In the same cross that is used in this study, Varshney *et al.* (2000) have identified a microsatellite marker associated with QTL for grain weight on chromosome 1AS. Monosomic analysis of seed weight in the same cross Rye Selection 111 X Chinese spring has indicated the presence of at least 2 QTLs on 1A and 7A for high TKW and 2 QTLs for low TKW on 6B and 7D (Singh and Dhaliwal, unpublished). In the present analysis, I have found 7 different chromosomal regions i.e. 1AS, 1DS, 2DL, 4BL, 5AL, 5BL and 6BL to be associated with grain size based on the previous maps or nullitetrasomic and ditelosomic analysis (Table-3.1). Four chromosomal locations viz. 1DS, 2DL, 5AL and 6BL, identified in this analysis but not reported in the past to carry grain weight QTLs, represent the additional new loci that are controlling this trait. Additional 6 loci which have been reported here, but for which the localization is not possible (Table-3.1), may or may not represent new loci contributing to grain size (TKW).

3.3.5 Implications in wheat grain quality improvement efforts:

The objective of this work was to provide information about the genetic control of the grain size in wheat and to identify molecular markers associated with this trait. This would help breeders to construct beneficial allelic

combinations and accelerate breeding programs as these markers can be used as an indirect selection tool. The low phenotypic contribution by some of the identified markers in the present work and the untapped variation could be due to various factors such as incomplete genome coverage, loose linkages between the marker loci and QTLs for grain size, interaction between grain size QTLs which are too small to detect; and environmental factors. Four chromosomal locations identified in my study that are associated with grain weight are reported for the first time to carry genes controlling grain size in wheat. Polygenes controlling important metric traits such as grain size are usually distributed among several quantitative trait loci (QTLs) which may not be linked to one another (Law and Worland, 1973, Fatokun *et al*. 1992). The low level of contribution to the phenotypic variation explained by individual markers in this data confirms the quantitative nature of grain weight inheritance and implies that transfer of grain size trait can not be approached by easy way through conventional breeding programs. Moreover, the identification of QTLs contributing to 39% of total phenotypic variation indicates that more than half of the phenotypic variance is yet to be tapped. However, the magnitude of seed size variation explained by these markers is substantial, in view of the quantitative nature of the trait.

Chapter 4

Dissection of grain shape and size traits in wheat using PCR-based DNA markers

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Part of the content of this chapter has been communicated as a full-length paper to Heredity

ABSTRACT:

Wheat kernel traits determine the end use quality and thereby economic value of wheat. Grain size and shape are important kernel traits for the visual preference of the customer. Therefore, deciphering the genetic factors governing grain size is an important wheat breeding objective. After analyzing the QTLs involved in controlling the grain size in the form of TKW, I was further interested to identify the traits contributing to grain shape and size such as grain length, width and density. Hence, to detect putative QTLs (pQTLs) controlling grain shape and size, the same recombinant inbred line population of 113 individuals developed from a cross between Rye Selection 111 and Chinese Spring as described in the previous chapter, was used. Both the parents differed significantly in the kernel traits namely, grain length, grain width and form density. The parents alongwith the 113 lines were genotyped using PCR based markers and evaluated for the same traits. Transgressive segregants were observed for these characters and genetic correlation studies were performed. The number of significant ($p<0.05$) markers associated with pQTLs for each trait ranged from 3 to 10. The percentage of phenotypic variance explained by each pQTL associated marker ranged from 2.9 to 9.5%. Many pQTLs were shared by all the traits, indicating probability of either pleiotropic effect of a single QTL and/or their linkage.

4.1 INTRODUCTION:

The economic value of wheat crop is determined by quality of the grain, which depends in part on the appearance of the grain (Campbell *et al.* 1999). In a preliminary survey of the market, it is observed that bold grains with attractive and uniform shape, size and amber colour fetch higher price. Grain shape and uniformity are two important factors, which influence the milling quality of wheat (Campbell *et al.* 1999). Berman *et al.* (1996) have shown that these two factors account for nearly 66% of the milling quality variation, wherein components of grain shape like grain length, width and area are each associated with 40% of the variation, while test weight is associated only with 17% of the variation. As the mixture of small and large grains affects the flour yield during the milling process, Gaines *et al.* (1997) have suggested separation of the grain lot based on the kernel size to improve the flour yield. Thus development of cultivars with large grain and better shape that fit into specific global market needs has become a major objective of wheat breeding programs.

Wheat grain size and shape are the final products of the manifestation of several components, such as grain length, width and density. Although there is some success in manipulating these traits, the underlying genetic basis is not clearly understood. Genetic analysis of seed size in wheat is complicated because of its quantitative nature (Giura and Saulescu 1996; Campbell *et al.* 1999) Till recently, most of the genetic studies on seed size have confined to estimation of heritability of the trait (Blanco *et al.* 1999). Not much is known about the genetic control of grain shape and its components like grain length, width and density in wheat. The dissection of such important traits by means of marker assisted QTL analysis will be of great significance in helping plant breeders to improve the economic value of wheat. However, progress in genetic mapping of wheat has been comparatively slow, mainly because of lack of informative markers and narrow genetic base (Roder *et al.* 1998). Consequently, the number of grain quality related genes/QTLs that have been identified are relatively few, compared to those in maize, barley and rice (Parker *et al.* 1999). With the recent development of highly informative and technically

simple PCR-based markers molecular analysis of agronomically important traits in wheat has gained momentum (Roder *et al.* 1998).

After deciphering the QTLs involved in grain size (TKW), as described in previous chapter, the present study describes the genetic analysis of grain shape into component traits in terms of identification, localization and estimation of the contribution of each putative QTL based on a RIL population used in previous analysis.

4.2 MATERIALS AND METHODS:

4.2.1 Plant material and phenotypic measurements:

Recombinant inbred line population of 113 plants developed by making a cross between, Rye Selection 111 and Chinese Spring was used in this analysis. These two parents differed significantly in grain length, width and factor form density. Phenotyping for grain length and width was done by measuring the length and width of 30 grains from the parents and each RIL with Vernier Calipers and an average of 30 grains was considered as the value of each sample. A "factor" referred to as form-density (Giura and Saluescu 1996) describing the differences in the grain structure (density) and the deviation from the cylindrical form was calculated as

$$\text{Form density} = \frac{\text{Grain weight}}{\text{Grain length X Grain width}}$$

4.2.2 DNA isolation:

DNA extractions from leaf tissue of each RIL along with the parents were done according to the protocol described by Anderson *et al*. (1992) from 15 days old seedlings as given in Chapter-3 (section 3.2.2).

4.2.3 Screening of parents and RILs with different DNA markers:

A total of 100 ISSR primers (University of British Columbia, Vancouver, Canada) were used for amplifying the DNAs according to the protocol described in Chapter-2 (section 2.2.3A). In RAPD analysis, 480 primers (380 primers were

from Operon technologies, USA and 100 primers were from University of British Columbia, Canada) were involved in PCR experiments according to protocol given in Chapter-2 (section 2.2.3B). Amplified products of ISSR and RAPD were resolved on 1.5% agarose gel followed by ethidium bromide staining.

One hundred and three STMS primer pairs were used as detailed in Chapter-3 (section 3.2.5) for the PCR amplifications using the cycling conditions as reported in Chapter-3 (section 3.2.5) in Gene-Amp 9700 thermal cycler (Perkin Elmer, U.S.A.). The amplified products were resolved on 3% metaphor agarose (FMC Bio Products, U.S.A.) gels in 1xTAE buffer, followed by ethidium bromide staining.

4.2.4 Data analysis:

The genotype of each RIL with ISSR, RAPD and STMS was scored and linkage analysis was performed as given in Chapter-2 (section 2.2.4). Molecular marker linkages were determined with MAPMAKER v3.0 (Lander *et al.* 1987) at LOD 3.0. Single point analysis to determine molecular marker-putative QTL associations was done using computer program QGENE (Nelson, 1997) based on simple linear regression. Markers with significant P values (<0.05) and/or LOD score (>2.0) were interpreted as putative QTLs (pQTL). Significant marker loci were then combined in a multiple linear regression model to determine the combined effect.

4.2.5 Chromosome assignment:

Nullitetrasomic (Sears, 1966) and ditelosomic (Sears and Sears 1978) lines of Chinese Spring wheat were used to localize ISSR and RAPD markers as reported in Chapter-2 (section 2.2.5).

4.3 RESULTS AND DISCUSSION:

4.3.1 Frequency distribution of grain traits:

The two parents selected in the present analysis, Rye Selection 111 and Chinese Spring, were phenotyped for the grain shape traits. Rye Selection 111 had 7.3 mm of grain length, 3.6 mm of grain width and 2.162 mg/mm^2 of form density whereas Chinese Spring possessed 5.1 mm of grain length, 2.4 mm of grain width and 1.939 mg/mm^2 of form density. The values of grain length, width and form density in the RIL population ranged from 5.1 to 7.4 mm, 2.1 to 3.6 mm and 1.49 to 3.3 mg/mm^2, respectively indicating a continuous variation which is a characteristic of polygenic nature of these traits (Fig-4.1a, b, c). Significant transgressives towards either of the parental alleles for form density and towards lower parental alleles for grain width were observed, whereas no trangressive segregation was found with grain length. The evidence of transgressive segregation indicated that Rye Selection 111 and Chinese Spring contained alleles associated with both high and low values of the grain shape traits.

4.3.2 Identification of putative markers linked with grain shape traits:

In previous chapter, I have shown approximately 32.7% of the tested ISSR primers gave polymorphic loci while in case of RAPD and STMS analysis, 23% and 31.2% primers respectively, were polymorphic in the parental analysis. All these polymorphic loci were further used for selective genotyping and markers that showed phenotype-specific segregation were used for analyzing the whole population. Figures 4.2a and 4.2b give representative photographs of segregation analysis of one ISSR marker UBC848$_{850}$ and one STMS marker Xgwm644. It can be seen in Fig 4.2a that a marker band of 850bp is present in Chinese Spring (lane b), absent in Rye Selection 111 (lane a) and it is present in 11 RILs while absent in remaining 14 RILs. Similarly, as shown in Fig 4.2b, a band of 150bp from Xgwm644, is present in Rye Selection (lane a), its allele of 145bp present in Chinese Spring (lane b) and out of 26 representative RILs,

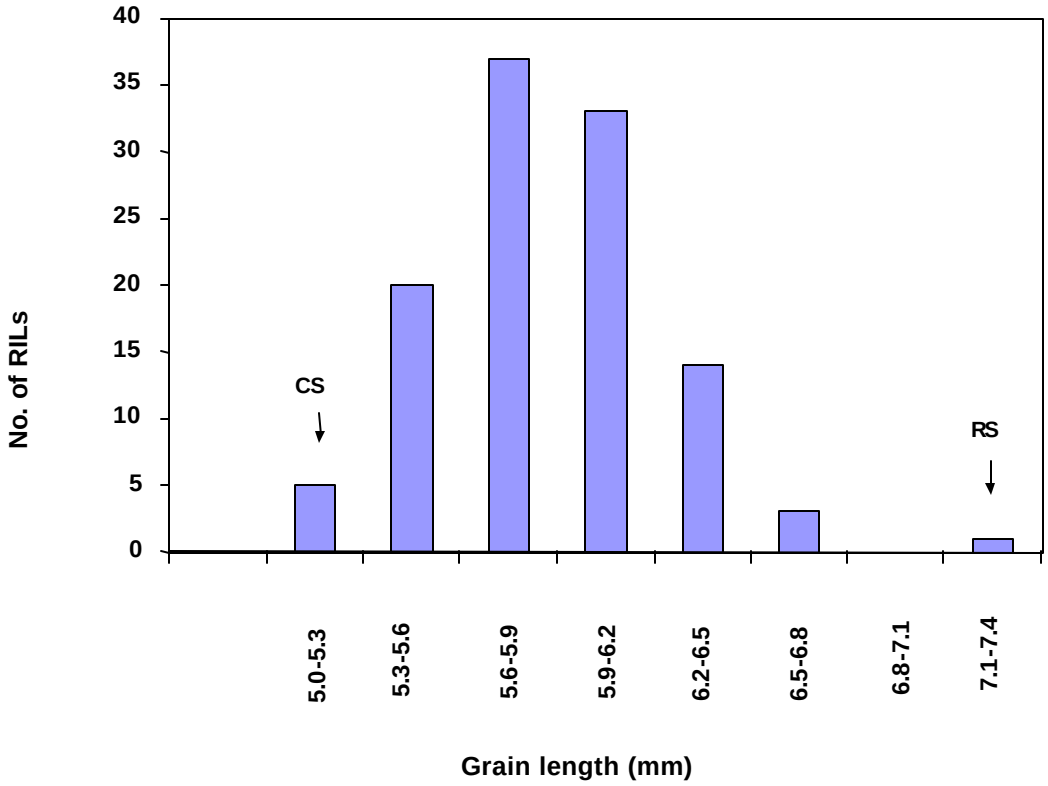

Fig. 4.1a: Frequency distribution of grain length in the RILs of Rye Selection 111 X Chinese Spring. Arrows indicate parental values.

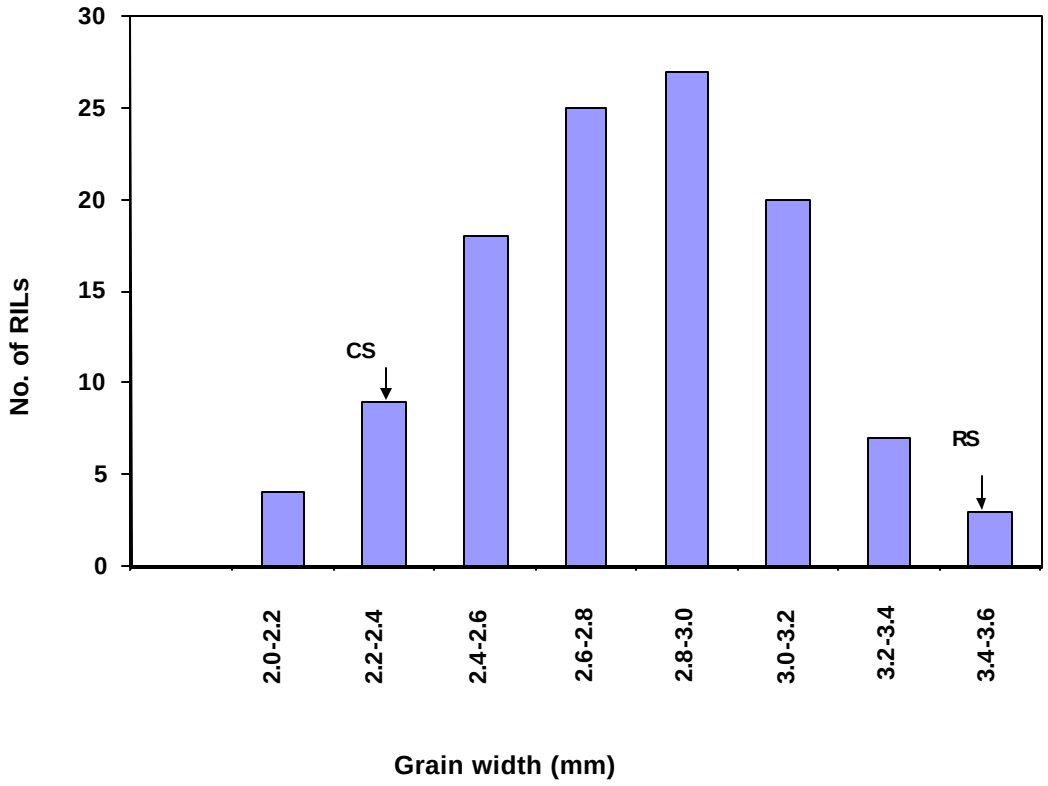

Fig. 4.1b: Frequency distribution of grain width in the RILs of Rye Selection 111 X Chinese Spring Arrows indicate parental values.

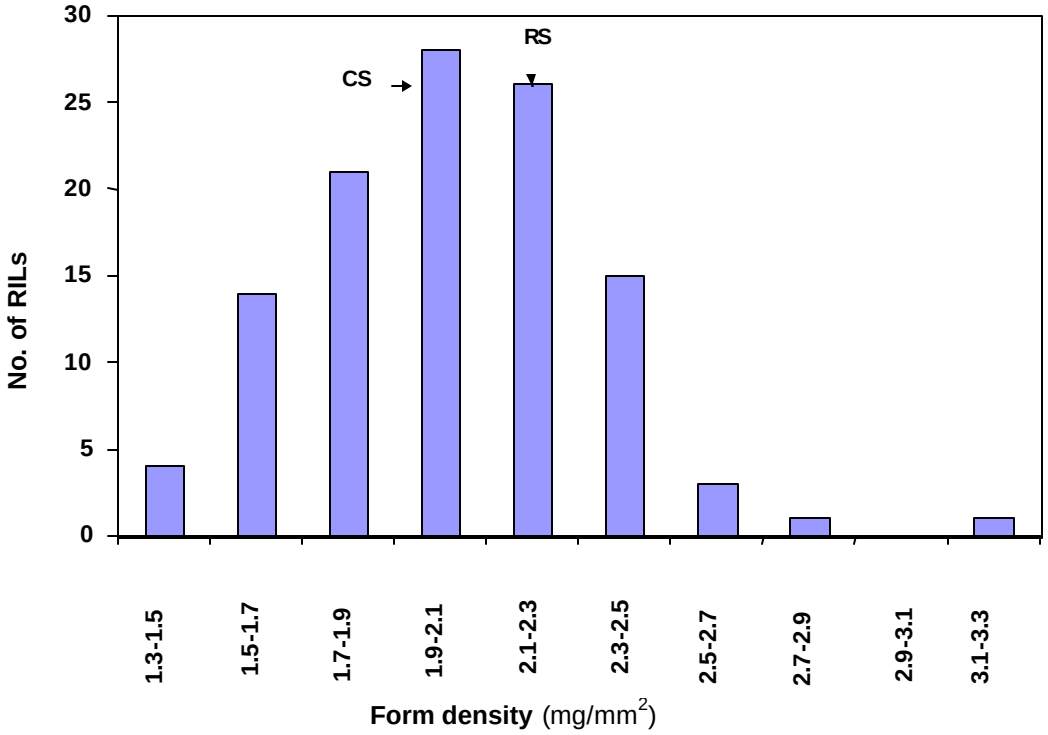

Fig. 4.1c: Frequency distribution of form density in the RILs of Rye Selection 111 X Chinese Spring Arrows indicate parental values.

15 show presence of 150bp marker band while remaining 11 RILs have the allele of 145bp. Single point regressions ($p<0.05$) using QGENE software served as the primary method of detecting associations between markers and QTLs. The DNA marker data obtained for each kernel trait is described below.

Grain length:

Total 10 markers were found to be associated significantly with the grain length (Table-4.1). Most of the markers that were associated with grain length also exhibited significant association with grain width. An additional marker, which showed contribution to grain length only, was a microsatellite marker Xgwm334 with 5.37% of total contribution and this was mapped on chromosome 6A by Roder *et al.* (1998). The contribution of individual marker to grain length varied between 4.7% to 9.46% (Table-4.1) and the best multiple regression model for grain length explained 23.1% of the phenotypic variance.

Grain width:

Six markers namely UBC $815_{850,}$ UBC843_{1200}, UBC815_{800}, UBC848_{850}, Xgwm644 and Xgwm156 showed association with grain width (Table-4.1). All the markers except UBC848_{850}, with a contribution of 2.91%, were shared by grain weight as described in Chapter-3, whereas markers UBC815_{850}, UBC815_{800} and Xgwm644 were shared by grain length, indicating that the same chromosomal regions might have influence on these traits. The contributions of each pQTL associated markers for grain width ranged from 2.91% to 7.6%. The best multiple regression model explained 12.5% of the phenotypic variation.

Form density:

Besides grain length and width, variation in grain weight can be due to differences in the grain form as indicated by higher or smaller deviations from a regular geometrical form (Giura and Saluescu 1996). Three markers viz. UBC814_{750}, UBC815_{800} and UBC848_{1500} were significantly associated with factor form density (Table-4.1). One of the markers UBC815_{800} was also associated with grain weight, length and width. UBC814_{750} was associated with grain weight whereas UBC848_{1500} was associated only with form density. The

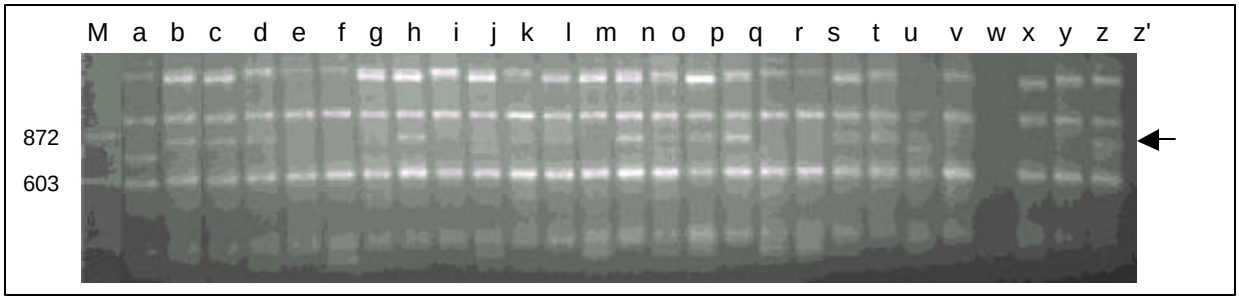

Fig 4.2a: Segregation of marker UBC848$_{850}$ in the RILs of Rye Selection 111 X Chinese Spring.

M= ϕX174/ *Hae* III digest, Lane a: Rye Selection 111, Lane b: Chinese Spring, Lanes c- z': RILs. An 850bp band, indicated with an arrow, is present in Chinese spring and absent in Rye Selection 111.

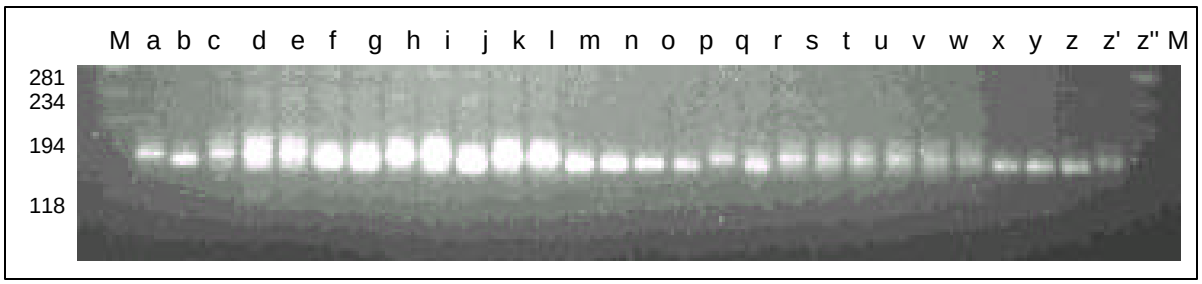

Fig 4.2b: Segregation of marker Xgwm644 in the RILs of Rye Selection 111 X Chinese Spring.

M= fX174/ *Hae* III digest, Lane a: Rye Selection 111, Lane b: Chinese Spring, Lanes c- z": RILs. A marker band of 150bp is present in Rye Selection 111 and its allele of 145bp is present in Chinese Spring.

Table-4.1: Summary of the marker-trait associations and their contributions.

Trait	Markers	R^2 x100	Chromosome location	P	Multiple regression R^2 x100
Grain length	$UBC818_{1000}$	9.5	-	0.0009	23.1
	$OPC5_{1400}$	8.4	-	0.003	
	$UBC815_{800}$	7.1	6BL	0.004	
	$UBC842_{600}$	6.8	-	0.005	
	Xgwm499	6.1	5BL	0.03	
	Xgwm334	5.4	6A	0.02	
	$UBC815_{850}$	5.2	2DL	0.01	
	$UBC812_{750}$	5.2	1DS	0.01	
	Xgwm251	5.0	4BL	0.04	
	Xgwm644	4.7	6BL	0.04	
Grain width	Xgwm644	7.6	6BL	0.009	12.5
	$UBC815_{800}$	6.5	6BL	0.006	
	Xgwm156	4.5	5AL	0.04	
	$UBC843_{1200}$	4.0	-	0.04	
	$UBC815_{850}$	3.8	2DL	0.03	
	$UBC848_{850}$	2.9	-	0.02	
Form density	$UBC814_{750}$	7.6	-	0.003	14.5
	$UBC815_{800}$	4.9	6BL	0.01	
	$UBC848_{1500}$	3.8	-	0.03	

best multiple regression model explained about 14.5% of the phenotypic variation.

4.3.3 Detection of new loci contributing to grain shape and size:

Identifying the individual genetic factors involved in grain size and shape is very useful for grain quality improvement as it would facilitate the manipulation of grain size and shape using genes even with relatively minor effects. Many researchers have carried out genetic analysis of grain size in wheat and these have revealed the existence of genes/QTLs controlling grain shape/ size on chromosomes 1A, 1B, 1D, 2A, 2D, 3B, 4A, 4B, 5A, 5D, 6B and 6D for grain weight, on chromosomes 1B, 2B, 2D, 3A, 3B, 4A and 4B for grain length, on chromosomes 1A, 1B, 2A, 3D, and 5A for grain width and on chromosomes 2B, 4B, 6A, 6D and 7A for factor form density (Halloran 1976; Petrovic and Worland 1988; Giura and Saluescu 1996; Campbell *et al.* 1999; Varshney *et al.* 2000). However, the chromosomal regions of several genes for grain size and shape still remain to be determined. Moreover, the parents in most of these studies do not differ significantly in the kernel traits. It is suggested that populations of parents with greater differences favor efficient identification of QTLs (Parker *et al.* 1999). The parents used in the present study namely, Rye Selection 111 and Chinese Spring differ significantly in these traits and several new chromosomal regions have been identified, which are not reported previously and this include 1D, 5B, 6A and 6B for grain length, 2D and 6B for grain width and 1A and 2D for form density.

4.3.4 Genetic correlation among grain shape traits:

Based on the phenotypic data available for grain weight (as detailed in Chapter-3), length, width and form density in RILs, an attempt was made to study interrelationship among these traits. Positive genetic correlation above 0.5 was observed for grain weight with grain width, length and factor form

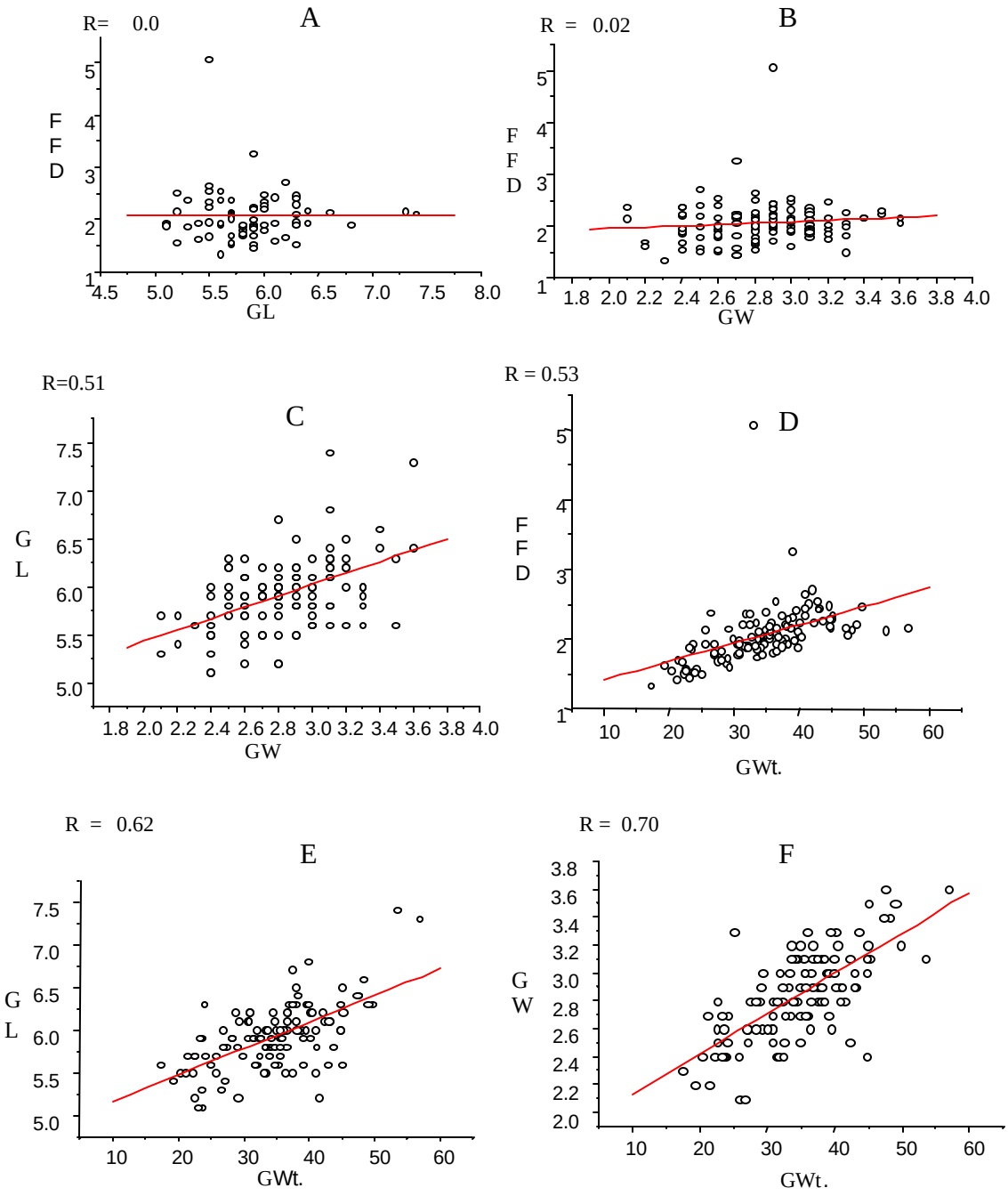

Fig 4.3: Correlation plots of among the kernel traits. GL - grain length, GW - grain width, GWt - grain weight and FFD - factor form density

ensity (Fig-4.3). Similarly, positive correlation was observed between grain length and width, which was in contrast with the report of Campbell *et al.* (1999) where a negative correlation between kernel length and width was reported. Campbell *et al.* (1999) used hard white wheat cultivar, Clark's Cream, with long and slender grain as superior parent and NY6432-18, soft wheat, with short and bold grain for the development of RIL population. In the present study, the superior parent (Rye Selection 111) had long and bold grain with more width as compared to the other parent (Chinese Spring) used for the development of RIL population. This could be the probable reason for the contrasting results obtained in my analysis. No correlations were observed between grain width and factor form density as well as between grain length and factor form density and these results were supported by the QTL analysis. Putative QTLs for grain weight also influenced the length, width and factor form density (see Table-3.1 and 4.1). On the other hand, one marker $UBC848_{850}$ associated with grain width was also associated with factor form density although they were not correlated. Marker $UBC815_{800}$ contributed to all the traits, although no correlation was observed for factor form density with grain length and width, indicating that the two parents involved in this study additionally possessed separate trait specific positive alleles for the grain shape/size.

Some of the putative QTLs identified in this work contribute for grain weight, length, width and factor form density, indicating that these pQTLs affect all the parameters. Nevertheless, one microsatellite marker Xgwm334 was associated only with grain length, one maker $UBC848_{850}$ for grain width and one marker $UBC848_{1500}$ for form density, indicating probable involvement of these loci in grain shape variation, although the contributions of respective loci were lower. As has been demonstrated by Alber *et al.* (1991), Paterson *et al.* (1991 and 1995), Veldboom *et al.* (1994), Xiao *et al.* (1996), Lin *et al.* (1996) and Campbell *et al.* (1999), genetically correlated traits have often QTLs located on to the same chromosomal regions. In present analysis, putative QTLs sharing similar locations have been observed for all the correlated traits and the direction of correlations are consistent with the effect of the QTLs on the traits.

Trait correlations may be due to either pleiotropy and/or from linkages among several QTLs controlling the traits. Pleiotropy can be assigned to the effect of $UBC815_{800,}$ which has a significant control on all the traits under consideration. The low variances explained by pQTLs of grain length, width and form density in the study suggest the possible involvement of additional loci which have not been detected at present. The latter may be either due to reasons such as smaller size of population or due to incomplete genome coverage or due to loose linkages between QTLs and markers. In summary, a locus identified using marker $UBC815_{800}$ probably shows pleotropic effect on grain shape traits and has potential for use in marker-assisted selection programs for wheat grain quality improvement. More such loci contributing to higher percentage of phenotypic variance, however, need to be identified.

Chapter 5

Yellow berry tolerance in wheat: Inheritance and molecular marker studies

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The contents of this chapter have been accepted as a full-length paper in Euphytica (2001) (In Press)

ABSTRACT:

Yellow Berry (YB) is a serious seed disorder in durum wheat, bread wheat and triticale, which arises due to deficiency in nitrogen concentration in the soil. YB seriously affects the grain protein content (GPC) thereby affecting bread making quality in bread wheat and pasta making quality in durum wheat. In order to study the inheritance and to identify DNA markers associated with YB tolerance, two parents namely, Rye Selection111 (RS111) and Chinese Spring (CS) were phenotyped and found to be tolerant and susceptible to YB, respectively. A RIL population of 113 individuals developed by making a cross between these two parents as discussed in previous chapters was used. Phenotyping of this population to YB incidence indicated that, at least one major gene/QTL and few minor genes governed the tolerance to YB. DNA marker analysis revealed linkage of two microsatellite markers Xgwm174 and Xgwm190 from chromosome 5D, while one ISSR marker $UBC842_{600}$ and one RAPD marker $OPR8_{1000}$ with YB tolerance When association of YB tolerance with that of GPC was analyzed using the markers associated with YB tolerance it was found to be reciprocal in this population in accordance with the previous reports.

5.1 INTRODUCTION:

Yellow berry (YB) is an important physiological disorder in wheat, which causes the production of undesirable and off-color grains (Gianibelli *et al.* 1990). YB is recognized by its softer, light colored and starchy endosperm, which lacks the vitreous texture characteristic of normal grains (Sharp, 1927) and is commonly referred to as starchiness or mealiness. YB grains have been reported to contain higher moisture content, a higher percentage of starch and a lower level of grain protein content (GPC) in comparison with vitreous grains (Hubbard *et al.* 1977; Dexter *et al.* 1989). Wheat that is grown in rainfed area is usually not affected with YB, but wheat grown in irrigated land with less nitrogen application is more prone to YB. Poor nitrogen availability is considered to be the most critical factor that leads to the incidence of YB in wheat (Robinson *et al.* 1979). It has been observed for many years that starchiness in wheat is associated with a shortage of soil nitrates. Nitrogen stress on the kernel at the early stage of kernel development may not only depress the total GPC but may also depress the proportion of gliadins (Gianibelli *et al.* 1990). The estimation of vitreous kernel content in hard common and durum wheats is an important international grading factor because of its importance in milling and baking and pasta cooking quality (Dexter *et al.* 1989). The reduced GPC affects the baking and milling quality of bread and durum wheat while mottled YB kernels (even less than 10%) disqualify for export purpose.

Durum wheats are more severely affected due to YB as compared to bread wheat. Since the desired product is coarse semolina and flour is a lower value byproduct, the presence of starchy kernels is considered to be undesirable in durum wheat which leads to reduced semolina yield (Dexter *et al.* 1989). YB also affects the composition of amino acids and proteins particularly gliadin fraction. Waines *et al.* (1978) compared the isoelectric focussing patterns of albumin and gliadin of the YB kernels and normal brown kernels and observed protein differences to be quantitative and not qualitative. Raath *et al.* (1995) demonstrated that though YB decreased GPC by 4% than that of the normal brown kernels, it was the gliadin fraction that was significantly

reduced rather than the albumin and globulin fraction. As a result, the gliadin and glutenin ratio, which is widely used as an important parameter of bread making quality of wheat (Blackman and Payne, 1987) was affected, thereby seriously lowering the bread making quality.

Several studies have shown that there is a negative correlation between YB and grain seed size in durum wheats, bread wheat and triticale (Moss 1968, Alessandroni *et al.* 1976, Waines *et al.* 1978, Robinson *et al.* 1979, Dhaliwal *et al.* 1981, Sharma *et al.* 1983), however, not much is known about the genetic basis of YB. Dhaliwal *et al.* (1986) reported for the first time the inheritance of YB in 6 intervarietal crosses in bread wheat wherein either 2 or 3 dominant genes control the trait. They also used monosomic analysis to identify at least 2 major genes on chromosomes 1A and 7A and 4 modifiers on 4A, 4B, 6A and 6D, which influenced the expression of YB in bread wheat. The lack of genetic information about the control of YB has seriously impeded the development of cultivars resistant to YB. Similarly, there are no reports so far

on tagging of gene(s) or QTLs controlling YB. This is the first study in which an attempt has been made to identify and map the gene(s)/QTLs controlling YB in wheat, using DNA markers. This will enhance our knowledge about the genetic basis of YB and increase the breeding efficiency to develop YB resistant cultivars.

5.2 MATERIALS AND METHODS:

5.2.1 Plant material and field experiments:

A recombinant inbred line population of 113 individual plants was developed by Single Seed Descent method by making a cross between Chinese Spring (CS), which is susceptible to YB and Rye Selection 111 (RS 111) which is highly tolerant to YB as described in Chapter-3 (section 3.2.1). RILs and parents were grown with random block design in the field at Punjab Agriculture University (P.A.U), Ludhiana. 60kg N_2/ha was applied to all the plants at vegetative stage, as there is a maximum expression of the YB disorder

in the susceptible genotype at this level of fertilizer application (Dhaliwal *et al.* 1981). RILs were harvested individually with 50 plants representing each line and selection of the yellow kernels was done by visual examination of each and every kernel. The appearance of YB incidence for each RIL was scored on 0-10 scale i.e. 0 as no YB incidence and 10 as all grains affected. All kernels with yellow or mottled appearance were classified according to the percentage of incidence of YB. 50 plants from each of the RIL were evaluated for the YB disorder and GPC was determined by Kjeldahl method (AACC, 1983).

5.2.2 DNA extractions:

From 15 days old seedlings of parental genotypes as well as RIL population, DNA extractions were done according to the protocol of Anderson *et al.* (1992) as described in Chapter-3 (Section 3.2.2),

5.2.3 PCR analysis:

One hundred ISSR primers (University of British Columbia, Vancouver, Canada) were used for the analysis. ISSR amplifications were carried out according to the protocol described in Chapter-2 (Section 2.2.3A). Amplified products were resolved on 1.5% agarose gels followed by staining with ethidium bromide. In RAPD analysis, total of 480 primers were used for this study (380 primers were from Operon Technologies, USA and 100 primers were from University of British Columbia, Canada). The PCR was performed and amplified products were checked as given in Chapter-2 (Section 2.2.3B). The microsatellite analysis was carried out with total of 106 STMS primer pairs as described in Chapter-3 (section 3.2.5). The amplified products of STMS analysis were resolved on 3% metaphor agarose (FMC Bio Products, U.S.A.) gels, followed by ethidium bromide staining.

5.2.4 Data analysis:

The genotype of each RIL with ISSR, RAPD and STMS was scored and linkage analysis was performed as given in Chapter-2 (section 2.2.4). Single

point analysis to determine molecular marker-putative QTL associations was done using QGENE software (Nelson, 1997) based on simple linear regression and markers with significant P values (<0.05) and/or LOD score (>2.0) were interpreted as putative QTLs.

5.3 RESULTS AND DISCUSSION:

5.3.1 One major and few minor genes govern YB tolerance in wheat:

In the present analysis, the selected two parents Rye Selection 111 and Chinese Spring differed significantly with respect to percentage of YB incidence where RS111 showed 0% incidence while Chinese Spring had 100 % YB incidence under nitrogen deficient conditions. The percentage of YB incidence in the RIL population ranged from 5% to 100%. A histogram (Fig. 5.1) developed based on these values indicated a near bimodal distribution suggesting the presence of at least one major gene, with few minor genes. Dhaliwal *et al.* (1986) studied the inheritance of YB in 6 intervarital crosses in bread wheat. They crossed 6 YB tolerant and varied GPC varieties namely WL711, HD2281, WG357, PH132, PH133 and PH138 with HD2009 a high yielding YB susceptible variety. Analysis of segregating F_2 populations from these crosses indicated that the trait was governed by either 2 or 3 dominant genes on chromosomes 1A and 7A and four modifiers on 4A, 4B, 6B and 6D, which influenced the expression of YB in bread wheat using monosomic analysis. So far, there are no reports of inheritance of YB in durum wheat and triticale. Genetic and molecular analysis of YB tolerance in this study may accelerate more serious efforts in this direction.

5.3.2 Identification of DNA markers associated with YB tolerance:

A total of 115 polymorphic markers representing 18 ISSR, 73 RAPD and 24 microsatellites were previously identified in analysis of Rye Selection 111

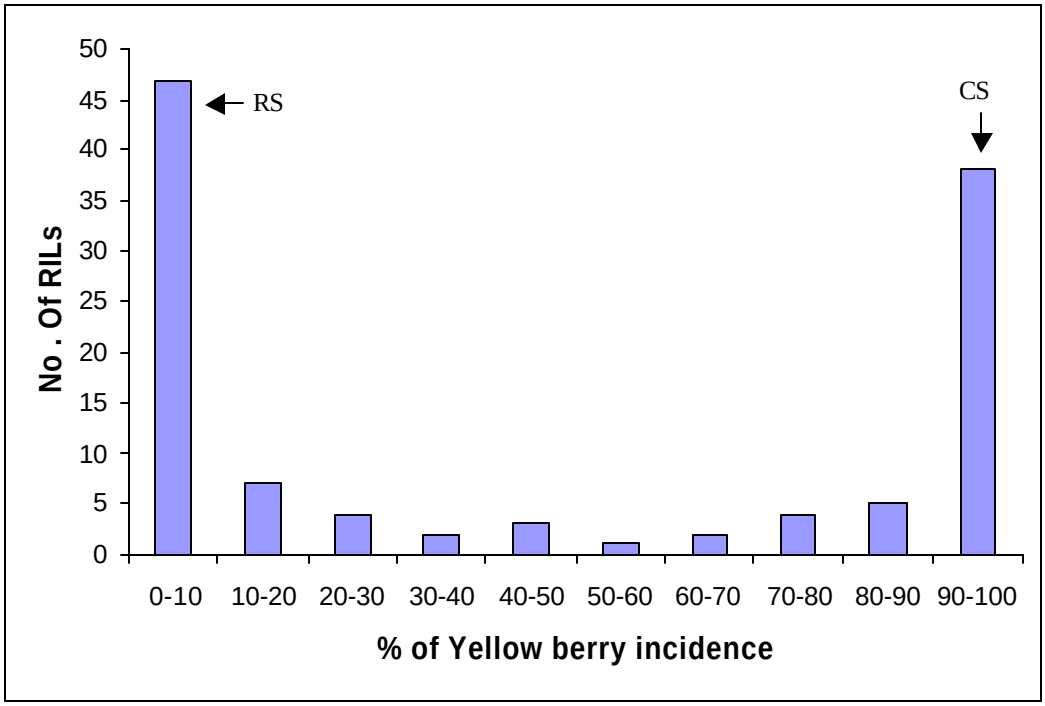

Fig 5.1: Histogram showing frequency distribution of yellow berry in the RILs of Rye Selection 111 X Chinese Spring. Arrows indicate parental values.

and Chinese Spring (Chapter-3). These markers were employed to selectively genotype 13 individuals each with tolerance and susceptibility to YB and markers co-segregating in phenotype specific manner were further used to screen the whole population. Figure 5.2 gives a representative photograph of segregation analysis of one STMS marker Xgwm190. It can be seen that a band of 210bp from is present in Rye Selection 111 (lane a), its allele of 215bp in Chinese Spring (lane b) and out of 26 RILs, the marker locus of 210bp is present in 15 RILs while remaining 11 RILs have an allele of 215bp. Single point regression using QGENE software was employed as the primary method of detecting associations between markers and QTLs/gene(s).

Two microsatellite markers Xgwm190 and Xgwm174 were found to be associated with YB tolerance with contribution of 19.4% and 5.3%, respectively. One ISSR marker $UBC842_{600}$ and one RAPD marker $OPR8_{1000}$ were also found to be associated with YB tolerance with contribution of 4.6% and 4.5%, respectively in repulsion (as indicated by negative values of the phenotypic contribution) (Table-5.1). The microsatellite markers Xgwm174 and Xgwm190 were previously mapped on chromosome 5D (Roder *et al.* 1998), while I was unable to localize markers $UBC842_{600}$ and $OPR8_{1000}$ due to their absence in Chinese Spring in which background the nullitetrasomic and ditelosomic lines were developed. Furthermore, the distance between the pairs of markers was more than 30 cM, and hence it is necessary to carry out interval mapping using more markers in these regions. Markers linked in repulsion phase to genes of interest were also observed elsewhere. For example, susceptibility linked markers were identified for bc-3 Bean Golden Mosaic Virus (BGMV), the co-1 and co-6 anthracnose resistance, Ur-3 rust resistance and fusarium wilt resistance (Haley *et al.* 1994; Jung *et al.* 1996; Ratnaparkhe *et al.* 1998). Furthermore, repulsion phase linkages were observed for QTLs affecting Bean Golden Mosaic Virus (BGMV) and Common Bacterial Blight (CBB) in common bean (Miklas *et al.* 1996).

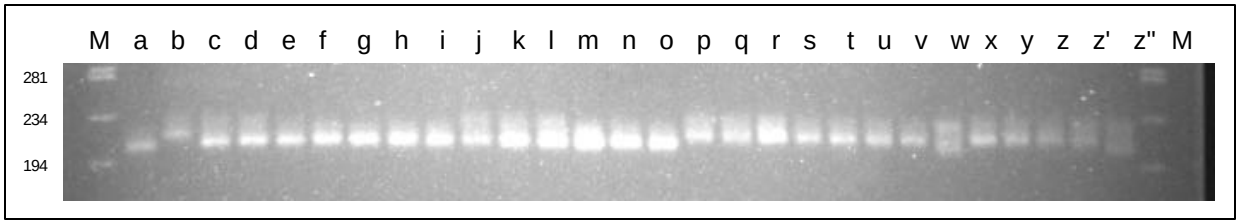

Fig 5.2: Segregation of marker Xgwm190 in the RILs of Rye Selection 111 X Chinese Spring

M = f X174/*Hae*III digest, Lane a: Rye Selection 111, Lane b: Chinese Spring, Lanes c-z" : RILS. A marker band of 210 bp is present in Rye Selection 111 and its allele of 215 bp is present in Chinese Spring

Table 5.1: Markers showing association with yellow berry tolerance.

Marker	R^2 X 100	P value	Chromosome
Xgwm 190	19.4	0	5DS
Xgwm 174	5.3	0.02	5DL
$UBC842_{600}$	4.6	0.02	-
$OPR8_{1000}$	4.5	0.02	-

5.3.3 Correlation of YB and GPC based on molecular data:

Several reports suggest that YB is negatively correlated with GPC and for a 10% increase in YB there is 1% decrease in GPC (Raath *et al.* 1995). In order to study this hypothesis at molecular level, the population was phenotyped for GPC (RS111 and CS differ significantly in GPC) and checked for the linkage and contribution of genes associated with YB tolerance to GPC using the YB linked molecular markers. Unlike YB, GPC showed continuous variation characteristic of a polygenic trait (Fig. 5.3). Further, genetic correlation analysis between GPC and YB showed a very low negative correlation (r = -0.02), in contrast to the available reports, which may be due to the small size of the population. The microsatellite marker Xgwm190 had a significant association with a contribution of 7.2% ($P<0.01$) to GPC and RS111 allele was probably the source for increasing GPC. Similarly, the microsatellite marker Xgwm174 was found to be associated with GPC with a contribution of 4.3%, and Chinese Spring allele probably contributed to increasing GPC, indicating that these marker loci had opposite effects on YB incidence and GPC, strongly supporting the previously reported genetic correlation data.

As an initial step towards the understanding of the genetic and molecular basis of tolerance to YB, this chapter describes the identification of DNA markers associated with YB tolerance. The locus-specific identification of tolerance to YB using microsatellite marker such as Xgwm190 (indirect selection tool) would be of immense help in accelerating marker assisted breeding for YB tolerance and pyramiding such loci into elite backgrounds.

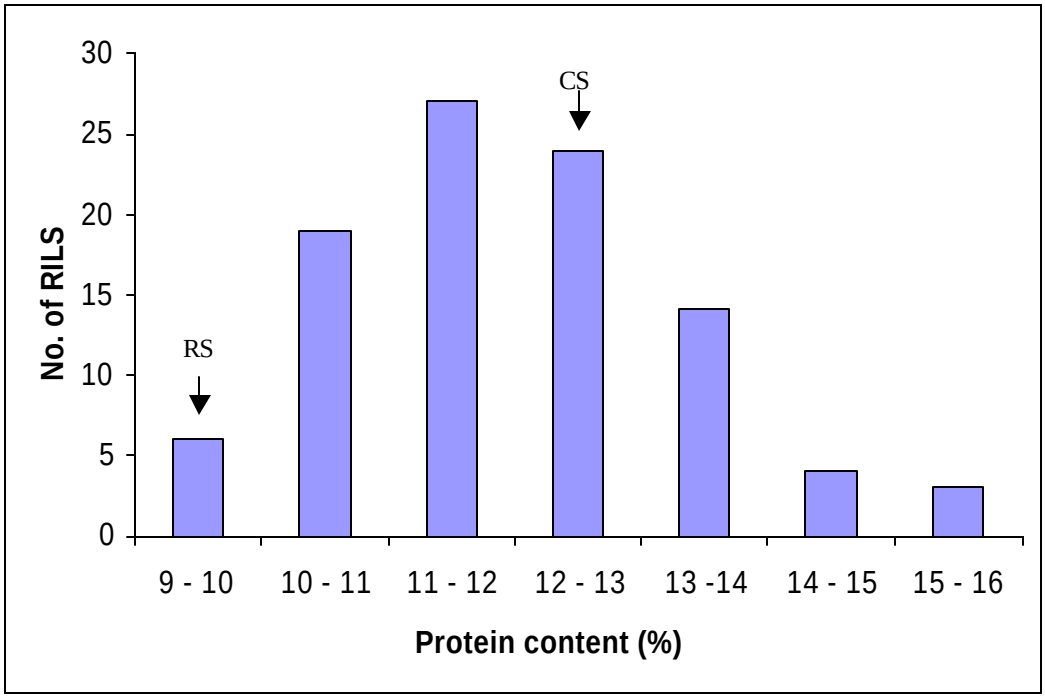

Fig 5.3: Histogram showing frequency distribution of protein content in the RILs of Rye Selection 111 X Chinese Spring. Arrows indicate parental values.

Thesis Summary and Future Directions

Most of the grain quality traits are governed by QTLs due to their polygenic inheritance and hence, it becomes difficult to identify and isolate the genes governing these oligo/polygenic characters through conventional methods of plant breeding. Molecular markers have received considerable attention in recent years and have been extensively used for genetic diversity analysis, phylogenetic and evolutionary studies, and mapping and tagging of genes/QTLs of agronomic importance in different plant species. However, progress in genetic mapping of wheat has been slow but with the development of highly informative and technically simple PCR-based markers like RAPD (Randomly Amplified Polymorphic DNA), ISSR (Inter Simple Sequence Repeat), AFLP (Amplified Fragment Length Polymorphism) and microsatellites, marker-based analysis of agronomically important traits in wheat has gained momentum. Molecular markers associated with a number of genes/QTLs for disease resistance, physiological responses and yield related traits have been identified in wheat as well as in other crops.

The quality of wheat grains, which was not well attended earlier, has become a very important criterion in global market. Indian wheat, in particular, needs to be improved in terms of quality, not only for the domestic use but also for meeting international standards for export purpose. Present-day Indian wheats have optimum characters needed for *Chapati* making, but are not ideal for industrial food products like biscuits, bread and noodles. To have a good bread, characters like high loaf volume, uniform and small grains in the crumb, smooth crust and hardness of grain should be present optimally in a wheat variety. Protein content and grain quality are especially two important characters that determine the overall bread making quality of wheat variety. As dissection of important grain quality traits by means of molecular marker assisted QTL analysis will be of great significance for plant breeders in designing their breeding strategies to improve wheat quality, I have used PCR based markers to identify loci governing grain quality traits and some of the highlights of this work are as follows:

Protein content in wheat: A trait analyzed at two agroclimatic zones in India using molecular markers

Grain protein content (GPC) of hexaploid wheat is one of the important factors that determines the end use quality (higher the GPC, harder the grain which in turn gives good milling recovery) as well as plays a pivotal role in human nutrition. However, GPC is governed by a complex genetic system and is influenced by environment. To identify DNA based markers linked to GPC, 106 RILs were developed from a cross between the two wheat cultivars PH-132 (high GPC) and WL-711 (low GPC) differing significantly in GPC. The RIL population was phenotyped for GPC at two agroclimatic locations, namely, Pune and Ludhiana and the parents were screened with different PCR based markers. The selective genotyping and whole population analysis revealed 15 markers associated with the trait. Two markers were observed to be associated with the trait in both the locations, whereas 6 markers were found to be specific to Pune and 7 markers were specific to Ludhiana, together accounting for 18.8% and 24.0% of phenotypic variation, respectively.

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Molecular marker analysis of wheat grain size, a determinant of consumer preference

The end use quality of wheat is greatly influenced by grain size, which can be measured by TKW (Thousand-kernel weight). Grain size is an important wheat breeding objective as it is phenotypically the most stable yield component and is positively correlated with flour yield. Grain size is a complex character in wheat and any information on its genetic control, is useful in increasing breeding efficiency. The present study was undertaken to identify molecular markers associated with grain size in hexaploid wheat, using 113 RILs developed from a cross of Rye Sel-111 (having bold grain) and Chinese spring (having small grain). Single markers analysis with QGENE software revealed total fourteen markers, which were associated with grain size. While individually these markers contributed 2.6% to 14.8% of total phenotypic variation, they showed total 39%

phenotypic contribution to grain size in multiple regression analysis. This information will help wheat breeders in selection of grain size at early generation and designing strategies to improve wheat grain quality.

Dissection of grain shape and size traits in wheat using PCR-based DNA markers

The economic value of wheat is determined by quality of the grain, which depends in part on appearance of the grain. Grain shape and uniformity, the two important factors which influence the milling quality of wheat, are the final manifestation of several components such as grain length, width and density. To detect putative QTLs (pQTL) controlling grain shape, the RIL population of 113 plants, developed from a cross of Rye Sel-111 and Chinese spring, was screened with different PCR based markers. These 113 RILs were genotyped with polymorphic marker loci and were evaluated for three kernel traits viz. grain length, width and density. The number of significant ($p<0.05$) markers associated with pQTLs ranged from 3 to 10. The percentage phenotypic variation explained by each pQTL associated with marker ranged from 2.9 to 9.5% and many pQTLs were shared among the traits indicating either pleiotropic effect of single QTL and/or tight linkage between QTLs. The information of genetic basis of these traits and identification of markers associated with them is the first step in the final objective of wheat improvement.

Yellow berry tolerance in wheat: Inheritance and molecular marker studies

Yellow berry (YB) is an important physiological disorder in wheat, which causes production of undesirable and off colored grains. YB arises primarily due to deficiency of nitrogen concentration in soil and is recognized by its softer light colored starchy endosperm, which lacks the various texture characteristics of normal grains. YB seriously affects the GPC, thereby affecting bread-making quality in common wheat and pastamaking quality in durum wheat (*T. durum*). In order to study inheritance and to identify molecular markers associated with YB tolerance, the RIL population of 113 plants, developed from a cross of Rye Sel-

111 (YB tolerant) and Chinese spring (YB susceptible), was phenotyped for YB tolerance. The data of YB incidences indicated that atleast one major gene/ QTL and few minor genes govern the tolerance to YB. DNA marker analysis revealed one major locus on chromosome 5D and three markers showed presence of minor loci for YB tolerance. Association of YB tolerance with GPC was analyzed using markers associated with YB tolerance and was found to be reciprocal in this population in accordance with previous reports.

Future of grain quality research with wheat genomics:

Wheat has a large genome size with high proportion of repeat sequences, making it difficult to isolate gene(s) of interest. To improve these efforts and to develop gene enriched genomic libraries, 'Genetic filtration' can be used as an alternative strategy which has been successfully employed in maize with the use of methylation restrictive *E. coli* host strains (Rabinowicz *et al.* 1999). During the last decade, comparative mapping efforts have shown similarity in gene order between wheat and rice and information generated with the complete sequencing of rice genome will be extremely useful for genetic studies in *Triticeae.* However, application of this information to some of the critical genetic problems specific to wheat such as breadmaking quality, pastamaking quality, winter hardiness and vernalization requirements is yet be ascertained (Lagudah *et al.* 2001). Also, positional cloning efforts in *Triticeae* using rice genomics information may have limited application as various reports have shown hybridization of cDNA clones to non-colinear regions in wheat and rice.

Efforts are underway for functional genomics and proteomics approach in wheat. Several labs around the globe are currently engaged in developing the BAC library of tetraploid and hexaploid wheats. During the initial stage in functional genomics of wheat, large numbers of ESTs is being generated and are currently available in the GenBank stems from the co-ordinated efforts of public institutions, under the International Triticeae EST Consortium (http://wheat.pw.usda.gov/genome/index.html). A consortium of thirteen USA-labs has recently been funded to assign 10,000 ESTs

(http://wheat.pw.usda.gov/NSF/home.html). The available information of ESTs can be utilized in assigning the gene function and assembly of a large number of wheat ESTs on solid-media (micro-arrays) can be useful in global gene expression studies in different tissues and developing stages. For example, micro-arrays of grain ESTs will improve our knowledge of the expression of genes affecting starch, gluten and lipid synthesis with their combined effect on grain quality (Lagudah *et al.* 2001). High-resolution 2-dimensional protein electrophoresis with improved heterologus systems for expressing isolated genes from wheat grain will provide proteomic approach, complementing the functional genomics in wheat. Both these approaches are currently being utilized to identify novel genes that may have role in the differential initiation and synthesis of wheat starch A and B granules (Lagudah *et al.* 2001). Availability of molecular markers for the main genes affecting quality has opened new avenues for commercial development of different wheat products. For example, varieties with altered starch properties can be selected using markers for the SGP-1 (starch granule protein-1) and GBSS (granule bound starch synthase) mutants. Also, hardness of the grain can be altered with introgression of 'softness' alleles and gluten strength can be modified with the simultaneous use of molecular markers for high and low molecular weight glutenins (Lagudah *et al.* 2001).

Despite the availability of a large number of molecular markers in wheat, their application in practical breeding involving marker-assisted selection (MAS) is still in its infancy and very few reports are available on MAS for wheat breeding such as Ogbonnaya *et al.* (1998) who have reported usefulness of molecular markers in selection and pyramiding genes for cereal cyst nematode resistance in wheat. My thesis work also provides a base to carry out marker-assisted selection in wheat. For example, the phenotypic data of the two populations i.e. of GPC and grain size, which are grown in different agroclimatic zones can facilitate to identify stable as well as environment specific loci governing the grain quality traits. Similarly, the markers, which are reported to have more than 10% of contribution to the phenotypic variations for these traits, can be used in different populations that are segregating for these characters for the marker

validation and application in MAS. Finally, the ISSR and RAPD markers that are linked to the traits can be converted into SCAR and employed in further population analysis. Thus, taking into account the fact that over 50% of the agricultural productivity of the world has been achieved through conventional breeding and the sensible use of modern tools of biotechnology, as put forth by Nobel Laureate, Prof. Norman E. Borlaug; the close interaction between plant breeders and biotechnologist will accelerate the efforts to meet the challenges of agricultural productivity and food security as well as consumer oriented world food market.

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