

**DEVELOPMENT OF NEAR-ISOGENIC LINES
WITH DIFFERENT SEED STORAGE PROTEIN
ALLELES FOR QUALITY ANALYSIS IN WHEAT
(*Triticum aestivum* L.)**

THESIS

Submitted to the
G.B. Pant University of Agriculture & Technology
Pantnagar-263 145, Uttaranchal, INDIA



by

Meenal Rathore

M.Sc. (Biotech.)

*IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF*

**DOCTOR OF PHILOSOPHY
(Molecular Biology and Biotechnology)**

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*DEDICATED TO
MY PARENTS
WHO ENCOURAGED ME
AND
MY TEACHERS
WHO ENABLED ME*

ACKNOWLEDGEMENTS

As I sit back to write this little piece, I think of the innumerable hands that have steered me through this arduous journey and put me on the right path of learning and enlightenment.

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January, 2002
Pantnagar

Meenal Rathore
(Meenal Rathore)

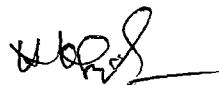
Dr. N.K. Singh
Associate Professor

Department of Molecular Biology and
and Genetic Engineering
College of Basic Sciences and Humanities,
G.B. Pant University of Agric. & Tech.,
Pantnagar-263 145 Uttaranchal (INDIA)

CERTIFICATE

This is to certify that the thesis entitled “**Development of Near-Isogenic Lines with Different Seed Storage Protein Alleles for Quality Analysis in Wheat (*Triticum aestivum* L.)**”, submitted in partial fulfilment of the requirements for the degree of **Doctor of Philosophy** with major in **Molecular Biology and Biotechnology** and minor in **Biochemistry** of the College of Post-Graduate Studies, G.B. Pant University of Agriculture and Technology, Pantnagar, is a record of *bona fide* research carried out by **Ms. Meenal Rathore**, Id. No. 22985, under my supervision and no part of the thesis has been submitted for any other degree or diploma.

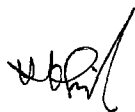
The assistance and help received during the course of this investigation and sources of literature have been duly acknowledged.



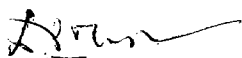
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CERTIFICATE

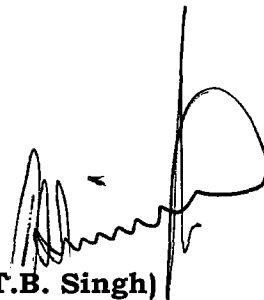
We, the undersigned, members of the Advisory Committee of **Ms. Meenal Rathore**, Id. No. 22985, a candidate for the degree of **Doctor of Philosophy** with major in **Molecular Biology and Biotechnology** and minor in **Biochemistry**, agree that the thesis entitled "**Development of Near-Isogenic Lines with Different Seed Storage Protein Alleles for Quality Analysis in Wheat (*Triticum aestivum* L.)**", may be submitted ⁱⁿ partial fulfilment of the requirements for the degree.



(N.K. Singh)
Chairman
Advisory Committee



(D.P. Mishra)
Member



(T.B. Singh)
Member

(G.K. Garg)
Member

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CHAPTER 1

INTRODUCTION

Wheat was the major crop responsible for development of man because of its intrinsic values for human health, convenience of production and storage and the varied end-uses of the flour it gives. This is because only wheat flour has the unique ability to form dough when mixed with water and retain gas during fermentation or by chemical leavening to give a leavened product. Apart from nutrition that it provides, wheat gives fibre and gluten with film producing properties. Indian foods are in great demand overseas, and the production of varied end products requires enhanced wheat production.

With sustained wheat production over the years, India has become the second largest wheat-producing nation in the world (**S. Nagarajan, 1999**). Being self-sufficient in wheat production, it is high time to address problems related to wheat quality for both domestic consumption as well as promotion of export potential. Wheat quality is affected by physical and chemical properties of the endosperm (**Halverson and Zeleny, 1988**). Amongst carbohydrates, lipids, proteins and additives, seed storage proteins are the most important factors affecting wheat quality. Protein quantity and quality contribute to the functionality of wheat flour.

Protein content/quantity is a function of the environment and can be measured precisely but protein quality is complex and difficult to measure. Storage proteins of wheat are actually

responsible for the variation in rheological properties in flour. Four classes of seed storage proteins are found in the wheat endosperm, namely-glutenins, gliadins, albumins and triticin. Sixty percent of the variability in quality characteristics can be attributed to HMW subunits of glutenin (**Payne, et al., 1987a**). Other proteins and their subunits, too, are known to effect wheat quality but more detailed study is required in this aspect. These proteins are encoded by at least 15 different major genes located on different homologous chromosomes of wheat (**Payne, et al., 1987a**). Each gene has several allelic forms. Thus, for all seed storage proteins a million combinations are possible! Each allele, individually, exerts an effect on the rheological properties of wheat flour. Numerous studies have been done for correlating allelic variation to dough property using different methods- chemical fractionation and reconstitution (**Finney, 1943; MacRitchie, 1985**), use of recombinant inbred lines (**Vazquez, et al., 1996; Pogna, et al. 1990**), genetic engineering (**Shewry et al., 1995b; Barro et al., 1997**), screening of varieties (**Ng and Bushuk, 1988; Autran, et al., 1986**) and use of near isogenic lines (**Payne, 1987a; Kozub, et al., 1994**). Each method has its own advantages and disadvantages. Till date, sporadic reports have been acknowledged for correlation studies. There is no report available for a comprehensive study of relative effect of alleles on the rheology of dough.

With this background, the present study was initiated with the following objectives.

1. Comprehensive cataloging of alleles present at different loci in wheat seed storage proteins and the standard varieties from different countries in which they are reported/initially identified. (Based on literature survey).
2. Screening of released Indian wheat cultivars for study of allelic variation at different loci in seed storage proteins (especially for gliadins and globulins).
3. Production of a comprehensive set of near isogenic lines in HD2329 with different seed storage protein alleles using molecular marker assisted accelerated backcrossing.

CHAPTER 2

REVIEW OF LITERATURE

Wheat, the miracle crop of the twentieth century, was originally a wild grass. Its intrinsic value for the human and animal health coupled with the convenience of its production and storage, enabled man to change some 6000-7000 years ago from being a nomadic hunter to a settled farmer and so, provided the basis for the rise of many centres of civilization. Evidence exists that it first grew in Mesopotamia and in the Tigris and Euphrates River Valleys in the Middle East nearly 10,000 years ago. Once settled, man began experimenting with the grain.

People gathered and began to develop ways to grind more grain at one time. At first, a couple of good stones were used, then sloping stones were used. This was very physical labour and the coarsely ground grain also wore down peoples teeth. Then sieves began to be used to separate flour from coarse pieces of grain around 6000 B.C. Still, the stone ground wheat took a toll on teeth. Later, mills developed ...early mills used two horizontal, disc shaped stones that rubbed and later ground against each other. This was the hand powered quern. Then, the millstones came. Windmills developed late in France- in 1180 A.D. Development of water driven and wind driven mills changed agriculture. Then came electricity and today, there are many types of mills serving to give finely ground, good quality flour in larger amount.

Once the flour was in hands, experiments begun and as early as 6700 B.C. , the Swiss lake dwellers used wheat in flat bread. It was the Egyptians who discovered how to make yeast-leavened breads between 2000-3000 B.C. Workers who built the pyramids were paid in bread. In 150 B.C. , the first baker guild was formed in Rome. By 1202, England adopted laws to regulate the price of bread and limit the profits of bakers. The bakers mark were among the first trademarks.

So, the civilizations established and developed. Wheat was the major crop responsible for development of man, and why not, more foods are made from wheat than from any other cereal grain. This is because only wheat flour has the unique ability to form dough when mixed with water and retain gas during fermentation or by chemical leavening, and thus give a leavened product.

2.1 USES OF WHEAT

Nutrition had been wheat's first role and is still overwhelmingly the primary role today. A glance at table 2.1 listing the nutritive value of wheat and other cereals reveals the advantages of its consumption in our daily diet-

Table 2.1 Nutritive Value of Cereals (per 100gm.)

Crop	Proteins (g)	Carbohydrate (g)	Fat (g)	Crude fiber (g)	Mineral matter	Calcium (mg)	Phosphorus (mg)
Wheat	11.8	71.2	1.5	1.2	1.5	41.0	306.0
Rice	6.8	78.2	0.5	0.2	0.6	10.0	160.0
Barley	11.5	69.6	1.3	3.9	1.2	26.0	215.0
Maize	11.1	66.2	3.6	2.7	1.5	20.0	348.0
Sorghum	10.4	72.6	1.9	1.6	1.6	25.0	222.0
Pearl millet	11.6	67.5	5.0	1.2	2.3	42.0	296.0

Mangla Rai and S. Mauria(1999)

Wheat foods are available sources of incomplete protein. But within the cereal group, wheat contains more protein than rice or corn and very little fat. Trace minerals selenium and magnesium are present. Other vitamins present are thiamine, iron, zinc and riboflavin.

Fiber is the indigestible carbohydrate in food which acts like a broom to sweep our digestive tract. With 1.2g of crude fiber in 100gm, wheat, with a variety of dietary fibers, provides protection against large bowel cancer (**Graeme P. Young, 1991**).

Besides being used as human food, wheat is also used as animal feed and as seed for growing the next crop. It is also extensively used for research purposes e.g. for breeding high yielding varieties or making disease resistant transgenics etc.

Research has also given products like VITACEL[®] wheat fibre which is white , micro-fine dietary fibre made from wheat plant using

a special production process. Its multifunctional and physiological properties make Vitacel the perfect choice for a wide range of applications in the food industry. This fibre is a food which can be added to all other foods, provided that no other special instructions have to be observed due to the composition of these foods (**Wieser,H; Kieffer,F. and Lelley,T., 2000**).

Wheat gluten, well known for its viscoelastic properties , also has film producing properties. **Wiegels and Latenster Zetmeel (1996)** have developed a non-food application of wheat – an organic solvent free and stable gluten dispersion that has excellent film forming properties. Work on using gluten for making edible/nonedible and/or biodegradable films and coatings has been underway (**Gontard and Guilbert, 1996**).

Wheat weaving is another area of use of wheat and it is the modern name of an old folkart. Objects made from dried straw are known to have been made in the earliest civilization, practised throughout Europe, Asia and South America. Harvest rituals occurred in every country where grain is grown in order to please the spirits of the crop. Abstract shapes or religious symbols made from straw were believed to ensure prosperity and good luck in the next growing season. Today, the art is so famous that Straw Art Classes are being offered in esteemed universities and a National association of Wheat Weavers is established in several countries.

The industrial end-uses of wheat are numerous and varied. Considering food enduses, wheat can be consumed in grain form or whole wheat can be flaked, shredded or otherwise heated to make breakfast cereals. Softened or malted grain is incorporated into baked products. But almost all human consumption of wheat follows the milling of wheat which is an industry inevitable to India. Wheat flour, sieved or not, may be leavened or unleavened and can be used with various quantities of salt, fat, sugar, eggs and probably other food constituents to prepare a variety of food e.g. breads, waffles, biscuits, rusks, muffins, cakes, pastries, crackers, pasta, macaroni, semolina etc. In India, we cannot ignore products like the daily chapati, paratha, puri, halwa, laddoo, sweets etc. And the production of all these is industrialized for there is great demand not only in the local market but also in the international market. This heritage of India is in great demand overseas and stores supplying a variety of Indian food are now a common affair abroad.

The increasing fame and demand of Indian food required enhanced production of varied endproducts and indirectly, asked for more production of wheat. There has been rise in the total area cultivated with wheat over the last decade. Lately, there has been sustained increase in wheat production without a corresponding increase in area thanks to the improved per hectare productivity. By harvesting more than 65 million tonnes for three years in a row,

India has emerged as the second largest wheat producing nation in the world (**S. Nagarajan, 1999**).

2.2 PRODUCTION OF WHEAT

The world wheat supply and demand data depict that though the total world wheat demand has increased by 11.02 mmt from 97/98 to 00/01 (Fig 2.1), there has not been a relative rise in wheat production. On the other hand, the total world wheat production is assumed to fall by 33.53mmt (USDA, 2000).

The comparative chart of the top five wheat producing nations from 1996-99 (Table 2.2) gives proof of the rise in India's position from a 62×10^6 mt producer in 1996 to the worlds second largest producer of wheat by 1999.

The area outlined (in green) on the map of India (Fig 2.2) indicates the main wheat growing areas in the country.

Punjab and Haryana have reached a high level of productivity and this has helped India to see a sustained increase in wheat production over the years (Fig 2.3). And so, India now shares 12.97 % of the share in total world wheat production leaving America behind with only 11.48%. China is still the largest producer of wheat in the world (Fig 2.4).

2.3 THE INDIAN SCENARIO: MILLING AND BAKING

Nearly fifty five percent of the total wheat produced is marketable surplus and of this only 12 million tonnes is needed for the roller flour mill (RFM) industry. Wheat flour is the first point of

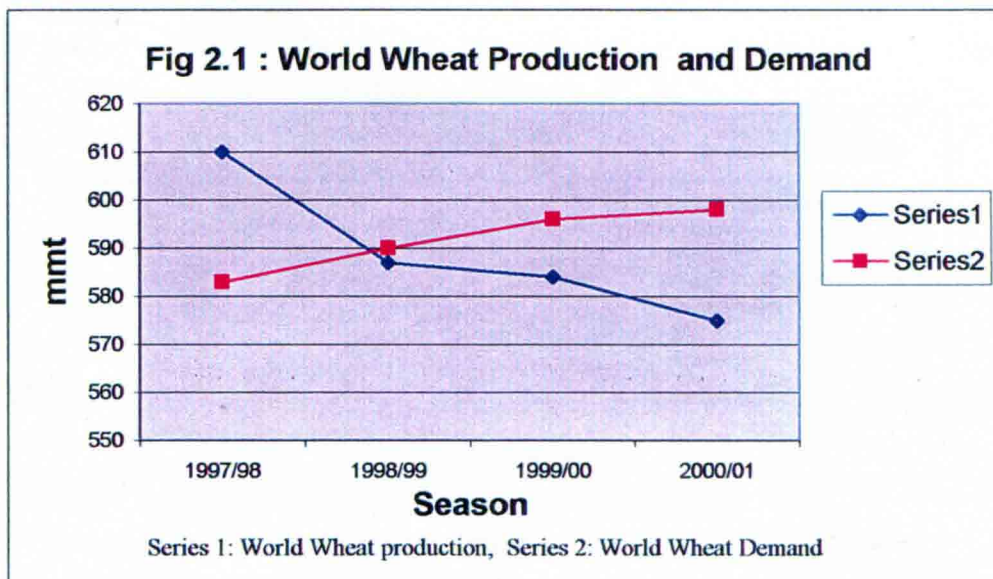


Table 2.2: Top Wheat Producing Nations, 1996-1999 (metric tons)

Wheat Production (mt)	Year			
	1996	1997	1998	1999
China	110,569,193	123,290,085	109,726,066	114,400,066
India	62,097,400	69,350,200	66,345,000	70,778,496
United States of America	62,000,000	67,523,000	69,327,000	62,662,000
France	35,948,900	33,847,000	39,793,000	37,009,248
Russian Federation	34,916,680	44,257,720	27,011,880	30,960,000

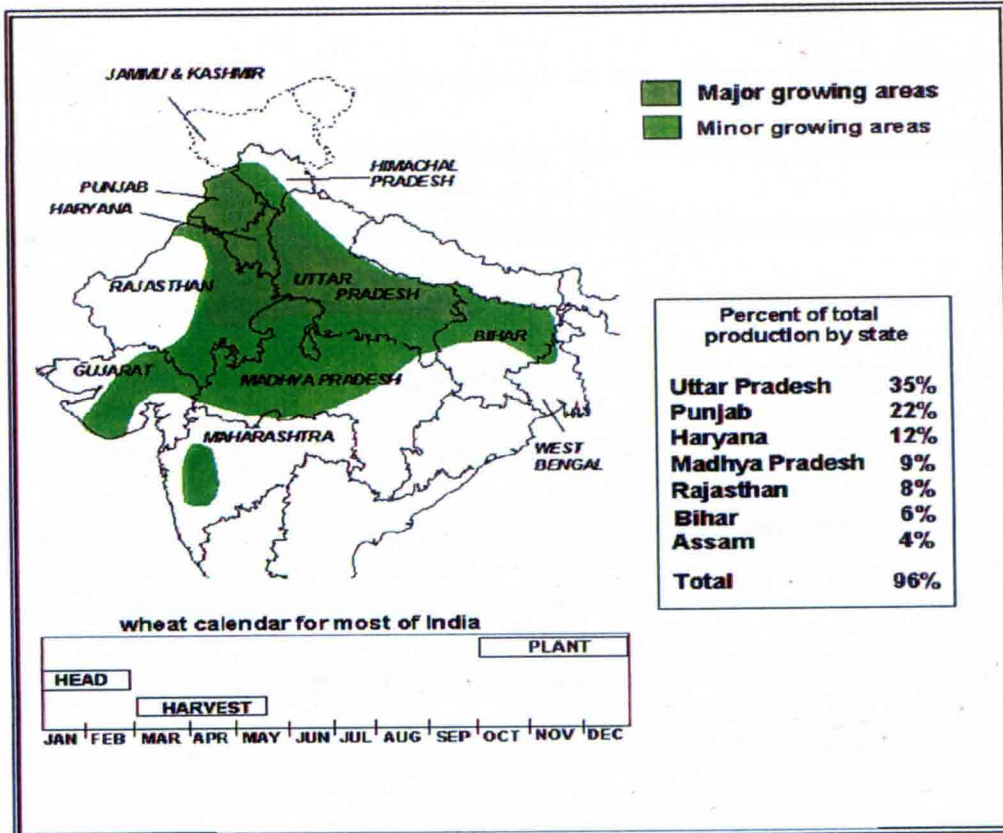


Fig.2.2: Wheat Growing Areas in India

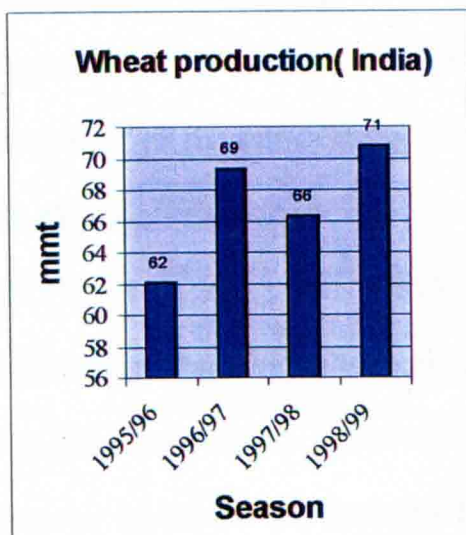


Fig. 2.3

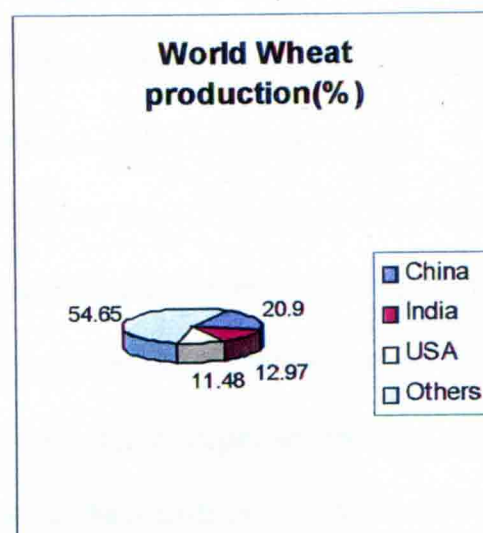


Fig. 2.4

value addition though the value added is low. Wheat flour serves as the raw material for processed foods. The semolina serves as base material for extruded products. In most of the metropolitan cities, high quality flour is needed for flat bread. A substantial quantity of wheat flour is also exported from India.

The 800 RFMs in India have an installed capacity of 20 million tonnes. But since they are heavily dependant on the state run procurement systems , only 60% of the installed capacity is used by the RFMs. By 2001 end, India will annually produce 3.6 million tonnes of biscuits, 0.5mt of vermicilli and 0.15 mt of noodles. Currently the annual growth rate of the food processing industry is around 5%. The crop improvement programme has taken note of this and accordingly come out with several new genotypes that are suited to the needs of the processing industry.

2.3.1 Milling efficiency

The grain size of wheat varies between varieties and in general, harvests from Madhya Pradesh and further south have larger grain and higher hectolitre value (HLV). The HLV represents the grain density or how well the endosperm is packed and is an index of flour recovery. By reducing the size of the embryo and making the aleuron layer thin, milling efficiency can be substantially improved. The All India Wheat Improvement Project is involved in the development of milling efficient, high quality wheat varieties suited for industrial needs.

2.3.2 Quality spots

The grain quality group of the Wheat Improvement Programme analyzed several samples and noted that at some locations, good quality grain is produced that suits bread, biscuit and pasta quality. Therefore, by growing the correct varieties at these sites and by procuring and milling the produce separately the industry will benefit. Good quality value added products can be procured by making small corrections in the manner that wheat is produced, procured and supplied to the mills. The loaf volume of bread is dependant on the grain protein level and the high molecular weight (HMW) glutenin value. The grain protein can be modulated by growing wheat at the favourable site and by developing new genotypes more suited to the processing industry.

2.4 WHEAT QUALITY

Wheat quality may mean different things to different people. To the cereal chemist, a quality wheat is one that will produce the most acceptable end product for that particular class (**D'Appolonia, 1993**). In speaking of flour, the terms 'quality' and 'strength' are frequently used as if they have the same meaning, which is not true. The term strength has a very clear definition, although other meanings are sometimes added to this term. "Quality" clearly defines its suitability for the intended purpose... a flour of high quality for pastries is a flour of low strength while a flour of high quality for

the production of very white bread or for mixing to improve the strength of a weak flour must be of high strength.

Nutritional quality of wheat refers to the various nutrients present in wheat flour such as proteins, minerals and vitamins. Details of these nutrients naturally present in wheat flour has already been discussed earlier (Table 2.1). The amount of nutrients varies little between varieties. However, there is difference in the amount of aminoacids present in seed proteins and so a larger fraction of a particular protein type shall indicate more of its constituent aminoacids.

Apart from nutritional quality, in analyzing the quality of wheat, one must understand this from two point of views- the millers and the bakers. These two frequently confuse the terms 'milling quality' and 'baking quality'. The miller wants above all to obtain a high yield of flour, while the baker requires a flour with the appearance and strength for its particular purpose. Wheat of excellent quality, therefore when milled, may produce flour that is utterly inedible, while a poor sample of some shrivelled wheat may produce a small yield of flour with excellent baking qualities. Hence, wheat quality is also known as "end-use quality" and its definition is universally accepted to be identical to the cereal chemists "wheat quality".

Suitability of a wheat for any given enduse depends on the various factors that effect the rheological properties of wheat flour.

These include physical and chemical properties of wheat which are in turn effected by environmental factors and their genetic makeup. The quality criteria in most cases refers to the characteristics of the endosperm (**Halverson and Zeleny, 1988**).

2.5 FACTORS AFFECTING WHEAT QUALITY

The obvious physical characteristics of wheat kernels include-

2.5.1 Physical Properties:

The obvious physical properties of wheat kernels include shape, size, color, bulk density or test weight , vitreousness, and hardness. Three physical characteristics that have traditionally been associated with flour milling quality are bulk density or test weight , mean kernel weight and kernel size distribution.

Bulk density or test weight, measured by weighing the amount of wheat that will fill a container of given volume when loaded in a strictly prescribed manner, is widely used as a primary specification in wheat grading and has also been considered to be correlated directly with flour yield. Test weight is influenced by factors such as weathering, wheat moisture content, kernel quantity and packing factors that would not be expected to relate to milling yield.

Kernel size distribution is of great importance to the flour miller particularly in the cleaning house where he may have difficulty in removing impurities that are of similar size and shape to millable

wheat kernels. Modern concentrators can separate dirty wheat into two streams in order to minimize cleaning losses.

Vitreousness and hardness are two other properties that can detect flour quality. Their relationship may be described by making two statements. The first is that vitreous kernels are always hard, but hard kernels are not always vitreous. Also, whereas vitreousness can be seen, hardness must be measured. Vitreousness has long been used to grade wheat grain having higher protein content (and hence higher value) or to assist in distinguishing between wheat classes. E.g. for durum wheat which is to be milled for granular semolina a high degree of vitreousness is desirable because non vitreous kernels tend to produce more flour during milling. Knowledge of hardness is essential for the miller e.g. soft wheat break down more quickly on the mill and need more surface at the front end of the mill flow unlike hard wheat. Knowledge of the degree of wheat hardness allows the miller, if necessary, to adjust his flow, sieve clothing and feed rates for optimum milling performance.

Hardness can be determined by either measuring the mean hardness of bulk samples or hardness of single kernels. The former gives indication of tempering requirements while the latter gives idea on the degree of heterogeneity within a bulk sample. Factors which can effect wheat hardness besides the genetic makeup of the variety are wet harvest conditions, frost damage during kernel development and protein content. Excess rainfall when mature wheat is in the

field can cause weathering and softening of the endosperm. And frost damage during growing period can cause kernels to become extremely hard and difficult to mill. Milling yield is reduced, flour colour deteriorates and starch damage is increased.

2.5.2 Chemical Properties:

Wheat chemical characteristics can be discussed by considering the composition and structure of the basic constituents and how they effect end-use processes. These can be categorized into four groups-

1. Carbohydrates
2. Lipids
3. Proteins
4. Additives.

2.5.2.1 Carbohydrates:

- *starch (damaged starch)
- *other complex carbohydrates
- *sugars

Damaged starch has been studied because of its close association with water absorption and starch itself, but it is not generally considered an important quality determining factor unless germination has caused degeneration of a portion of the granules. As such there are no big differences in starch properties between wheat varieties.

Other polysaccharides, notably cellulose and hemicellulose are important nutritionally. Hemicellulose has been demonstrated to promote oxidative gelation but its importance in bread making has not yet been established.

Non starch carbohydrates, pentosans (arabinoxylane and arabinogalactans) are contributors to textural variation and end use functions in soft wheat. Pentosans don't appear to have a large texture modifying effect in hard wheats. Within soft wheats, membrane associated pentosans are most influential in explaining variation in softness. Membrane associated pentosans also explain 69% of the variation in water holding capacity of wheat flour –a widely used measure of wheat quality. Total pentosans, plus flour protein explain 87% of the variation in cookie quality within the soft wheat class, thus providing information needed to guide the processing of wheat (**Bettge,A.G. and Craig, M.;1999**).

2.5.2.2 Lipids:

As ingredients, lipids provide structure, lubricate and separate dough sheets and produce the derived organoleptic properties of flavour and texture. Soft wheat starch has associated with its surface a number of polar lipids while hard wheat and durum starch have very little or none. (**Morris Craig, 1999**).

Lipids modify extractability of gluten proteins and their aggregation, as they relate to breadmaking quality (BMQ). Much evidence indicates that lipids can suppress (**Simmonds et al, 1972**)

or enhance (**Charbonnier, 1973**) the solubility of isolated or flour proteins and thus depends on the quantities and types of lipids in the association of proteins. **Chung and Pomeranz (1979)** did experiments on proteins, lipids and their interactions showed that—

i) Less protein lipid interaction occurs in poor than in good bread making flours. ii) Proteins from good flours more strongly bind lipids than as proteins from poor flour. iii) glutenin bind more lipids and are more hydrophobic than gliadins. iv) Hydrophobic properties of acid soluble proteins from good and poor bread making flour are different. v) The mechanisms, as they relate to protein-lipid interaction, of optimum dough mixing time, mixing tolerance, and loaf volume potential may be different.

Chung (1982) and coworkers were the first to show strong inverse correlation between loaf volume and the ratio of free nonpolar lipids to free polar lipids in bread making. For such interaction, the lipids must have a partner for best results, that partner must be, among others, protein of high quality. Consequently, polar lipids can only slightly effect the protein quality of flours of poor bread making potential (**Pomeranz, Y.; 1985**). However, efforts to use lipids to enhance consumer acceptance have been done e.g. a new function of di-oleyle-glycerol was added to the flour which greatly softened the bread (**Inaoka,S.; 1991**).

2.5.2.3 Proteins:

* quality- seed proteins

* quantity

The ability of wheat flour to form a viscoelastic dough on mixing with water depends largely on the physiochemical properties of its proteins. Based on protein content, dough strength and grain hardness and their relation to different endusage , wheat cultivars can be classified as follows (Fig 2.5). **Dough Strength**

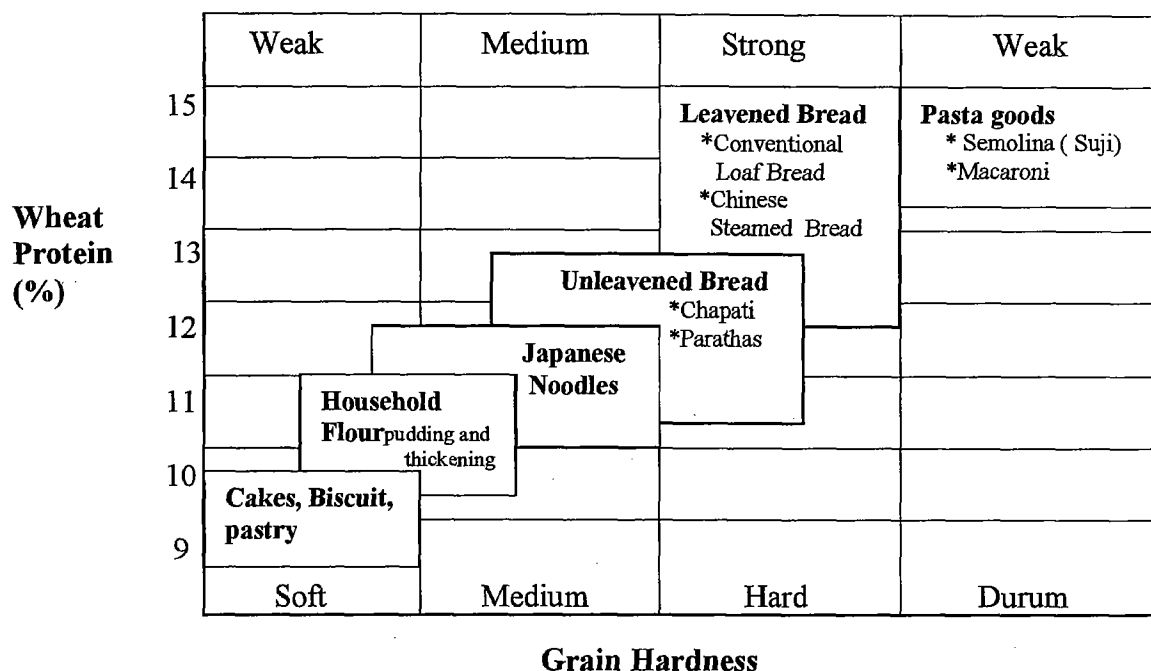


Fig 2.5. The range of wheat types for different endusage (Based on Australian bread making Handbook)

Thus, it is clear that wheat proteins contribute to the functionality of wheat flour in two ways-

- 1) The flour must have a relatively high protein content
- 2) The protein must have the right quality.

Protein content may be determined precisely but protein quality is extremely complex and difficult to measure. Quantity is largely a function of the environment depending on agronomic and weather conditions while protein quality is primarily a genotypic trait.

However, quantity is also influenced by the wheat variety and quality is also affected by the environment.

When water is added to flour, the proteins with some other flour constituents, rapidly become hydrated. Both protein quantity and quality are important factors in determining the rate and capacity of water absorption by flour. The individual hydrated flour particles are forced together by the mixing action to form the continuous network of dough. It is the unique protein of wheat that plays a key role in the transformation of flour into dough. Subsequent mixing transforms the agglomerated flour particles into a developed dough with just the right combination of physical properties for maximum loaf volume with appropriate crumb texture and grain. During dough development, protein aggregates, which at first appear to be particulate or fibrillar in physical structure, are converted into continuous films or membranes that have the right combination of rheological properties for optimum expansion and gas retention during baking. The transformation of hydrated flour proteins into a developed dough depends, to a large extent, on the nature of flour proteins.

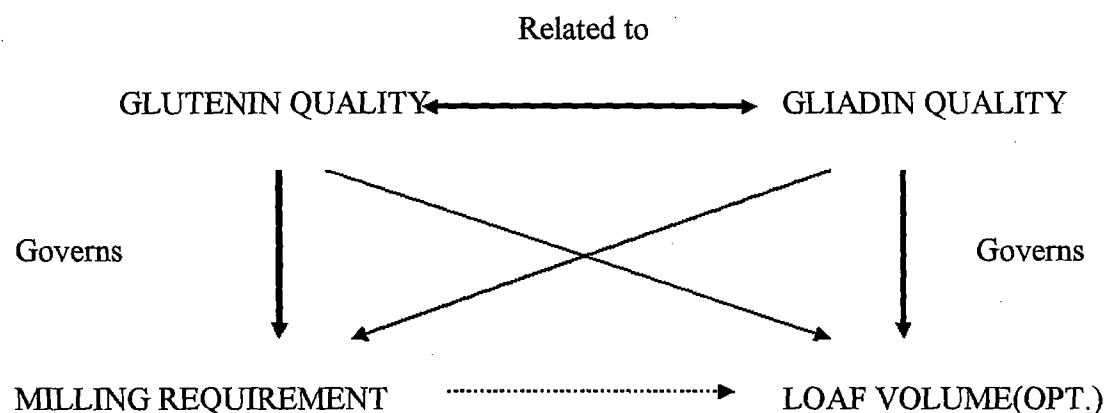
The function of flour protein in bread making is not over with denaturation during baking. Increasing evidence shows that both content and quality of flour particles contribute to the retention of freshness in bread. Finally, protein contributes substantially to the eating quality of bread and to the nutritive quality of the overall diet.

2.5.2.3a Quality of the Seed Proteins

All three types of proteins, defined by their physiological function as metabolic, storage and structural, are present in wheat

flour. Technologically and in relation to functionality, storage proteins play the key role in structure of gluten and dough, and are the most important.

With the right combination of constituents and ingredients, optimum dough structure is developed and good bread is made. Apart from presence or absence of a protein, the combination of their alleles is another factor important in determining the flour quality. Probable relationships between and within functions, properties and protein fractions is also an important consideration in this context. Loaf volume is a function of Gliadin quality and mixing requirement is a function of glutenin quality. The relationship is shown in the figure as follows (Fig 2.6):



Broken lines indicate indirect relationship

The broader the line, the stronger the relationship.

Fig 2.6: Relationship between loaf volume milling requirement, gliadin and glutenin quality (Rosalind, 1984).

Also, cytogenetic studies have revealed (Rosalind, 1984) gliadin and glutenin to be located nearby on the same chromosome.

Therefore, a direct relationship between the qualities of gliadin and glutenin were expected and together with the other direct relationships between glutenin quality and mixing requirement and between gliadin quality and loaf volume, the indirect relationships indicated by the broken lines is supported.

Some of the most notable advances in understanding the role of gluten proteins begun from the work of Dr. Payne and his colleagues and when they based their experiments on earlier pioneer work done in this aspect. They showed that high molecular weight glutenin subunits are associated with various quality characteristics, notable dough strength and loaf volume. Using separate isogenic lines, **Payne *et al.* (1987b)** found that up to 60% of the variability in quality characteristics of varieties could be attributed to these HMW subunits. With development of new techniques, refinement of old ones, more extensive study has been done and is underway in this aspect. More of this shall be dealt with later in this manuscript.

Factors affecting the solubility of proteins indirectly affect the functionality of flour proteins in determining flour quality for bread making. The differences in solubility result from a combination of differences in molecular properties and the related physical properties e.g.

i) Amino acid composition- variation in amino acid composition of glutenin fractions is well documented (**Khan, K.; 1979**). Accordingly total glutenins of wheat varieties of different qualities ought to vary

in this aspect. However, significant differences have not been proven by direct analysis.

ii) Molecular weight- fractionation studies of glutenin in strongly dissociating solvents (**Kobrehel,K.; 1984**) indicates that glutenin of better quality wheat varieties have a higher average M.wt. than do the glutenins of lower quality wheat.

iii) Primary, Secondary and tertiary structures- all three levels of molecular structure contribute to physical properties, e.g. solubility. Presumably this applies to higher levels of structure, such as aggregates and films, both of which are vitally important to the functionality of flour proteins in breadmaking.e.g. Thompson and coworkers found the location of a cysteine residue near the amino terminal of the glutenin subunit. In the actual glutenin molecule, the sulphydryl group of this residue forms a disulphide crosslink with a similar residue of another subunit. Such a terminal location of an inter polypeptide disulphide bond would be consistent with the recent molecular models of glutenin and with the well known effect of reducing agents on the rheological properties of glutenin and dough.

2.5.2.3b Quantity of the Seed Proteins

The effect of quantity on the rheological properties of flour and its bread making functions can be measured easily. Studies on this aspect have been ongoing for quite some time. The different effects are-

- i) When protein decreases below 12% mixing time increases, primarily because lower the protein, the greater the difference in forming a continuous phase (**Finney, K.F.; 1967**).
- ii) Physical dough properties of the mixogram are generally poor indices of dough handling properties e.g. the desirability of handling usually lessens with decreasing mixing requirement and tolerance.
- iii) As protein content increases within a variety, water absorption increases (**Finney et al., 1945**).
- iv) Oxidation requirement of bread wheat is a function of both quantity and quality of wheat (**Shogren et al., 1974**).
- v) Studies (**Finney, K.F. 1943; Finney and Barmore, 1948; Finney and Fryer, 1958**) have revealed that loaf volume increases with higher protein content within a variety. Loaf volume and protein content of a single sample of wheat flour establish its relative protein (gas retention) quality.
- vi) Crumb grain of bread is effected both by quality and quantity of protein. The effect of growth conditions also effects BMQ indirectly by effecting flour protein components and dough properties (**Pechanek et al, 1997**).

Thus, on a whole, the quantity and quality of seed storage proteins determines the bread making quality to a large extent. And, as if this is not enough, these endosperm components don't act in isolation during end processing. Powerful new techniques help

unravel and study some of these complex interactions between protein, carbohydrates and lipids.

2.5.2.4 Additives

Numerous additives are used in the baking industry. The commercially available flours in India do respond to additives and are thus used. Bromate, ascorbate, proteases, amylases, L-cysteine, emulsifiers, metabisulphites, etc do their specific corrections to dough rheology. Bleaching agents are used to give the endproduct a desired colour. However, the list of additives in India is not as elaborate as can be seen in most of the other countries. A few of the commercially available additives finding place in the baking industry of countries are-

- i) Lecithins from Ross and Rowe, Division of Archer Daniell Midland Co., Illinois- this when added to dough or batter results in reduced emulsifier cost and more rapid and homogenous mixing of ingredients, easier handling and significantly improved texture (Ross and Rowe, 1985)
- ii) Doh-tone- an alternative of malt. A lower amount of Doh-tone (1gm) substitutes 60gm of malt thus reducing storage, handling and shipping costs. Doh-tone becomes inactive prior to starch gelatinization and indirectly enhances shelf life of endproduct (**Pennwalt, 1985**).

2.6 SEED PROTEINS

Protein content of wheat seeds grown under normal conditions varies from 8-15% on dry weight basis. The time of protein biosynthesis varies- albumins and globulins are preferentially synthesized in initial stages of grain development and other storage proteins in later stages (**Galova et al.1999**). The seed storage proteins are deposited in the developing starchy endosperm. Thus, their only biological role is to provide a store of amino acids for germination. The proteins are synthesized on the rough endoplasmic reticulum (ER) and are directed into the lumen via a standard signal peptide mediated mechanism. They are then deposited in protein bodies which may be derived from direct accumulation within the ER lumen or transport via the golgi apparatus to the vacuole: the precise pathway and mechanism is still unclear and may vary between proteins and stages of development (**Rubin, R.1992; Shewry, P. R., 1995a, b**). **Hara Nishimura I et al (1998)** showed that novel vesicles accumulate a large amount of proprotein precursors of storage proteins {and are called precursor accumulating (PAC) vacuoles} which mediate the transport of a major storage protein ,here, pro2S albumin, to protein storage vacuoles in developing seeds. However, as endosperm cells fill with starch , the protein bodies (now in a ~20µm range of diameter) disrupt and finally coalesce to form a matrix of storage protein surrounding the starch granules in mature dry tissue (**Shewry. P.R. and Tatham, A.S., 1995b**).

2.6.1 Classification

Wheat endosperm consists of 80-85% alcohol soluble protein fraction, which are kernel prolamins and 10-20% of albumins/globulins which are water/salt soluble proteins. The major prolamins fraction is traditionally sub-divided into gliadin and glutenin based on their solubility. Gliadins are monomeric proteins constituting 25-45% of the total flour proteins and are known to contribute viscosity to the dough upon hydration. Glutenins on the other hand are polymeric in nature and constitute 35-45% of the total flour proteins and play a major role in conferring elasticity to the dough. The gliadins are further subdivided into α , β , γ , and ω gliadins, whereas glutenins are made up of high molecular weight (HMW) and low molecular weight (LMW) subunits, based on their electrophoretic mobilities in Acid-PAGE and SDS-PAGE, respectively. The biochemical and functional properties are discussed later in this chapter. Amongst albumins/globulins, triticin is a comparatively newly identified legumin like storage protein constituting a small proportion of wheat flour proteins (**Singh and Shepherd, 1985, 1988b**).

2.6.1.1 Classification based on the solubility of wheat proteins:

A major obstacle in the characterization of wheat proteins has been the lack of suitable methods to solubilize the total proteins due to the presence of large number of nonpolar side chains. The seed proteins are classified according to their solubility as water soluble

albumins, salt soluble globulins, alcohol soluble prolamins and dilute acid/alkali-soluble glutelins (**Osborne, 1907**). However, a change in extraction temperature can alter the class to which a given protein is assigned and enhance the yield. Still, complete solubilization is not possible and some 10-30% of the protein remains undissolved. Repeated extractions can reduce this further, but a portion of residual protein always remains un-extracted (**MacRitchie, 1987**). This insoluble protein is the HMW polymeric glutenin protein, which is made up of a number of different polypeptide chains by intermolecular disulfide linkages. This particular character distinguishes it from other gluten proteins, gliadins and non-prolamins, which consist of a single polypeptide chain.

A number of cationic, anionic detergents like CTAB, SDS have been used to solubilize the gluten proteins. Though solubility increases considerably a significant amount of protein is still not dissolved. Use of urea (**Pomeranz, Y., 1965**), CTAB and acetic acid-the AUC solvent (**Meridich and Wren, 1966**) solubilized 80-90% of the protein but not all proteins. More techniques like combination of gel filtration chromatography with electrophoresis developed to resolve the proteins but **Huebner and Wall (1976)** still got only four peaks for HMW glutenin, LMW glutenin, monomeric gliadins and albumins/globulins respectively.

The stronger the dough properties of the flour the more protein remains in insoluble residue, this has been the basis for the residue protein test for assessing the dough properties in breeding lines (**Orth and Bushuk, 1972; Kurowska, 1988**). Sonication has been found to be particularly convenient for achieving close to complete (95-100%) solubilization of wheat proteins (**Singh and MacRitchie, 1989; Singh *et al*, 1990b; Batey *et al*, 1991**). In this system the largest glutenin polymers breakdown into smaller polymers, thus facilitating their extraction. These can then be separated from other proteins by size-exclusion-HPLC (SE-HPLC). Electrophoretic analysis revealed the first peak to have HMW and LMW glutenins, second to have gliadins and third to have albumins/globulins. The proportion of glutenin can be measured by percent area of the first peak, which showed significant positive correlation with the breadmaking quality (**Singh *et al*, 1990b**).

So, even after sonication no new protein class was identified and on the basis of solubility seed proteins are still classified as

1. Water soluble **albumins**
2. Salt soluble **globulins-triticin**
3. Alcohol soluble prolamins/**gliadins**
4. Acid/alkali soluble **glutenins**.

2.6.1.2 Classification of wheat seed proteins based on their molecular structure

Even today the Osborne classification is widely used for subdivision into four groups. However, this classification is not entirely satisfactory because of significant overlap between different fractions. The main difference between gliadins and glutenins is that gliadins are monomeric while glutenins are made up of multiple chains linked together by disulfide bonds. **Shewry *et al* (1986)** revised the classical Osborne classification when he noted that when disulfide bonds break glutenins also solubilize in aqueous alcohol and hence, they too, should be considered as prolamins. This is further justified considering similarity in their amino acid composition and conserved sequence. Hence **Shewry *et al* (1986)** classified cereal prolamins based on their amino acid composition and sequence similarities into three groups. (Fig 2.7).

1. Sulphur-rich(S-rich)
2. Sulphur-poor(S-poor)
3. HMW prolamins.

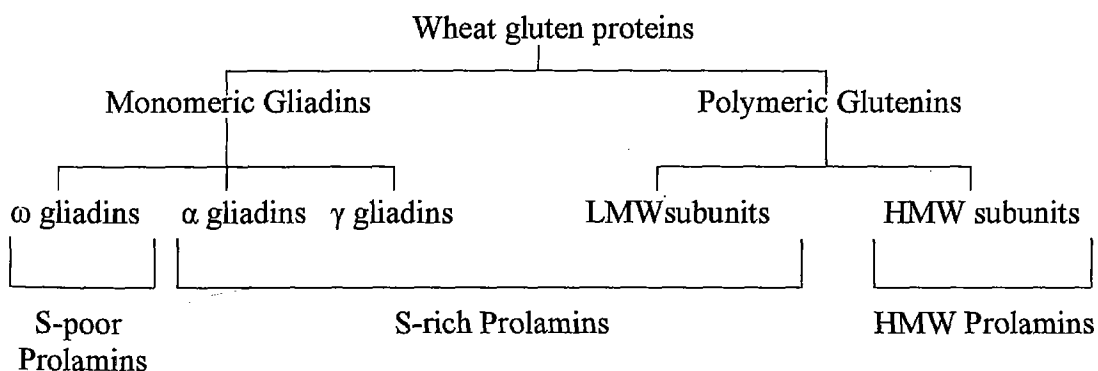


Fig.2.7 Classification of gluten proteins based on structural homologies and genetic relationships. (Schofield, 1994)

There is no further division or grouping for albumins and globulins in this context.

2.6.2 Structure

We are now aware of four major classes of seed proteins namely, glutenin, gliadin, globulin (triticin) and albumin whose structure we shall now study in detail.

2.6.2.1 Glutenins:

Glutenins are further divided into high molecular weight (HMW prolamins) and low molecular weight (LMW=aggregated S-rich prolamins) subunits.

2.6.2.1.1 High molecular weight prolamins

In the prolamins complex, the HMW subunits account for about 10% of the total prolamins with 3-5 individual components (**Lawrence and Shepherd, 1980; Shewry *et al*, 1992**). These are further classified into two types based on their M_r values and sequences. The “X” type migrate more slowly on SDS-PAGE and have molecular weights (based on sequence data) of about 83-88kDa and “Y” type migrate faster and have a molecular weight of 67-74kDa. All the HMW subunits hence a distinct domain structure i.e. the N-terminal sequence (81-104 residues) and C-terminal 42 residues flanking a central repetitive domain (Fig 2.8). The non-repetitive domains of X-type subunits have got three cysteine residues and that of Y-type subunits have five cysteine residues in

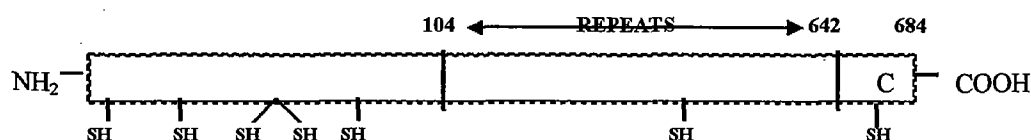
the N-terminal domain and in C-terminal domain. The repetitive domain is based on five different motifs as shown in the Table 2.3 given below. The structure prediction of HMW subunits indicates that the N-termini and C-termini have got more alpha helix, while the central repetitive domain forms regularly repeated beta turns (**Tatham *et al.*, 1984; Tatham *et al.*, 1985**). These subunits are responsible for the elastic properties of dough and hence are associated with the bread making quality.

Table 2.3 :Consensus repeat motifs of wheat prolamins

Prolamins				
HMW X type	HMW Y type	S-poor	S-rich	Aggregative S-rich
PQQPFPQQ PGQGQQGQQ PGQGQQGQQ GYYP TSPQQ PGQGQQ	PQQPFPQ PGQGQQ GYYP TSLQQ	PQQPPF S PQQPY	PGQGQQ PQPGFPF PQQPY	PGQQQQ QQQQPVL
1	1	2	3,4,5,6	3,4,5,6

1. Shewry *etal*,1989, 2. Shewry *etal*,1990, 3. Shewry *etal*,1983,
4. Okita *etal*,1985, 5. Tao *etal*,1989, 6. Colot *etal*, 1989.

Fig 2.8: Schematic diagram of structure of HMW prolamins:



Small angle X-ray scattering of HMW-GS revealed it to be a highly extended rod-like particle in solution with a length of ~69nm and diameter~6.4nm (**Thomson, 1999**). **Roels and Delcour (1996)**

reported that glycosylation in HMW-GS is unlikely while in the same year Tilley suggested that partial glycosylation is possible. Previously reported work had led to the conclusion that HMW subunits-2,7,8, and 12 of cv Chinese Spring had mannose and N-acetylglucosamine bound to them. Experiments by **Bollicker and Schofield (1996)** reveal that HMW subunit 1 and 7 are not glycosylated data detectable level. The controversy, however, still remains.

2.6.2.1.2 LMW (aggregated S-rich) prolamins

These consist of LMW subunits of glutenin and are polymeric stabilized by interchain disulfide bonds. Because of the lack suitable procedures to isolate and fractionate them, these are less well studied. Based on their electrophoretic mobility these have been divided into two main groups viz. "B" subunits (M_r 40-60kDa) and "C" subunits (M_r 30-40kDa) (**Payne and Corfield, 1979**).

These have a short non-repetitive sequence which may or may not contain a single cysteine residue, followed by a proline rich repetitive domain of interspersed repeats based on two consensus motifs as given in Table 2.3, Fig 2.9.

The CD spectra and structure prediction show C-terminal domain of LMW subunits to be rich in alpha helix and N-terminal domain to be rich in beta turns (**Tatham *etal*, 1990**)

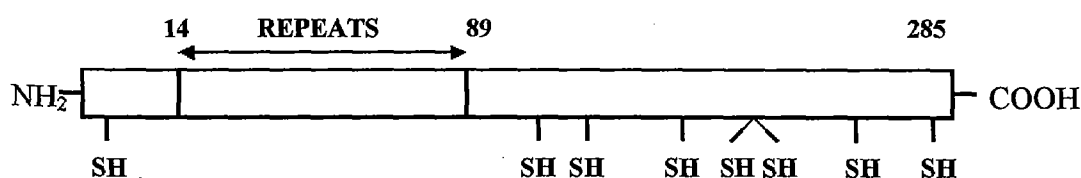


Fig 2.9: Schematic diagram of structure of LMW subunit:

A number of proposed models of glutenin structure suggest that the glutenin polymers consist of high and low molecular weight subunits which are linked via cysteine residues (**Moonen *et al.*, 1985; Graveland *et al.*, 1985; Kasarda, 1989**). The polymeric structure has substantial effects on the functionality of wheat dough.

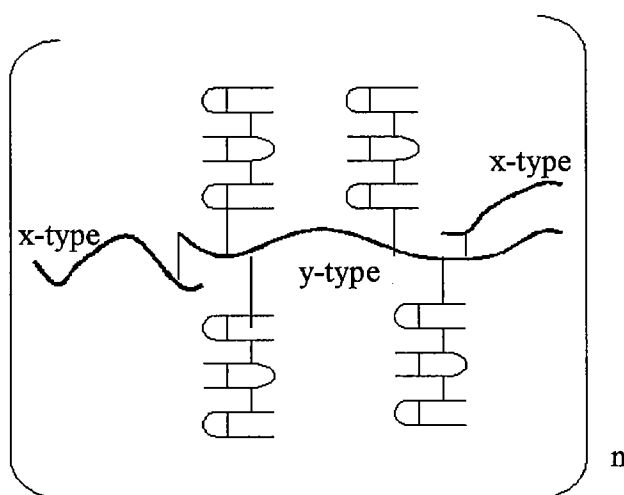
Ewart (1968, 1977, 1979) proposed his linear glutenin theory which envisaged that glutenin comprised of 'concatenations' of polypeptides, that are linked head to tail by disulfide bonds in random order (Fig. 2.10a). Later **Graveland *et al.* (1985)** proposed a different model, in which the different types of glutenin polypeptides occur in a repeat unit comprising of a 'Y' type HMW subunit attached to two different 'X' type HMW subunits by disulfide bonds. Also attached to the 'Y' type subunit are four side chain clusters, each containing three LMW subunits. In turn, these subunits are linked by disulfide bonds between N- and C-terminal of the 'X' type HMW subunits resulting in a structure containing a backbone of HMW subunits with many sidechains of LMW subunits (Fig. 2.10b). **Kasarda (1989)** proposed another model in which the subunits of glutenin are linked by interchain disulfide bonds. The LMW subunits are linked to each other by disulfide bonds in the non-repetitive C-terminal domain (tail to tail). The HMW subunits are linked into glutenin polymers via LMW subunits (Fig. 2.10c).

Fig 2.10. Models proposed for the polymeric structure of glutenin:

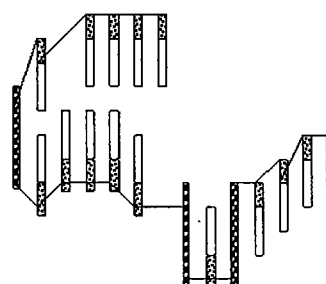
(a) Ewart's model:



(b) Graveland's model:



(c) Kasarda's model:



2.6.2.2 Gliadins

Monomeric gliadins can be divided into

- (1) S-rich prolamins = α -type and γ -type gliadins
- (2) S-poor prolamins = ω -gliadins for convenience.

Small angle X-ray scattering studies reveal that all gliadins appear as prolate ellipsoids with extended conformation (**Thomson et al., 1999**).

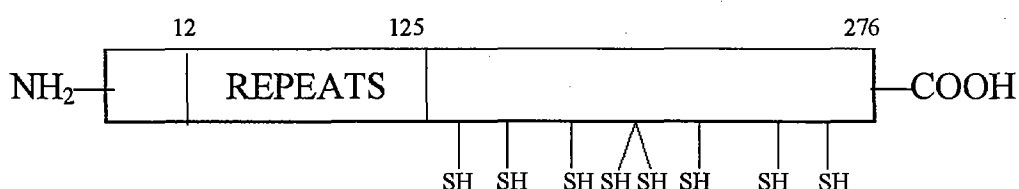
2.6.2.2.1 S-rich prolamins

The S-rich prolamins are quantitatively the most abundant group in wheat accounting for about 80% of the total prolamins. Their M_r

values ranges between 36 kDa and 40 kDa on SDS-PAGE. These appear to have similar basic structure with an N-terminal domain proline rich repeats and C-terminal domains containing most of the cysteine residues. These are conveniently divided into two families based on the divergence in their structure.

In wheat gamma type S-rich prolamins are monomeric with intra chain disulfide bonds (Fig 2.11). These have a unique N-terminal domain of 12 amino acid residues preceding a series of repeats based on the consensus motif PQQPFPQ. The proline-poor non-repetitive domain contains all the cysteine residues. The CD spectra of gamma gliadin fractions dissolved in 70% ethanol showed higher content of alpha helix (**Tatham and Shewry, 1985, Tatham *et al.*, 1989**) than beta turns.

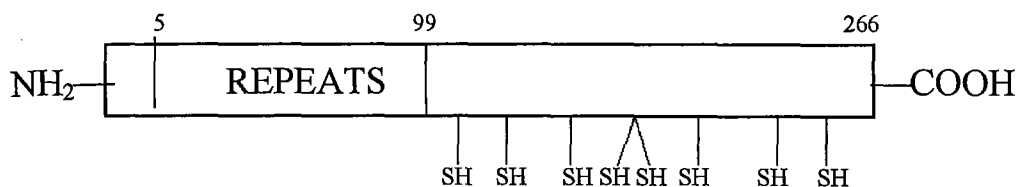
Fig 2.11 γ -type Gliadin of Wheat



The Alpha/Beta type S-rich prolamins are also monomeric but their repetitive domain is less conserved contain interspersed sequences based on two consensus motifs PQQPFPF, PQQPY. In addition the C-terminal domain of the alpha type prolamins contain two poly-glutamine regions. Infrared and CD studies revealed predominance of beta turns and low alpha helical content in the N-terminal region. The C-terminal domain showed predominance of

alpha helix (**Purcell *et al.*, 1988**). Schematic diagram showing structure of alpha type gliadin (Fig 2.12)

Fig 2.12: α -Type Gliadin of Wheat

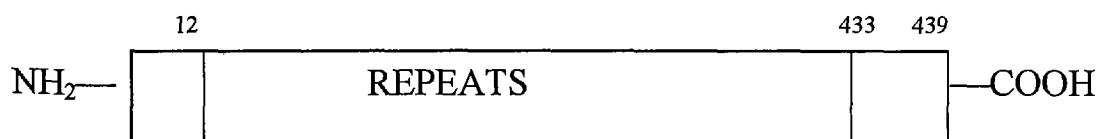


2.6.2.2.2 S-poor prolamins

These are omega gliadins, which have M_r in the range of 44-78 kDa as determined by SDS-PAGE (**Kasarda *et al.*, 1983**). These proteins are completely extracted by aqueous alcohols with reducing agent and contain no cysteine. They account for 10-20% of the total prolamin fraction of wheat, barley and rye (**Shewry and Miflin, 1985**). In these proteins glutamate/glutamine, proline and phenylalanine together account for about 80% of the total amino acid residues. The N-terminal sequences are homologous to omega secalins of rye and C-hordeins of barley. These polypeptides have a short non-repetitive domain followed by several repeated peptides on two consensus motifs PQQPPPFs, PQQPY. The CD spectra of Omega gliadins indicate beta turn rich structures (**Tatham and Shewry, 1995; Tatham *et al.*, 1990**). Schematic diagrams of S-poor prolamins is show in Fig 2.13.

Fig 2.13: S-poor prolamins

C Hordein of Barley



2.6.2. 3 Albumins

These proteins are water-soluble and occur as a group of bands in SDS-PAGE (**Gupta et al., 1991c**). Their elution with polymeric glutenin and tritacin in peak I of SE-HPLC suggests that they are also disulfide linked molecules. Immunological assays of albumin has confirmed the HMW albumins 69, 63 and 60 kDa protein to be β -amylase, but not the lower one (**Gupta et al., 1991c**); while the lower ones e.g. ~13, 16 kDa are found to be α -amylase inhibitors (**Singh, J. et al., 2000a**) e.g. the band of M.wt. 16 kDa and pI 7.3 shows 90% homology with α -amylase inhibitor with its amino terminal and internal sequence to be SGPFMCYPGQ and it has swiss-prot accession no. P01085. Several other examples are present which show albumin to be precursor of certain enzymes e.g. polypeptide with M.wt. 37 kDa and internal sequence AEPPVARGLS is 87% homologous with peroxide BP- Precursor (Swiss Prot accession Q40069).

These proteins have yet not been studied in great detail and demand attention keeping in view its involvement in bakers' asthmas (**Posch et al., 1995 and Amano et al., 1998**).

2.6.2. 4 Triticin

Triticin protein has striking common features between their subunit structures and that of 11-12S globulin storage proteins of legumes. There were some conflicting early reports on whether such

proteins occurred in the wheat seed (**Burguss *et al.*, 1986, Robert *et al.*, 1985**) but their presence was later confirmed by the amino acid sequence data supported by immunochemical analysis using anti-oat 12S globulin antibodies (**Singh *et al.*, 1988b, 1993**).

Triticin bands occurred in cathodal half of the gel as a set of three bands (triplet bands) in a zone of heavy background streaking during SDS-PAGE of unreduced sample. The reduction of triticin bands with β -mercaptoethanol revealed the heterotetramer nature of the triplet bands. Two dimension electrophoresis confirmed four subunits designated D (M.wt. 58 kD), δ (22 kD), A (52 kD) and α (23 kD), where Tri-1, the slowest moving band = $D\delta D\delta$; Tri-2, the intermediate band = $D\delta A\alpha$; Tri-3, the fastest moving band = $A\alpha A\alpha$. With very low concentration of β -mercaptoethanol the tetramers dissociated into dimeric subunit pairs ($D\delta$, $A\alpha$), the monomers being observed with higher concentration of β -mercaptoethanol (**Singh and Shepherd, 1985, Singh *et al.*, 1991a**).

The relative amounts of three proteins are influenced by the dosage of these chromosomes in the endosperm. The purified D and δ polypeptides showed amino acid composition and isoelectric points similar to those of the large and small polypeptides of legumin.

Amino acid sequence data on triticin revealed 49% sequence homology (**Singh *et al.*, 1988a**) and provided definite evidence that triticin belongs to the legumin super family. From the nutritional point of view, wheat triticin has a very good balance of essential

amino acids but it makes up only about 5% of the total seed protein (**Singh et al., 1991**).

2.6.3 Nomenclature and gene symbols for storage protein genes

All seed storage proteins are either prolamins or nonprolamins. They all have characteristic amino acid composition and are rich in prolamine and glutamine, hence the name prolamins (**Singh and Shepherd, 1985**). Based on solubility, they are then divided into glutenin, gliadin, albumin and triticin (globulin).

The symbols used to denote a particular gene locus and its allele for a protein type are based on a basic nomenclature system-

- 1) The gene complexes or compound loci that are genetically linked, such as glutenin loci in wheat should be assigned one symbol. The individual genes that compose gene complexes may be designated by adding a hyphen (-) and an Arabic numeral to the locus designation e.g. Glu-A1 designates chromosome 1 of A genome.
- 2) Different alleles of individual genes may be designated with lower-case letters each following the gene designation e.g. *Glu-A1*, a designates chromosome 1 of A genome which codes for the allele 'a'.

Those subunits which have not been associated to a particular gene and locus are identified by their M_r or molecular weight. These M_r wt. are calculated against a standard run in an SDS-PAGE. e.g. as in the case with albumins and LMW triticins. Studies, however, are upsurge for identification of chromosomal location of albumin polypeptides.

Earlier, the denotation of allelic forms was different and varied from person/place to person/place. This led to several discrepancies in allotting allelic symbols. And due to this fault problems are still faced today. e.g. initially with the numbering system, band 1 was allocated to the HMW subunit having the slowest electrophoretic mobility during SDS-PAGE and the rest were assigned consecutive numbers with increasing mobility (**Payne and Lawrence, 1983**). This practice had two limitations-

1. Any subsequent work from the original study may present new bands of intermediate mobilities to those already described, without the relevant numbers being available to describe them logically.
2. When the work of two different groups is compared, even though the same principle of numbering has been used, one is not certain that the same numbers in different studies refer to the same subunits.

The aforesaid limitations, still, pose a problem for today's research.

2.6.4 Genetics of wheat seed storage proteins and their effect on wheat quality

2.6.4.1 Gene location

The genetic information in wheat is effectively triplicated due to the presence of three different genomes namely A, B and D, each with seven pair of chromosomes making the total number of chromosomes to 42 (Fig 2.14a). Each chromosome has a long and a

Basic chromosome no. $X = 7$
 Gametic chromosome no. $n = 21$
 Somatic chromosome no. $2n = 42$

	Genomes		
	A	B	C
1	1A	1B	1D
2	2A	2B	2D
3	3A	3B	3D
4A	4B	4D	4D
5	5A	5B	5D
6	6A	6B	6D
7	7A	7B	7D

Fig 2.14a: Genomic organization of hexaploid bread wheat.

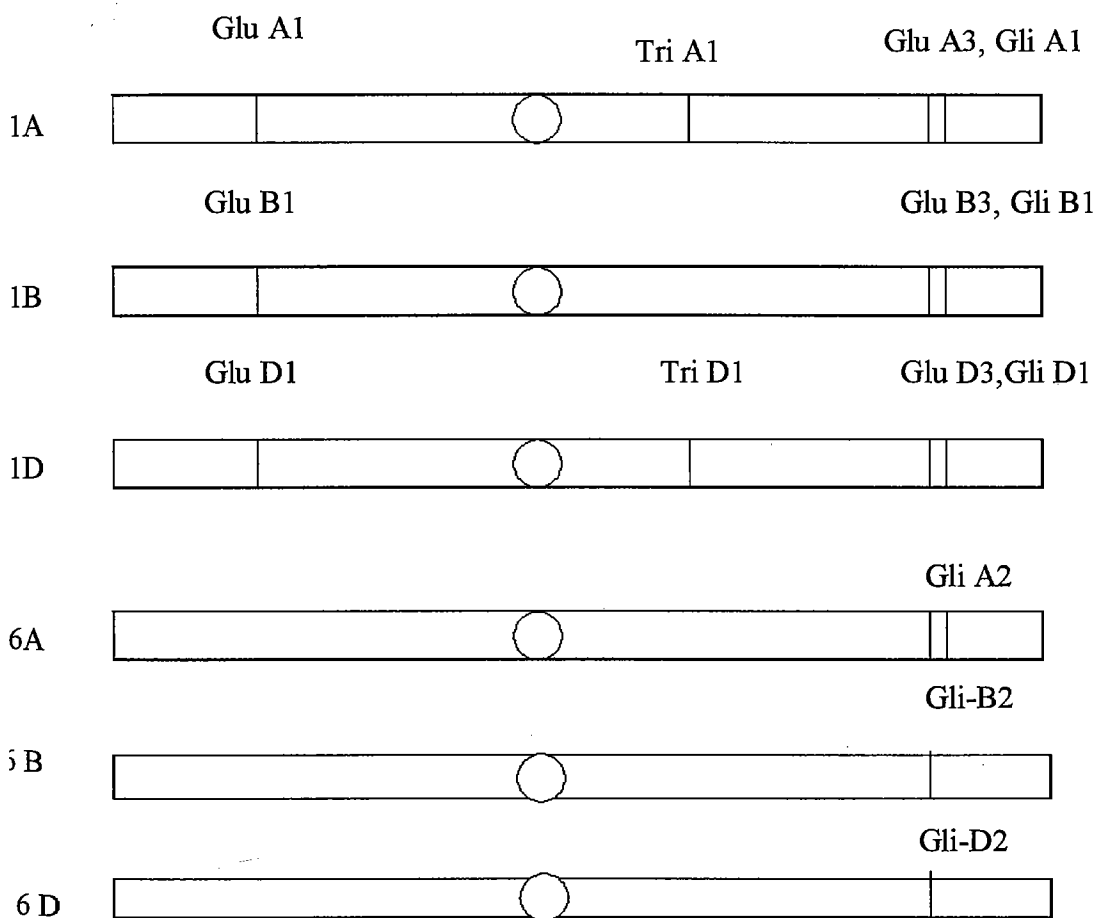


Fig 2.14b: Chromosomal location of some of major genes encoding wheat endosperm proteins.

short arm, which are joined at the centromere (**Law, 1981**).

Spontaneous partial or full chromosome deletions occur quite frequently and are not lethal in the hexaploid wheat. The chromosomal location of genes for a particular protein can be determined by the use of special cyto-genetic stocks viz. nullisomic (N), tetrasomic (T) lines of variety Chinese spring (CS) in combination with electrophoresis (**Sears, 1966; 1969**). The chromosome arm location of the genes can be determined by analysis of ditelocentric (Dt) lines (**Sears, 1954; 1978**). The genes encoding most of the seed storage proteins of wheat have already been located to specific chromosome arms and their positions on the chromosome have also been mapped.

The seed storage proteins of wheat are encoded by at least 15 different major genes located on the homeologous chromosomes of group 1 and group 6 (**Payne, 1987**). The major homeoloci are designated as Glu-1, Glu-2, Glu-3, Gli-1, Gli-2, Gli-3 and Tri-1 all of which are triplicated with one gene on each of the three genomes (Fig 2.14 b). However, the 1B chromosome encoded triticin protein product is not yet identified (**Singh and Shepherd, 1988a**), and only 1B-encoded acidic LMW subunit (Glu-2/ Gli-3 locus) has been mapped (**Jackson *et al.*, 1985**).

2.6.4.2 Allelic Variation and Wheat Quality

The viscoelasticity of the dough determines the suitability of wheat flour for preparing a variety of food products e.g. bread,

chapati, biscuit, etc. During the last 17 years, it has been possible to associate the functional properties of the dough from durum and bread wheats, with the allelic variation controlling the variations in components of gliadins and glutenins amongst other seed storage proteins also.

2.6.4.2.1 Glutenin

Variation has been observed at each locus for glutenin subunits, be it HMW or LMW. 104 alleles are known for HMW subunits and 43 for LMW glutenin subunits are known. Table 2.4 summarizes the genes and alleles encoding different glutenin subunits.

Table 2.4: A summary of the genes and alleles encoding HMW and LMW subunits of glutenin (Gupta *et al.*, 1998).

Sl. No.	GENE LOCUS		NUMBER OF ALLELES
1.	HMW	<i>Glu-A1</i>	22 (Glu-A1a to Glu-A1t, 2,*, 2.1)
2.		<i>Glu-B1</i>	56 (Glu-B1a to Glu B1l, Glu-B1aa to Glu-B1au, others)
3.		<i>Glu-D1</i>	26 (Glu-D1a to Glu-D1s, others)
4.	LMW	<i>Glu-A3</i>	14 (Glu-A3a to Glu-A3h, others)
5.		<i>Glu-B3</i>	15 (Glu-B3a to Glu-B3l, others)
6.		<i>Glu-D3</i>	07 (Glu-D3a to Glu-D3e, others)
7.		<i>Glu-D4</i>	03 (Glu-D4a to Glu-D4c)
8.		<i>Glu-D5</i>	0.2 (Glu-D5a to Glu-D5b)
9.		<i>Glu-B2</i>	02 (Glu-B2a to Glu-B2b)
10.		<i>Glu-B4</i>	-----
11.		<i>Glu-B5</i>	-----

In Fig 2.15 the location of the genes/bands in SDS-PAGE are indicated. Arrowheads indicate polymorphism in bands between different Indian varieties.

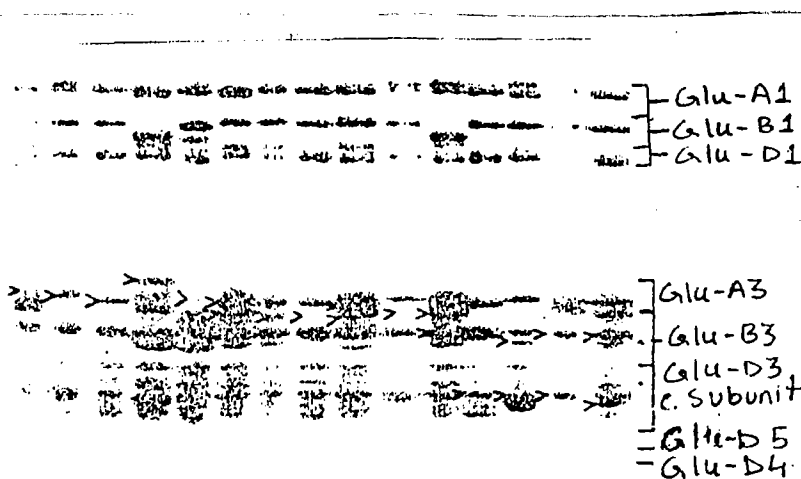


Fig 2.15: SDS-PAGE pattern of glutenin representing different wheat cultivars.

Immense variation is available at each of the HMW glutenin loci. But there is variation in number of alleles detected at each locus and the frequencies of different alleles among wheat cultivars examined. The most frequent alleles at the *Glu-1* loci (**Gupta et al., 1998**) are.

Glu-A1: null, 1 and 2*

Glu-B1: 7+8, 6+9, 20, 16 and 13+18

Glu-D1: 2+12 and 5+10

The variation in the expression of the genes at the above loci determines the levels of HMW glutenin content, and the combinations of alleles at these loci determine its quality. It has been

shown that the presence of HMW protein subunit encoded by alleles of the *Glu-A1* locus (this locus often has a null allele) increases the total extractable protein from 8 % in lines with four HMW subunits to about 10 % in lines containing five subunits. This indicated that the association of *Glu-A1* encoded HMW protein content results in a higher amount of elastic polymer, it leads to improved quality. The demonstration of the effect of gene dosage on the amount of HMW subunit protein suggests to the possibility of engineering plants for improved quality by adding additional copies of HMW subunit genes that should express.

The variation in quality also arises firstly, due to a particular combination of HMW protein subunits and secondly, due to the qualitative differences in the properties and structure of the individual HMW subunits (**Burnouf and Bouriquet, 1980, Payne et al, 1979, 1981, Moonen et al., 1983, Payne, P.I. 1987**).

Payne et al. (1987a) established that the subunits 5+10 contribute to good quality whereas 2+12 gives poor quality. **Greene et al. (1988)** while making comparison between 5/2 subunits and 10/12 subunits further demonstrated that the additional cysteine residue present in subunit 5, compared with subunit 2 would promote a differential cross-linking, thus endowing dough with increased strength and superior performance. This additional cysteine residue perhaps also leads to higher polymerizing capacity giving greater proportion of large polymers in the combination 5+10

than in 2+12 (**Lafiandra et al., 1993**). These large particles also add to the dough strength.

In durum wheat, it has been shown that the HMW glutenin subunits 7+8 (encoded by *Glu-B1-1a* and *Glu-1i-2a*) determine larger SDS sedimentation volumes and higher gluten elasticity than subunits 6+8 (encoded by *Glu-B1-1d* and *Glu-B1-2a*) and 20 (encoded by *Glu-B1-1e*) (**Pogna et al., 1990**).

Allelic differences in the LMW glutenin subunits have also shown to be significantly related to flour qualities in bread (**Gupta and Shepherd 1987, 1988, Gupta et al., 1989, Payne et al., 1987a, b, Singh et al., 1990b**) and durum wheat (**Autran et al., 1987, Pogna et al., 1988**). **Gupta et al. (1989)** studied the effects of allelic variation at *Gli-A1*, *Glu-A3* and *Glu-A1* loci on dough resistance and extensibility. For this purpose they examined the F₆ families from a cross between bread wheats MKR (111/8) and Kite. It was shown that the *Glu-A3m* and *Glu-Alb* (*Glu-A1-lc*) alleles were associated with larger effects on dough resistance and extensibility than the null alleles *Glu-A3k* and *Glu-Alc* (*Glu-A1-a*) respectively. Their effects were also found to be additive. The effect of the LMW glutenin allele *Glu-A3m* on both dough resistance and extensibility was relatively larger than that of the HMW glutenin allele *Glu-Alb* (*Glu-A1-lc*). Some other LMW glutenin alleles such as *Glu-A3b* and associated gliadins have also shown to be associated with dough extensibility in bread wheat (**Gupta and Shepherd, 1988**). For instance, *Glu-A3a*

was superior to *Glu-A3e* (**Payne et al., 1987b**); *Glu-A3b* was superior to *Glu-A3c* (**Gupta and Shepherd, 1988**) and *Glu-A3c* was superior to *Glu-A3e* (= *Glu-a3k*) of Kite and Hope (**Payne et al., 1987b, Gupta et al., 1989**). However more information is required on the relationships between the different LMW glutenin subunit alleles and technological qualities of wheat flours. This will allow selection for improved quality on the basis of LMW glutenin subunit composition.

2.6.4.2.2 Gliadins

In all 12 *Gli* loci (*Gli-A1*, *Gli-B1*, *Gli-D1*, *Gli-A2*, *Gli-B2*, *Gli-D2*, *Gli-A3*, *Gli-B3*, *Gli-D3*, *Gli-A4*, *Gli-A5* and *Gli-B5*) are recognized. Most of the ω - and γ -gliadins are encoded by *gli* genes on short arm of group 1 chromosomes. Some other ω -type gliadins are encoded by *gli* genes on the short arm of 1B. The α - and β -gliadins on the other hand, because of structural similarity in their N-terminal sequences, are grouped into the same class designated α -type (**Kasarda et al., 1987**) and are encoded by genes on the short arm of the homoeologous group 6 chromosomes. The specific components of gliadin proteins have been associated with the technological properties of the dough.

Allelic variation at the gliadin loci is summarized in Table 2.5.

Table 2.5: Summary of the genes and alleles encoding different gliadins (Gupta *et al.*, 1998).

Sl. No.	Gene Locus	Number of Alleles
1.	<i>Gli-A1</i>	20 (Gli-A1a to Gli-A1s, a*)
2.	<i>Gli-B1</i>	22 (Gli-B1a to Gli-B1p, Others)
3.	<i>Gli-D1</i>	14 (Gli-D1a to Gli-D1m, null)
4.	<i>Gli-A2</i>	27 (Gli-A2a to Gli-A2x, others)
5.	<i>Gli-B2</i>	30 (Gli-B2a to Gli-B2v, others)
6.	<i>Gli-D2</i>	23 (Gli-D2a to Gli-D2v, new)
7.	<i>Gli-A3</i>	1 (Gli-A3a)
8.	<i>Gli-B3</i>	3 (Gli-B3a to Gli -B3c)
9.	<i>Gli-D3</i>	-
10.	<i>Gli-A4</i>	-
11.	<i>Gli-A5</i>	-
12.	<i>Gli-B5</i>	-

Fig 2.16 shows SDS-PAGE pattern of gliadin proteins of HD-1941 and indicates the block of bands corresponding to a locus.

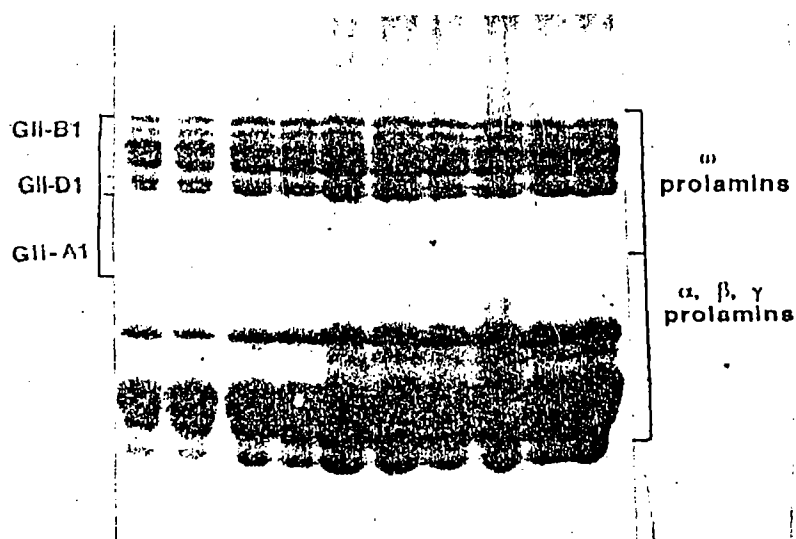


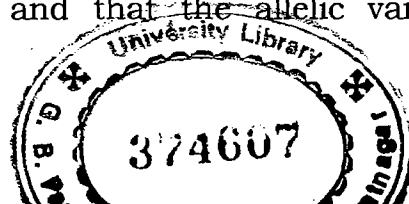
Fig.2.16 : SDS-PAGE pattern of gliadins of HD 1941

The *Gli-1* loci code for omega and gamma gliadins are known to be tightly linked with the *Glu-3* loci (Singh and Shepherd, 1988a).

Gli-2 locus codes for mainly α/β gliadins which are designated as Gli-A2, Gli-B2 and Gli-D2 located on chromosomes 6A, 6B and 6D respectively.

Effect of omega gliadins on bread making quality has been analyzed by several groups in correlation studies, reconstitution studies and analysis of S-deficient wheats. Correlation studies by **Wrigley** and co-workers (**1982**) showed that omega gliadin bands 2 and 4 correlate with strong dough and bands 9 and 16 with weak dough. **Branlard and Dardavet (1985)** demonstrated that the ω -gliadins had negative effects. However, they reported that out of 24 ω -gliadins five to six (ω -11, 13.5, 16, 32, 32) correlated positively and four (ω -14.5, 17, 27.5, 35.5) negatively.

The varieties having γ -gliadin component designated as γ -45 have gluten with good technological properties, whereas those containing a γ -42 have inferior gluten quality (**Damidaux et al., 1978; Kasmolak et al., 1980**). These two γ -gliadin components are allelic variants at the *Gli-B1* locus, and are closely linked to the different LMW glutenin subunits as well as to specific ω -gliadin components (**Payne et al., 1984**). Subsequently, however, following the analysis of Italian durum wheat cv Barillo, which contains a genetic recombination between γ -gliadin and LMW glutenins, it has been demonstrated that the γ -gliadin components 42 and 45 are only genetic markers and that the allelic variation among the LMW



glutenin subunits is actually responsible for difference in gluten viscoelasticity (**Pogna *et al.*, 1988, 1990**).

Overall, the role of gliadins in determining bread-making quality is variable. Correlative studies indicate both positive and negative effects and reconstitution; nutritional studies indicate predominantly negative effects. The positive effects in correlation studies may be mainly due to linkage between the gliadins and LMW glutenin genes.

2.6.4.2.3 Albumins

Limited studies have been undertaken to characterize the albumins and genes for some water-soluble proteins have been unambiguously assigned to individual chromosomes (**Fra-mon, P. *et al.*, 1984**). The genes controlling these highly polymorphic water-soluble endosperm proteins were mapped on long arm of chromosome 7 by isoelectric focusing (**Liu, CJ. *et al.*, 1989**).

However, still, there is no detailed published data on the chromosomal control of several albumin polypeptides and none of them have been assigned universally accepted allelic symbols. The protein subunits are still identified by their M.wt. in a gel. **Singh, J. *et al.* (2000b)** have studied and worked in this direction and accumulated information regarding the same.

The systematic study by **Singh, J.** and co-workers of the water soluble proteins (albumin) and salt soluble (globulin) proteins of wheat flour, by analysis of null genetic lines using different

methods of protein separation has established location of their gene to individual chromosomes (Table 2.6)

Table 2.6: Chromosomal control of albumin polypeptides analyzed using different protein separation methods (Singh, J. *et al.*, 2000b personal communication)

Chromosome	Molecular Weight	Estimated PI	Other Reports in Reference
2A	-	6.80	Masoje, P. <i>et al</i> (1993)
2B	-	6.85	Masoje, P. <i>et al</i> (1993)
2D	16,000	6.83	Masoje, P. <i>et al</i> (1993)
2D	42,000	5.80	-
3AL	32,000	7.25	-
3BS	13,000	5.44	Sanchez-Monge, R. <i>et al.</i> (1989)
3BS	13,000	7.11	Sanchez-Monge, R. <i>et al.</i> (1989)
3DS	16,000	7.30	Konarev, A.V. (1978)
3DS	20,000	6.20	-
4A	56,000	5.50	-
4A	52,000	5.25, 5.4	-
4A	51,000	5.30	-
4BS	62,000	5.25	-
4BS	66000	5.40	Aragoncillo, C. <i>et al.</i> (1975)
4DL	64000	5.30	Payne, P.I. <i>et al</i> (1985); Forsyth, S.A. <i>et al.</i> (1992)
4DL	16000	5.85	Aragoncillo, C. <i>et al.</i> (1975)
4DL	28000	7.00	-
5DL	38000	6.0-7.10	Gupta, R.B. <i>et al.</i> (1991a)
6BS	41000	-	Fra-mon, P. <i>et al.</i> (1984)
6BS	48000	-	Fra-mon, P. <i>et al.</i> (1984)
6DS	42000	-	Fra-mon, P. <i>et al.</i> (1984)
6DS	-	6.38	Sanchez-Monge, R. <i>et al.</i> (1989)
7A	56000	-	-
7BS	46000	6.71	Aragoncillo, C. <i>et al.</i> (1975)
7BS	45000	7.40	Aragoncillo, C. <i>et al.</i> (1975)
7BS	39000	7.60	Aragoncillo, C. <i>et al.</i> (1975)
7BS	19000	6.60	Rodriguez-Loperena, M.A. <i>et al.</i> (1975)
7DL	-	7.20	-
7D	44000	-	Aragoncillo, C. <i>et al</i> (1975)

*M.wt. based on SDS-PAGE

There is no information available as to correlate bread making quality with particular albumin polypeptides.

2.6.4.2.4 Triticin

The genetic control of triticens has been studied by **Singh and Shepherd (1985)** using nullisomic-tetrasomic and ditelocentric lines of the variety Chinese spring. These proteins are encoded by genes on short arm of chromosomes 1A and 1D. The slowest moving band (Tri-1) of the triticens is controlled by genes on chromosome 1DS and the fastest moving band (Tri-3) is controlled by genes on 1AS. The band with intermediate mobility (Tri-2) is a hybrid aggregate of the subunits controlled by 1 DS and 1AS. Fig 2.17 marks the positions of the bands in discussion. The 1B-encoded triticin to not yet identified.

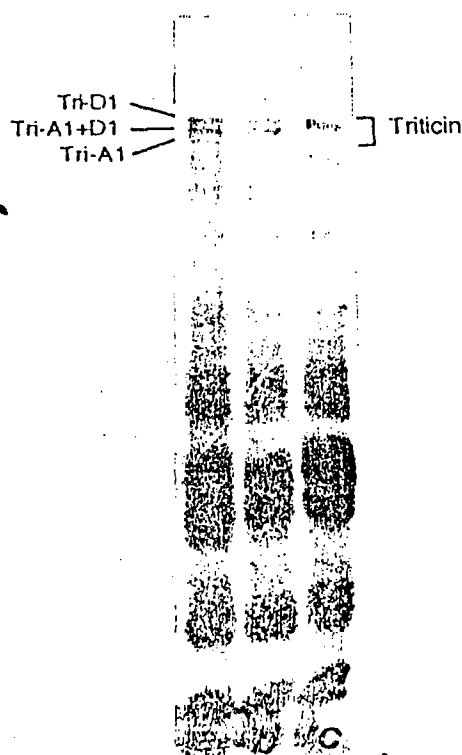


Fig 2.17: SDS-PAGE patterns of triticin in wheat cultivars a) N-8223 (Tri-A1b and Tri-D1a), b) NI-4 (Tri-A1b and Tri-D1b) c) NI-917 (Tri-A1a and Tri-D1b).

The allelic forms present at the two loci having genes encoding for triticin are given in Table 2.7.

Table 2.7. Number of alleles present at *Tri-A1* and *Tri-D1* in wheat

S. No.	GENE	NUMBER OF ALLELES
1.	<i>Tri-A1</i>	2 (Tri-A1a and Tri-A1b)
2.	<i>Tri-D1</i>	2 (Tri-D1a and Tri-D1b)

The effects of these proteins on the functionality of wheat dough is not clear as yet. Literature survey indicates that study in this direction has not begun. However, these proteins contain higher lysine and are assumed to play an important role in determining the nutritional quality of wheat.

2.7 METHODS FOR CORRELATING FLOUR COMPONENTS WITH WHEAT QUALITY

The correlation between varied flour components and wheat flour functionality involves a thorough study into the factors controlling it. Knowledge of the seed storage proteins, their structure and molecular biology is essential to be able to correlate them with the rheological properties of dough and end-use quality. There are several different and complementary methods that can be followed to carry out correlation studies, namely:

1. Studying/ screening varietal sets.
2. Chemical fractionation and reconstitution studies
3. Using F2 derived lines i.e. recombinant inbred lines (RILs)

4. Genetic engineering (transgenic lines)
5. Genetic reconstitution by breeding methods e.g. Near Isogenic Lines.

2.7.1 Screening of varietal sets

Proteins from different varieties are extracted and seen on SDS-PAGE. This allelic composition is studied. Flour of there varieties is kneaded into dough and rheological studies done. The flour components of a variety are then correlated to its rheological properties. e.g. work by **Autran et al., 1986** revealed that rheological-type characteristics (gluten firmness and elasticity, and sodium dodecyl sulfate sedimentation) where strongly associated, typically variety-dependent and independent of protein content. Polyacrylamide gel electrophoresis showed that when the gamma-gliadin allelic block 45 (also containing genes coding for low molecular weight-2 glutenins) was present at the *Gli-B1* locus, rheological characteristics were significantly better that when block 42 (with genes coding for LMW-1 glutenins) was present.

Ng and Bushuk (1988) studied 26 Canadian wheats by SDS-PAGE and correlated proteins to loaf volume by developing a statistical model based on multiple regression analysis. They concluded that for a family narrow groups of bread wheat verities grown under reasonably uniform environment, most of the inter-varietal variability in leaf volume can be explained on the basis of difference in composition of HMW glutenin subunits

A study was made (**Gupta, et al., 1991b, c**) of low and high molecular weight (LMW and HMW) glutenin subunit composition in 2 sets of material, Australian varieties and 69 genotypes from around the world, in relation to dough quality traits measured on an extensograph. Results showed that allelic variation in LMW glutenin subunits is important for explaining dough quality variations and suggested that dough quality can be improved by selecting for the alleles *Glu-1* and *Glu-3* together more effectively than by selecting either of them alone.

However, the method was not suitable as the effect of several alleles, cumulatively, is studied. It is very much possible that the positive effects exerted by presence of an allele on the rheological properties of flour are cancelled/ diminished due to presence of another allele at some other locus. The relative effect of each allele cannot be studied.

2.7.2 Chemical Fractionation and Reconstitution studies:

The aim in reconstitution experiments is to separate wheat flour components and then either to (1) vary their amounts in a given flour or to (2) interchange equivalent components between flours of contrasting properties. The success of the method depends on carrying out the different steps without altering the functional properties of the components (**Finney, 1943; MacRitchie, 1985**). By using the first approach, **Finney (1943)** showed that bread loaf volume, in an optimized baking test, increased linearly as flour

protein content increased from 8 to 22%. The slopes of the regression lines varied from one variety to another, indicating the differences in baking quality between varieties were due to differences in protein composition. This has been generally confirmed using the second approach of interchanging the protein (more particularly the gluten protein) between flours of differing baking quality (**MacRitchie, 1978**). Similar reconstruction experiments have shown the gluten protein to be responsible for the rheological and mixing properties of flour dough (**Finney, 1979; MacRitchie, 1980a**). Once it has been established that it is largely responsible for differences in functionality, the protein can then be further fractionated in order to evaluate the contributions of specific fractions (using the first approach) and to pinpoint the fraction(s) that contribute(s) most to the difference (second approach.). However, this method is not foolproof as the first obstacle to come is change of functional properties of components after fractionation and reconstitution of flour.

2.7.3 Use of RILs:

Production of recombinant inbred lines has been a commonly used breeding technique and it has been exploited over the years for correlation studies also. In these studies, crosses are made and then these F_2 , F_3 onward generations are studied for correlating flour components to rheological properties. e.g. the durum wheat cultivars Ferox, Laota, Penefiel and Blatfort, differing in LMW glutenin subunit

patterns and glutenin quality, were used to make 3 crosses. Grain storage proteins from the F_2 were analyzed. Three new alleles at 3 different loci were found: the gliadin encoding allele Gli-B1, and the LMW glutenin subunit encoding alleles Glu-B3 and Glu-A3. An analysis F_3 grain showed that allelic differences at the Glu-B1 or Glu-A3 loci had no significant effect on gluten strength while all variation at Glu-B3 affected gluten quality significantly (**Vazquez, J.F. *et al.*, 1996**).

Local Ukrainian varieties and the first varieties bred in the Ukraine (local Krynka [wheat from the Crimea] variety Zemka, Kooperatora [Cooperator] and Odessa 3) had the allele Gld -A1, which is associated with good frost resistance but poor flour quality. Later, wheats with gld 1A2 were bred (P-iboi [Suef], Stepnya [Wheat from the STEPpe] and Chaika [Seagull]) with Hostianum 237 as donor. This allele ensured fairly good baking quality and a moderate degree of frost resistance. The allele 1A4, for which Bezostaya1 (Awnless 1) is the usual donor gives good baking quality but relatively low frost resistance. Combining gld 1A2 and gld1A4 in one variety (Odessa 51), high-yielding material with good baking quality and fairly good resistance has been obtained (**Sobko, T.A., 1981**).

Though this method is most widely used, it has certain limitations. Firstly, a relatively much long time is required to breed these lines and use them for correlation studies. Also, a large

number of progeny plant/ seeds are required to study segregation and pick up samples of our choice.

2.7.4 Genetic engineering

With recent developments in recombinant DNA technology in plant molecular biology research, including plant transformation and regeneration, alternative molecular approaches can now be implemented to alter the composition of seed proteins for improved wheat quality.

The demonstration of the effect of gene dosage on the amount of HMW protein suggests the possibility of engineered plant for improved quality by adding additional copies of HMW subunit genes that should express. This approach is being followed in several laboratories to develop transgenic plants using currently available genes controlled by their own endosperm- specific promoters **(Shewry *et al.*, 1995b, Atpelter *et al.*, 1996, Barro *et al.*, 1997).**

Similarly transgenic plants may be used for correlation studies. Addition of genes responsible for encoding different allelic forms may be introduced into a plant as the gene may be deleted and the effect studied in flour of such engineered plants. The whole scenario is obviously very complex, expensive and requires intense work and effort. Working through this approach when we still have better, cheaper and easier methods at hand is baseless.

Functional studies of individual glutenin subunits involving the partial reduction of flour proteins and the subsequent incorporation

of the proteins into the polymeric glutenin structure by reoxidation have been very useful in assessing the effect of particular subunits on functional properties of dough (**Bekes *et al.*, 1994 a,b**). This approach combined with the expression of individual glutenin subunits in *E.coli* and the availability of microscale testing to evaluate dough properties, facilitated understanding of the affect of particular glutenin subunits in wheat quality (**Bekes, F. and Gras, 1999**).

2.7.5 Production of near isogenic lines

NILs are produced by backcross breeding which is a system of breeding where by recurrent backcrosses are made to one of the parents of a hybrid, accompanied by selection for a specific character(s) in the following generation(s). This cycle is usually repeated for 5-6 generations. Selfing at this stage produces homozygosity in terms of the character under transfer. By this time all other characters are homozygous as well.

Selection in this population for the desired character gives a variety, which is very much like the recurrent parent, but bears the transferred character, for which the backcross programme was undertaken. This whole procedure, usually, takes 8-10 years and then the NILs are ready for analysis. All the lines would be identical but different in one protein/ allele. Flour from these lines could easily be studied for rheological properties and be directly correlated to the transferred allele, without any possibility of spurious results

with respect to presence of other factors varying between lines. Much work has already been done in this direction.

Payne, P.I. *et al.* (1987a) developed five NILs by crossing the parents to give F1 & back crossing for five generation before selfing them. Studie on loaf volume, loaf score, mean sedimentation volume and farinograms were done to study the rheological properties. They found 2+12 to give better loaf volume than null allele but poorer effect in comparison to 5+10.

In another study by **Kozub, N.A. *et al.* (1994)** a set of near-isogenic line bred by backcrossing the bread wheat Bezostaya 1 with 22 donors of different gliadin alleles was analyzed. Some gliadin blocks were identified and a catalogue of allelic variants of gliadin loci in the homeologus group was compiled. Similarly, many studies were done.

A modification in this method is integration of DNA profiling after BC-3 generation. Analysis of BC-3 seeds using AFLP to select our sample (obviously only when knowledge of molecular biology of desired character is present) decreases the total time period of production of NILs. The selected samples may be grown and selfed for 2-3 generations before the lines are established. Correlation studies using NILs in the most favourable and precise method to follow.

Hence, in this study production of NILs using molecular breeding was preferred over other available methods. This was

possible because preliminary knowledge and literature on molecular biology of seed storage proteins is available and much work has already been done in this direction.

2.8 OBJECTIVES OF THE PRESENT STUDY

With this background, the present study was begun with the following objectives.

1. Comprehensive cataloging of alleles present at different loci in wheat seed storage proteins and the standard varieties from different countries in which they are reported/initially identified.
(Based on literature survey).
2. Screening of released Indian wheat cultivars for study of allelic variation at different loci in seed storage proteins (especially for gliadins and globulins).
3. Production of a comprehensive set of near isogenic lines in HD2329 with different seed storage protein alleles using molecular marker assisted accelerated backcrossing.

CHAPTER 3

MATERIALS & METHODS

3.1 GENETIC STOCKS

Seeds of various wheat varieties were obtained from Pantnagar and Karnal while several accessions were from abroad (Table 3.1). genetic stocks were maintained by growing cultivars in the field of College of Basic Sciences and Humanities, Pantnagar.

Table 3.1: List of cultivars used in the present study-

Serial No.	Cultivars	Source
1.	Bodallin	1
2.	Bungulla	1
3.	Calidad	1
4.	CPAN 1796	2
5.	CPAN 3004	3
6.	CS 1A-1B	1
7.	CS 1BL	1
8.	Girija	2
9.	HD 2135	2
10.	HD 2329	3
11.	HP 1633	3
12.	HUW 55	3
13.	HUW 234	3
14.	Kalyansona	5
15.	K 9523	5
16.	K 68	3
17.	MG 7249	4
18.	PBW 266	5
19.	Raj 3077	3
20.	Sonalika	5
21.	Sunkota B	1
22.	UP 115	5
23.	UP 1109	5
24.	UP 2121	5
25.	UP 262	5
26.	UP 319	5
27.	UP 301	5
28.	VL 401	3
29.	VL 404	2
30.	VL 784	5
31.	WH 147	3

1-Dr. K.W. Shepherd, Adelaide, Australia

2- DWR, Karnal

3- CFTRI, Mysore

4- D'Lafiandra, Viterbo, Italy

5- Dr. T.B.Singh, Deptt. Of Genetics and Plant Breeding, College of Agric., Pantnagar

3.2 CHEMICALS

Chemicals used during the study were either of Molecular Grade or analytical grade, supplied largely by Sigma, SRL, Merck and Qualigens.

The Large Genome Analysis System I kit was from Gibco-BRL. (γ -³³P)

ATP came from BARC, Mumbai.

3.3 EQUIPMENT

- (i) Glassware (Borosil, india)
- (ii) Eppendorf tubes and microtips (Tarson, India)
- (iii) Vertical Slab gel electrophoresis Apparatus (Hoefer, USA)
- (iv) Power supply for electrophoresis unit (Hoefer, USA)
- (v) Hybridisation oven/shaker (Amersham, UK)
- (vi) Microfuge (Sorvall Instruments, USA)
- (vii) Refrigerated centrifuge (Remi and Sorvall, USA)
- (viii) Oven (Scintronic instruments, India)
- (ix) pH meter (Systronics, India)
- (x) Cyclomixer (Remi, India)
- (xi) Micropipette (Tarsons, India)
- (xii) Fume cupboard (Atlantis, India)
- (xiii) Electrical balance (Adair Dutt and Co., India)
- (xiv) Deep Freezer (Blue Star, India)
- (xv) Gel Documentation System (UVP, MacRascol)
- (xvi) Stabilizers (Blueline, India)
- (xvii) Horizontal Gel electrophoresis assembly (Biotech, India)
- (xviii) RC-5C (Sorvall, USA)
- (xviv) Horizontal gel electrophoresis apparatus (Bangalore Genei, India)
- (xvv) Thermal Cycler(Perkin Elmer 9600)
- (xvvi) Sequencing gel apparatus (Hoefer, USA).

3.4 SEQUENTIAL EXTRACTION OF WHEAT SEED STORAGE PROTEINS

The sequential extraction of wheat seed storage proteins albumins, gliadins, glutenins and tritacin was performed according to **Sreeramalu (1996)** with a few modifications.

3.4.1 Solutions

- A. 1M NaCl
- B. 1M tris-Cl
- C. Tritacin extraction buffer
- D. 70% (v/v) ethanol
- E. Prolamine extraction buffer[50% (v/v)propan-2-ol]
- F. Glutenin extraction buffer
- G. 0.5m Dithiothreitol
- H. 1.4% (v/v) 4-vinylpyridine
- I. Sample buffer, pH=8.0
- J. β -mercaptoethanol
- K. Distilled water (dH₂O)
- L. Glacial acetic acid

3.4.2 Procedure

3.4.2.1. Albumins

1. The endosperm half of a single wheat kernel was crushed to a fine powder between a folded butterpaper with hammer.
2. Distilled water (200 μ l) was added to sample, vortexed and kept at room temperature for one hour.

3. Centrifuged at 12,000g for 10 min., and 100 μ l of supernatant was transferred to a fresh eppendorf tube.

4. 100 μ l of sample buffer (pH=8.0) and 8 μ l of β -mercaptoethanol was added to the tube with supernatant. This step was done inside a fumeboard.

--Mixed well and kept at room temperature before loading.

3.4.2.2. Triticin

5. dH₂O (1ml) was added to the residue left behind after albumin extraction, vortexed and centrifuged at 12,000g for 2min. Supernatant was then discarded.

6. Triticin extraction buffer (1ml) was added to the residue, vortexed and incubated at 55°C overnight.

7. Centrifuged at 12,000g for 10 min. and total supernatant was transferred to a fresh eppendorf tube.

8. Glacial acetic acid (6 μ l) was added and after brief vortexing allowed to stand for one hour at room temperature.

9. After centrifugation at 12,000g for 15 min. the supernatant was decanted.

10. The pellet was washed with 70% ethanol to remove traces of gliadin.

11. Centrifuged at 12,000g for 10 min. and supernatant was then discarded.

12. 100µl of sample buffer (pH=8.0) was added to the eppendorf tube with pellet.

-- It is kept at 65°C for 15 min. for SDS-complexing and centrifuged at 12,000g for 10 min. before loading for SDS-PAGE.

3.4.2.3. Gliadins

13. Distilled water (1ml) was added to the residue left behind after tritacin extraction, vortexed and centrifuged at 12,000g for 2 min. Supernatant was discarded to remove excess salt.

14. 200µl of solution E was added to the residue and kept at 37°C for 30 min.

15. Vortexing was done and the sample was further incubated at 37°C for 30 min.

16. After centrifugation at 12,000g for 10 mins, 100µl of supernatant was mixed with 100µl of sample buffer, pH=8.0 in a fresh eppendorf tube.

--Mixed well and kept at 65°C for 30 min., centrifuged at 12,000g for 10 min. before loading for SDS-PAGE.

3.4.2.4. Glutenins

17. Solution E was added to the residue, vortexed and incubated at 65°C for 30 min.

18. After centrifugation at 12,000g for 15 min., the supernatant was discarded.

19. Steps 17 and 18 were repeated twice more.
 20. After washing, 100µl of solution F was added. 13µl of solution G was also added, vortexed and incubated at 65 °C for 30 min.
 21. After centrifugation at 12,000g for 5 min. at room temperature, 100 µl of solution F was added, vortexed and incubated at 65 °C for 30 min.
 22. Centrifuged at 12,000g for 5 min. at room temperature.
 23. Supernatant (100 µl) was added to 100 µl of sample buffer (solution I) in a fresh eppendorf tube.
- Mixed well and kept at 65 °C for 30 min for SDS-complexing.
- Centrifuged at 12,000g for 10 min. before loading for SDS-PAGE.

3.5 SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

SDS-PAGE was done on a vertical electrophoresis system according to the method described by **Lawrence and Shepherd (1980)** and as modified by **Singh (1991b)**

3.5.1. Solutions

- A. 10% ammonium persulfate
- B. 2X separating gel buffer, pH=8.9
- C. Stock acrylamide for separating gels
 - a—0.8%
 - b—1.5%
- D. 2X stacking gel buffer, pH=6.8
- E. Stock acrylamide for stacking gel
- F. 10X electrode buffer, pH=8.6

- G. Staining solution
- H. Destaining solution
- I. TEMED

3.5.2. Procedure

3.5.2.1. Preparation of separating gel (60ml for two gels)

Solution B	30ml
Solution C-a/b	20ml
dH ₂ O	10ml
TEMED	100μl
10% APS	120μl (added just before pouring)

3.5.2.2. Preparation of stacking gel (9ml for two gels)

Solution D	4.5ml
dH ₂ O	3.5ml
Solution E	1.0ml
TEMED	30μl
10% APS	60μl (added just before pouring)

3.5.2.3. Method

Casting of gel

Before gel preparation, the glass plates have to be assembled to make a leak proof gel cassette. All the glass plates should be spotless. The commercial unit from Hoefer has its own sealing mechanism and hence, silicon grease was not needed for the same. Two glass slabs were

set on the base strand provided and locked. These gave us gels with dimension—

150 x 120 x 1.5 mm.

1. Separating gel solution was prepared from stock solutions as described earlier. Immediately after adding APS, it was mixed and poured into the glass plate assembly with a 30ml glass/plastic syringe
2. It was filled up to a mark 2.5cm from the top of the glass plate.
3. The syringe was immediately rinsed to prevent blocking it due to polymerization of residual acrylamide solution.
4. dH₂O (0.5ml) was carefully layered on top of the gel using a syringe to seal off the air which inhibits gel polymerization.
5. After 25-30 min. when the gel is fully set, a clear interface will be visible between the top of the gel and the water layer.
6. Excess water was removed from top of the gel. The comb was inserted in place leaving a 1.5cm gap between the comb ends and separating gel.
7. APS was added to the stacking gel solution, mixed well, and poured between the glass plates using a syringe.
8. Stacking gel sets in 10-15 mins.

Loading of sample

9. The comb was removed once the gel was set, the wells were cleaned and then 1X electrode buffer was filled into the wells.

10. The samples of protein prepared as described in section 3.4.2. were loaded in individual wells with a micro syringe in the following volumes- Albumins-30 μ l, tritacin-40 μ l, gliadins-20 μ l and glutenin-25 μ l for a 20 well comb.

11. The gel casting apparatus was fixed with the upper tank in the gel casting assembly and about 600-700ml of 1X electrode buffer was added to it.

12. Air bubbles under the gel, if any, were carefully removed by agitating the lower tank buffer with a glass rod.

Electrophoresis conditions

13. Gels were run at a constant current of 40mA/gel until the tracking dye reached the bottom end of the gel (in about three hours). Voltage was initially ~ 120V and gradually increased to ~320V by the end of the run.

NOTE: - For running tritacin protein samples in SDS-PAGE –

a) 0.8% stock acrylamide for separating gel was used. Rest of the composition was same as mentioned before.

b) The dye front was allowed to come out of the gel during the run. Thereafter a 25 min. overrun was given.

Staining and destaining of the gels

14. The gels were stained for a minimum of 5 hours to a maximum of overnight duration in staining solution. Destaining was done in 3% NaCl solution till the background was fairly reduced.

3.6 CROSSING SCHEME FOR THE PRODUCTION OF NILS

The strategy followed was as follows:

Recurrent parent × Donor Parent → F₁

F₁ × Recurrent parent → BC1

Electrophoresis (for segregation analysis of the alleles)

BC1 × Recurrent parent → BC-2

Electrophoresis

BC 2 × Recurrent parent → BC-3

Electrophoresis (Productions of >100 seeds)

DNA profiling to select plants closest to recurrent parent

Selfing to homozygosity.

In the F₁ generation, we obtain 50% of the genes from both the parents. With each backcross we regain 50% more genes of the recurrent parent which were present in the last cross. Hence in BC-1 we regain 75% genes of the recurrent parent, and by the BC-3 stage, we have 93.75% of the genes from the recurrent parent along with our desired allele. After BC-3, the increase in percentage of gene background regained from the recurrent parent is minimal e.g. ~3.0% then 1.5% and so on. Hence in our strategy we stopped backcrossing after the BC-3 stage and opted for DNA profiling to select the seeds closest to the recurrent parent. This helps us choose plants bearing our desired allele

in a background with maximum similarity to the recurrent parent at the molecular level. By selfing, homozygosity is attained.

Once the lines are ready, the relative effect of each allele on the rheological properties of wheat flour may be studied. The ground rules thus identified can be used as guidelines to select genotypes useful in wheat quality breeding programmes for enhanced bread, pasta, noodle & chapati-making quality.

HD 2329 was chosen as the recurrent parent as it gives flour of poor quality in context to milling & baking industry.

Ears of the chosen to be recurrent parent (HD 2329) were emasculated and pollinated by selected donor varieties. One ear was cross-pollinated by pollen from one donor variety only. Fertilized ovaries gave seeds of the F1 generation. The wheat ear has several spikelets and awns. The middle floret of each spikelet is removed first; thereafter the awns are cut away. Florets on either side are opened by applying a slight pressure on its top by the index finger while supporting its base with the thumb. Each floret has three anthers, which are carefully removed using sharp forceps.

E emasculated ears are covered by butter paper bags to prevent undesired cross-pollination. Date of emasculation was written on these bags. Emasculation was preferably done in the afternoons.

Florets were opened by applying pressure with the index finger and thumb. Turgid, yellow anthers were picked up using forceps and used to pollinate the emasculated ears. On an average, one anther was sufficient for pollination of 2-3 florets. Details were written on the paper bags. Pollination was done in the morning hours, as anthers were then ready for dehiscing.

These F1 seeds were germinated on Whatman filterpaper in petridishes before transplanting them in pots. HD 2329 was also grown in the same way. Pollen of HD 2329 was now used to pollinate the emasculated heads of the F1 plants. Fertilized ovaries, thus, gave BC-1 seeds. The endosperm halves of BC-1 seeds were used for the electrophoretic study segregation analysis of the desired alleles. Embryo halves of the sample with the band of the desired allele were then germinated on Whatman filter paper in petridish and later transplanted into pots. Backcrossing was done to give BC-2 seeds. Similarly, BC-3 seeds were obtained.

Fieldwork was conducted in the fields and glasshouse of College of Basic Science & Humanities, Pantnagar. Geographically, Pantnagar falls in the humid, sub-tropical zone at the foothills of Shivalik ranges of the Himalayas, located between 29.5° N latitude and 79.2° E longitude with an altitude of 243.84 m

The soil type was clay loam. Soil from Crop Research Center, and cow dung from Nagla were mixed in a 1:1 ratio to fill the pots. Pots were watered before transplantation of the seedlings.

Transplanting of the seedlings (F1/BC) began near mid-November. Each pot had five seedlings planted in clockwise manner. The first of them was marked properly and the pot was given a number. Details of the pot, seedlings and date of transplantation were all recorded in the planting book. HD 2329 was also transplanted at regular intervals (every 2nd -3rd day) throughout the season so as to provide pollen for backcrossing.

Distance between the plants in a pot of radius 10cm was ~7.5-9cm. Donor varieties maintained in the fields were sown as half a row in a plot. Each plot thus, had approximately 24 varieties sown in it.

Table 3.2: Time schedule of germination and transplantation of BC-1 seeds in winter season of 199798

Date of Germination	Date of Transplantation	Material	Notes
13/11	15/11	HD 2329	The no. of transplantations varied. The no. of lines to be transplanted was fixed to five at one time
	18/11	e.g. for Backcross-2	
	18/11	Line-1	
	20/11	Line-2-5	
	22/11	HD2329	
18/11		HD2329	
		HD2329	
		HD2329	
18/11	23/11	Lines-6, 7,8,9,10	
	24/11	HD 2329	
	26/11	HD 2329	

24/11	29/11 30/11 31/11	Lines-11, 12,13,14,15 HD 2329 HD 2329	*Generally, HD 2329 was germinated in a large no. (enough for 2-3 transplantation events) stored at 4-8°C. These were picked up and transplanted on the desired days.
29/11	4/12 6/12 7/12	Lines-16, 17---20 HD 2929 HD 2329	
4/12	8/12 9/12	Lines 21-26 HD 2329	
8/12	13/12 13/12 16/12	Lines 27-31 HD 2329 HD 2329	
13/12	18/12 19/12 21/12	Lines 32-37 HD 2329 HD2329	
18/12	23/12 24/12 27/12 30/12	Lines 38-42 HD2329 HD2329 HD 2329	
	1/1 3/1 7/1 10/1 15/1 18/1 21/1 27/1	HD 2329 HD 2329 HD 2329 HD 2329 HD 2329 HD 2329 HD 2329 HD 2329	*For HD2329, only 10 transplantations were done in a day i.e. 2 pots only.

However, ideally, transplantation of lines ought to be distributed throughout the season e.g. from mid-November to December end (Table 3.2). Thus, then, one line could be transplanted daily. However, the pattern of HD2329 transplantation followed actually was good, as it gave

pollen throughout the season for timely backcrossing. However, it was also planted in the fields so as to provide a back-up source for pollen.

BC-3 seeds were analyzed for segregation analysis. The positive embryo halves were germinated and then transplanted directly in the fields. The distance between two plants was ~17 cm; some were planted in pots also.

The pots were initially kept in the glasshouse. When the seedlings grew up and became a bit strong, the pots were transferred into the open, near the fields.

Storage

After harvesting, seeds of the donor varieties were stored in duplicates. Seeds in polythene bags were stored inside the seed cabinet at RT while those in paper bags were kept at ~10°C. However, the latter were also sealed in polythene bags. Seeds from the backcross were also stored in properly labeled papers bags and kept ~10°C after sealing them in polythene bags.

3.7 GENOMIC DNA ISOLATION

Genomic DNA was isolated from etiolated levels of germinated seeds by modified CTAB extraction method (**Doyle and Doyle, 1990**)

3.7.1. Solutions

- A. CTAB DNA extraction buffer
- B. Phenol. Chloroform: isoamylalcohol buffer

- C. Chloroform: isoamylalcohol mixture (24:1) (%)
- D. 100% Isopropanol
- E. 70% ethanol
- F. TE, pH= 8.0
- G. RNase (100mg/ml)
- H. 3M sodium acetate, pH= 5.2
- I. 100% ethanol
- J. T₁₀ buffer, pH=8.0
- K. β -mercaptoethanol

3.7.2 Procedure

1. Etiolated leaves from 7-10 germinated seeds were cut to small pieces and crushed to fine power in a precooled mortar and pestle using liquid nitrogen. Powder was transferred to eppendorf tube.
2. Prewarmed DNA extraction buffer (60°C) with 0.2% β -mercaptoethanol was added to the eppendorf tube and kept at 60°C for an hour with intermittent swirling
3. The tubes were centrifuged at 5000rpm for 3 minutes and supernatant transferred carefully into another eppendorf tube by decantation.
4. Phenol :chloroform: isoamyl alcohol treatment was given followed by a chloroform, isoamylalcohol treatment. Mixing was done by gently inverting the tubes during treatment.

5. Centrifuged at 18000rpm for 10 minutes at room temperature and aqueous phase transferred to a fresh tube.
6. Chilled isopropanol was added so that tube is full to the top. Mixing was done by gentle inversion till precipitation began.
7. DNA was spooled out or centrifuged at 10,000 rpm for 10 minutes at 4°C. Supernatant is decanted.
8. Precipitate washed with 70% ethanol and air dried. TE (200µl) is added to dissolve DNA.
9. RNase treatment was given after DNA dissolved in TE buffer. RNase was added and kept at 37°C for 1 hour.
10. Equal volume of chloroform: isoamylalcohol was added and tube inverted for mixing.
11. Centrifuged at 10,000 rpm for 5 minute at room temperature. Aqueous layer collected in fresh eppendorf tube.
12. One-Tenth volume of 3M Na-acetate (pH =5.2) was added followed by two volumes of absolute alcohol. Mixed gently by inversion.
13. Centrifuged at 5000 rpm for 3 minutes at 4°C
14. Pellet washed with 70% alcohol and air dried.
15. T₁₀ buffer (100µl) was added to dissolve DNA.

3.8 AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis of the isolated genomic DNA was performed to know about the quality and quantity of DNA.

3.8.2 Solutions

- A. 50X TAE
- B. Ethidium bromide (10mg/ml)
- C. DNA loading dye

3.8.2. Procedure

1. The open ends of a clean dry plastic tray supplied with the electrophoresis apparatus are sealed with autoclave tape so as to form a mold. The mold is set on a horizontal section of a bench.
2. Agarose gel was prepared by dissolving approximate amount of agarose in 1X TAE. A comb with adequate number of wells is inserted to make the wells, the thickness of the gel was kept less for easy and clear resolution of the DNA.
3. After the gel is completely set, comb is removed carefully with the autoclave tape and gel is mounted into electrophoresis tank.
4. DNA loading dye was mixed with the DNA sample in 1:6 ratio and loaded into the gel with micropipette.
5. Electrophoresis was done at constant volts.
6. The gel was put in staining solution with ethidium bromide concentration of 0.5 µg/ml for 5 minutes.
7. The gel was then destained for 5-10 minutes and visualized in ultraviolet light.

3.9 AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP)

AFLP is a DNA fingerprinting technique that combines both classical, hybridization based fingerprinting and PCR- based fingerprinting.

3.9.1 Solutions and additional requirements

A. AFLP analysis system I

Core reagent kit

Starter primer kit

B. Taq DNA polymerase and buffer with MgCl_2 .

C. X-ray film

D. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ for radio active detection

E. Developer and fixer (Kodak)

3.9.2 Procedure

A) Restriction Digestion of Genomic DNA

1. The following were added to a PCR tube.

Component	amount/volume/ reaction
5x reaction buffer	2.5 μl
H_2O	to make 12.5 μl
Sample DNA (200-250ng)	≤ 9 μl
EcoRI/MseI	1.0 μl

	12.5 μl

2. Mixed gently and collected by brief centrifugation. Incubated at 37°C for 2 hours
3. Incubated the mixture at 70°C for 15 minutes. Tubes placed on ice and contents collected by brief centrifugation

B. Ligation of adapters

1. Following were added to the digested DNA from step 3 of section 3.9.2.A

Component	Volume
Adapter ligation solution	12 µl
T4 DNA Ligase	0.5 µl

2. Mixed gently at RT, centrifuged briefly and incubated at 20°C ± 2°C for 2 hours.
3. Incubated at 70°C for 15 minutes
4. A 1:5 dilution of the ligation mixture was performed as follows
10 µl of reaction buffer was added to 40µl of autoclaved dH₂O (available in kit)
5. Unused portion of reaction mixture may be stored at -20°C

This is a possible stopping point in the protocol.

C. Preamplification reactions

1. The following were added to a 0.2 ml PCR tube/ reaction

Component	Volume
Diluted template DNA	2.5 µl
(from step 3 of section 3.9.2 B)	

Pre-amp primer mixture	20.0 μ l
10X PCR buffer plus Mg	2.5 μ l
Taq DNA polymerase (3 μ / μ l)	0.1 μ l
	25.0 μ l

2. Mixed gently and centrifuged to collect contents

3. PCR was performed as follows

94°C for 30s

56°C for 60s

72°C for 60s

A total of 20 cycles were performed, soak temperature was 4°C. In last cycle, the extension temperature 72°C was extended for 5 min.

4. A 1:20 dilution were performed as follows 2.5 μ l of template in 47.5 μ l of dH₂O. Both unused diluted and undiluted reactions can be stored at
- 20°C.

This is a possible stopping point in the protocol.

D. Primer labeling and marker labeling

1. The following components were added in a 0.2ml PCR tube
volumes given are per reaction

Component	Volume
EcoR I primer (select one)	0.09 μ l
dH ₂ O	0.05 μ l
5X kinase buffer	0.05 μ l
(γ - ³³ P) ATP (2000 ci/mmol)	0.05 μ l
T4 kinase	0.01 μ l
	0.25 μ l

The primer amount required for total number of reactions is calculated and the volumes in above step changed accordingly.

20 bp ladder was also labeled to be used during gel analysis.

dH ₂ O	3.425 μ l
5X kinase buffer	1.35 μ l
Marker	0.35 μ l
T ₄ kinase	0.275 μ l
[γ - ³³ P] ATP	0.85 μ l
	6.25 μ l

This is sufficient for loading 2-3 times.

2. Mixed gently, centrifuged and incubated at 37°C for 1 hour.
3. Heat inactivated at 70°C for 10 minutes
4. Centrifuged briefly to collect contents

E. Selective AFLP amplification

The primer pairs used in present study are given as follows.

1. E-AAC + M-CAA
2. E-AAC + M-CAC
3. E-ACT + M-CAA
4. E-ACT + M-CAC
5. E-AGC + M-CAC

1. For each Mse I primer to be used, a master mix was prepared as follows with volumes indicated for setup of single reaction

Component	Volume
dH ₂ O	3.95µl
10X PCR buffer	1.00µl
Mse I primer	2.25 µl
Taq DNA polymerase	0.083 µl

mixed and centrifuged gently to collect contents.

2. 0.25 µl of labeled EcoRI primer was added per reaction.
3. Master mix, labeled EcoRI primer were added to diluted DNA temperature of 2.5 µl. The total reaction volume per sample was 10 µl.
4. Mixed gently and centrifuged to collect contents.
5. PCR was done as follows.

- a) One cycle at 94°C for 30s, 65°C for 30s and 72°C for 60s was performed.
- b) The annealing temperature was lowered in each cycle by 0.7°C during 12 cycles. This gave a touch down phase of 13 cycles.
- c) 23 cycles were then performed at
 - 94°C for 30
 - 56°C for 30
 - 72°C for 60

Total time taken for selective amplification is 2 hr. 2 min.

F. Gel Analysis

1. After PCR, an equal volume (10 µl) of formamide dye was added to each reaction samples were heated at 90°C for 3 min and immediately placed on ice.
2. A 5%-6% polyacrylamide gel was poured.

Urea	45 gm
dH ₂ O	10 ml
5 ×TBE	20 ml
20:1 acrylamide: Bisacrylamide	30 ml
	100 ml

and used in the Hoefer apparatus available for running gels. 0.4 mm spacers and sharks tooth combs were used.

3. Gel was pre-electrophoresed at constant power (60 W) for ~20-30 mins.
4. 3 μ l of each sample, including markers, was loaded on gel.
5. Electrophoresis at constant power was done until xylene cyanol (Slower dye) ran 2/3 down the length of the gel.
6. Gel was dried and exposed to X-ray film (Kodak® SB) at -80°C for 4-5 days.
7. Film was developed, fixed, dried and analyzed after developing.

CHAPTER 4

RESULTS & DISCUSSION

The seed storage proteins of wheat are coded by atleast fifteen major genes located on the homeologous chromosomes of group 1 and 6. Each of these genes has several allelic forms as shown by electrophoretic studies. Our aim was to produce near isogenic lines with different seed storage protein alleles in a common background of Indian wheat variety HD 2329. This was chosen as the recipient variety for its medium dough quality so as to be able to detect the effect of both deleterious and positive alleles in wheat. For this, different varieties, mainly of Indian origin, were screened for polymorphism at these loci, to be used as donor parents.

The study was carried out on all the four classes of seed storage proteins namely, glutenins, gliadins, albumins and triticin. We begin our results with glutenin.

4.1 Glutenins

4.1.1 High molecular weight subunits

The HMW subunits of glutenin have three loci, namely *Glu-A1*, *Glu-B1* and *Glu-D1*. The number of alleles present at each locus are shown in Table 4.1.

Table 4.1 : Summary of alleles for HMW subunits and known Indian varietal standards

Locus	Total No. of alleles	Common/rare alleles	Indian standard varieties for common alleles		
			Allele	band	Variety
<i>Glu-A1</i>	22	3/19	a	1	UP 301
			b	2*	UP 262
			c	Null	HD 1941
<i>Glu-B1</i>	56	5/52	b	7+8	UP 262
			c	7+9	Sonalika
			e	20	C 306
			f	13+16	UP 301
<i>Glu-D1</i>	26	2/24	i	17+18	Kalyansona
			a	2+12	CPAN 1922
			d	5+10	K 68

1. Sreeramalu, G (1994,1996)

Detailed information on the alleles present at each locus and the standard varieties (foreign/Indian) in which they were first identified is available in the form of tables attached as Annexures- A (for *Glu-A1*), B (for *Glu-B1*) and C (for *Glu-D1*) at the end of this chapter.

The varieties in which the alleles were first identified are generally of foreign origin and these varieties are treated as international standards for these allelic forms. There are very few Indian varieties which have been analyzed for the presence for presence of an allele at a locus in context to seed storage proteins in wheat. Keeping this in mind, we studied the polymorphism in HMW glutenin subunits in several Indian wheat varieties and some foreign accessions which were available with us (Fig. 4.1). The electrophoretic pattern of HMW glutenin subunits from different Indian varieties reveal existing polymorphism e.g. UP-301 and UP-2121 both show band 1 at the *Glu-A1* locus which is absent in HD 2329 and several other varieties. Varieties K-68, UP-319 and Calidad are similar for the *Glu-A1* locus for band 2*. The *Glu-B1* locus revealed extensive polymorphism while at the *Glu-D1* locus, variation was comparatively less. Once the varieties available with us were screened for polymorphism at HMW glutenin loci we cross-checked them with the F_1 crosses available with us. Crosses that had already been made with parents showing polymorphism were chosen and used for further backcrossing

Fig. 4.1 : SDS-PAGE of Glutenin protein extracted from single whole seed of different wheat varieties using the sequential extraction method

Lane 1	HD 2329
Lane 2.	Bungulla
Lane 3.	Bodallin
Lane 4.	Calidad
Lane 5.	Millewa
Lane 6.	Aroora Sr 22
Lane 7.	WL 711
Lane 8.	WH 147
Lane 9.	Kalyansona
Lane 10.	Sunkota B
Lane 11.	HUW 55
Lane12.	K-68
Lane 13.	PBW 65
Lane 14.	PBW 175
Lane 15.	UP 301
Lane 16.	UP 2121
Lane 17.	UP 115
Lane 18.	UP 319
Lane 19.	UP 262
Lane 20.	Chinese Spring



The details of the lines that were chosen as donors for different HMW glutenin subunit alleles are shown in Table 4.2.

Table 4.2: Source of different alleles for the production of NILs for HMW glutenins in HD 2329.

Sl. No.	Locus	Allele	Donor variety	Allele present in HD 2329
1.	<i>Glu-A1</i>	1	UP-2121	2*
2.	<i>Glu-B1</i>	7+8	UP-1109	7+9
3.	<i>Glu-B1</i>	7+8*	CS1A-1B	7+9
4.	<i>Glu-B1</i>	17+18	Kalyansona	7+9
5.	<i>Glu-D1</i>	5+10	K-68	2+12
6.	<i>Glu-D1</i>	Null	CS 1 BL	2+12

* The band 7 from Chinese Spring has much lighter intensity compared to UP 1109.

Though we used mainly Indian varieties as donors for this study, some of the alleles were obtained from Chinese Spring wheat so that effect of the created variation on rheological properties of dough may also be studied later.

These were the six lines that were back crossed for three seasons with screening by SDS-PAGE after each backcross for segregation analysis of the desired alleles. Figs. 4.2, 4.3 and 4.4. show the electrophoretic pattern for 17+18 allele at the *Glu-B1* locus for BC-1, BC-2 and B-3 generations, respectively.

In Fig. 4.2 the segregating pattern of *Glu-B1*(17+18) vs. 7+9 and *Glu-D1*(5+10) vs. 2+12 is very clear. Nine out of 18 samples had the bands representating allele 17 + 18. This indicates a fifty per cent segregation which is expected as per Mendels law. For allele (5+10)

12/18 samples had the bands for 5 + 10 i.e., 66% had them. This is because the figure shows only 18 samples of 85 that we had for analysis. However, on a whole, 1:1 segregation was observed in all cases. Of these segregating samples, only these were further used which had the allele of interest in maximum background of the recipient parent i.e. HD 2329.

The corresponding embryo halves were germinated in petriplates and then transferred to soil in pots for growth. Back-crossing was done when heads were ready for pollination and this gave BC-2 seeds. These were properly labelled and kept for analysis. Segregation analysis again revealed 50% segregation and we chose samples with our allele of interest in a background of the recipient parent (Fig. 4.3). Unlike that observed at BC-1, the band 5+10 was not segregating at BC-2 because of background selection.

As done earlier, following the same steps we generated BC-3 seeds. Fig. 4.4 represents the electrophoretic pattern of the segregation analysis of BC-3 seeds of 17 + 18 allele at the *Glu-B1* locus. The corresponding embryo halves of the positive samples of the BC-3 seeds were germinated in the next season and planted directly into field. This time self fertilization was permitted (Fig. 4.5). Seeds of the BC-3 selfed generation were collected, properly labelled and stored at 10°C in the cold chamber, to be used later for analysis at the molecular level.

Fig. 4.2 : SDS-PAGE of Glutenin subunits showing segregation at *Glu-B1*, 17+18 in seed from lines at BC-1 stage

Lanes 1-9 : Samples 1 to 9
Lane 10 : Recipient (HD 2329) (R)
Lane 11 : Donor (Kalyansona) (D)
Lanes 12-20 : Samples 10 to 18

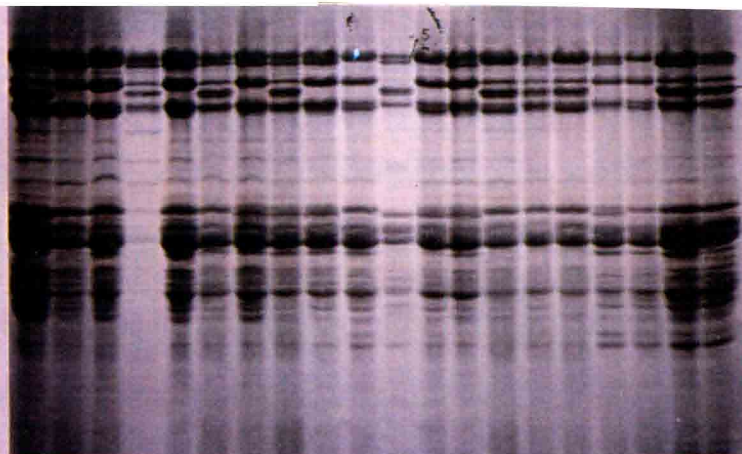
Fig. 4.3 : SDS-PAGE of Glutenin subunits showing segregation at *Glu-B1* 17+18 in BC-2 seeds

Lanes 1-9 : Samples 1 to 9
Lane 10 : Recipient (HD 2329) (R)
Lane 11 : Donor (Kalyansona) (D)
Lanes 12-20 : Samples 10 to 18

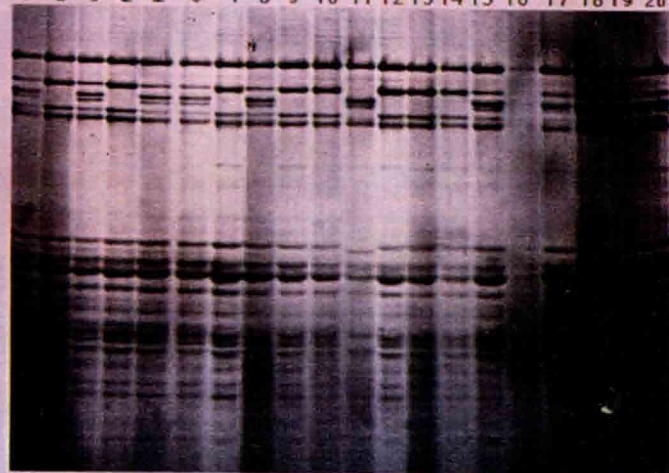
Fig. 4.4 : SDS-PAGE of Glutenin subunits showing segregation at *Glu-B1*, 17+18 in BC-3 seeds

Lanes 1-9 : Sample No. 47-56
Lane 10 : Recipient (HD 2329) (R)
Lane 11 : Donor (Kalyansona) (D)
Lanes 12-20 : Sample No. 56-64
* indicates the band for allele in consideration

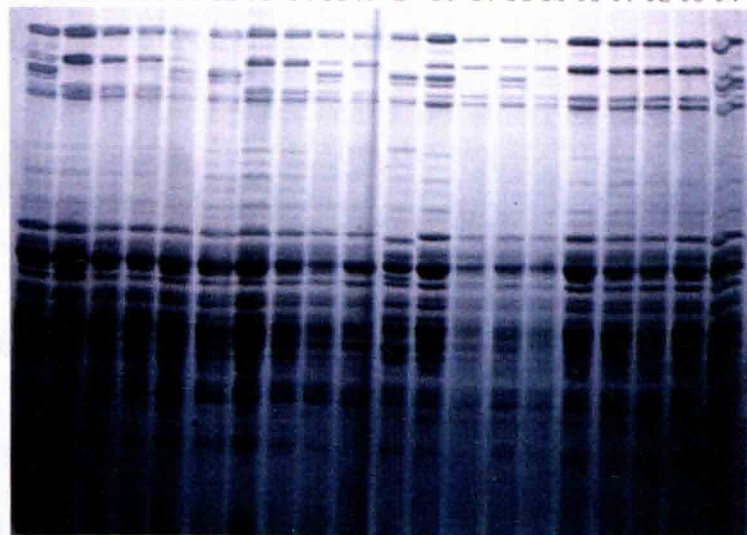
Fig. 4.5 : Plants growing in field from germinated BC-3 seeds that were allowed to self-pollinate in the season ('00-'01).



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20



47 48 49 50 51 52 53 54 55 R D 56 57 58 59 60 61 62 63 64



An important observation during the process of back-crossing was the reduced number of seed settings per head. This led to an initial decrease in total number of seeds obtained after back cross 1& 2. (Table 4.3). To overcome this reduction, (1) we planted more embryos, whenever possible depending on the number of samples screened in during segregation analysis. (2) As seen in previous generations, pots placed in open fields instead of the glasshouse grew healthier and gave better seed sets. Hence, pots were placed in the field. (3) Also, technical support from people with knowledge of back-crossing helped us to use all the available pollen in early morning when the pollen was at correct stage for maximum seed set.

All this helped to increase the number of seed sets drastically at BC-3 as is indicated from figures in Table 4.3.

Table4.3: Number of backcross seeds obtained at different stages of backcrossing during the development of NILs for HMW glutenins

Locus	Allele/donor	Total number of seeds set at			
		F ₁	BC-1	BC-2	BC-3
<i>Glu-A1</i>	Band-1 (UP 2121)	99	84	32	183
	7+8 (UP 1109)	81	45	31	150
<i>Glu-B1</i>	7+8*(CSIA-1B)	50	45	45	100
	17+18 (Kalyansona)	100	85	45	129
<i>Glu-D1</i>	5+10 (CS Dt 1BL)	80	70	12	98
	Null (Kalyansona)	100	90	50	133

All the lines that were to be developed for HMW glutenin subunits were made in similar fashion as explained above and each line showed ideal 1:1 segregation pattern. Representative gels for the segregation of alleles (indicated by presence/absence and/or

intensity of band) for different loci that have been developed are shown by Figs. 4.6. { for *Glu-D1*(5+10)} and 4.7 { for *Glu-D1*, null}.

4.1.2 Low molecular weight subunits

The low molecular weight subunits of glutenin are controlled by genes at 8 loci. The number of alleles present at each locus and the known Indian standard varieties for these are given in the chart 4.4.

Table 4.4: Summary of alleles for LMW subunits and known Indian varietal standards for them

Locus	Total No. of alleles	Common/ rare alleles	Known Indian standards varieties		
			Symbol	band pattern	Variety
<i>Glu-A3*</i>	14	4/10	a	6	Sonalika
			b	5	CPAN 1922
			c	6+10	C 306
			d	6+11	HD 1941
			e	11	UP 2121
			a	2+4+15+19	GW 120
<i>GluB3*</i>	12	2/10	b	8+9+13+16	J 24
			c	2+4+14+15+19	DL-20-9
			d	2+4++15+17+19	C 306
			e	2+4+15+16+18	CPAN 1922
			f	2+4+15+17	CPAN 1796
			b	NA	C-306
<i>Glu D3*</i>	7	2/5	c		CPAN 1796
			d		HP 1633
			e		DL-218-6
<i>GluD4**</i>	3	2/1	a	narrow	J 24 ¹
			b	Wide	PBW 154 ¹
			c	Null	NI4 ¹
<i>GluD5**</i>	2	2/0	a	Narrow	J 24 ¹
			b	Null	K-68 ¹
<i>GluB2*</i>	2	1/1	a	12	NA
			B	Null	

*Annexures D, E and F, G attached at end of this chapter for details of other alleles present at locus *Glu-A3*, *Glu-B3*, *Glu-D3* and *Glu-B2*, respectively.

**Study and identification of these loci /alleles done for the first time and done in India. (Sreeramalu, 1996)

NA-Information not available in literature

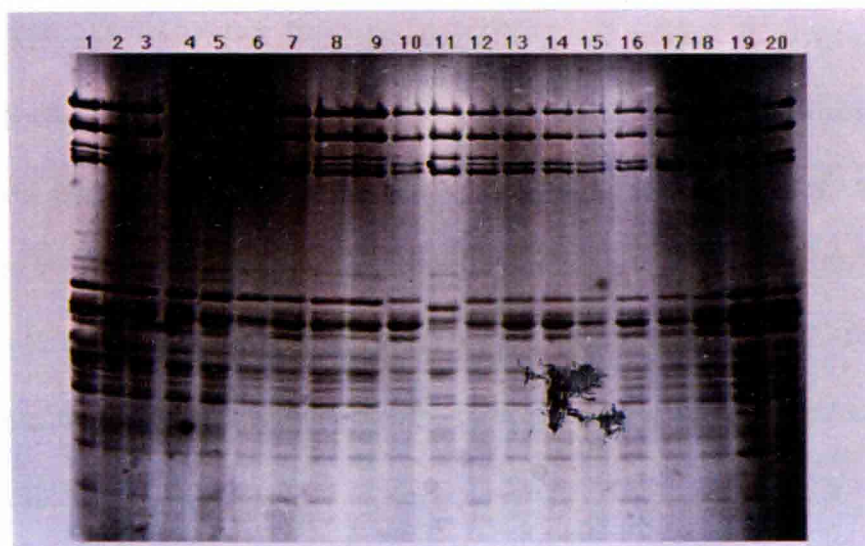
1. Sreeramalu, G. (1996) Ph.D. thesis
2. Nieto-Taladriz *etal*, 1997)

Fig. 4.6 : SDS-PAGE of Glutenin subunits showing segregation at *Glu-D1*, 5+10 in BC-3 seeds

Lane 1-9	:	Sample No. 57 to 65
Lane 10	:	Donor (K-68) (D)
Lane 11	:	Recipient(HD 2329) (R)
Lane 12-20	:	Sample No. 66 to 74

Fig. 4.7 : SDS-PAGE of Glutenin subunits showing segregating null allele at *Glu-D1* locus from seeds at BC-2 stage

Lane 1-9	:	Samples 1 to 9
Lane 10	:	Recipient(HD 2329) (R)
Lane 11	:	Donor (CS 1BL) (D)
Lane 12-20	:	Samples 10 to 18



The pattern of study for low molecular weight glutenin subunits is similar to that followed for high molecular weight subunits. Thus, initially a SDS-PAGE was run to see the polymorphism available within low molecular weight glutenin subunits in Indian wheat varieties, some foreign cultivars and substitution lines were also used (Fig. 4.8). Immense variation is seen for the low molecular weight subunits. For the *Glu-A3* locus, the mobility of band in Kalyansona was higher/faster in comparison than HD 2329 (Fig. 4.8, lanes 1 and 9, also Fig. 4.9.). A band present in HD 2329 at *Glu-B3* was actually absent in HUW-55 (Fig. 4.8., lanes 1 and 11). Band present at *Glu-D3* locus in K-68 was absent in HD 2329 (Fig. 4.8, lanes 1 and 12). There are many differences between varieties for low molecular weight subunits at the genetic level which is revealed by the electrophoretic pattern shown in Fig. 4.8. We then examined the F1 crosses available with us to screen those that had been crossed with donor varieties showing allelic variation for low molecular weight subunits. These screened F1s were then used for the development of near isogenic lines with different low molecular weight glutenin subunits.

Table 4.5 gives us the details of the near isogenic lines that were chosen to be developed with different low molecular weight subunits of glutenin.

Table 4.5: NILS being developed for the LMW glutenin subunits

Serial No.	Locus	Allele	Donor variety	Allele present in HD 2329
1.	<i>Glu-A3</i>	a	CS 1BL	b
2.	<i>Glu-A3</i>	c	Kalyansona	
3.	<i>Glu-A3</i>	d	UP 319	
4.	<i>Glu-A3</i>	d	UP 1109	
5.	<i>Glu-A3</i>	e	UP 115	
6.	<i>Glu-B3</i>	d	HUW 55	hd
7.	<i>Glu-D3</i>	e	HUW 55	C
8.	<i>Glu-D3</i>	d	CSIA-1B	
9.	<i>C-subunit</i>	ks	Kalyansona	hd
10.	<i>C-subunit</i>	sb	Sunkota B	
11.	<i>Glu-D4</i>	a	CS 1A-1B	b
12.	<i>Glu-D5</i>	b	K-68	a
13.	<i>ω-type</i>	ks	Kalyansona	Null

Again, some foreign cultivars and a substitution line were used to prepare a set of lines near isogenic for different alleles.

These were the thirteen lines that were back-crossed continuously for three generations, again with screening by SDS-PAGE at each stage to screen in samples within the cross for the presence of allele/band of interest in a background of the recipient parent. Figs. 4.9 and 4.10 represent electrophoretic patterns in BC-1 and BC-2 stage for the *Glu-A3*, a from CS1BL. As can be made out from the Fig. 4.9, 7 out of 18 samples in the BC-1 seed have the allele a, representing a 1 : 1 segregation. But of these only 3 samples (Fig. 4.9 lanes 9, 11 and 18) had the allele a in a maximum background of the recipient variety HD 2329. These samples were marked and the corresponding embryo halves were sown in coming season to produce BC-2 seeds.

Fig. 4.8 : SDS-PAGE of Glutenin protein extracted from single whole seed of different wheat varieties using sequential extraction method

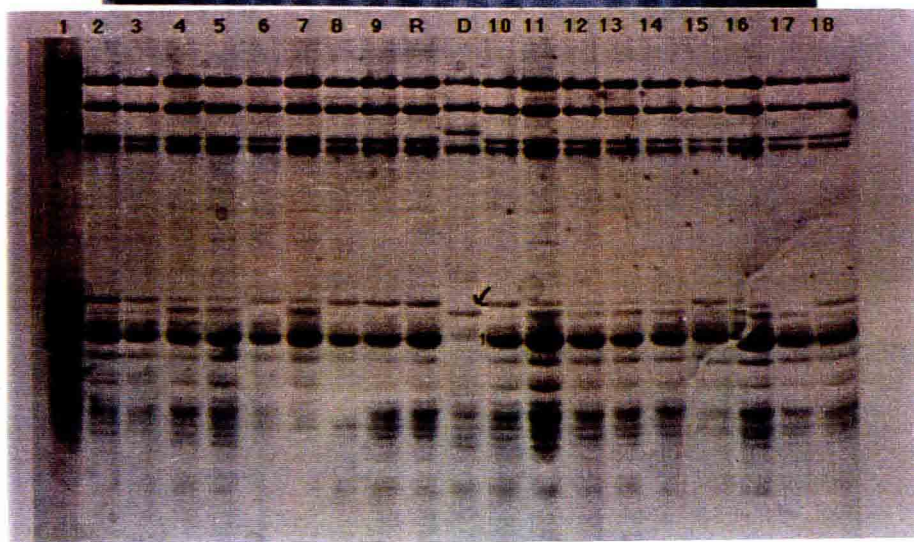
Lane 1.	HD 2329
Lane 2.	Bungulla
Lane 3.	Bodallin
Lane 4.	Calidad
Lane 5.	Millewa
Lane 6.	Aroona Sr. 22
Lane 7.	WL-711
Lane 8.	WH 147
Lane 9.	Kalyansona
Lane 10	Sunkota B
Lane 11.	HUW 55
Lane 12.	K 68
Lane 13.	PBW 65
Lane 14.	PBW 175
Lane 15.	UP 301
Lane 16.	UP 2121
Lane 17.	UP 115
Lane 18.	UP 319
Lane 19.	UP 262
Lane 20.	Chinese Spring

Fig. 4.9 : SDS-PAGE of Glutenin protein showing segregating *Glu-A3*, a from BC-1 stage seeds

Lane 1-9	: Samples 1to9
Lane 10	: Recipient (HD 2329) (R)
Lane 11	: Donor (CS1BL) (D)
Lane 12-20	: Samples 10 to18

Fig. 4.10 : SDS-PAGE of Glutenin protein showing segregating *Glu-A3*, a for BC-2 stage seeds

Lane 1-9	: Samples 1 to 9
Lane 10	: Recipient (HD 2329) (R)
Lane 11	: Donor (CSIBL) (D)
Lane 12-20	: Samples 10 to 18



Segregation analysis of BC-2 seeds again showed 50% segregation as approximately half the samples had the alleles of interest. As seen from Fig. 4.10, all the samples with allele a at the *Glu-A3* locus had maximum background similar to HD 2329. This change or enhancement of recipient background in BC-2 is expected because we chose samples at the BC-1 stage with maximum recipient background alongwith our desired allele.

Again, samples chosen after the BC-2 seed segregation analysis were used to produce BC-3 seeds. Analysis of BC-3 seeds was then done and the corresponding embryo halves sown in field and allowed to self-fertilize. Seeds of this season were then packaged, labeled and stored for future analysis and use. Fig. 4.11 shows SDS-PAGE of BC-3 seeds for *Glu-A3*, c. Here, 5 of 18 samples had the desired allele *Glu-A3*, c in the recipient background.

As with the high molecular weight glutenin lines, less number of seed were produced at F1, BC-1 and BC-2 for continuing the backcross after verification at each stage (Table 4.6). However, more seeds were produced at BC-3 so that an enriched recipient background could be selected from these using DNA markers. For this measures were taken like keeping pots in the open field instead of glasshouse, as given earlier. This enhanced the total number of seeds obtained in BC-3 generation (Table 4.6).

Table 4.6 : The number of seed produced at each generation of backcrossing for the production of NILs for LMW glutenin

Locus	Allele	Donor	Total number of seed set in			
			F ₁	BC-1	BC-2	BC-3
<i>Glu-A3</i>	a	CSIBL	87	61	57	91
	c	Kalyansona	100	85	20	89
	d	UP 319	86	50	49	105
	d	UP 1109	81	45	45	188
	e	UP115	58	58	35	104
<i>Glu-B3</i>	d	HUW55	94	66	25	206
<i>Glu-D3</i>	d	CS1A-1B	50	45	21	148
	e	HUW 55	94	66	26	131
<i>C-subunit</i>	ks	Kalyansona	100	85	29	118
	sb	Sunkota-B	60	50	39	199
<i>Glu-D4</i>	a	CS1A-1B	50	45	38	137
<i>Glu-D5</i>	b	K-68	80	70	25	134
<i>ω-type</i>	ks	Kalyansona	50	44	35	97

Each line given in Table 4.6 was developed on the same pattern as with the high molecular weight glutenin lines. Again, segregation of the bands followed Mendelian pattern and overall fifty per cent of the seeds had our allele of interest with background of recipient. Representative gels showing segregation for alleles at different loci of low molecular weight glutenin subunits are shown in Figs 4.12, (for *Glu -A3*, d), Fig 4.13 (*Glu-B3*, d), Fig. 4.14 (*Glu-D3*, d), Fig. 4.15 (c-subunit in Kalyansona), Fig. 4.16 (for *Glu-D4*, a), Fig.4.17 (*ω-type*).

4.2 Gliadins

In all, 12 Gli loci are recognized. Table 4.7 gives a summary of the genes and alleles encoding different gliadins alongwith a list of

Fig. 4.11 : SDS-PAGE of Glutenin protein showing segregation at *Glu-A3*, c for BC-3 stage seeds

Lanes 1-9 : Samples 75 to 83
Lane 10 : Recipient (HD 2329) (R)
Lane 11 : Donor (Kalyansona) (D)
Lanes 12-20 : Samples 84 to 92
* indicates the band for allele in consideration

Fig. 4.12 : SDS-PAGE of Glutenin protein showing segregation at *Glu-A3*, d in BC-2 stage seeds

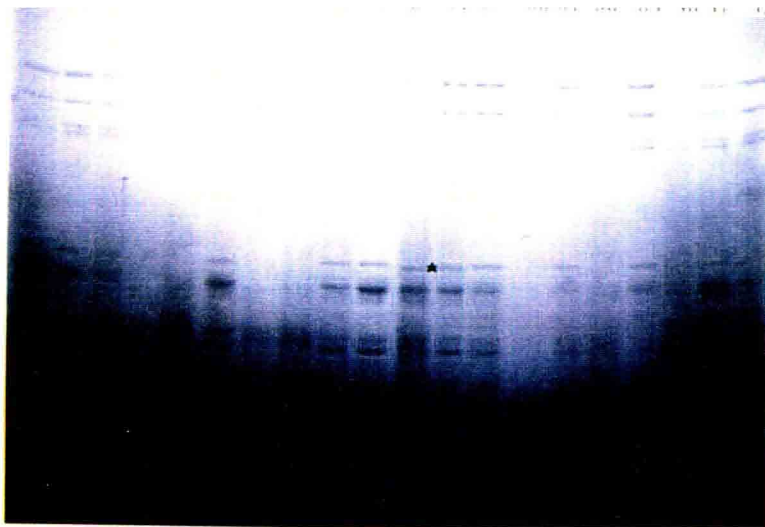
Lanes 1-9 : Samples 106 to 114
Lane 10 : Recipient (HD 2329) (R)
Lane 11 : Donor (UP 1109) (D)
Lanes 12-20 : Samples 115 to 123

Fig. 4.13 : SDS-PAGE of Glutenin protein showing segregation at *Glu-B3*, d from BC-2 stage seeds

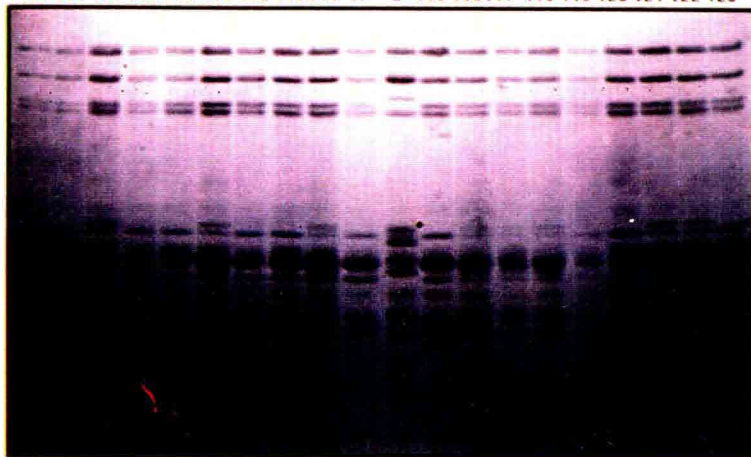
Lanes 1-9 : Samples 91 to 99
Lane 10 : Recipient (HD 2329) (R)
Lane 11 : Donor (HUW 55) (D)
Lanes 12-20 : Samples 100 to 108

Fig. 4.14 : SDS-PAGE of Glutenin subunit showing segregation at *Glu-D3*, d from BC-2 stage seeds

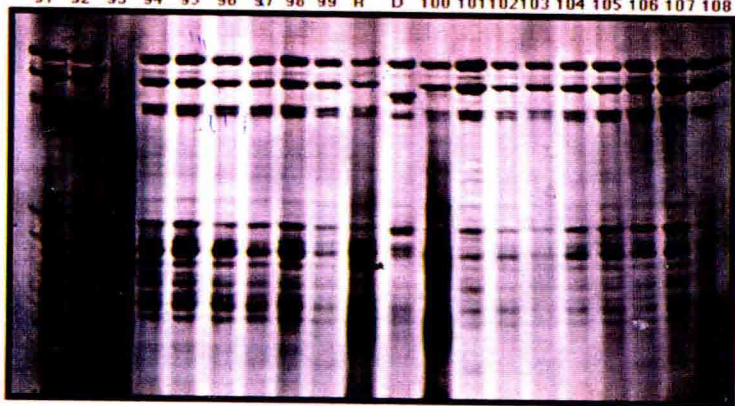
Lanes 1-9 : Samples 91 to 99
Lane 10 : Recipient (HD 2329) (R)
Lane 11 : Donor (CSIA-1B) (D)
Lanes 12-20 : Samples 100 to 108



106 107 108 109 110 111 112 113 114 R D 115 116 117 118 119 120 121 122 123



91 92 93 94 95 96 97 98 99 R D 100 101 102 103 104 105 106 107 108



91 92 93 94 95 96 97 98 99 R D 100 101 102 103 104 105 106 107 108



Fig. 4.15 : SDS-PAGE of Glutenin subunits showing segregating c-subunit from seeds of lines at BC-2

Lanes 1-9 : Samples 1 to 9
Lane 10 : Recipient (HD 2329) (R)
Lane 11 : Donor (Kalyansona) (D)
Lanes 12-20 : Samples 10 to 18

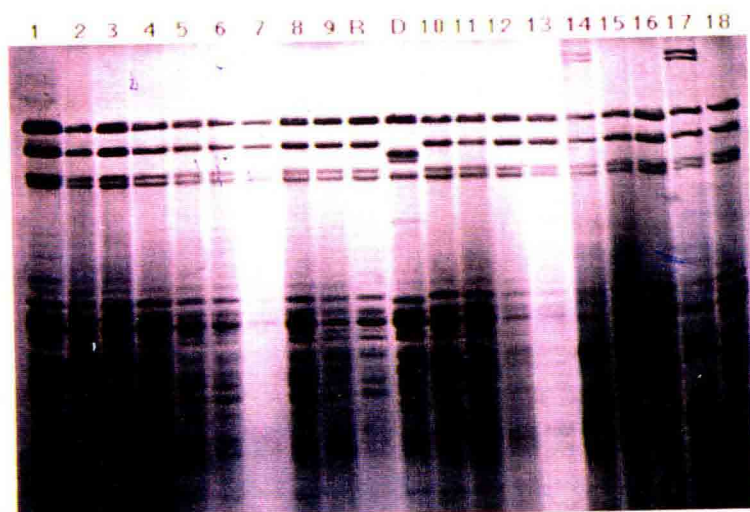
Fig. 4.16 : SDS-PAGE of Glutenin subunits showing segregating *Glu-D4*, a from BC-2 stage seeds

Lanes 1-9 : Samples 37 to 45
Lane 10 : Recipient (HD 2329) (R)
Lane 11 : Donors (CSIA - 1B) (D)
Lanes 12-20 : Samples 46 to 54

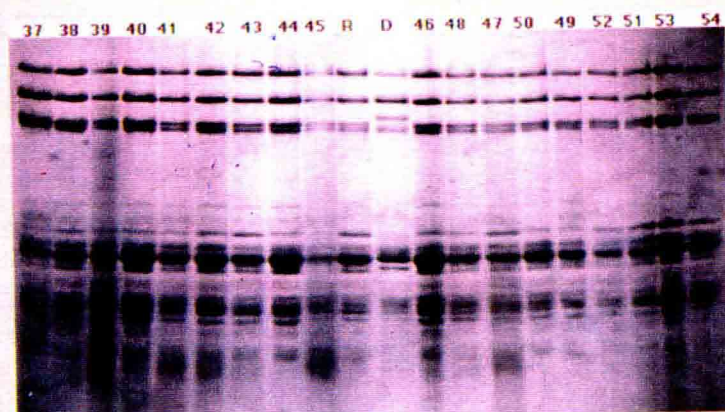
Fig. 4.17 : SDS-PAGE of Glutenin protein from BC-2 lines for contaminating gliadin allele in glutenin subunits

Lanes 1-9 : Samples 1 to 9
Lane 10 : Recipient (HD 2329) (R)
Lane 11 : Donor (Kalyansona) (D)
Lanes 12-20 : Samples 10 to 18

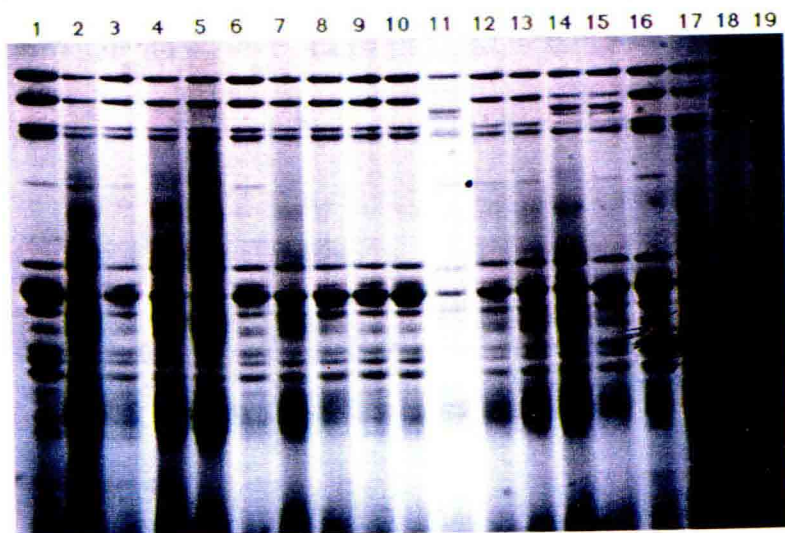
indicates the band for allele in consideration



mr-14a for c subunit D KS



mr-18c for Glu D49a) n-42



ql in Glu

be-2

Indian wheat varieties which can be treated as standards for a particular alleles.

Table 4.7 : Summary of common gliadin alleles present in Indian wheat varieties alongwith the known standards for them

Locus	Total no. of alleles ¹	Common/ Rare	Known Indian standard varieties	
			Symbol	Variety
Gli-A1*	20	3/17	a	CPAN 1796
			b	C 306
			c	CPAN 1922
Gli-B1*	22	7/15	a	DL-20-9
			b	CPAN 1796
			c	DL-377-8
			d	DL-784-3
			e	CPAN 1992
			f	DL-218-6
			g	C 306
Gli-D1*	14	2/12	a	CPAN 1796
			b	C 306

1. Gupta, P.K. *et al.* (1998)

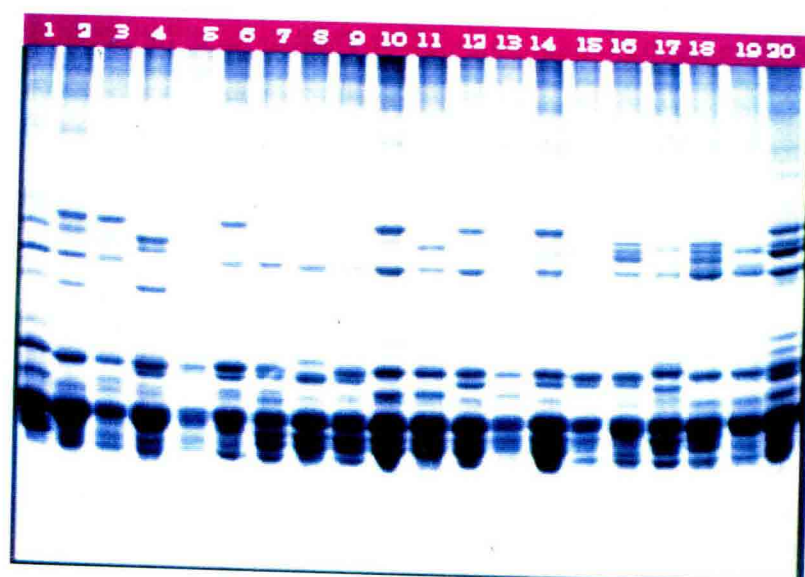
2. Sreeramalu, G. (1996)

*Annexures for the locus are attached at end of chapter (for details of alleles.)

As done before for the glutenin subunits, polymorphism for different allelic forms of gliadin loci was studied in Indian varieties and some foreign acquisitions (Fig. 4.18). As is clearly visible, Bodallin, Aroona Sr.22, K-68 and PBW 175 have the same band pattern at the top (at *Gli-B1* locus) (Fig. 4.18, lanes 3,6,12 and 14). UP-2121 and UP-319 have identical band pattern at this locus. But this allelic form is different from those present in K-68 and PBW-175. Variation at *Gli-D1* locus amongst the varieties studied was less. But *Gli-D1* (b) was found in WH 147 as an extra band present below the single *Gli-D1* band present in HD 2329 (allele a). Further down the lane, WH-147 had a faster moving band in comparison to the recipient parent at the *Gli-A1* locus. This is the *Gli-A1,g* allele against the *Gli-A1,c* present in HD 2329 (Fig. 4.18, lane 8vs.1). *Gli-B1*, *Gli-*

Fig. 4.18 : SDS-PAGE of Gliadin protein extracted from single whole seed of different wheat varieties using sequential extraction method

Lane 1.	HD 2329
Lane 2.	Bungulla
Lane 3.	Bodallin
Lane 4.	Calidad
Lane 5.	Millewa
Lane 6.	Aroona Sr. 22
Lane 7.	WL-711
Lane 8.	WH 147
Lane 9.	Kalyansona
Lane 10.	Sunkota B
Lane 11.	HUW 55
Lane 12.	K 68
Lane 13.	PBW 65
Lane 14.	PBW 175
Lane 15.	UP 301
Lane 16.	UP 2121
Lane 17.	UP 115
Lane 18.	UP 319
Lane 19.	UP 262
Lane 20.	Chinese Spring



D1 and *Gli-A1* loci have genes coding for ω -gliadins. The low molecular weight gliadin (α , β and γ -gliadins) have greater variation in terms of band pattern and hence allelic forms. Fig. 4.18 reveals immense variation in low molecular weight gliadins. After studying variation in the available varieties we chose the F_1 crosses that had combined different alleles from the various donor varieties.

Table 4.8 gives details of the varieties and alleles chosen to make NILs for gliadin protein.

Table 4.8 : Details of near isogenic lines being developed for different gliadin alleles

Sl. No.	Locus	Allele	Donor variety	Allele present in HD 2329
1.	<i>Gli-A1</i>	G	WH 147	c
2.	<i>Gli-A1</i>	h*	UP 115	
3.	<i>Gli-B1</i>	A	CS1A-1B	c
4.	<i>Gli-B1</i>	B	UP 2121	
5.	<i>Gli-B1</i>	c*	UP 1109	
6.	<i>Gli-B1</i>	d**	Kalyansona	
7.	<i>Gli-D1</i>	B	WH 147	a
8.	γ <i>Gli</i>	Ma	UP 1109	
9.	γ <i>Gli</i>	Mb	WH 147	
10.	γ <i>Gli</i>	Mc	UP 319	hd
11.	γ <i>Gli</i>	Md	UP 115	
12.	γ <i>Gli</i>	Me	Sunkota B	

1. Sreeramalu, G. (1996)

**Gli-A(h)* is linked to *Glu-A3 (e)* in UP 115

***Gli-B1(d)* is linked to a low molecular weight gliadin in Kalyansona

A point to be considered is that at the *Gli-A1* locus, WH 147 has allele c and so does HD 2329, as per studies done by **Sreeramalu, G. (1996)**. However, when we studied the polymorphism existing at this locus, we found the band pattern to be different from that in HD 2329 and similar to that of allele 'g' in HUW 55 (Fig. 4.19). Segregation analysis studies also proved this allele to be different from that in HD 2329. Similar was the case at the *Gli-D1* locus. WH 147 was found to have allele 'b' and not 'a' at the locus *Gli-D1* as represented by **Sreeramalu, 1996**. The band for *Gli-B1(c)* in UP 1109 also varies from the actual allele 'c' of HD 2329. But because no literature is available for this allele type, we have marked it as allele c* so that in future, the discrepancy may be sorted out.

In total, twelve near lines were developed for gliadin alleles at different loci. These were developed using the strategy as mentioned in materials and method (which had been followed for developing lines for glutenin alleles also). Screening for the segregating alleles was done after each backcross by SDS-PAGE and samples were screened in for further use. All the revealed fifty per cent Mendelian segregation (within experimental error limits). After backcross-3, the screened in samples were marked and corresponding embryo halves were germinated and transplanted into field and allowed to self fertilize. Seeds were then carefully collected, labelled and stored for further analysis and quality testing.

Sufficient number of BC-3 seeds were produced in order to apply DNA marker assisted selection for the recipient genetic background (Table 4.9).

Table 4.9 : The number of seed produced in each generation during production of NILs for gliadins

Locus	Allele	Donor	Total number of seed set in			
			F ₁	BC-1	BC-2	BC-3
<i>Gli-A1</i>	g	WH 147	111	73	63	143
	h	UP 115 ¹	58	58	60	121
<i>Gli-B1</i>	a	CSIA - 1B	50	45	25	95
	b	UP 2121	9	90	15	85
	c*	UP 1109	81	45	33	97
	d	Kalyansona	100	85	43	123
<i>Gli-D1</i>	b	WH 147	111	73	26	73
<i>γ-Gli</i>	ma	UP 1109	81	45	17	101
	mb	WH 147	111	73	27	78
	mc	UP 319	86	50	39	105
	md	UP 115	58	58	58	133
	me	Sunkota B	60	50	11	122

Representative gels of segregation analysis in different lines being developed for each locus are given in Figs. 4.19 (for *Gli-A1*, g), Fig. 4.20 (for *Gli-B1* b), Fig. 2.21 (for *Gli-D1* b), Fig. 4.22 (*γ-Gli*, mb), Fig. 4.23 (*γ-Gli*, mc) and Fig. 4.24 (*γ-Gli*, mc).

4.3 ALBUMINS

Recent studies by **Singh et al. (2000b)** (personal communication) have revealed the chromosomal control of albumin

Fig. 4.19 : SDS-PAGE of Glutenin protein showing segregating *Gli-A1*, g for BC-2 stage seeds

Lanes 1-9 : Samples 1 to 9
Lane 10 : Recipient (HD 2329) (R)
Lane 11 : Donors (WH 147) (D)
Lanes 12-20 : Samples 10 to 18

Fig. 4.20 : SDS-PAGE of Glutenin protein showing segregating *Gli-B1*, b from BC-2 stage seeds

Lanes 1-9 : Samples 1 to 9
Lane 10 : Recipient (HD 2329) (R)
Lane 11 : Donors (UP 2121) (D)
Lanes 12-20 : Samples 10 to 15

Fig. 4.21 : SDS-PAGE of Glutenin protein showing segregating *Gli-D1*, b from BC-2 stage seeds

Lanes 1-9 : Samples 1 to 9
Lane 10 : Recipient (HD 2329) (R)
Lane 11 : Donors (WH 147) (D)
Lanes 12-20 : Samples 1 to 18

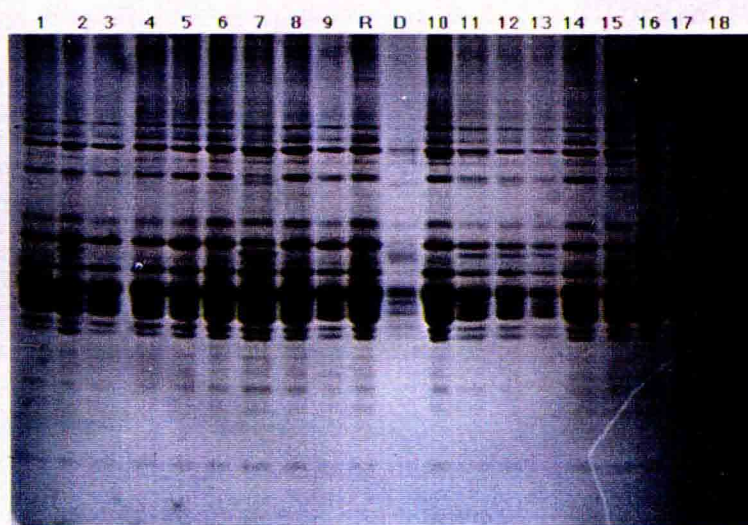
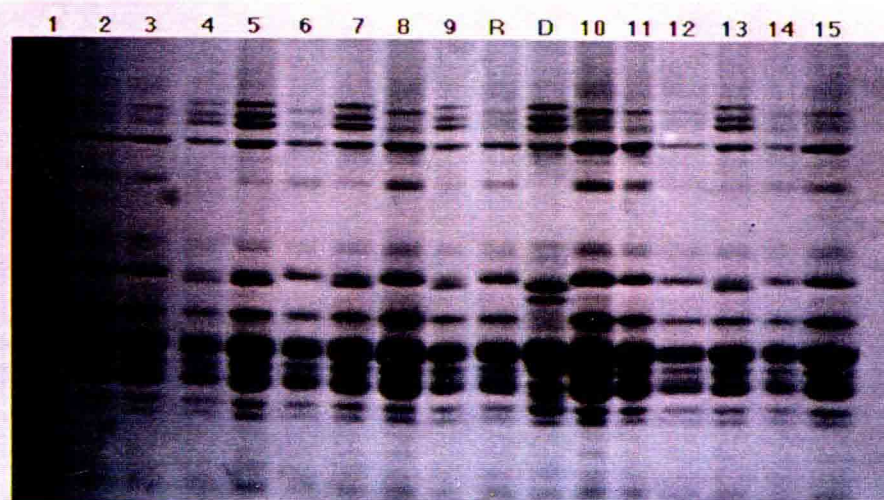
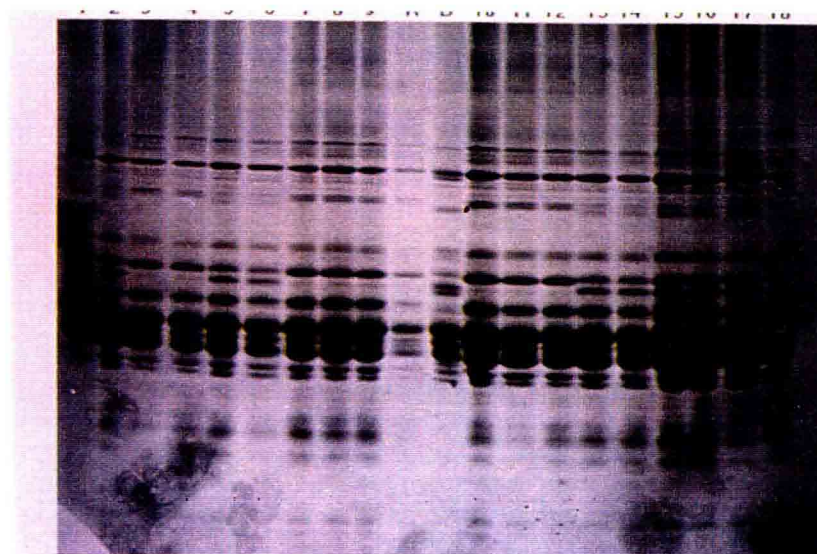


Fig. 4.22 : SDS-PAGE of gliadin protein showing segregating γ -
gliadin, mb at BC-2 stage

Lanes 1-9 : Samples 1 to 9
Lane 10 : Recipient (HD 2329) (R)
Lane 11 : Donors (WH 147) (D)
Lanes 12-20 : Samples 11to 18

Fig. 4.23 : SDS-PAGE of gliadin protein showing segregating γ -
gliadin, mc at BC-2 stage

Lanes 1-9 : Samples 1 to 9
Lane 10 : Recipient (HD 2329) (R)
Lane 11 : Donors (UP 319) (D)
Lanes 12-20 : Samples 10 to 18

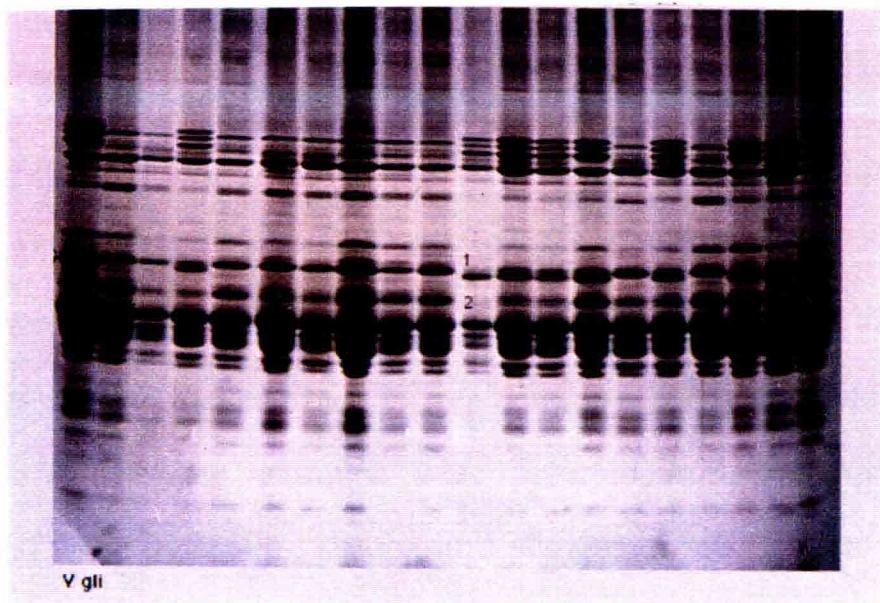
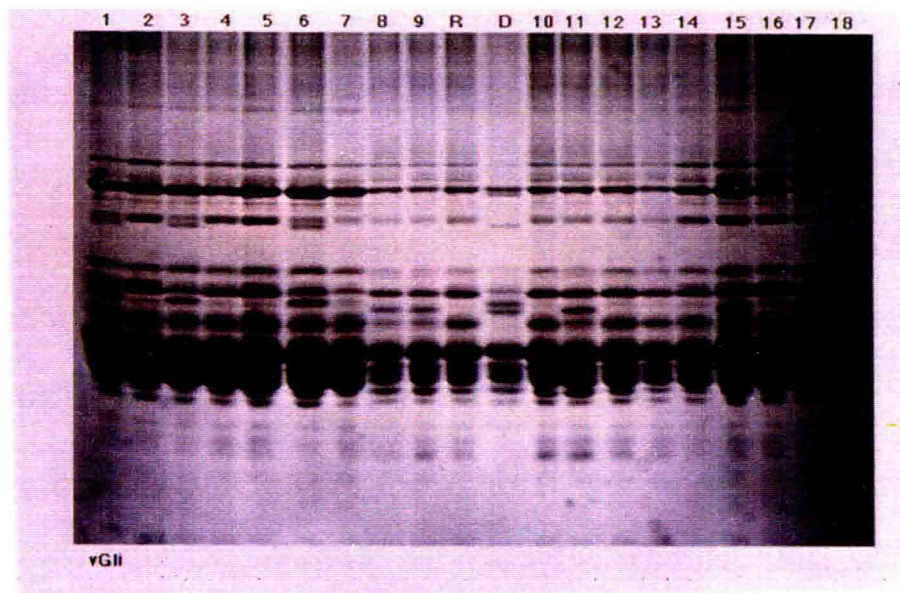
- 1- indicates the band for allele in consideration
- 2- indicates the bands studied to compare recipients background

Fig. 4.24 : SDS-PAGE of gliadin protein showing segregating γ -
gliadin, me at BC -2 stage.

Lanes 1-9 : Samples 1 to 9
Lane 10 : Recipient (HD 2329) (R)
Lane 11 : Donors (Sunkota B) (D)
Lanes 12-20 : Samples 10 to 18

Arrowheads mark the band for allele in consideration

* indicates the band /allele in consideration



polypeptides. But, still, different polypeptides are identified by their molecular weight or Mr and no allelic symbol has been assigned to them. In this study we have marked the position of bands showing variation amongst different varieties for convenience as shall be seen in coming figures.

Varietal polymorphism for albumin polypeptides was studied and the detected polymorphic band(s) were recorded (Fig. 4.25). F1 crosses from the corresponding donor parents were chosen for further backcrossing. Segregation analysis was done after each backcross and samples having our band of interest in a background closest to the recipient parent was chosen. Corresponding embryo halves were grown in the coming season. Backcrossing was then done again. The procedure was repeated till the third backcross generation. Analyzed samples were selected and corresponding embryo(s) sown in coming season and allowed to self fertilize. At the end of season, selfed seed were collected and stored for further studies.

Table 4.10 summarizes the details of near isogenic lines being developed for different water-soluble polypeptides. We have given symbols to the bands in consideration. No gene symbol had been assigned previously to any of these polypeptides.

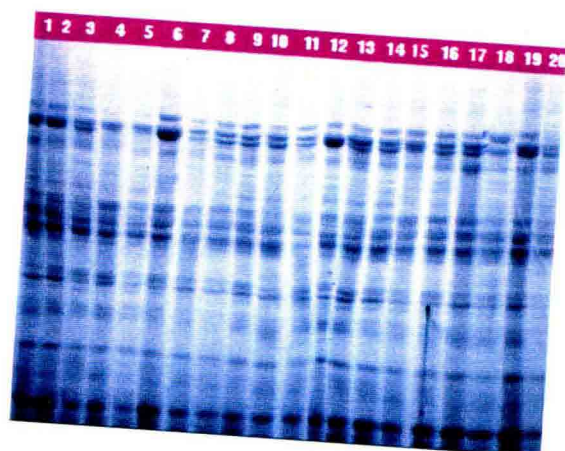


Fig. 4.25 : SDS-PAGE of water soluble albumin proteins from single whole seed of different wheat varieties

Lane 1	HD 2329
Lane 2.	Bungulla
Lane 3.	Bodallin
Lane 4.	Calidad
Lane 5.	Millewa
Lane 6.	Aroora Sr. 22
Lane 7.	WL 711
Lane 8.	WH 147
Lane 9.	Kalyansona
Lane 10.	Sunkota B
Lane 11.	HUW 55
Lane 12.	K-68
Lane 13.	PBW 65
Lane 14.	PBW 175
Lane 15.	UP 301
Lane 16.	UP 2121
Lane 17.	UP 115
Lane 18.	UP 319
Lane 19.	UP 262
Lane 20.	Chinese Spring

The black dot in lane no. 17 denotes allele mc and the red dot denotes allele md. For UP 115, similarly, black dot in lane 12 denotes allele mg while red dot indicates band/allele mi for K-68

Table 4.10 : Details of near isogenic lines being developed for different albumin polypeptides

BAND/ALLELE SYMBOL¹	DONOR VARIETY	ALLELE/BAND (PATTERN) PRESENT IN DONOR VARIETY
1. ma	UP 301	Lower band present (Fig. 4.26)
2. mb	UP 301	Dark middle band (Fig. 4.26)
3. mc	UP 115	Intense upper band (Fig. 4.27)
4. md	UP 115	Darker upper band in the pair of bands (Fig. 4.27)
5. me	UP 319	Lower band present in the band pair pattern (Fig. 4.28)
6. mf	UP 1109	Presence of a band
7. mg	K-68	Presence of upper (Fig. 4.25) band a band pair pattern
8. mh	K-68	Middle band present (Fig. 4.25)
9. mc (repeat)	UP 1109	-

¹ - Symbol assigned during this study. No symbol assigned to band/allele for albumin till date

Ideally, we are developing eight lines with different polypeptides/alleles (mb-mh). We had begun our experiments with nine lines but later we found that the line being developed with UP 1109 as donor has the same band as UP 115 (mc). However, the line is still being maintained for further quality tests. The total number of seeds produced for each near isogenic line at different stages of backcrossing is shown Table 4.11)

Table 4.11 : Total number of seeds set in each generation during development of NILs for albumins

ALLELE SYMBOL	DONOR	TOTAL NUMBER OF SEED SET IN			
		F1	BC-1	BC-2	BC-3
ma	UP 301	51	40	22	179
mb	UP 301	51	40	29	136
mc	UP 115	58	58	50	131
md	UP 115	58	58	51	248
me	UP 319	86	50	37	135
mc	UP 1109	81	45	48	137
mf	UP 1109	81	45	35	110
mg	K-68	80	70	31	122
mh	K-68	80	70	23	126

Representative gels showing segregation analysis in different near isogenic lines being developed for different albumin subunits are given by Fig. 4.26 (for ma, mb), Fig. 4.27 (for mc) and Fig. 4.28 (for me).

4.4 Triticin

Two loci, namely, *Tri-A1* and *Tri-D1* are recognized for the high molecular weight triticin protein that occur as a triplet band in SDS-PAGE. Table 4.12 gives the alleles found at each locus and standard Indian variety in which they are present.

Table 4.12 : Summary of alleles present in triticin and known Indian standards for them

LOCUS	NO. OF ALLELES	COMMON/ RARE	KNOWN INDIAN STANDARD	
			SYMBOL	VARIETY
<i>Tri-A1</i>	2	2/0	a	HS 277
			b	CPAN 3004
<i>Tri-D1</i>	2	2/0	a	HS 277
			b	DWR-39

It is known that our recipient parent, HD 2329 has alleles 'b' for both *Tri-A1* and *Tri-D1* loci. Chinese spring and K-68 have *Tri-A1*, a and *Tri-D1*, a. Figure 4.29 shows the triticin protein from Chinese Spring lines and K-68. Substitution lines were seen to observe the pattern they show in SDS-PAGE. Chinese Spring and K-68 give an intense upper band of *Tri-D1*, a and also have the band for *Tri-A1*, a, both of which are absent in HD 2329. The lane for CSDtAL gives the band only for *Tri-D1*, a while CSDt1DL gives the band only for *Tri-A1*, a, as expected. However, these lines were not used for developing

Fig. 4.26 : SDS-PAGE for albumin polypeptides showing segregating (ma) and (mb) alleles from seeds at BC-1 stage

Lanes 1-9 : Samples 1 to 9
Lane 10 : Recipient (HD 2329) (R)
Lane 11 : Donor (UP 301) (D)
Lanes 12-20 : Samples 10 to 18

The black line between samples D and lane 10 indicates the band for alleles (ma) and the one near sample 18 makes the allele (mb) in UP 301

Fig. 4.27 : SDS-PAGE for albumin polypeptides showing segregating (mc) alleles from seeds at BC-2 stage

Lanes 1-9 : Samples 1 to 9
Lane 10 : Recipient (UP 115) (R)
Lane 11 : Donors (UP 301) (D)
Lanes 12-20 : Samples 10 to 18

Arrowhead indicates band corresponding to allele (mc) in UP 115
Black dot indicates band corresponding to allele (md) in UP 115

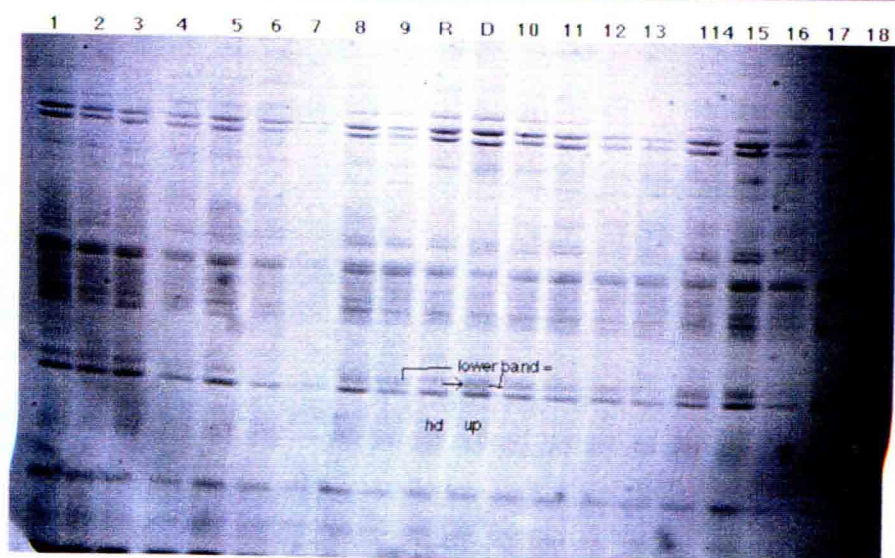
Fig. 4.28 : SDS-PAGE for albumin protein showing segregating (me) alleles from seeds at BC-2 stage

Lanes 1-9 : Samples 1 to 9
Lane 10 : Recipient (HD 2329) (R)
Lane 11 : Donors (UP 319) (D)
Lanes 12-20 : Samples 10 to 18

Arrowhead marks the lower band corresponding to allele (me) in UP 319



BC-1 for Alb.(2) D: Up 301



NILs as they lack the entire short arm of chromosome 1A and 1D, which carry several other important genes for seed proteins including *Glu-3* and *Gli-1*.

Table 4.13 summarizes the details of the NILs being produced for triticin with different alleles.

Table 4.13 : Details of NILs being developed with different triticin alleles

S. NO.	LOCUS	ALLELE	DONOR PARENT	ALLELE PRESENT IN HD 2329
1.	<i>Tri-A1</i>	a	K-68	a
2.	<i>Tri-D1</i>	a	K-68	b

The two lines were developed in a manner similar to the lines developed for other proteins. After back-crossing the total number of seeds produced at different stages for each NIL produced is shown in Table 4.14.

Table 4.14 : Total number of seeds set in each generation during development of NILs for triticin

LOCUS	ALLELE	DONOR PARENT	TOTAL NUMBER OF SEED SET IN			
			F1	BC-1	BC-2	BC-3
<i>a</i>	b	K-68	80	70	23	144
<i>Tri-D1</i>	a	K-68	80	70	20	130

Fig. 4.30 represents the SDS-PAGE of triticin protein samples from seeds at BC-1 stage. It is at this stage that samples were sorted out and separate lines for the loci began development.

Fig 4.31 shows pots with BC-2 plants in them. Butter paper bags cover the heads that have been pollinated by HD 2329 pollen to produce BC-3 seeds. The plants were healthy and showed good tillering as they had been placed in the field during this season (1999-2000).

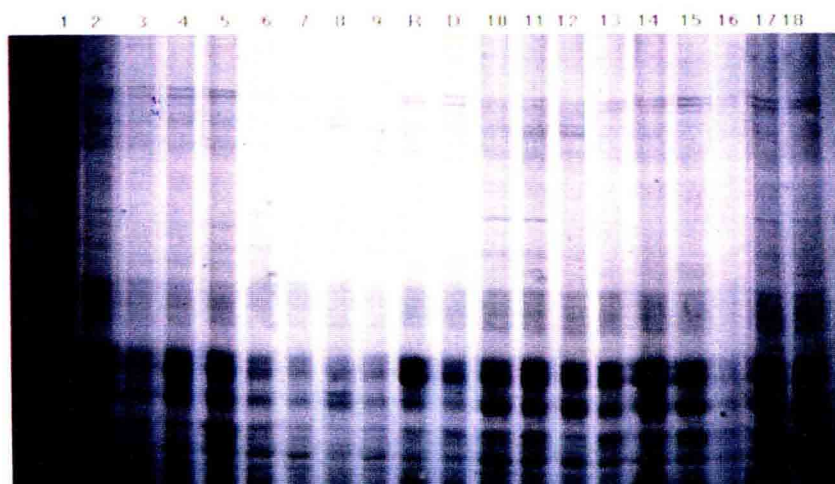
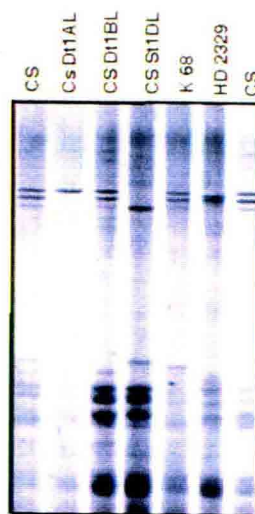
Fig. 4.29 : SDS-PAGE of salt-soluble tritacin protein from single whole seed of different wheat varieties and lines

Lane 1 : Chinese spring
Lane 2 : CS Dt1AL
Lane 3 : CS Dt1BL
Lane 4 : CS Dt1DL
Lane 5. : K-68
Lane 6. : HD 2329
Lane 7. : Chinese spring

Fig. 4.30 : 0.8% SDS-PAGE showing samples of tritacin protein from seeds at BC-1

Lanes 1-9 : Samples 1 to 9
Lane 10 : Recipient (HD 2329) (R)
Lane 11 : Donors (K-68) (D)
Lanes 12-20 : Samples 10 to 18

Fig. 4.31 : Pots with BC-2 plants of lines for tritacin in glasshouse. Back-crossed heads are covered with butter paper bags



BC 1 for In D1 and In A1 D K 68



Once the BC-3 seeds were collected, they were analyzed for the presence of donor tritacin alleles. The corresponding embryo halves were sown in fields and allowed to self fertilize. Seeds obtained from these plants were then used for analysis at the molecular level as follows.

AFLP analysis of BC-3 F₁ plants

Genomic DNA was extracted from etiolated leaves of BC-3 F₁ seeds germinated in petriplates. We analyzed a total of 10 samples for NILs with *Tri-A1*, a and 13 samples for NILs with *Tri-D1*, a. DNA from parents was also extracted in a similar manner. The genomic DNA was of good quality and each sample had DNA of concentration 50 ng/μl or above (Fig. 4.32).

This DNA was used for AFLP analysis. Five primer pair combinations, given below, were used in the study.

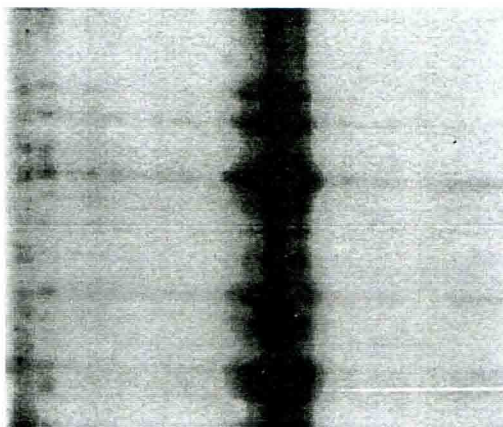
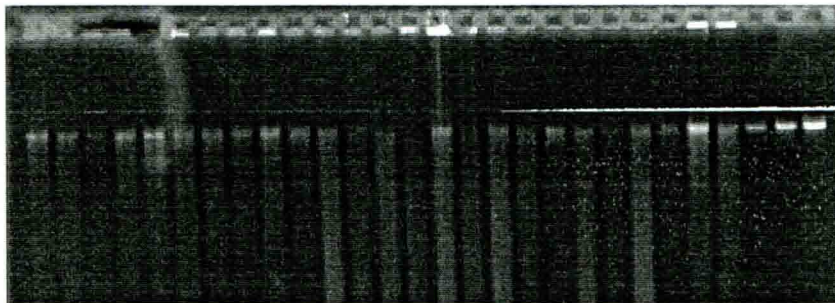
1. E-AAC + M-CAA
2. E-AAC + M-CAC
3. E-ACT + M-CAA
4. E-ACT + M-CAC
5. E-AGC + M-CAC

AFLP was done as given in the materials and methods (section 3.9). The primer combination no. 5 did not work well as with this primer set, only few samples gave some bands while others did not give any at all (Fig. 4.33).

Fig. 4.32 : 0.8% agarose gel with genomic DNA from 23 triticin lines NILs

Lane 1.	3/32
Lane 2.	20/32
Lane 3.	26/32
Lane 4.	27/32
Lane 5.	33/32
Lane 6.	40/32
Lane 7.	46/32
Lane 8.	57/32
Lane 9.	68/32
Lane 10.	100/32
Lane 11.	6/60
Lane 12.	9/60
Lane 13.	10/60
Lane 14.	11/60
Lane 15.	13/60
Lane 16.	14/60
Lane 17.	17/60
Lane 18.	56/60
Lane 19.	68/60
Lane 20.	116/60
Lane 21.	118/60
Lane 22.	119/60
Lane 23.	121/60
Lane 24.	HD 2329
Lane 25.	K-68
Lane 26.	Uncut Lambda DNA (50 ng)
Lane 27.	Uncut Lambda DNA (100 ng)
Lane 28.	Uncut Lambda DNA (200 ng)

Fig. 4.33 : Autoradiogram showing AFLP gel (6%) of 23 samples from the 2 triticin NILs with primer pair number 5 (E-AGC + M-CAC)



The other four primer pair combinations worked well and a number of bands were seen. The number of polymorphic bands were noted (Table 4.15)

Table 4.15 : Number of polymorphic bands seen with each primer pair used with parental lines K-68 and HD 2329

	Primer pair	No. of polymorphic band(s) detected	Remarks
1.	E-ACC + M-CAA	(4)	Polymorphic bands were not distinct and occur at top and lower region of gel
2.	E-AAC + M-CAC	(4)	Polymorphic bands were not very distinct and occur at top and lower region of gel
3.	E-ACT + M-CAA	(0)	No polymorphism detected
4.	E-ACT + M-CAC	(6)	Polymorphic bands are comparatively more distinct and present in middle of gel
5.	E-AGC + M-CAC	(1)	Primer pair did not work well

Table 4.15 shows that primer pair no. 4 worked well and hence samples were analyzed using this combination of primers. Of the 6 polymorphic bands identified between the parents, only 3 could be studied for sample analysis due to improper resolution and faint band pattern in the gel. Table 4.16 summarizes the polymorphism detected amongst samples as the 3 polymorphic bands studied.

Table 4.16 : Summary of polymorphism detected by AFLP in BC-3 F₁ samples

S.No.	SAMPLE DETAILS (PLANT NO./LINE NO.)	NIL FOR	BAND1	BAND2	BAND3
1.	3/32	Tri-A1(b)	+	-	+
2.	20/32		+	+	+
3.	26/32		+	+	+
4.	27/32		+	+	-
5.	33/32		-	-	-
6.	40/32		+	+	+
7.	46/32		-	-	+
8.	57/32		-	+	+
9.	68/32		-	+	+
10.	100/32		-	-	-
11.	6/60	Tri-D1(a)	-	-	+
12.	9/60		-	-	+
13.	10/60		-	+	+
14.	11/60		-	+	+
15.	13/60		-	+	+
16.	14/60		-	+	+
17.	17/60		-	+	+
18.	56/60		-	+	+
19.	68/60		-	-	-
20.	116/60		-	-	+
21.	118/60		-	+	+
22.	119/60		-	+	+
23.	121/60		?	+	+
24.	HD 2329		-	+	+
25.	K-68		+	-	-

Analysis at BC-3 stage had been done and only those samples used for further studies which had our allele of interest of course, in the background of the recipient parent. Hence, the BC-3 F₁ samples being analyzed already had the allele of interest and analysis was done to chose samples closest to the recipient parent by AFLP analysis.

In the lines being developed for *Tri-A1*, a two samples (57/32) and 68/32) were found to bear bands similar to HD 2329 at all three polymorphic positions. Four other samples had bands similar to HD 2329 only at 2 positions while the third was like that present in K-

68. Four samples were more like K-68 at these polymorphic positions. With the help of AFLP analysis these seeds could be discarded at an early stage of backcrossing.

For *Tri-D1*, a lines analyzed, eight samples were identical to HD 2329 for the polymorphic bands. Four were similar to HD 2329 for two polymorphic bands while one was like K-68.

Fig. 4.34 shows region of the AFLP gel where polymorphic bands were detected.

AFLP is an efficient and precise technique for DNA fingerprinting as it combines classical hybridization and PCR methods for fingerprinting of DNA. Using AFLP, plants closest to the recurrent parent at the genetic level can be easily screened in and this helps us in reducing the total time span needed to produce NILs by molecular breeding. Classical methods to produce NILs take ~10 years for the same.

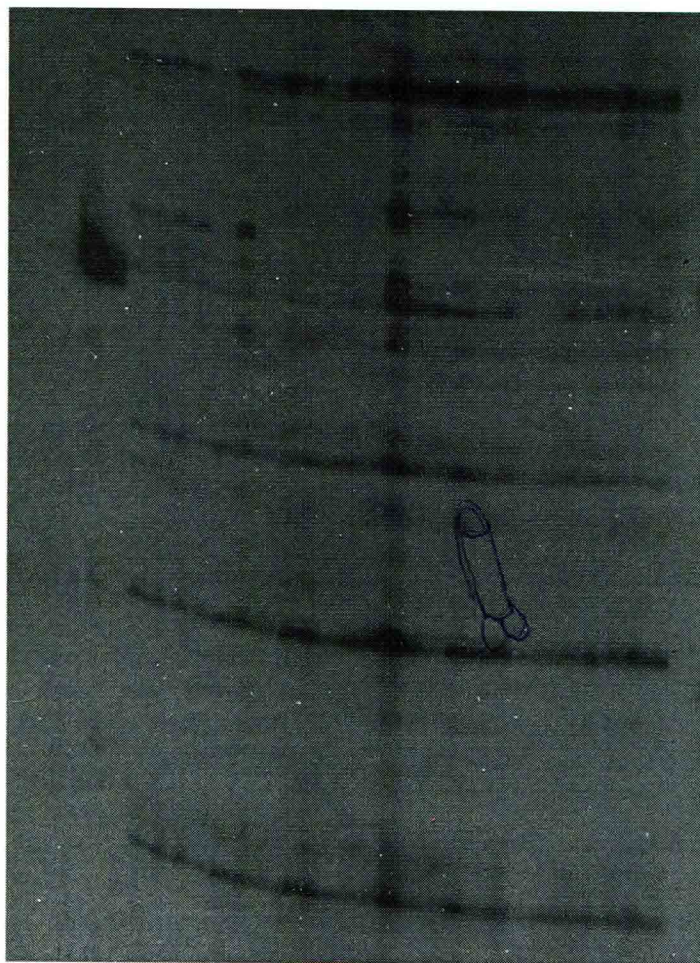
It was observed that the plants grown from screened in embryo halves during the last segregation analysis were morphologically more similar to the recipient parent. Each one had dark brown heads like HD 2329 unlike the white heads in K-68. The plant height was also reduced -a trait similar to the recurrent parent.

4.5 Production of fresh F₁ crosses for additional NILs

Though a total of 42 NILs were being produced in this study, many more could still be developed, including some with the common seed protein alleles. As more alleles were found in donor

Fig. 4.34 : Detection of autoradiogram of AFLP gel (6%) showing polymorphic bands (1, 2 and 3)

Lane 1	Marker
Lane 2.	Marker
Lane 3.	3/32
Lane 4.	20/32
Lane 5.	26/32
Lane 6.	27/32
Lane 7.	33/32
Lane 8.	40/32
Lane 9.	46/32
Lane 10.	57/32
Lane 11.	68/32
Lane12.	100/32
Lane 13.	6/60
Lane 14.	9/60
Lane 15.	10/60
Lane 16.	11/60
Lane 17.	13/60
Lane 18.	14/60
Lane 19.	17/60
Lane 20.	56/60
Lane 21	68/60
Lane 222	116/60
Lane 23	118/60
Lane 24	119/60
Lane 25	121/60
Lane 26	HD 2329
Lane 27	K-68



varieties available with us on literature survey, we made fresh crosses so that a more comprehensive set of NILs could be made for future studies. The various alleles of seed storage protein for which NILs can be made from the fresh F₁ crosses are given in Table 4.16.

Table 4.6 : Selected alleles for which NILs can be made with the fresh F₁ crosses

Protein (locus/allele) Donors	HMW glutenin subunits	LMW glutenin subunits	Gliadins	Albumins
Girija		<i>Glu-B3</i> , f	<i>Gli-A1</i> , e	
HUW 234		<i>Glu-D3</i> , b	<i>Gli-D1</i> , b	
UP 2121	<i>Glu-B1</i> , f			
	<i>Glu-A</i> , c			
HD 2135		<i>Glu-D3</i> , c	<i>Gli-D1</i> , e	
HP 1633			<i>Gli-B1</i> , f	
Sonalika		<i>Glu-A3</i> , a*		
		<i>Glu-B3</i> , b		
K-68	<i>Glu-B1</i> , e			
UP 262			<i>Gli-A1</i> , h*	
VL-404			<i>Gli-A1</i> , d	
CPAN 1796			<i>Gli-A1</i> , a	
K-9523				<i>Alb</i>
Bungulla				<i>Alb</i>
PBW-266				<i>Alb</i>
VL-401				<i>Alb</i>
UP 319				<i>Alb</i>
VL 784				<i>Alb</i>

*Lines already being developed for this allele but donor is of foreign origin

Thus, using sixteen donors, nineteen more new near isogenic lines can be developed for glutenin, gliadins and albumins. Also, in the NIL being developed at present with *Gli-A1*,h the allele was found to be linked to *Glu-A3* (e) in the donor UP 115. By making another line for the same allele using a different donor UP 262 which has *Glu-A3* (d), linkage between the two loci can be studied.

Discussion

As such, production of near isogenic lines by classical breeding methods takes approximately ten years. But, by combining it with molecular biology techniques, the total time span can be reduced to ≈ 6 years, saving time of 4 years! Also, analysis at the molecular level and then selection of samples for future use provides more specific and precise results. With the same effort, lines can be produced in a shorter time span.

In the process of transfer of qualitative characters by backcrossing, we recover germplasm of the recurrent parent in each back-cross. The F_1 of a cross between the recipient parent and the donor parent has 50% germplasm from each of the two parents. With every backcross, the proportion of the germplasm of the recurrent parent is increased by one-half, while that of the donor parent is decreased by one-half. Thus with 5-6 back-crosses the resultant population has 98.4% and 99.2% of the genome of the recurrent variety respectively. By BC-3 we have 93.8% of the germplasm from recurrent parent. This shows that by increasing the number of backcrosses beyond BC-3 we do not make much gain in the subsequent generations in context to genome recovery from recipient parent. Using molecular marker techniques like AFLP we can identify plants which are more similar to the recurrent parent at the genetic level using large number of BC-3 seeds to select from.

Thus, we can reduce the number of back-crossing required before selfing to regain the recurrent parents germplasm. Fifty per cent segregation was invariably visible during analysis on SDS-PAGE. Moreover, doubtful sample were screened out on analysis. Thus, the NILs were developed following the rules and laws of genetics.

Literature study gave immense information about the alleles at different loci in wheat seed storage proteins. Alleles for high molecular weight glutenin, ω -gliadins and tritacin have been assigned symbols/number as the case be. No symbols, however, have been assigned to albumin protein. They are still identified on the basis of their molecular weights. We, thus, in our study have given symbols to the albumin protein bands for which NILS were made

Another point to be careful is the discrepancies in nomenclature of alleles (**Gupta et al., 1998**). The same allele is assigned different symbols by different workers which poses problems for future workers in this area. However, care has been taken in this study to not to repeat alleles and if lines with the same allele have been developed, then they are properly identified e.g. **Sreeramalu, G. (1996)** identified the band at *Gli-A1* in WH 147 to be allele 'c' while we found it to be actually allele 'g'. This was done by comparing the band pattern of WH 147 gliadins to band patterns of different varieties and the allelic symbols assigned to them (**Sreeramalu, 1996**). Similarly, again in WH 147, *Gli-D1,a* was the

symbol assigned to the allele which is similar to that in HD 2329. But, during our study we found that the band for this allele (in WH 147) migrates faster than in HD 2329. A probable reason of this difference may be variability between stocks or it may be due to incorrect identification.

No symbol has been identified in literature for γ -gliadins and albumin. We, in this study, have given the bands/alleles for which NILs are made a symbol.

Analysis of triticin BC-3 F₁ lines was done by AFLP. DNA used for AFLP had been dissolved in T₁₀ instead of the usual TE and all dilutions made during AFLP were in water i.e. use of TE was avoided. This was because EDTA had been found to hinder during amplification (**Swain, personal communication**). The AFLP autoradiogram showed bands in all lanes/samples for four primer pairs but one primer pair did not work well. Some samples gave a number of bands with this primer pair while others did not give a single band. Any problem with the samples is ruled out as the same samples were used for amplification with other primer pairs. A possible reason could be improper labelling of the EcoRI primer E-AGC.

The autoradiogram reveals intense major bands while the minor bands are very faint. This is possibly because of low temperature of the gel during electrophoresis. The gel was run on a chilly night at constant power of 60 W. The usual temperature at

which gels are run is 55°C. And this temperature is maintained naturally, during summer. Low temperature during electrophoresis may a possible cause for occurrence of faint bands. Also, it was seen that more number of bands were found at the top of the gel which suggests in complete restriction digestion of the samples.

Only six polymorphic bands were detected with the best primer pair (best in terms of the number of polymorphic bands). Of these six, only three could be studied amongst samples as they were clearly resolved and hence visible. Bands at the other 3 positions were very faint and indistinct. This number of polymorphic bands is very less to come to any conclusion. Also, a larger number of primer pairs need to be screened and used for detailed analysis of the BC-3 F₁ seeds of the various lines developed before inferring anything and coming to a conclusion. As a result, AFLP study done reveals that the protocol followed works well but a few points need to be kept in mind during electrophoresis. Also, more work needs to be done at this level so that the lines may be screened and developed further perhaps using microsatellite and SNP markers in addition to AFLP.

Annexure 'D'
Glu-A3 locus

COUNTRY		AUSTRALIA ^{1,2}	FRANCE ³	INDIA ⁴	JAPAN ⁵	SPAIN ⁶ (a)
ALLELES						
SYMBOL ¹⁵	BAND/ PATTERN ⁶					
a	6			Sonalika		Mexicali
b	5			CPAN1922		Langdon
c	6+10			C 306		Cocorit
d	6+11			HD 1941		Alaga
e	11			UP 2121		Blatfort
f	6+11+20					Clarofino
g	6+10+20					Claro de balazote
h/k	null	Kite				Jiloca
m	-		Marengo			
p	-	MKR(111/8)	Prinqual			
N61	-				Norin 61	
Asakaze	-				Norin 12	
Aoba	-				Norin 67	

a = *T. durum*

1. Gupta *et al.* (1989)
2. Singh *et al.* (1991)
3. Nieto-Taladriz *et al.* (1994)
4. Sreeramalu (1996)
5. Nagamine *et al.* (2000)
6. Nieto-Taladriz *et al.* (1997)

PORTUGAL 20
ALVADOR 20

[illegible]

Glu-A1

1. Moonen *et al.* (1983)
2. Jurkovic *et al.* (2000)
3. Souza *et al.* (1994)
4. Mir Ali *et al.* (1999)
5. Ghong *et al.* (1992)
6. Labuschagne *et al.* (1995)
7. Lawrence (1986)
8. Sreeramalu and Singh (1994)
9. Payne and Lawrence (1983)
10. Groger *et al.* (1997)
11. Lafiandra *et al.* (1997)
12. Tahir *et al.* (1996)
13. Gupta *et al.* (1998)
14. Rodriguez-Quijano and Carrillo (1996)
15. Graybosch (1992)
16. Kuktaite *et al.* (2000)
17. Ivanov *et al.* (1998)
18. Bronneke *et al.* (2000)
19. Morgunov *et al.* (1990)
20. Rodriguez-Quijano *et al.* (1998)

[illegible]

YCHARCHIA
65 A. C

HARDI
NEWANA
PRINQUAL
HILOUR

ABADIA

 $\gamma; 20(\kappa)$

Glu-B1

1. Souza *et al.* (1994)
2. Lawrence (1986)
3. Groger *et al.* (1997)
4. Zhong *et al.* (1992)
5. Jurkovic *et al.* (2000)
6. Payne and Lawrence (1983)
7. Bronneke *et al.* (2000)
8. Bronneke *et al.* (2000)
9. Sreeramalu (1996)
10. Gupta *et al.* (1998)
11. D'Ovidip and Anderson (1994)
12. Kuktaitte *et al.* (2000)
13. Labuschagne *et al.* (1995)
14. Moonen *et al.* (1983)
15. Tahir *et al.* (1996)
16. Rodriguez-Quijano *et al.* (1998)
17. Mir Ali *et al.* (1999)
18. Ivanov *et al.* (1998)
19. Graybosch (1992)
20. Morgunov *et al.* (1990)

	ABERDEEN 14 71006	AUSTRALIA BAYONET-A	AUSTRIA COMPASS	AFGHANISTAN	CHINA DU 3MAL-1	CROATIA KVA	FRANCE CHAMPLEN	GERMANY HERZOG	G. BRITAIN AQUILA	INDIA CEAN-1922	ITALY N-STAMPOLI	JAPAN DANCHI	ME LITHUANIA ALBA	ZER
(2+12)														
(3+12)	ID 71320		DOMINUS		YUMAI 5		CHAMPLEN		HOBBIT					
c (4+12)		BODALIN			YUNAN 15		REMAN	ALIDOS		CEAN-3004	BE20STAYA-1		NANDU	
d (15+10)	ID 71027	SPICAD	ERLA KOLEBN		DONGXIE 3	ANA	KLINOR				MG 7249	DANCHI		
e (2+10) R											BT2288			
f (2.2+12) R		TERRA			ZHEMAL 1						FIORILLA			
g (5+9)											MG27095/133			
h (5+12)														
i (null)														
j (2+12) 7														
k (2)					KEHAN 5									
l (12)				AUS 10037										
m (10)				AUS 13673										
n (2.1+10)				AUS 14653										
o (2.1+13)				AUS 19519 (d)										
p (35)														
q (2+11)														
r (2.3+12)														
s (38)														
5*											FIORILLA			
5**12											FIORILLO			
2**12*											KG 7631			
2.2**12											MG 315			
12*											MG 27111			
3+10											EG 27078/304			
2+12,														

d=T. macha

[illegible]

Glu-D1

1. Souza *et al.* (1994)
2. Sreeramalu (1996)
3. Pogna *et al.* (1990)
4. Kuktaite *et al.* (2000)
5. Labuschagne *et al.* (1995)
6. Graybosch (1992)
7. Payne and Lawrence (1983)
8. Rodriguez-Quijano and Carrillo (1996)
9. D'Ovidio and Anderson (1994)
10. Gupta *et al.* (1998)
11. Tahir *et al.* (1996)
12. Moonen *et al.* (1983)
13. Jurkovic *et al.* (2000)
14. Lawrence (1986)
15. Groger *et al.* (1997)
16. Mir Ali *et al.* (1999)
17. Zhong *et al.* (1992)
18. Ivanov *et al.* (1998)
19. Rodriguez-Quijano *et al.* (1998)
20. Morgunov *et al.* (1990)
21. Bronneke *et al.* (2000)

Annexure 'E'
Glu-B3 locus

COUNTRY		AUSTRALIA ^{1,2}	INDIA ³	JAPAN ⁴	SPAIN ⁵ (a)
ALLELE					
SYMBOL ^{1,4}	BAND PATTERN ⁵				
a	2+4+15+19	Chinese spring	GW 120		Mexicali
b	8+9+13+16	Gabo	J 24		Langdon
c	2+4+14+15+19	Insignia	DL-20-9		Siloca
d	2+4+15+17+19	Orca	C306		Mundial
e	2+4+15+16+18	Cheyenne	CPAN 1922		Granja badajoz
f	2+4+15+17	Radja	CPAN 1796		Ardente
g	2+4+15+17	Bungulla			Claro de balazote
h	1+3+14+18	Thatcher			Blatfort
i	7+8+14+18				
j	-				
k	-				
l	-				
N61	-			Norin 61	
Gogatsu	-			Norin 67	
Toyohu	-			Norin 46	

a = *T. durum*.

1. Gupta *et al.* (1990)
2. Singh *et al.* (1991)
3. Sreeramalu (1996)
4. Nagamine *et al.* (2000)
5. Nieto-Taladriz *et al.* (1997)

Annexure 'F'
Glu-D3 locus

COUNTRY ALLELE	AUSTRALIA ^{1,2}		INDIA ³	JAPAN ⁴
	SYMBOL ^{1,4}			
a	Chinese spring		-	
b	Gabo		C306	
c	Insignia		CPAN1796	
d	Norin 61a		HP 1633	
e	Orca		DL-218-6	
N61				Aobakomugi
Asakaze				Asakazekomugi

1. Gupta *et al.* (1990)
2. Singh *et al.* (1991)
3. Sreeramalu (1996)
4. Nagamine *et al.* (2000)

Annexure 'G'
Glu-B2 locus

COUNTRY		SPAIN ¹ (a)
ALLELE		
SYMBOL	BAND/PATTERN	
a	12	Mexicali
b	Null	Langdon

a = *T. durum*

1. Nieto-Taladriz *et al.* (1997)

Annexure 'H'
Gli-A1 locus

COUNTRY		FRANCE ²	INDIA ³	ITALY ⁴
ALLELE				
SYMBOL ^{1,2,4}	PATTERN ¹			
a	d8	Arbon	CPAN 1796	Chinese Spring
b	d9	Apexal	C 306	Alpe
c	d8d9	Pernel	CPAN 1922	-
d	d8d9d10	-	VL 404	-
e	-	Open	Girija	-
f	-	Aiglon	Hira	Bolero
g	-	-	Huw 55	-
h	-	-	DL-784-3	-
i	-	Proqual	UP 1109	-
j	-	-	-	-
k	-	Abo	-	Salmone
l	-	-	-	Liocorno
m	-	Armada	-	-
n	-		-	-
o	-	Admiral	-	Etruria
p		-	-	-
q		-	-	-
r		-	-	-
s ³		-	-	Asiago
A*, ³		-	-	Farneto

1. Metakovsky *et al.* (1998)
2. Nieto-Taladriz *et al.* (1994)
3. Sreeramalu (1996)
4. Vaccino *et al.* (1995)

Annexure 'I'
Gli-B1 locus

COUNTRY		FRANCE ¹	INDIA ³	ITALY ⁴
ALLELE				
SYMBOL ^{1,4}	PATTERN ^{2,4}			
a	d1d5d6	-	DL-20-9	Chinese Spring
b	d2d4	Abo	CPAN 1796	Alpe
c	d4	Prinqual	HD 2329	Loreto
d	d3	Chopin	DL-784-3	Tiberio
e	d5	Aiglon	CPAN 1992	Aurelio
f	d5d6	Aboukir	DL-218-6	Ardito
g	-	Albatros	C 306	Adria
h	-	Floreal	PBW 65	-
i	-	-	HUW 55	-
j	-	-	Kharchia 65B	-
k	-	Magnif 27	-	Aquileja
l	-	Admiral	-	Fiocco
m	-	Faust	-	Constantino
n	-	-	-	-
o	-	-	-	-
p	-	-	-	-
q	-	Goya	-	Gallo
Null	null	Touzelle	-	-
m*	-	-	-	Pandas
r*	-	-	-	Sal 8 ^a
s	-	-	-	Salmone
n*	-	-	-	Spada

a- Sal 8 is a mixture of two biotypes differing at Gli-D1

1. Nieto-Taladriz *et al.* (1994)
2. Metakovsky *et al.* (1998)
3. Sreeramalu (1996)
4. Vaccino *et al.* (1995)

Annexure 'J'
***Gli-D1* locus**

COUNTRY	FRANCE¹	INDIA²	ITALY³
ALLELE SYMBOL^{1,4}			
a	Arfort	CPAN 1796	Chinese Spring
b	Abo	C 306	Alpe
c	-	HUW 55	-
d	-	DL-20-9	Aquileja
e	-	HD 2135	-
f	Aboukir	HPW 42	Ardito
g	Beaver	-	-
h	Fortin	-	Produttore
i	-	-	-
j	Artiside	-	-
k	Cargimarec	-	Adria
l	Admiral	-	-
null	Darius	-	-
m	Heurtebise	-	-

1. Metakovsky *et al.* (1998)

2. Sreeramalu (1996)

3. Vaccino *et al.* (1995)

4. Gupta *et al.* (1998)

Annexure 'K'
Gli-A2 locus

COUNTRY	FRANCE¹	ITALY²
ALLELE SYMBOL^{1,2,3}		
a	-	Chinese Spring
b	Beaver	Alpe
c	Prinqual	Eridano
d	-	-
e	Apexal	Brasilia
f	Gala	Farneto
g	Abo	Adria
h	Apollo	N. Strampelli
i	-	-
j	Aiglon	-
k	Eureka	-
l	Aboukir	Bolero
m	Rex	-
n	-	-
o	Castan	Chiarano
p	Artaban	Inallettibile 96
q	-	-
r	Admiral	-
s	-	-
t	Arfort	-
u	Titien	-
v	-	-
w	-	-
x	-	-
New ¹	Proqual	-
t ¹	Rescler	-
Z ²	-	Gallo

1. Metakovsky *et al.* (1998)
2. Vaccino *et al.* (1995)
3. Gupta *et al.* (1998)

Annexure 'L'
Gli- B2 locus

COUNTRY ALLELE SYMBOL^{1,2,3}	FRANCE²	ITALY³
a	-	Chinese Spring
b	Abo	Etruria
c	Arfort	Farneto
d	Friedland	-
e	Arsenal	Produttore
f	Master	-
g	Admiral	-
h	Belaviso	Mec
i	-	-
j	-	Farnese
k	-	-
l	Albatross	-
m	Amelio	-
n	-	-
o	Aboukir	Adria
p	Champital	Artu
q	-	-
r	Arminda	-
s	-	-
t	-	-
u	-	-
v	Declic	brasilgia
ac	Artaban	-
ad	Champion	-
ae	E. de choisy	-
e*	Floreal	-
new	Magnif 27	-
ab	Orep i	-
y	-	Alpe
aa	-	Salmone

1. Gupta *et al.* (1998)
2. Metakovsky *et al.* (1998)
3. Vaccino *et al.* (1995)

Annexure 'M'
***Gli-D2* locus**

COUNTRY		
ALLELE	FRANCE²	ITALY³
SYMBOL^{1,2,3}		
a	Abo	Chinese Spring
b	Apexal	Alpe
c	-	Eridano
d	-	-
e	Lodi	-
f	Creneau	-
g	Admiral	Inallettibile 96
h	Amelio	Bolero
i	-	-
j	Arcane	Adria
k	-	-
l	-	-
m	Arfort	Farneto
n	Alto	Frassineto
o	-	Salmone
p	-	-
q	Aristide	-
r	-	Aquileja
s	-	-
NEW	Albatros	-
v	Arbon	-
u	Martial	Fiorello
t	-	Golia

1. Gupta *et al.* (1998)
2. Metakovsky *et al.* (1998)
3. Vaccino *et al.* (1995)

CHAPTER 5

SUMMARY & CONCLUSIONS

Thirty one wheat varieties were screened by SDS-PAGE to study polymorphism in glutenin subunits (HMW and LMW), gliadin, albumin and tritacin proteins. Significant polymorphism was detected among varieties for each seed storage protein. F1 crosses already made in winter season of 1996-97 were available with us. The F1 crosses involving polymorphic bands and different loci were selected for further study. Seeds of the chosen F1 crosses were then germinated and later transplanted to pots. When heads appeared in the F1 plants, they were emasculated and covered with butter paper bags to prevent undesired cross-pollination. Ready pollen from HD 2329 was taken and used to pollinate the emasculated heads. BC-1 seeds set in after fertilization were harvested on maturity. These BC-1 seeds were then analyzed by SDS-PAGE for segregation of the desired allele in a background similar to the recurrent parent (HD 2329). Samples having the desired allele in the background of recurrent parent were selected and their corresponding embryo halves germinated in the coming season. The germinated plants were then transferred to pots and back-crossed. The BC-2 seeds were harvested on maturity, analyzed and embryo halves corresponding to samples screened in marked. These were sown in coming season and backcrossed to produce BC-3 seeds. By this time ~94% of the genes of recurrent parent has been recovered.

The BC-3 seeds were then screened as stated above and the corresponding embryo halves germinated. They were later transferred to fields. The plants, which developed, were allowed self-pollination. On maturity, these were harvested, labeled and stored at 10°C.

AFLP analysis of BC-3 F1 plants for the two triticin lines was done. Genomic DNA was extracted by the CTAB method, quantified against known standards on agarose gel (0.8%), and used for restriction digestion. There were ten samples for the NIL with *Tri-A1, a* and thirteen samples for the NIL with *Tri-D1, a*. Five different primer pairs were used of which one gave good and clear polymorphism. Three primer pairs gave 4 polymorphic bands each. One primer pair gave inconsistent results, may be due to improper labelling of the EcoRI primer. The primer pair (E-ACT + M-CAC) gave six polymorphic bands between the donor and recurrent parent and was the best amongst all primer pairs used. But, when compared within the 23 samples studied by AFLP, only three polymorphic bands resolved clearly and these only were analyzed.

Results show that 2 out of 10 samples of NIL with *Tri-A1, a* are identical to HD 2329 while 8 out of 13 are identical in samples of NIL with *Tri-D1, a*. However, we cannot draw any conclusion from the results of AFLP and more work is needed in this direction using a large number of primer pair combinations or other molecular marker techniques.

Seventeen fresh F1 crosses were made which are to be used in back-crossing. The donors, however, had different alleles at different loci. Thus, these F1 crosses can be used to make 19 more NILs with different seed storage protein alleles, taking the total to 61 NILs.

Once the NILs are ready, the effect of different protein bands on end-use quality may be studied individually or in combination by intercrossing using different NILs. The ground rules thus identified can be used as guidelines for the selection of genotypes useful in wheat quality breeding programs for enhanced bread, pasta, noodles and chapati-making quality.

Finally, we can summarize the results and conclude:

1. A total of forty-two NILs were developed for different wheat seed storage proteins. These included -

HMW glutenin (HMW)	06
LMW glutenin (LMW)	13
gliadins (GLI)	12
albumins (ALB)	09
triticin (TRI)	02

2. The different loci and the alleles for which the NILs were developed are given in Table 5.1. Each NIL has been identified by assigning it an Arabic numeral prefixed by the abbreviated name of protein.
3. Alleles for which no symbol was available in literature were assigned symbols.

4. AFLP analysis of 23 samples from two triticin NILs by five primer pairs revealed only one of them to work properly with six polymorphic bands. Work in this direction using more primer pairs is required before any conclusion can be drawn. Other technologies e.g. use of microsatellites and SNPs are also an alternative.
5. Seventeen fresh F1 crosses were made with different donors to give a more comprehensive set of NILs. Details are given in Table 4.16 (chapter 4).

Table 5.1: Summary of NILs developed with different seed storage protein alleles during the course of this study.

Sl. No.	Line Name	Locus/Allele Band symbol	Donor Parent
1.	HMW-1	<i>Glu-A1 (a)</i>	UP2121
2.	HMW-2	<i>Glu-B1 (b)</i>	UP1109
3.	HMW-3	<i>Glu-B1 (b*)</i>	CSIA-1B
4.	HMW-4	<i>Glu-B1</i>	Kalyansona
5.	HMW-5	<i>Glu-D1(d)</i>	K-68
6.	HMW-6	<i>Glu-D1</i>	CS1 BL
7.	LMW-1	<i>Glu-A3(a)</i>	CS 1BL
8.	LMW-2	<i>Glu-A3(c)</i>	Kalyansona
9.	LMW-3	<i>Glu-A3(d)</i>	UP319
10.	LMW-4	<i>Glu-A3(d)</i>	UP1109
11.	LMW-5	<i>Glu-A3(e)</i>	UP115
12.	LMW-6	<i>Glu-B3(d)</i>	HUW55
13.	LMW-7	<i>Glu-D3(e)</i>	HUW 55
14.	LMW-8	<i>Glu-D3(d)</i>	CS1A-1B
15.	LMW-9	c-subunit (KS)	Kalyansona
16.	LMW-10	c-subunit (sb)	Sunkota B
17.	LMW-11	<i>Glu-D4 (a)</i>	C1A-1B
18.	LMW-12	<i>Glu-D5 (b)</i>	K-68
19.	LMW-13	Gli in glu	Kalyansona
20.	GLI-1	<i>Gli-A1(g)</i>	WH147
21.	GLI-2	<i>Glu-A1(h)</i>	UP115
22.	GLI-3	<i>Gli-B1(a)</i>	CSIA-1B
23.	GLI-4	<i>Gli-B1(b)</i>	UP2121

24	GLI-5	<i>Gli-B1(c)</i>	UP1109
25	GLI-6	<i>Gli-B1(d)</i>	Kalyansona
26	GLI-7	<i>Gli-D1(b)</i>	WH147
27	GLI-8	v Gli (ma)	UP1109
28	GLI-9	v Gli (mb)	WH147
29	GLI-10	v Gli (mc)	UP319
30	GLI-11	v Gli (md)	UP115
31	GLI-12	v Gli (me)	Sunkota B
32	ALB-1	ma	UP301
33	ALB-2	mb	UP301
34	ALB-3	mc	UP 115
35	ALB-4	md	UP115
36	ALB-5	me	UP319
37	ALB-6	mf	UP 1109
38	ALB-7	mg	K-68
39	ALB-8	mh	K-68
40	ALB-9	mc	UP 1109
41	TRI-1	<i>Tri-A1 (b)</i>	K-68
42	TRI-2	<i>Tri-D1(a)</i>	K-68

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LITERATURE CITED

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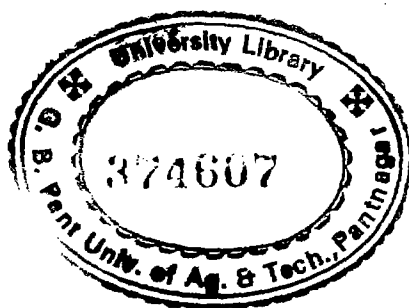
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APPENDIX



APPENDIX

NaCl (1 M) (100 ml)

NaCl	5.8 g
dH ₂ O	90 ml

Make up to 100 ml, autoclave and store at RT

Tris-Cl (1 M), pH 8.0 (100 ml)

Tris-Cl	15.75 g
dH ₂ O	80 µl

Adjust pH=8.0 with concentrated HCl, make up volume to 100 ml. Autoclave and store at RT.

Triticin Extraction Buffer (100 ml)

1 M NaCl	99 ml
1 M Tris-Cl	1ml

Makes 100 ml. Autoclave and store at RT

Prolamine Extraction Buffer (100 ml)

Propan-2-ol	50 ml
dH ₂ O	50 ml

Store in screw capped bottle, store in fridge.

Glutenin Extraction Buffer (100 ml)

Tris base	9.68 g
Propan-2-ol	50 ml
dH ₂ O	40 ml

Bring pH=8.0 by adding concentrated HCl. Make up to 100 ml, store in screw capped bottle in a fridge.

DTT (0.5 M)

DTT	1.545 gm
Sodium acetate	20 ml
(pH=5.2, 0.01 M)	

Do not autoclave DTT or solutions containing DTT. Sterilize by filtration. Dispensed into 1 ml aliquots and stored at -20°C.

Ethanol (70%) (100 ml)

100% ethanol	70 ml
dH ₂ O	30 ml

Makes 100 ml. Keep at RT/4°C

Sample buffer, pH=8.0 (100 ml)

Tris base	9.68 gm
SDS	2 gm
Glycerol	40 gm=32 ml
Bromophenol blue	2 mg
dH ₂ O	50 ml

Bring pH=8.0 with concentrated HCl and make up to 100 ml.
Store at RT.

Ammonium Persulfate (10%) 1 ml

APS	0.1 gm
dH ₂ O	1 ml

Ideally, made fresh daily, but keeps for 7 days in fridge.

2X separating Gel Buffer (pH=8.9) (500 ml)

Tris Base	45.4 gm
SDS	1 gm
dH ₂ O	460 ml

Add concentrated HCl drop wise till pH falls to 8.9. Store in fridge.

Sodium acetate (3 M), pH=5.2 (100 ml)

Sodium acetate	24.6 gm
dH ₂ O	80 ml

Adjust pH to 5.2 with glacial acetic acid and make up volume to 100 ml. Autoclave and store at RT.

Stock Acrylamide for separating gel (250 ml)

0.8%C

Acrylamide	75 gm
Bisacrylamide	0.6 gm
dH ₂ O	181 ml

Makes 250 ml (30% acrylamide, 0.24% bisacrylamide 0.8%C). Store in dark bottle at 4°C.

1.5% C

Acrylamide	75 gm
Bisacrylamide	1.15 gm
dH ₂ O	181 ml

Makes 250 ml (30% Acrylamide, 0.58% bisacrylamide, 1.5%C) store in dark bottle at 4°C.

2X stacking Gel Buffer (pH=6.8) (200 ml)

Tris base	6.06 gm
SDS	0.4 gm
dH ₂ O	190 ml

Add concentrated HCl drop wise till pH falls to 7.2 and then dilute HCl till pH falls to 6.8. Makes 200 ml store in fridge.

Stock Acrylamide for stacking gel (50 ml)

Acrylamide	15 gm
Bisacrylamide	0.4 gm
dH ₂ O	36 ml

Makes 50 ml (30% acrylamide, 0.8% bisacrylamide, 2.7%C) store in dark bottle at 4°C.

10X Electrode buffer (pH=8.6) (1000 ml)

Tris base	30.3 gm
Glycine	144.2 gm
SDS	10 gm
dH ₂ O	885 ml

Makes one liter. pH is approximately 8.6. Store at RT

Working Solution

For use, 1 part of this solution is mixed with 9 parts of dH₂O.

Staining solution (1000 ml)

Step 1:

Coomassie Brilliant Blue R	0.25 gm
dH ₂ O	25 ml

Step 2:

TCA	60 gm
dH ₂ O	720 ml
Methanol	180 ml
Glacial acetic acid	60 ml

Step 3:

Tip step 1 solution into step 2 solution and mix by stirring.
Makes 1 liter. Store in dark bottle at RT.

Destaining solution (1000 ml)

NaCl	30 gm
dH ₂ O	1000 ml

Store at RT

EDTA (0.5 M) pH = 8.0 (100 ml)

Sodium-EDTA	18.61
dH ₂ O	80 ml
NaOH pellets	~20

Adjust pH to 8.0 and make up volume.

Autoclave and store at RT.

NaCl (4 M) (100 ml)

NaCl	23.3 gm
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dH ₂ O	80 ml
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Make up volume to 100 ml.

Autoclave and store at RT.

CTAB (10%) (100 ml)

CTAB	10 gm
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dH ₂ O	80 ml
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Make up to 100 ml and store at RT.

DNA Extraction buffer (200 ml)

1 M tris-Cl	20 ml
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0.5 M EDTA	8 ml
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4 M NaCl	70 ml
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10% CTAB	40 ml
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β-mercaptoethanol 400 μl (to be added freshly).

dH ₂ O	60 ml
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Make up volume to 200 ml and use.

Buffer without β-mercaptoethanol may be prepared, autoclaved and stored at RT.

TE (pH = 8.0) (100 ml)

1 M tris-Cl 1 ml

0.5 M EDTA 200 μ l

Make up volume, autoclave and store at RT.

T₁₀ (pH = 8.0) (100 ml)

1M tris-Cl 1 ml

dH₂O 99 ml

Autoclave and store at RT

50 X TAE, pH = 8.0 (100 ml)

Tris base 24.2 gm

Glacial acetic acid 5.71 ml

0.5 M EDTA (pH = 8.0) 10 ml

dH₂O 60 ml

Adjust pH to 8.0 and make up volume.

Autoclave and store at RT.

Ethidium bromide (10 mg/ml)

EtBr 100 mg

dH₂O 10 ml

Dissolve and store at 4°C. Wrap in aluminum foil . Staining solution was prepared by adding 10 μ l of EtBr stock solution to 200 ml dH₂O.

Wear gloves while handling EtBr.

DNA Loading dye, pH =8.0 (10 ml)

Bromophenol blue	0.025 gm
Xylene cyanol FF	0.025 gm
Sucrose	4 gm
dH ₂ O	5 ml

Make up volume to 10 ml and set pH =8.0 with 1 M NaOH.

Store in aliquots at 4°C.

5X TBE, pH = 8.0 (200 ml)

Tris base	5.4 gm
Boric acid	2.75 gm
0.5 M EDTA (pH = 8.0)	2 ml

Make up volume, autoclave and store at RT.

Formamide dye (50 ml)

Formamide	49 ml
0.5 M EDTA	1 ml
Bromophenol blue	50 mg
Xylene cyanol	50 mg

Mix nicely, aliquot and store at 4°C.

Acrylamide: Bisacrylamide (20:1) (100 ml)

acrylamide	20 gm
bisacrylamide	1 gm
dH ₂ O	60 ml

Make up volume to 100 ml and store at RT.

VITA

Name : **Meenal Rathore**
Date of Birth : 20th October, 1974
Place of Birth : Pantnagar

Academic Record

Degree	Year	Division	Board/University
High School	1990	I	CBSE
Intermediate	1992	I	CBSE
B.Sc. (ZBC)	1995	I	Kanpur University
M.Sc. (Mol. Bio. & Biotech.)	1997	I	GBPUA&T, Pantnagar

Scholarships

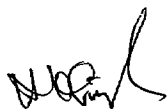
1. Recipient of DBT fellowship during Masters Programme
2. Qualified GATE 1996
3. Recipient of Junior Research Fellowship in DBT sponsored National Wheat Network project "Sub-project V"
4. Recipient of Senior Research Fellowship of CSIR

ABSTRACT

Name: Meenal Rathore Id. No. : 22985
Semester and year of admission: 1st semester, 1997-98
Degree: Ph.D Major: Molecular Biology and Biotechnology
Department: Molecular Biology and Genetic Engineering
Thesis title: Development of Near-Isogenic Lines with Different Seed Storage Protein Alleles for Quality Analysis in Wheat (*Triticum aestivum* L.)
Advisor: Dr. N.K. Singh

The end use quality of wheat largely depends on its seed storage protein composition. Water-soluble albumins, saline-soluble globulins, alcohol soluble gliadins and acid-soluble glutenins are the four major classes of wheat seed storage proteins. which are synthesized by genes at 15 major independent loci. Each locus has multiple alleles giving a possibility of over a million combinations. To study the relative effect of individual alleles on the end-product quality, NILs having a common genetic background but differing in only one allele of a protein are ideal study material. In the present study, HD 2329 was taken as the recipient parent. Donor parents of mostly Indian origin were used. Molecular marker assisted accelerated backcrossing of F1 crosses produced earlier using different donor varieties was followed and at present, we have 42 NILs at the BC-3F₂ stage (six NILs for HMW glutenin, thirteen for LMW glutenin, twelve for gliadins, nine for albumins and two for triticin protein). Bands for which no allelic symbol was available in literature were assigned symbols (γ -gliadins and albumins). AFLP analysis of 23 samples from triticin NILs was done. More primer pairs need to be screened for use before any further conclusive analysis can be reported. Also seventeen fresh F1 crosses were made to add to a more comprehensive set of NILs. Comprehensive cataloging of alleles for each protein locus and the variety/ cultivar in which they are reported was done on the basis of literature survey.

Once the NILs are ready, the relative effect of alleles on end- use quality may be studied directly or in combination by further crossing between lines. It is for the first time that NILs for albumin and triticin are being developed and shall be used for correlating allelic variation to quality analysis.



(N.K. Singh)
Advisor



(Meenal Rathore)
Authoress