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THE STRUCTURE AND GENETIC CONTROL OF ENDOSPERM PROTEINS IN WHEAT AND RYE

by

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Abstract

It is widely accepted that the storage proteins of the wheat endosperm are important determinants of the nutritional and organoleptic qualities of wheat flour. During the last decade, with the advent of improved electrophoretic techniques of fractionation, the structure and genetic control of these proteins have been studied intensively. However, most of these studies have focussed on the prolamins and glutelins. In the present work, a new class of storage proteins was identified in the wheat endosperm and the structure and genetic control of these new proteins, together with that of the prolamins and glutelins of wheat and rye, are reported in this thesis.

Sodium dodecyl sulphate — polyacrylamide gel electrophoresis (SDS-PAGE) of unreduced total protein extracts from the endosperm of hexaploid wheat revealed three high molecular weight protein bands, designated as "triplet" bands, in a zone of heavy background streaking. Since these proteins have not been described before, they were characterized in some detail. Electrophoretic examination of 137 diverse hexaploid wheat cultivars showed at least five different patterns of triplet bands. Nine durum wheat cultivars showed only a single band in this region of the gel. Analysis of aneuploid lines of 'Chinese Spring' wheat revealed that the slowest moving band (Tri-1) of the triplet was controlled by gene(s) on chromosome arm 1DS and the fastest moving band (Tri-3) was controlled by 1AS. The band with intermediate mobility (Tri-2) was shown to be a hybrid aggregate of the subunits controlled by 1DS and 1AS.

The composition of both the triplet bands and the background streak was determined by using a non-reducing/reducing form of 2-dimensional (2-D) SDS-PAGE. With this method, triplet bands were shown to be heterotetramers composed of four subunits designated as D (M.W. 58,000), δ (22,000), A (52,000) and α (23,000) where Tri-1 = DδDδ, Tri-2 = DδAα and Tri-3 = AαAα . Furthermore, it was revealed that the background streak, observed after electrophoresis of unreduced extracts, was due to a continuous array of different-sized aggregates involving subunits of glutenin and triplet proteins. Using this 2-D method, the LMW glutenin subunits were efficiently separated

from the monomeric gliadins and the genes controlling many of the LMW glutenin subunits were located on chromosome arms 1AS, 1BS and 1DS.

The triplet proteins were located in the protein bodies of the developing endosperm of wheat, proving that they are in fact storage proteins. However, they are insoluble in distilled water, 0.04M sodium chloride solution, 70% aqueous ethanol and 0.1M acetic acid. Thus they do not correspond to any of the four classical solubility classes for wheat seed proteins defined by Osborne. In fact, their molecular structure resembles that of the subunit pairs present in the globulins of oats, rice and some legumes.

The non-reducing/reducing form of 2-D SDS-PAGE was also applied to protein extracts from cereal rye (*Secale cereale* L.) it was found that this cereal does not possess any major LMW subunit, like glutenin or triplet proteins, with an ability to form intermolecular disulphide bonds. However, three intermediate-sized subunits of 75kd γ-secalins were shown to be involved in disulphide bonding and the genes controlling these proteins were located on chromosome arm 2RS. These secalins formed disulphide-linked oligomers which were detected as discrete bands in the SDS-PAGE patterns of unreduced protein extracts from the rye endosperm. However, no discrete oligomeric bands were obtained in the analysis of extracts from a wheat-rye amphiploid and the appropriate addition lines. The possible reasons for this behaviour are discussed in relation to disulphide bonding between wheat and rye subunits.

A translocation mapping procedure was used to map gene-centromere distances, for the loci controlling endosperm proteins on each of the long and short arms of homoeologous group 1 chromosomes (1A,1B, 1D and 1R) of wheat and rye. The genes controlling triplet proteins were found to be very closely linked to the centromere on chromosome arms 1AS and 1DS but loosely linked to the respective gliadin genes on the same arms. No recombination was observed between the genes controlling LMW glutenin subunits and gliadins on 1AS and 1DS. However, the analogous genes on 1BS showed rare recombination. The genes controlling HMW glutelin subunits, on the long arm of chromosomes 1A, 1B, 1D and 1R, were closely linked to the relevant centromeres

whereas the genes controlling prolamins, on the short arm of these chromosomes, were very loosely linked to the centromeres. The gene on chromosome arm 1RS, controlling stem-rust resistance, was found to be distal to the secalin locus, and closely linked to it. The map distances obtained with translocation mapping were compared with those obtained with telocentric mapping methods.

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(Nagendra K. Singh)

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Chapter 1

GENERAL INTRODUCTION

The storage proteins of the wheat endosperm are perhaps its most important constituent in determining the inherent end-use quality of the derived flour (Elton and Ewart, 1967). Proteins are important in influencing both the nutritional quality and the aesthetic and organoleptic quality of the final product e.g. leavened bread. Both protein quantity and quality are involved in these aspects of flour quality. While protein quantity is easily and precisely measured by several methods of analysis, protein quality is defined depending on whether the assessment is made for nutritional or organoleptic quality. The work presented in this thesis is related to the second parameter for two main reasons: first, protein quality is less subject to environmental influence than is protein quantity and second, the protein quantity is usually negatively correlated with grain yield. Thus from the plant breeder's view point to improve quality it is more amenable to breed for improved quality than for improved quantity of proteins

Wheat proteins are deficient in certain essential amino acids and may induce adverse reactions such as coeliac disease in some people (see Wrigley, 1982); hence, a better biochemical and genetic understanding of these proteins may help in the future to breed wheat that has better amino acid balance and is safer for the people showing hypersensitivity to wheat products.

Bread-making quality, on the other hand, is much more difficult to assess objectively because it depends on a combination of compositional, ingredient and processing factors. The influence of both amount and type of proteins on bread-making quality was studied by Finney and Barmore (1948) and Bushuk *et al.* (1969). The latter authors clearly demonstrated that loaf volume, the most accurate single index of bread-making quality, of cultivars Manitou and 11463-A increased with increase in protein quantity. However, a difference in protein quality of the two cultivars was evident by the fact that for an identical protein content, cultivar Manitou always showed significantly higher loaf volume than the cultivar 11463-A and, furthermore, the increase in the loaf volume per unit increase in the

protein content was comparatively higher in Manitou.

Although very little is known about the precise cause of such differences in protein quality of different cultivars, it is reasonable to assume that these differences might be traced back to the molecular level in the primary structure of the protein components, protein—protein and protein—non-protein (carbohydrates and lipids) interactions. Some interesting results have already been obtained in this direction by correlating specific protein components in durum and bread wheat cultivars to pasta- and bread-making quality, respectively (Damidaux *et al.*, 1978; Payne *et al.*, 1979). Although further investigations are required to understand the nature of these correlations between specific protein bands and flour quality, it could be explained, at least partly, by better aggregating ability of some protein components (Payne *et al.*, 1984c).

Besides providing a basis for relating differences in protein type between cultivars to differences in nutritional or organoleptic quality, an understanding of the structure and genetic control of storage proteins in wheat and related species have other important implications as outlined below. They could provide:

(i) a better knowledge of the genetic and evolutionary relationships between particular wheat chromosomes and chromosomes in related species;

(ii) a useful tool in cytogenetics where by storage protein markers can be used as effective tags for the presence or absence of particular chromosome segments in chromosome restructuring by centric fusion (Shepherd, 1973) and for the controlled transfer of useful homoeoallelic characters from alien species into wheat by induction of homoeologous pairing (Koebner and Shepherd, 1985) and;

(iii) an efficient aid to cultivar identification (Wrigley *et al.*, 1982a).

A general literature review of the current knowledge on the classification, fractionation, chromosomal control, linkage mapping and quality associations of wheat and rye proteins is presented in Chapter 2.

The initial aim of the work presented in this thesis was to map the glutelin and prolamin genes located on the chromosomes of homoeologous group 1 in wheat and rye, with respect to their relevant centromeres. The reason for including rye was that this

species is a diploid and is being used in this laboratory as a model alien species for introgression of useful disease resistance gene(s) into wheat without adversely affecting the yield and quality of the recipient wheat parent. Furthermore, rye is the only other species, apart from wheat, which yields a flour capable of producing a significant amount of visco-elastic protein complex.

However, when these investigations were initiated in October, 1981, whilst examining the electrophoretic patterns of unreduced extracts of total endosperm proteins from Chinese Spring wheat, in an attempt to visualize the gliadin proteins using the sodium dodecyl sulphate — polyacrylamide gel electrophoresis (SDS-PAGE), three electrophoretically slow-moving bands were observed in a zone of heavy background streaking. Similar bands were detected in a diverse range of hexaploid wheat cultivars and they were consequently designated as "triplet" bands. When it was realised that these were a new type of wheat proteins; the main focus of attention turned towards characterizing them further including an analysis of their structure and their genetic control. A two-dimensional (2-D) elctrophoretic procedure, similar to that used by Wang and Richards (1974) for the analysis of disulphide-linked membrane proteins, was used to determine their structure. There is strong resemblance between the structure of triplet proteins and the globulin storage proteins of oats and some legumes (see Walburg and Larkins, 1983) which may have important implications on the genetic and evolutionary relationships of the storage proteins in cereals and legumes.

The 2-D electrophoretic method adopted not only proved useful in determining the structure of the triplet proteins but also was efficient for separating disulphide-linked low molecular weight (LMW) glutenin subunits from the monomeric gliadins which have similar electrophoretic mobility in one-dimensional (1-D) SDS-PAGE system. A modification of this 2-D system is described which was used in the linkage mapping experiments for fractionating up to 20 samples in a single gel for screening disulphide-linked high molecular weight (HMW) and LMW subunits of glutenin. The endosperm proteins of rye were subjected to the same treatment and analytical procedures as the wheat proteins, and the results of these investigations on triplet proteins and other

covalently interacting proteins in wheat and rye are presented in Chapter 3 of this thesis. These results have given an interesting insight into the nature of disulphide bonding in the endosperm proteins of wheat and rye.

The second major topic of the thesis (Chapter 4) constitutes the data obtained from the linkage mapping experiments designed to map the positions of the seed protein loci, including genes controlling the synthesis of HMW and LMW glutelin subunits, the prolamins and the triplet proteins, located on each of the long and short arms of homoeologous group 1 chromosomes in wheat and rye. A translocation mapping procedure was used for this purpose and these results were compared with those obtained from the conventional telocentric mapping procedure both in the present study and independently obtained by other researchers as recently reviewed by Payne *et al.* (1984b).

These results are discussed separately in each of the two sections (Chapters 3 and 4) and a general discussion on the significance of these findings is presented in Chapter 5.

Chapter 2

LITERATURE REVIEW

2.1 CLASSIFICATION AND CHARACTERIZATION OF WHEAT PROTEINS

In his comprehensive studies early this century, Osborne (1907) divided wheat proteins into four classes based on solubility critreria. These are: albumins, soluble in water; globulins, insoluble in water but soluble in dilute salt solution; gliadins, insoluble in water or dilute salt solution but soluble in 70 % ethanol; and glutenin, insoluble in water, dilute salt solution or aqueous ethanol but soluble in dilute acid or alkali solutions. The protein fraction that remains unextracted after the above treatments is called residue protein. The terms gliadin and glutenin are usually reserved for wheat proteins, and Osborne suggested that prolamin and glutelin be used as the generic names for the equivalent protein fractions from the other cereals. Although this classification has been retained upto the present time, difficulties have been encountered in recent years because new techniques of protein separation have shown that each of these solubility classes contains a complex mixture of proteins with much overlap of components. Furthermore, Osborne's (1907) classification does not provide for the complex proteins such as lipoproteins and glycoproteins which are now known to play an important role in determining the technological properties of the wheat flour (Pomeranz, 1980; McMaster and Bushuk, 1983; Békés *et al.*, 1983; Zawistowska *et al.*, 1984).

In a recent development in the nomenclature of the cereal proteins, Miflin *et al.* (1983) and Shewry *et al.* (1984b) consider that all of the storage proteins of barley, rye and wheat should be termed prolamins. This is because they are all soluble in aqueous alcohol (especially propan-1-ol) after reduction of disulphide bonds. Furthermore, they are all storage proteins which are deposited in the protein bodies during endosperm development (Miflin *et al.*, 1981, 1983) and they are not synthesized in any other tissue. Also they all have characteristic amino acid composition and are rich in proline and glutamine, hence the name prolamins. These prolamins are further divided into three groups on the basis of

their chemical characteristics, notably their molecular weight and amino acid composition and chromosomal location of their structural genes. These are: (i) high molecular weight prolamins, (ii) sulphur-poor prolamins and (iii) sulphur-rich prolamins. The structural and evolutionary relationship of these proteins in wheat, rye and barley have been discussed in detail by Shewry *et al.* (1984b).

Lásztity (1984) has outlined the other possibilities of the classification of cereal seed proteins. These are, (i) on the basis of morphology: proteins of the endosperm , the aleurone layer and the embryo; (ii) on the basis of chemical composition: simple proteins and complex proteins such as lipoproteins and glycoproteins; and (iii) on the basis of biological function: metabolically active/cytoplasmic proteins and storage proteins. The best description of a particular protein component would of course be the one that takes into account each of these criteria. Nevertheless, from the recent biochemical studies at the molecular level it seems that the classification based on biological function, is most acceptable from a scientific point of view. Metabolically active proteins in this classification correspond roughly to the albumins and the globulins of Osborne's solubilty fractions whereas storage proteins comprise mainly the prolamins and the glutelins. However, some overlapping of the properties and the functions is possible as many of the globulins appear to be storage proteins that occur in the protein bodies located in the cells of the aleurone layer and some of the cells of the embryo (Danielsson, 1949; Buttrose, 1963). Furthermore, it is important to note here that the major storage proteins in oats and many legumes are the 11-12S globulin (Derbyshire *et al.,* 1976; Peterson, 1978; Nielsen, 1984). However, similar proteins have not been reported in wheat.

2.2 DISULPHIDE BONDING IN WHEAT ENDOSPERM PROTEINS

It is well established now that all of the Osborne's original solubility fractions are complex mixture of different polypeptides or protein subunits. Moreover, it is generally accepted that the gluten, the visco-elastic protein complex comprising more than 80% of the storage proteins in wheat, is mainly composed of gliadins and glutenins linked together by

covalent and non-covalent bonds and their interactions. Various models have been proposed to elucidate how the components of gliadin, glutenin and residue proteins interact and aggregate into bigger and more complex molecules (Ewart, 1968, 1979; Kasarda *et al.*, 1976a; Bietz and Huebner, 1980; Bietz and Wall, 1980; Bushuk *et al.*, 1980; Graveland *et al.*, 1984; Lásztity, 1984). However, further studies are needed to confirm, reject or modify these models and also to determine the exact role of the non-protein components such as lipids and carbohydrates in the formation of the gluten complex.

Although several kinds of forces including electrostatic interactions, hydrogen bonding and hydrophobic interactions are involved in the formation of the visco-elastic protein matrix which is thought to confer the unique visco-elastic properties on dough (see review by Lásztity, 1984). It has been shown that the disulphide bonds have a major influence on the structure of native glutenin (Pence and Olcott, 1952; Nielsen *et al.*, 1962) and on dough properties (Jones *et al.*, 1974; Bloksma, 1975). Useful data have been obtained on the nature and role of disulphide bonds by oxidation-reduction experiments and by measuring the changes in rheological properties of the proteins or dough due to these treatments.

Although all the cysteine sulphur in the gliadins occurs as disulphide, the average molecular weight of gliadins was shown by Pence and Olcott (1952) to change only slightly upon reduction, suggesting that the disulphide bonds in gliadins are predominantly intramolecular. Woychik *et al.* (1964) showed that the intermolecular disulphide bonding was present to a limited extent in the gliadin fraction. Based on additional studies on changes in physico-chemical properties of gliadins after reduction, Beckwith *et al.* (1965) showed that reduction of disulphide bonds did not involve any perceptible change in the molecular weights of gliadins. However, some of the gliadin bands showed slightly decreased mobility in the gels which was explained by the change in conformation. An increase in viscosity and decrease in solubility was also accounted for by the same reason. When reduced gliadins were reoxidized in acid solvents at low protein concentrations, the chemical and physical properties of the native gliadin were practically recovered. In further studies, Beckwith *et al.* (1966) separated 70% ethanol-soluble gliadins into two fractions

by using gel filtration chromatography and later Nielsen *et al.* (1968) demonstrated that the properties of these two fractions were quite different. The higher molecular weight fraction (HMW-gliadin), present in small quantity -approximately 6 % of the total gliadins- had the properties similar to low molecular weight native glutenin and therefore they designated this fraction as LMW glutenin. The viscosity of this fraction decreased drastically upon reduction of disulphide bonds and also its molecular weight dropped from about 125,000 to around 37,000. This indicates that the disulphide bonds present in this fraction are predominantly intermolecular as in glutenin. However, the LMW fraction of gliadin (purified gliadin) showed no change in viscosity after reduction. Thus, they concluded that disulphide bonds present in purified gliadins are intramolecular.

Glutenin was first fractionated by Jones *et al.* (1959) who observed that glutenin had only one major peak when separated by moving boundary electrophoresis. Ultracentrifugal analysis showed that native glutenin was heterogeneous, consisting of molecules with molecular weights ranging from about 50,000 to several million, with an average MW of 1.5-2 million. Molecular weights remained constant in several dissociating solvents suggesting that chemical bonds, rather than physical aggregation, were responsible for the large molecular size of glutenin (Jones *et al.*, 1961, 1964). Nielsen *et al.* (1962) not only confirmed the molecular weight distribution of native glutenin but also they showed that after reduction of disulphide bonds, the MW of glutenin was drastically reduced to a value etimated to be 20,000. However, this molecular weight was later shown be an underestimate (Bietz and Wall, 1972). Furthermore, reduced glutenin had lost its visco-elastic properties, showing that the disulphide cross links are important in maintaining the glutenin's native structure. Using starch gel electrophoresis in aluminium lactate buffer at acid pH, Woychik *et al.* (1961) and Jones *et al.* (1963) showed that the native glutenin could not enter the starch gel, but after reduction of disulphide bonds it separated into 20 or more components (Woychik *et al.*, 1964) some of which had similar electrophoretic mobility to gliadins. Thus these researchers proposed that the intermolecular disulphide bonding was a principal factor in the structure and rheological properties of glutenin. Similar results were obtained with wheat glutenin by Beckwith and

Wall (1966).

Kaczkowski and Mieleszko (1980) have suggested the presence of at least four types of disulphide bonds in gluten, which are as follows: (i) intramolecular S-S bonds which can be reduced only after unfolding of the polypeptide chain; (ii) intermolecular S-S bonds, less accessible to the reducing agents; (iii) easily accessible intermolecular S-S bonds; (iv) intermolecular S-S bonds which occur only in the gel form of gluten or as the component of dough, to bind together the separate molecules forming the giant protein aggregates. They also reported some association between the levels of different types of disulphide bonds and wheat qualtiy. Detailed studies have been conducted on the reoxidation of reduced gluten proteins, under varying conditions by Lásztity (1984) who has also reviewed the literature on the role of disulphide bonds and their localization in wheat protein molecules.

2.3 FRACTIONATION OF PROTEIN COMPONENTS AND THEIR GENETIC CONTROL

Two approaches have been used to fractionate the wheat endosperm proteins. First, differential extraction methods are used to achieve preliminary fractionation based on solubility and the components of these fractions are then analysed by various chromatographic or electrophoretic techniques. Some modifications have been made to the original Osborne's procedure in order to achieve better protein extraction (Chen and Bushuk, 1970; Bietz and Wall, 1975; see review by Kasarda *et al.*, 1976a). In the second approach, attempts have been made to dissolve completely all the proteins of endosperm and to resolve the complex mixture by gel filtration based on molecular weight (Danno *et al.*, 1974; Huebner and Wall, 1976; Payne and Corfield, 1979; Bottomley *et al.*, 1982) or differential precipitation and centrifugation (Graveland *et al.*, 1979, 1982), followed by electrophoretic separation of different fractions. Solvents used for the total solubility methods are: (i) acetic acid (Jones *et. al.*, 1959); (ii) acetic acid — urea — cetyltrimethylammonium bromide [AUC] (Meredith and Wren, 1966; Orth and Bushuk,

1973); (iii) sodium dodecyl sulphate [SDS] (Danno *et al.*, 1974; Danno, 1980; Bottomley *et al*, 1982); (iv) sodium salts of fatty acids (Kobrehel and Bushuk, 1977, 1978; Wasik *et al.*, 1979); and most recently (v) propan-1-ol and 2-mercaptoethanol mixture (Miflin *et al.*, 1983). Although these techniques have given a new insight into the composition and genetic control of gluten proteins, there is no single undisputed system of relating the protein fractions obtained by the new techniques to Osborne's original nomenclature that is acceptable to all researchers.

From a genetical point of view, however, the first step is to develop a gel electrophoretic system that gives good separation of individual protein components in the protein fraction of interest, so that there is a minimum overlap of bands or spots. Usually the next step is to determine the chromosomal location of the genes controlling these protein components. In wheat this work has been relatively simpler because of the availability of suitable aneuploid stocks which lack only a particular chromosome or chromosome arm (Sears, 1954, 1966a; Sears and Sears, 1978). Intervarietal chromosomal substitution lines (Sears *et al.*, 1957; Morris *et al.*, 1966; Law and Worland, 1973; Law *et al.*, 1978) have also proved very useful. Monosomic analysis and the analysis of F_2 progeny of genetic crosses between appropriate parents, are the other procedures used for this purpose. The third step is to study the intervarietal variation and to work out the allelic relationships of the variant protein phenotypes. The final step is to map the position of the individual genes along the chromosome with respect to other genes or to the relevant centromere. Considerable progress has been made in the chromosomal location of the genes controlling endosperm proteins in wheat and some related species as reviewed by Garcia-Olmedo *et al.* (1982). These and some more recent findings on wheat proteins are summarized below.

2.3.1 Gliadins

Gliadin proteins are usually fractionated by electrophoresis in starch or polyacrylamide gels containing aluminium or sodium lactate buffer at acid pH (usually pH 3.1) after extraction

with 70 % ethanol, 2—6 M urea solution or aluminium lactate buffer. Typical gliadins were first classified into α, β, γ and ω components based on their electrophoretic mobility in a moving boundary electrophoretic system (Jones *et al.*, 1959). This nomenclature was further elaborated by Woychik *et al.* (1961) using starch gel electrophoresis, first applied to wheat proteins by Elton and Ewart (1960).

The first report on the chromosomal location of gliadin genes was by Boyd and Lee (1967) who reported that two slow-moving gliadin bands (ω-gliadins) disappeared when one arm of chromosome 1D was missing. The first comprehensive genetic study was done by Shepherd (1968) who, using nullisomic-tetrasomic, ditelocentric and tetrasomic lines of Chinese Spring wheat, concluded that 9 out of 17 major gliadin bands were controlled by the genes on chromosome arms1AS, 1BS, 1DS, 6AS and 6DS. Each of the remaining eight bands was presumably a mixture of gliadins whose genes were located on more than one chromosome. Wrigley (1970) fractionated wheat gliadins into 46 spots by combined isoelectricfocusing (IEF, pH 5—9) and starch gel electrophoresis at pH 3.2. Later, Wrigley and Shepherd (1973) confirmed and extended the earlier findings of Shepherd (1968) and 33 out of 44 possible components could be assigned to specific wheat chromosomes of homoeologous groups 1 and 6. One major component was difficult to assign probably because more than one chromosome were involved and the control of the remaining 10 minor protein spots was difficult to determine because their detection in a given stock depended on how much protein was loaded on the gel.

These early findings were also confirmed by Payne *et al.* (1982a) using the acid polyacrylamide gel electrophoresis (APAGE) method of Bushuk and Zillman (1978) in the first dimension, followed by SDS-PAGE in the second dimension. Other 2-D electrophoretic systems used for the separation of gliadins are: combined IEF (pH 4—7.5) and SDS-PAGE of O'Farrell *et al.* (1975) by Brown *et al.* (1979) and Brown and Flavell (1981); and electrophoresis at pH 3.2 followed by electrophoresis at pH 9.2 by Mecham *et al.* (1978) and Lafiandra *et al.* (1984). These and other studies have given convincing evidence that the genes encoding classical gliadins are located on the short arms of group 1 and group 6 chromosomes in wheat.

2.3.2 Glutenins

Unlike gliadins there are no internationally accepted standard definitions for glutenin proteins. However, glutenins have two main characteristics: (i) they are insoluble in water, dilute salt solution or aqueous ethanol at room temperature and (ii) they consist of polypeptides that are linked together by intermolecular disulphide bonds. This definition however, includes about 6 % of the ethanol-soluble fraction variously called HMW gliadin (Beckwith *et al.*, 1966; Bietz and Wall, 1980), LMW glutenin (Nielsen *et al.*, 1968; Bietz and Wall, 1980) and Glutenin III (Graveland *et al.*, 1982).

Glutenin was first fractionated by Jones *et al.* (1959) who observed that it had only one major peak when separated by moving boundary electrophoresis. However, using starch gel electrophoresis in aluminium lactate buffer, Woychik *et al.* (1964) separated reduced glutenins into 20 or more components some of which had similar electrophoretic mobility to gliadins. The next major advance in the fractionation of glutenin was the introduction of SDS-PAGE by Bietz and Wall (1972), who showed that glutenin was composed of subunits having at least 15 different apparent MWs, ranging from 11,600 to 133,000.

Orth and Bushuk (1974) were the first to study the genetic control of glutenin subunits. Using the SDS-PAGE technique, they showed that genes encoding four glutenin subunits with apparent molecular weights of 152,000, 112,000, 60,000 and 45,000 were located on chromosome 1D of Chinese Spring wheat. Bietz *et al.* (1975) used single kernel analysis based on the sequential extraction method of Bietz and Wall (1975) and after analysis of nullisomic-tetrasomic and ditelocentric lines of Chinese Spring by SDS-PAGE, they concluded that two glutenin subunits (MW 104,000 and 93,000) were controlled by the genes on long arm of chromosome 1B and two other subunits (MW 133,000 and 86,000) were similarly assigned to the long arm of chromosome 1D. Several LMW subunits from their glutenin preparation could not be assigned to particular chromosomes.

Brown *et al.* (1979) and Brown and Flavell (1981) used 2-D electrophoresis to fractionate the glutenin obtained by the modified Osborne procedure (Chen and Bushuk, 1970) and gel filtration method (Huebner and Wall, 1976; Payne and Corfield, 1979). They confirmed the results of Bietz *et al.* (1975) by locating the genetic control of two HMW subunits on chromosome arm 1DL, although the size of these molecules in the work of Brown *et al.* (1979) [125,000 and 88,000], were slightly different from those determined by Bietz *et al.* (1975). More recent work by Holt *et al.* (1981) has shown that the isoelectric points of the 1B subunits are higher than the pH range employed by Brown *et al.* (1979) in their study and this is the reason why they could not analyse the HMW subunits controlled by chromosome 1B. These results on the chromosomal location of the genes controlling HMW subunits of glutenin have since been confirmed and elaborated by Lawrence and Shepherd (1980, 1981), Payne *et al.* (1980, 1981a) and Galili and Feldman, 1983a, b) using an improved discontinuous system of 1-dimensional SDS-PAGE (Laemmli, 1970) to fractionate the total protein extracts from the endosperm halves of single kernels. Furthermore, these researchers have also shown that chromosome arm 1AL of wheat also carries genes controlling HMW glutenin subunits.

Unlike gliadins and HMW glutenin subunits, the genes controlling the LMW glutenin subunits have been difficult to assign to particular chromosome arms because of the lack of suitable techniques for fractionation. However, recently by combining two different 2-D electrophoretic systems (O'Farrell, 1975 and O'Farrell *et al.*, 1977), Jackson *et al.* (1983) have located the genes controlling some of these LMW subunits on chromosome arms 1AS, 1BS and 1DS. Singh and Shepherd (1984b, 1985) have obtained similar results using a non-reducing/reducing form of 2-D electrophoresis described in this thesis.

2.3.3 Albumins and Globulins

Albumins and globulins have not been studied in as much detail as the gliadins and glutenins, possibly because they constitute less than 20% of the total endosperm proteins. Furthermore, they have not been implicated specifically as determinants of bread-making

quality. Albumins and globulins as a group migrate well ahead of the gliadins, when separated electrophoretically in starch gel at acid pH. These proteins have been extracted by using solvents which do not extract glutenin such as distilled water, dilute salt solution, 1M or 2M urea, 25 % aqueous 2-chloroethanol, 70 % aqueous ethanol and aqueous dimethyl formamide. Unlike gliadins and glutenins, genes controlling the synthesis of these proteins are scattered throughout the genome and chromosomes from each of the seven homoeologous groups are shown to be involved in their genetic control. Shepherd (1971) located genes controlling albumins on chromomsome arms 5AL, 5BL and 5DL. Waines *et al.* (1973) showed that some water-soluble, ethanol-extracted seed proteins were controlled by chromosomes 2A, 4A, 6A, 2B, 3B, 4B, 2D, 3D, 4D, 6D and 7D. Aragoncillo *et al.* (1975) have shown the control of 70% ethanol soluble non-gliadin proteins by chromosomes 4Aß, 3BS, 6BS, 7BS, 3Dß, 4D, 5D and 7D. Recently, using 2-D electrophoresis (IEF, pH 4—9 × starch gel electrophoresis), genes controllig 14 out of 25 components of the dilute salt soluble proteins were assigned to chromosomes 1B, 3B, 3D, 4A, 4B, 5B, 6B, 6D, and 7D (FraMon *et al.*, 1984).

2.4 VARIATION OF ENDOSPERM PROTEIN PATTERNS AMONG CULTIVARS AND THE ALLELIC RELATIONSHIPS OF GENES CONTROLLING THEM

Various fractions of the wheat grain proteins have been assessed for their suitabiltiy in cultivar identification. Among these, albumins and globulins are suitable only for distinguishing between the different wheat species because there is not much variation in albumins in cultivars of the same species (Johnson, 1972; Caldwell and Kasarda, 1978; Cole *et al.*, 1981; Wrigley, 1982). However, there is substantial intervarietal variation for banding patterns for gliadins and HMW glutenin subunits. Recent work shows that variation exists among LMW glutenin subunits also (Payne *et al.*, 1984d; Singh and Shepherd, 1984b) but there is little information on this. The gliadins have proven ideal for discrimination at the cultivar level and have been used most often. In fact, electrophoretic analysis of gliadin composition now provides the main basis for segregating wheat on a

commercial scale in several countries (Wrigley *et al.*, 1982a; Lásztity, 1984).

The intervarietal variation at specific gene loci controlling endosperm storage proteins have been studied by analysis of large collections of wheat cultivars, intervarietal chromosomal substitution lines, monosomic analysis and by the analysis of segregating progeny of the appropriate crosses between cultivars with different banding patterns. These studies have proven useful in determining the allelic relationships between protein subunits or subunit combinations present in different wheat cultivars.

2.4.1 Gliadins

Gliadin patterns of the intervarietal substitution lines containing 'Cheyenne' chromosomes substituted in Chinese Spring, produced by Morris *et al.* (1966), were studied by Eastin *et al.* (1967) and later by Kasarda *et al.* (1976b). The genes controlling 13 of the 25 detectable gliadin components in Cheyenne, and 11 of the 22 components in Chinese Spring, were assigned to chromosomes of homoeologous groups 1 and 6. Solari and Favret (1967) studied the inheritance of gliadin patterns in crosses between different cultivars and concluded that at least 11 loci, belonging to three linkage groups (or chromosomes), were involved in their synthesis. The inheritance of intervarietal variation of gliadin patterns was studied by Doekes *et al.* (1973) and they established that gliadin patterns were inherited as unaltered 'blocks'. The genes controlling certain gliadin bands in the cultivar 'Odesskaya' were assigned to the chromosomes of group 1 and 6 by analysing progeny from crosses between this cultivar and Chinese Spring aneuploids (Rybalka, 1975). Sasek and Kösner (1977) studied progeny from crosses between Kavkaz and the 21 monosomics of Chinese Spring to determine the effect of Kavkaz chromosomes on individual Kavkaz gliadin bands. They assigned the genetic control of some gliadin bands to chromosomes 1B, 1D and 6B. The inheritance of gliadin components unique to three cultivars was investigated by Mecham *et al.* (1978) using 2-D electrophoresis and they concluded that many of the gliadin bands segregated as if they were controlled by a single gene. Linkage analysis provided evidence that genes

controlling gliadin components occurred as codominant alleles in a gene cluster. Payne *et al.* (1984b) used six different intervarietal substitution lines of chromosome 1A into Chinese Spring to locate the protein components controlled by chromosome 1A in these cultivars. The most extensive analysis of 'block' inheritance and allelic variation of gliadins has been carried out by Sozinov and coworkers in the USSR (Sozinov and Poperelya, 1980; Metakovsky *et al.*, 1984). Genetic analysis of F_2 seeds from 36 crosses involving 39 winter wheat cultivars, revealed 'blocks' with 1 to 6 gliadin components which are inherited as individual Mendelian traits. About 80 different variants of these blocks were detected. On the basis of tests for allelism these blocks were grouped into six series (loci) named *Gld1A* (15 alleles), *Gld1B* (18 alleles), *Gld1D* (8 alleles), *Gld 6A* (13 alleles),*Gld 6B* (11 alleles) and *Gld 6D* (10 alleles) and these alleles were confirmed to be inherited co-dominantly (Metakovsky *et al.*, 1984).

2.4.2 Glutenins

Intervarietal variation in HMW glutenin subunits was studied by Lawrence and Shepherd (1980) who reported that the number of bands in each of the 98 wheat cultivars ranged from three to five and at least 34 different banding patterns were observed. In these patterns, some bands or band combinations were mutually exclusive. That is, they behaved like alleles and could be assigned to three groups located on chromosomes 1A, 1B and 1D. Payne *et al.* (1980) observed similar results although they used only seven cultivars. However, using intervarietal substitution lines, they concluded that 2 subunits were controlled by chromosome 1D, 1 or 2 subunits by chromosome 1B and 0 or 1 subunit by 1A. Brown *et al.* (1981) analysed substitution lines from eight cultivars into Chinese Spring and found somewhat similar results but in their 2-D system, the subunits coded by chromosome 1B were not detectable. A catalogue of alleles for the complex loci *Glu-A1*, *Glu-B1* and *Glu-D1*, coding for HMW subunits of glutenin has been published by Payne and Lawrence (1983). Although variation in LMW glutenin subunits has been recorded (Payne *et al.*, 1984b; Singh and Shepherd, 1984b), only limited studies have been made

on these subunits so far.

In summary, it appears that the genes controlling gliadins and glutenins in hexaploid wheat occur at nine different complex loci. The loci *Glu-A1*, *Glu-B1* and *Glu-D1* are located on the long arm of chromosomes 1A, 1B and 1D; the loci *Gli-A1*, *Gli-B1* and *Gli-D1* are located on the short arm of chromosomes 1A, 1B and 1D and loci *Gli-A2*, *Gli-B2* and *Gli-D2* are located on the short arm of chromosomes 6A, 6B and 6D, respectively (Payne *et al.*, 1984b). Among these loci, *Gli-1* appear to be most complex, followed by *Gli-2* and *Glu-1*. Presumably, the variation occurring in different cultivars is due to allelic differences and that the proteins which are inherited as blocks are the products of a cluster of structural genes (isoloci) that have arisen from a single ancestral gene by duplication and mutation (Wrigley, 1982).

2.5 LINKAGE MAPPING OF THE GENES CONTROLLING WHEAT ENDOSPERM PROTEINS

Lawrence and Shepherd (1981b) estimated the recombination frequency between glutenin and gliadin genes on chromosome 1B. They analysed progeny of a triparental cross, the parents having electrophoretically different HMW glutenin subunits and ω-gliadins controlled by chromosome 1B, and concluded that the two genes were segregating independently. Payne *et al.* (1982b) showed two different recombination values of 47% and 42% between *Glu-A1* and *Gli-A1*, and also two different recombination values of 39% and 47% between *Glu-B1* and *Gli-B1*, using four different triparental crosses. In a study with F_2 progeny, Chojeki *et al.* (1983) found that *Glu-D1* and *Gli-D1* on chromosome 1D were segregating independently. In summary, all of these results suggest that the *Glu-1* loci on the long arms of group 1 chromosomes are well separated from the *Gli-1* loci on their short arms.

Linkage maps of the genes that are segregating independently can only be constructed when one or both of these genes can be shown to be linked with some intermediate gene(s) or a chromosome break point or a centromere. The results of Chojeki *et al.* (1983) are

significant in this respect since they observed 36.2 % recombination between *Glu-D1* and the *Gpi-D1* locus controlling a glucose phosphate isomerase isozyme on the chromosome arm 1DS, and 34.5% recombination between the *Gpi-D1* and *Gli-D1* loci. Thus the intermediate locus *Gpi-D1* showed significant linkage with each of the gliadin and glutenin loci. Using the telocentric mapping procedure of Sears (1962, 1966b), Rybalka and Sozinov (1979) showed 42% recombination between the centromere and the gliadin locus on chromosome arm 1BS. This indicated that *Gli-B1* must be located distally on chromosome arm 1BS. In fact, a recent detailed cytogenetic and biochemical study of a spontaneous mutant involving this chromosome has revealed that the *Gli-B1* locus must occur in the satellite region of chromosome 1B (Payne *et al.*, 1984a) .

Telocentric mapping has also been used by Payne *et al.* (1982b) to map genes controlling the HMW glutenin subunits. They obtained recombination values of 7.6%, 9.2% and 10.1% betweeen the centromere and the *Glu-A1*, *Glu-B1* and *Glu-D1* loci on the long arm of chromosomes 1A, 1B and 1D, respectively. In recent linkage studies, Payne *et al.* (1984d) have shown that each of the short arms of chromosomes 1A and 1B in wheat, contains a single complex locus, named *Gli-A1* and *Gli-B1* , respectively, which contains the genes for the ω, γ and β gliadins and the LMW glutenin subunits. They found no recombination between these different types of proteins, in 693 test-cross progenies analysed for the proteins coded by chromosome arm 1AS. In similar tests with the equivalent proteins coded for by chromosome arm 1BS, no recombinants were detected in 203 test-cross progenies but two possible recombinants were observed among 160 F_2 progeny. Singh and Shepherd (1984b) have also reported very close linkage between genes controlling gliadins and LMW glutenin subunits on each of the chromosome arms 1AS, 1BS and 1DS.

Recently Galili and Feldman (1984) reported that a gliadin component (B-30 according to their nomenclature) showed 25.5 % recombination with the other gliadin components and 23.5 % recombination with the HMW glutenin subunits. They concluded that this gliadin component is controlled by a separate locus (*Gld-B6*), located proximally to the major gliadin gene cluster on chromosome arm 1BS.

2.6 ASSOCIATION BETWEEN PROTEINS AND QUALITY CHARACTERS

It is widely accepted that to produce a good loaf of bread wheat flour must have a minimum quantity and quality of protein. Above these minimal requirements, either an increase in protein quantity or an improvement in protein quality can improve the bread-making quality of flour (Finney and Barmore, 1948; Bushuk *et al.*, 1969). Generally protein quantity is inversly related to grain yield and has low heritability, therefore, most effort has been given to searching for a correlation between protein quality and bread-making quality. Although certain types of allergenic activity can be readily associated with some individual cereal proteins following gel electrophoresis of crude mixtures (c.f. Wrigley, 1982), it has been more difficult to associate individual protein bands with bread- or pasta-making quality. This is mainly because of the difficulty in purifying individual proteins from the wheat endosperm to test their effect on dougn quality by addition. Instead researchers have used the alternative approach of seeking for an association between quality performance and protein composition in a range of genotypes, segregating progeny of appropriate genetic crosses or isogenic lines.

Early attempts to associate dough properties with specific gliadins (Doekes, 1968; Huebner and Rothfus, 1968; Orth and Bushuk, 1972) or glutenins (Huebner, 1970; Orth and Bushuk, 1972) did not succeed. However, recently, researchers in France (Damidaux *et al.*, 1978), Canada (Kosmolak *et al.*, 1980) and Australia (du Cros *et al.*, 1982) have reported that pasta-making quality in durum wheats is strongly associated with the presence of a specific gliadin component (band '45'). In contrast allelic gliadin (band '42') is associated with poor quality. Pattern analysis by 1-D and 2-D electrophoretic studies and quality data suggests that bands 42 and 45 are each part of a block of gliadins (du Cros *et al.*, 1982), which are also very closely linked with LMW glutenin subunits (Payne *et al.*, 1984c). The association of dough quality with a group of proteins rather than a single band provides a more plausibile explanation for the observed correlations. Specific gliadin components have also been associated with dough strength in bread wheat (Wrigley *et al.*, 1981), but it is not known whether these associations might be more related to linked

LMW glutenin subunits than the gliadin components themselves.

Payne *et al.* (1979, 1981b) found significant associations between certain HMW subunits of glutenin and bread-making quality as assessed by the SDS-sedimentation test (Axford *et al.*, 1979). Relationship between allelic HMW glutenin subunits and bread-making quality has also been reported by Burnouf and Bouriquet (1982) and Moonen *et al.* (1982,1983). Recently Payne *et al.* (1984b) have reviewed the relative importance of different alleles at the storage protein loci on quality and concluded that the order was: *Glu-1*> *Gli-1*> *Gli-2*. The rating of *Gli-1* above *Gli-2* is consistent with the findings of Sozinov and Poperelya (1980). However, Wrigley *et el.* (1982b) found that quality associations were stronger for gliadins than for glutenins, particularly with gliadin bands 2, 4, 14, and 19 of their nomenclature. Wrigley *et al.* (1981) have used computer based techniques for pattern analysis to overcome the problem of handling large amount of data. This approach has been further developed to include other attributes such as grain hardness, resistance and extensibility and considerations of the possible effect of pedigree in explaining protein quality associations. Using this technique it has been further indicated that associations involving grain hardness were mainly pedigree related but this was not so for protein-dough strength associations (Wrigley *et al.*, 1982c,d).

As mentioned earlier, *Gli-1* is a complex locus coding for ω, γ, β gliadins and LMW glutenin subunits. Since these genes are very tightly linked and usually do not recombine (Payne *et al.*, 1984d; Singh and Shepherd, 1984b), it is difficult to determine which of these groups are responsible for the differences in bread-making quality. However, because of their aggregating ability LMW glutenin subunits are likely to be more important. Despite the promising initial findings with HMW glutenin subunits and classical gliadins, no similar study has been carried out on the effect of LMW glutenin subunits on flour quality mainly because of the lack of suitable techniques for separating these proteins. Furthermore, to answer specifically the question of whether the observed quality-protein associations are due to close linkage between the structural genes for protein synthesis and quality-conferring genes or to the causal effect of these proteins themselves on dough properties, these studies need to be extended to include genotypes from a broader genetic

base and to analyse isogenic lines which vary only for particular protein component(s) in an otherwise uniform genetic bakground.

2.7 ENDOSPERM PROTEINS OF CEREAL RYE

Among the cereals, rye (*Secale cereale* L.) is closest to wheat in yielding a flour which forms cohesive-elastic dough. This feature is also possessed by the wheat-rye hybrid triticale, and is thought to result from the properties of the endosperm proteins in these cereals. The major storage proteins in rye are glutelins and secalins and the early work on these has been reviewed by Simmonds and Campbell (1976). Preston and Woodbury (1975) purified rye secalins into four subfractions by gel filtration and compared their amino acid composition and migration on SDS-PAGE. More recently, Field *et al.* (1982) and Shewry *et al.* (1982) also purified and characterized four major groups of components which they called ω-secalins, 40 kilodaltons (kd) γ-secalins , 75kd γ-secalins and HMW secalins and showed their relationship to storage proteins of wheat and barley. Fractions corresponding to 40kd γ- and ω-secalin groups were also purified and characterized by Charbonnier *et al.* (1981). The disulphide-linked HMW aggregates of rye proteins have been observed as discrete bands (Preston and Woodbury, 1975) or as heavy background streaking in gels (Charbonnier *et al.*, 1981; Field *et al.*, 1983a), which disappeared after reduction of proteins with 2-mercaptoethanol. Caldwell (1983a, b) has shown that most of the secalins are easily reduced to polypepetides of intermediate mobility ranging in size from about 82 to 92 kilodaltons, a size corresponding to that of 75kd γ-secalins. However, the real manner of aggregation of these subunits is not known.

The first genetic analysis of rye proteins was carried out on the monomeric prolamins (secalins) by Shepherd (1968) who examined the electrophoretic patterns of these proteins from 'King II' rye, 'Holdfast' wheat, their amphiploid and seven separate wheat-rye addition lines. It was concluded (Shepherd and Jennings, 1971) that only the short arm of chromosome V (=1R) carried the genes controlling the synthesis of prolamins. Similar results were obtained using addition lines involving 'Imperial' rye and Chinese Spring

wheat, where chromosome E (=1R) was solely responsible for the prolamin bands observed. In this respect, rye was different from wheat where two chromosome groups, numbers 1 and 6, are involved in prolamin synthesis (Shepherd, 1968; Wrigley and Shepherd, 1973).

Jagannath and Bhatia (1972) found that when chromosome 2R of Imperial rye is substituted for any of its homoeologous chromosome pairs in Chinese Spring wheat, the resulting lines contain elevated percentages of grain protein. However, they could not detect any extra protein band associated with chromosome 2R. Lawrence and Shepherd (1981a) not only showed the genetic control of two HMW glutelin subunits in rye by chromosome arm 1RL similar to their location in wheat, but also they showed for the first time that chromosome 2R controls an intermediate MW subunit which corresponds to the 75kd γ-secalins of Shewry *et al.* (1982). Recently these results have been confirmed by Shewry *et al.* (1984a) who have shown further that the locus which controls HMW glutelin subunits on 1RL is loosely liked (40.8% recombination) with the locus controlling 40kd γ-secalins. Genes controlling HMW glutelin subunits on chromosome arm 1RL, are very closely linked to the centromere with only 4.6 % recombination (Singh and Shepherd, 1984a). However, none of the other secalin genes have been mapped with respect to the centromere and furthermore, their is no published report on location of genes controlling 75kd γ-secalins on specific arm of chromosome 2R.

Chapter 3

CHROMOSOMAL CONTROL AND STRUCTURE OF ENDOSPERM PROTEINS IN WHEAT AND RYE

3.1 INTRODUCTION

A significant development in the characterization of the seed storage proteins of wheat during the last 20 years, and particularly in the last 5 years, has been the coupling of genetic studies with improved methods of protein fractionation [see Chapter 2 for a detailed review]. It has long been known that the Osborne (1907) fractions of the wheat seed proteins, based on differential solubility, each contains a complex mixture of proteins with much overlap of components. However, it was not until the development of improved electrophoretic methods for protein separation that it became possible to separate individual components in these fractions and then determine their genetic control. For example, the introduction of zone electrophoresis in starch gels at acid pH (Elton and Ewart, 1960; Woychik *et al.*, 1961) gave at least partial separation of unreduced monomeric gliadins. Application of this separation procedure to single kernels of aneuploid stocks of wheat allowed Shepherd (1968) and later Wrigley and Shepherd (1973), using 2-dimensional electrophoresis for better resolution, to determine the genetic control of α, β, γ and ω gliadins by the genes on the short arms of homoeologous group 1 and group 6 wheat chromosomes. Similarly, the introduction of SDS-PAGE provided a means for resolving glutenin, after reduction with 2-mercaptoethanol (ME), into fifteen separate subunits which have been classified into HMW and LMW glutenin subunits (Bietz and Wall, 1972). The genetic control of the HMW subunits was first investigated by Orth and Bushuk (1974) and Bietz *et al.* (1975), and subsequently many other workers have extended our knowledge of the number and chromosomal location of the genes controlling HMW glutenin subunits on the long arms of homoeologous group 1 chromosomes (Brown *et al.*, 1979, 1981; Lawrence and Shepherd, 1980, 1981a; Payne *et al.*, 1980, 1981a; Galili and

Feldman, 1983a,b).

In contrast to these two groups of relatively well characterized proteins, the LMW glutenin subunits have proven much more difficult to analyse. The genetic control of these subunits was not determined earlier because of the difficulty of resolving the subunits in one-dimensional electrophoresis. Although Jackson *et al.* (1983) recently succeeded in fractionating and characterizing the LMW glutenin subunits by using gel filtration and 2-D electrophoresis (O'Farrell, 1975; O'Farrell *et al.*, 1977), this approach lacks general application for rapid screening and inheritance studies because only one sample can be analysed per gel in the second dimension and furthermore, the LMW glutenin subunits cannot be directly distinguished from gliadins (see Payne *et al.*, 1984d).

It is widely accepted that aggregated proteins resulting from the disulphide linkage between various subunits in the wheat endosperm are important determinants of the bread-making quality of wheat flour. These disulphide-linked proteins, mainly glutenins, are thought to interact with the gliadins to form an elastic but resilient matrix which confers unique visco-elastic properties on dough. Although several different kinds of forces including electrostatic interactions, hydrogen bonding and hydrophobic interactions, are involved in the formation of this matrix (Lásztity, 1984), it has been demonstrated that disulphide bonds have a major influence on the structure of native glutenin (Pence and Olcott, 1952; Nielsen *et al.*, 1962) and on dough properties (Jones *et al.*, 1974; Bloksma, 1975). Two major classes of disulphide-linked proteins have been identified in wheat endosperm: firstly, the high molecular weight native glutenins which are composed of aggregates of HMW and LMW subunits (Bietz and Wall 1973); and secondly, low molecular weight native glutenins which are composed of aggregates of LMW glutenin subunits (Bietz and Wall, 1973, 1980). However, the precise manner of aggregation of these subunits is still not known.

When examining the SDS-PAGE patterns of unreduced total endosperm protein extracts from Chinese Spring wheat, in an attempt to screen for the monomeric gliadins by SDS-PAGE, three electrophoretically slow-moving bands were observed in a zone of heavy background streaking. These bands are readily reduced into smaller subunits by

trace amounts of ME and, since they are electrophoretically different from any of the previously characterized endosperm proteins of Chinese Spring, they were thought to be a new class of disulphide-linked proteins in the endosperm of wheat. These three bands, or a similar set of three bands, were found to be present in most hexaploid wheat cultivars surveyed and consequently they have been designated as "triplet" bands until they are more fully characterized. In this chapter, the results are presented from the studies aimed at defining the variation, genetic control and subunit composition of these proteins. A 2-D electrophoretic procedure employing non-reducing conditions in the first dimension and reducing conditions in the second dimension was used to analyse the structure of these triplet proteins. This non-reducing/reducing form of 2-D procedure has been used previously for the analysis of disulphide-linked proteins of cell membranes (Wang and Richards,1974; Haynes and Destree,1977), ribosomal proteins (Sommer and Traut (1975), and legumins (Matta *et al.*,1981). With some modifications, this 2-D method has also proven to be efficient for separating the subunits of the other disulphide-linked proteins in wheat endosperm, particularly the LMW glutenin subunits, and it has provided a new insight into the aggregation properties of the glutenin subunits of wheat.

These 1-D and 2-D electrophoretic procedures were also used to separate the total protein extracts from rye endosperm to investigate whether triplet proteins and LMW glutelin subunits are also present in cereal rye. The 2-D method has demonstrated how rye protein subunits aggregate by intermolecular disulphide bonds to produce a series of discrete oligomers in diploid rye, and also how they interact with wheat proteins. Results are also presented which locate the genes controlling 75kd γ-secalins on the short arm of chromosome 2R of Imperial rye.

A brief summary of some of this work was presented at the Second International Workshop on Gluten Proteins held at Wageningen (Singh and Shepherd 1984b) and also part of this work has been accepted for publication in Theoretical and Applied Genetics (Singh and Shepherd, 1985).

3.2 MATERIALS AND METHODS

3.2.1 Genetic Stocks Analysed

3.2.1.1 Chinese Spring aneuploids

The following ditelocentric (Dt), nullisomic-tetrasomic (NT) and tetrasomic (T) lines of Chinese Spring wheat (Sears 1954, 1966a; Sears and Sears, 1978), kindly donated by Dr. E.R. Sears and now maintained at the Waite Agricultural Research Institute, were used: Dt 1AL, Dt 1BL, Dt 1DL, NT 1A-1B, NT 1A-1D, NT 1B-1A, NT 1B-1D, NT 1D-1A, NT 1D-1B and T1A.

3.2.1.2 Wheat cultivars and breeding lines

A list of 137 hexaploid wheat cultivars and advanced generation breeding lines and 9 durum wheat cultivars, including representative cultivars from the main wheat growing areas of the world, used for the survey of triplet protein patterns, is given in Appendix A (page 126). These gneotypes are maintained at the Waite Institute and the seeds were kindly provided by Dr. A. J. Rathjen and Dr K.W. Shepherd.

3.2.1.3 Wheat-rye derivatives and diploid rye

The rye chromosome addition, substitution and translocation lines used in this study were present in the wheat cultivar Chinese Spring. The rye chromosomes in the wheat-rye amphiploid and addition lines E (=1R) and B (=2R) were derived from the rye cultivar 'Imperial' (see Driscoll and Sears, 1971). The original seeds were kindly supplied by Dr. E. R. Sears. The seeds for substitution line Chinese Spring 2R(2B) originally developed by Sears, translocation line 2BS-2RL and F_2 seeds from a translocation heterozygote (2BL-2RS/2B) together with another wheat parent used in this cross, were kindly provided

by Dr. C. E. May of the Department of Agriculture, Wagga Wagga, N.S.W. (see May and Appels, 1980).

3.2.2 Extraction of Seed Proteins

The endosperm halves from single kernels were placed in a folded glassine sheet [Lilley, Powder paper (glassine) 3 $^1/_2$" × 4$^1/_2$"] and crushed with a hammer. The crushed material was transferred to 1.5 ml Eppendorf tubes to which was added 0.1 ml or 0.2 ml (for 1-D and 2-D electrophoresis, respectively) of the SDS-Tris-HCl extracting buffer (pH 6.8). The buffer was prepared by freshly mixing the required amount of a stock solution, containing 0.125M tris(hydroxymethyl)aminomethane (Tris), 8.0% (w/v) SDS, 30% (v/v) glycerol and 0.005% (w/v) bromophenol blue made to pH 6.8 with HCl, with an equal volume of water. The samples were usually incubated overnight at near 40°C ror approximately 16 h. However, if samples were required in a minimum time,1-2 h incubation at 60°C was found sufficient for the extraction of the desired proteins. To extract reduced proteins, 1.5% (v/v) ME was included in the extracting buffer. Extraction in the presence of ME is expected to solubilize all of the different protein types in the grain.

For the non reducing acid PAGE × reducing SDS-PAGE form of 2-D electrophoresis, the endosperm proteins were extracted with 2M urea solution, with 1.0% (w/v) methyl green included as tracking dye, for 16 h at 4°C .

3.2.3 One-dimensional SDS-PAGE

The discontinuous system of SDS-PAGE was based on the method of Laemmli (1970). Vertical slab gels (145 × 100 × 1.2 mm) were prepared between two glass plates clamped to the side of a perspex stand similar to that described by Studier (1973) and modified by Lawrence and Shepherd (1980). The separating gel contained 10.0% (w/v) acrylamide and 0.08% (w/v) bisacrylamide (Bis), 0.1% (w/v) SDS, and 0.375M Tris made to pH 8.8 with HCl and the stacking gel contained 3% (w/v) acrylamide, 0.08% Bis, 0.1% (w/v)

SDS and 0.125M Tris made to pH 6.8 with HCl. Both gels were polymerized with N, N, N', N' - tetramethyl- ethylenediamine (TEMED) and ammonium persulphate. The electrode buffer, for both upper and lower tanks contained 0.1% (w/v) SDS and 0.025M Tris made to pH 8.3 with glycine. Before electrophoresis, samples were centrifuged in a Beckman Microfuge at 10,000 rpm for 1 min and 10 µl of clear supernatant was loaded into each slot. The electrophoresis was carried out with the cathode in the upper tank and at a constant current of 40 mA/gel for about 2 h (new electrode buffer) or 3 h (once-used electrode buffer) until the marker dye front reached the bottom of the gel.

3.2.4 Two-dimensional Electrophoresis

3.2.4.1 Non-reducing × reducing SDS-PAGE

Proteins were first extracted in the SDS (Tris-HCl, pH 6.8) solvent without ME as described above and then separated in SDS-polyacrylamide disc gels in glass tubes (120 mm long, 5 mm internal diameter). The compositions of the separating (90 mm) and stacking (10 mm) sections of the gel were identical to those of the 1-D SDS-PAGE slab gels. Samples were centrifuged at 10,000 rpm for 5 min in order to avoid any solid particles in the supernatant and, unless stated otherwise (in the legends of Figures), 50 µl of extract was layered onto the stacking gel. The samples were electrophoresed at 3 mA/tube for 45 min and then 2 mA/tube for 2 h. The gels were then removed from the tubes and incubated at 37°C for 30 min to 2 h in an equilibration mixture consisting of 1 % (v/v) ME freshly added to a solution of 10.3% (w/v) glycerol, 0.07M Tris, 2.4% (w/v) SDS made to pH 6.8 with HCl, to achieve protein reduction (c.f. Brown *et al.* 1979). Control gels were incubated in the same solution but without the addition of ME. After incubation, the proteins in the disc gel were subjected to electrophoresis in a direction at right angles to that of the first run. The gel rod was loaded horizontally onto the stacking gel portion of a 3.2 mm thick slab gel with similar constituents to the disc gel. The second dimension of electrophoresis was carried out at a constant current of 75 mA/gel for the first

45 min and then at 50 mA/gel for the remainder of the run (about 2 $^1/_2$ h). Later, this procedure was modified and disc gels with a reduced diameter (3mm) were loaded onto a second dimension slab gel of 2 mm thickness. In a still later modification, gel strips of 1 mm thickness and 10 mm width (thickness of the sample wells) were cut longitudinally from the slab gel after first dimension run and loaded onto slab gels of 1.2 mm thickness for the second dimension run.

3.2.4.2 Non-reducing acid PAGE × reducing SDS-PAGE

In the first step of this 2-D electrophoresis , proteins extracted from the rye endosperm with 2M urea, were fractionated in a disc gel containing 15.0% (w/v) acrylamide 0.12% (w/v) Bis, 0.0025% (w/v) ferrous sulphate, 0.01% (w/v) ascorbic acid and 2.5% (w/v) aluminium lactate made to pH 3.1 with lactic acid as modified from Bushuk and Zillman (1978). The electrode buffer contained 1.25 % aluminium lactate made to pH 3.1 with lactic acid. The electrophoresis was carried out with the anode in the top tank, at a constant voltage of 200 V for 5h. The equilibration of the disc gel and the second dimension of electrophoresis was identical to that described above for the non-reducing/ reducing SDS-PAGE system.

3.2.5 Staining and Destaining

Gels were stained overnight in a staining solution consisting of one part of 1% (w/v) Coomassie Brilliant Blue R mixed with 40 parts of 6 % (w/v) trichloroacetic acid in water: methanol:glacial acetic acid (80:20:7) as described by Lawrence and Shepherd (1980), but destaining was carried out in water for 48 h. Silver staining was carried out according to the following procedure as modified from Morrissey (1981): Coomassie Blue stained gels were destained for 24 h in water, methanol and glacial acectic acid (111:48:8) as used by Lawrence and Shepherd (1980). After destaining in the above solution prefixation, corresponding to the 'step 1' of Morrissey's procedure, was not needed. Destained gels

were fixed in 10 % glutaraldehyde for 30 min and then washed in several changes of deionized water for 2 h to remove the excess glutaraldehyde. The gels were then soaked in 5 μg/ml dithiothreitol for 30 min and after pouring off dithiothreitol, 0.1% silver nitrate was added to the gel. After 30 min of silver nitrate treatment, the gels were rinsed rapidly once with a small amount of deionized water and then twice with a small amount of developer (50 μl of 37% formaldehyde in 100 ml of 3% sodium carbonate). The gels were left in the developer until the desired level of staining was achieved and then citric acid was added to neutralize the developer and agitated for 10 min. After discarding this solution the gels were washed four times in deionized water over a period of 30 min. Before storage, the gels were soaked for 10 min in 0.03 % sodium carbonate to prevent bleaching. During this staining procedure the gels were handled only with rubber gloves to avoid fingerprints and all gel solutions were filtered to remove dust particles which can give spurious staining in the gel.

3.2.6 Molecular Weight Calibration

Apparent molecular weights of wheat proteins were determined by comparison with the mobilities of the following proteins used as standards in SDS-PAGE gels. Three sets were chosen to cover a wide range of molecular weights. First set was obtained from Pharmacia and included: phosphorylase B (94,000), bovine albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (21,000). The second set was obtained from SIGMA and included: myosin (205,000), β-galactosidase (116,000), phosphorylase B (97,000), bovine albumin (66,000), egg albumin (45,000) and carbonic anhydrase (29,000). The third set of standards obtained from Bio-Rad, included: phosphorylase B (92,500), bovine serum albumin (66,200), carbonic anhydrase (31,000) soybean trypsin inhibitor (21,500) and lysozyme (14,400).

3.2.7 Subcellular Isolation of Protein Bodies

Protein bodies were isolated from the developing endosperm using the procedure described by Miflin *et al.* (1981) as follows: Developing seeds, from Chinese Spring wheat grown in the glass house, were collected about 21 days after anthesis and the embryo portions were removed by hand dissection. Fresh endosperms from about 150 seeds (7.0 g) were chopped into 0.5 mm slices with a scalpel blade in 12 ml of an extraction buffer containing 0.05M tricine, 0.1M potassium acetate, 0.005M EDTA and 15% (w/v) sucrose, adjusted to pH 7.5 with potassium hydroxide or acetic acid. A further 4 ml of extraction buffer was added and the homogenate was filtered, with squeezing, through 4 layers of cheese cloth. The protein bodies were isolated from the filtrate by ultracentrifugation in the sucrose density gradients [16-60% (w/w)] made up in the above buffer, except 0.001M EDTA was used instead of 0.005M. The sucrose density gradients were prepared in the polyethylene centrifuge tubes which fitted a Beckman SW 27 rotor. Equal volumes (7 ml) of sucrose solutions with four different concentrations [16 %, 30 %, 45 % and 60 % (w/w)] were layered step wise into the centrifuge tubes. The lightest solution was poured first and then a syringe, with a long metal needle to reach the bottom of the tube, was used to inject the more concentrated solutions successively into the bottom of the tubes and thereby displacing the lighter solutions upwards. The centrifuge tubes with four steps of sucrose concentrations ranging from 60% at the bottom to 16% at the top were left undisturbed overnight (15-16 h) in a cold room (4°C) to obtain smooth gradients by diffusion (c.f. Hames, 1978). The endosperm homogenate (5.5 ml) was then layered on to these sucrose density gradients. After centrifugation in a Beckman SW27 rotor at 25,000 rpm for 2.5 h, the gradient was fractionated by piercing the bottom of the centrifuge tube (Hames, 1978) and fifteen 1.2 ml fractions were collected and weighed to determine the density. The heaviest fraction eluted first and protein bodies, forming a clearly visible white band near the bottom of the tube, corresponded to fractions 3, 4 and 5. Each of the fifteen fractions was diluted with an equal volume of distilled water and then centrifuged at 40,000 g for 30 min. After decanting the supernatant the pellets in each

tube were dissolved in 0.2 ml of SDS-Tris-HCl extraction buffer (pH 6.8) without ME and fractionated by 1-D SDS-PAGE. Later 3 µl of ME was added to each tube to obtain reduced proteins for SDS-PAGE analysis.

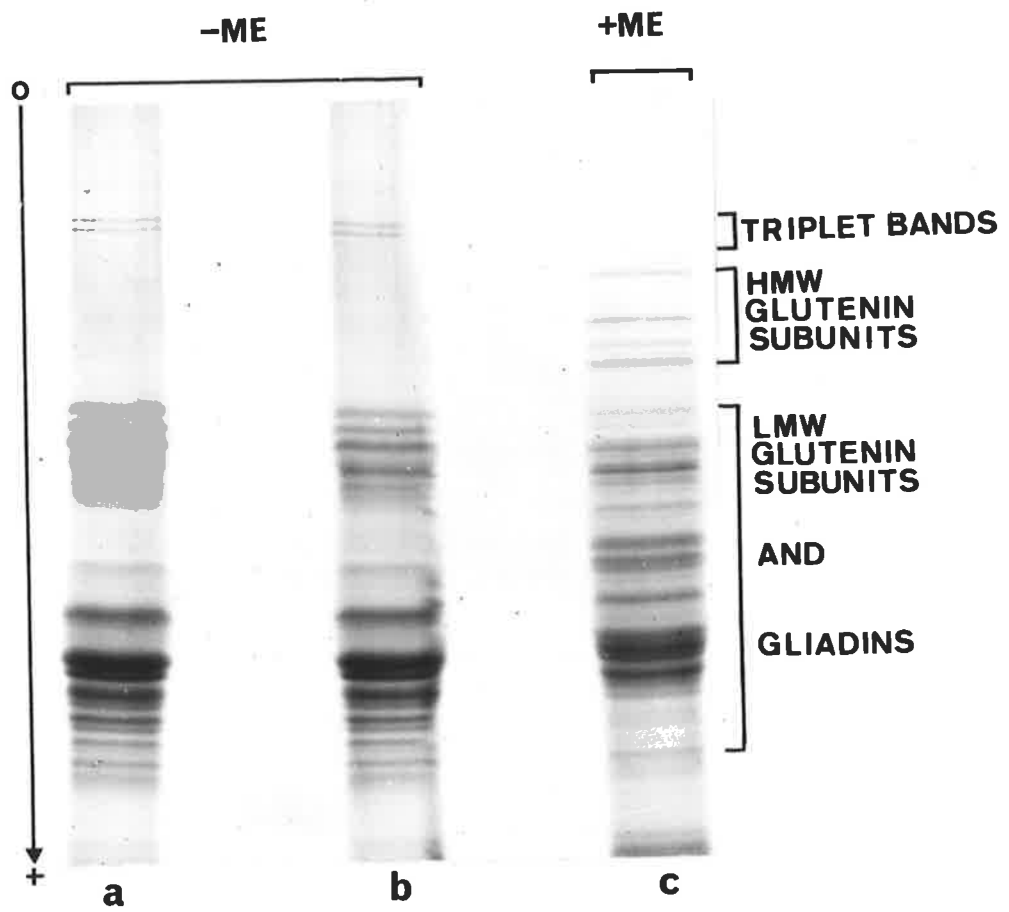
3.3 RESULTS

3.3.1 Triplet Proteins in Wheat and their Interaction with Glutenin

3.3.1.1 Detection of triplet bands and their genetic control

The one-dimensional electrophoretic patterns of unreduced and reduced extracts of total endosperm proteins of Chinese Spring wheat are shown in Fig. 3.1. The triplet bands, with relative staining intensities of approximately 4:4:1, occur in the cathodal (upper) half of the gel amongst heavy background streaking in the unreduced samples (Fig. 3.1, a, b). The other, faster moving, discrete protein bands are thought to be monomeric gliadins, albumins and globulins. The triplet bands and the background streak were absent in the samples reduced with ME (Fig. 3.1, c), suggesting that disulphide bonding was involved with both of these features of unreduced extracts. The appearance of many new bands after reduction, including the four HMW glutenin subunits and many less well resolved bands in the LMW region of the gel, was consistent with this conclusion. The triplet bands and the streak are extremely sensitive to reduction with ME, since diffusion of ME from track c has resulted in a pronounced edge effect in track b (Fig. 3.1).

The chromosomal control of the triplet bands was determined by analysis of Chinese Spring aneuploids. Seeds from stocks lacking chromosome 1A (e.g. NT 1A-1B), or the short arm of 1A (Dt 1AL), possessed only the slowest moving band (Tri-1) of the triplet (Fig. 3.2, b, c, d), whereas stocks lacking chromosome 1D (e.g. NT 1D-1A), or the short arm of 1D (Dt 1DL), had only the fastest moving band (Tri-3) (Fig. 3.2, j, k, l). The band with intermediate mobility (Tri-2) was absent whenever either chromosome arm 1AS or 1DS was absent. In contrast, the removal of complete chromosome 1B or its short arm did not have any obvious effect on the triplet pattern (Fig. 3.2, f, g, h). Besides these qualitative changes in pattern, some quantitative changes in band staining intensity were observed when the dosage of chromosomes 1A or 1D in the endosperm was varied. The approximate 4:4:1 ratio of staining intensities of bands Tri-1: Tri-2 :Tri-3 in euploid



-ME
+ME
o
+
a
b
c
TRIPLET BANDS
HMW
GLUTENIN
SUBUNITS
LMW
GLUTENIN
SUBUNITS
AND
GLIADINS

Chinese Spring, having equal doses of chromosomes 1A and 1D, was changed to 1:2:1 and 2:1:0(?) [or16:8:1] ratios when the dosage of these respective chromosomes was changed to 2:1 (NT 1B-1A, Fig. 3.2, g) and 1:2 (NT 1B-1D, Fig 3.2, h).

These observations indicate that the triplet bands are controlled by genes on the short arms of chromosomes 1A and 1D and that the relative amounts of these proteins are influenced by the dosage of these chromosomes in the endosperm. Furthermore, these observations are consistent with a hypothesis that the Tri-1, Tri-2 and Tri-3 bands are 'dd', 'da' and 'aa' dimers, respectively, produced by random association of 'd' and 'a' monomers controlled by chromosomes 1D and 1A, with the amount of 'd' monomer produced per chromosome being twice that of 'a' monomer. On the basis of this hypothesis, variation in the staining intensity of triplet bands associated with the change in the dosage of 1A and 1D chromosomes can be explained by the binomial expansions presented in Table-3.1.

Table 3.1 Model relating change of chromosome dosage to changes in the relative staining intensities of triplet bands

Chinese Spring stocks	Relative amounts of **d** and **a** subunits n_1*	n_2*	Binomial expansion for the dimer combinations $(n_1d+n_2a)^2$	Relative intensities of triplet bands Tri-1:	Tri-2:	Tri-3
Euploid	2	1	$(2d+a)^2$ = 4dd+4da+ aa	4	4	1
NT 1B-1A	2	2	$(2d+2a)^2$ = 4dd+8da+4aa	1	2	1
NT 1B-1D	4	1	$(4d+a)^2$ = 16dd+8da+ aa	16	8	1

*n_1 and n_2 are the relative amounts of **d** and **a** subunits of triplet bands

However, this model is an oversimplification since it was found later that triplet bands are tetramers rather than dimers (see section 3.3.5).

It is known from earlier work using aluminium lactate starch gels at acid pH (Shepherd 1968; Wrigley and Shepherd 1973) that the monomeric prolamins (gliadins) are also controlled by genes on the short arms of chromosomes 1A,1B and 1D and the patterns

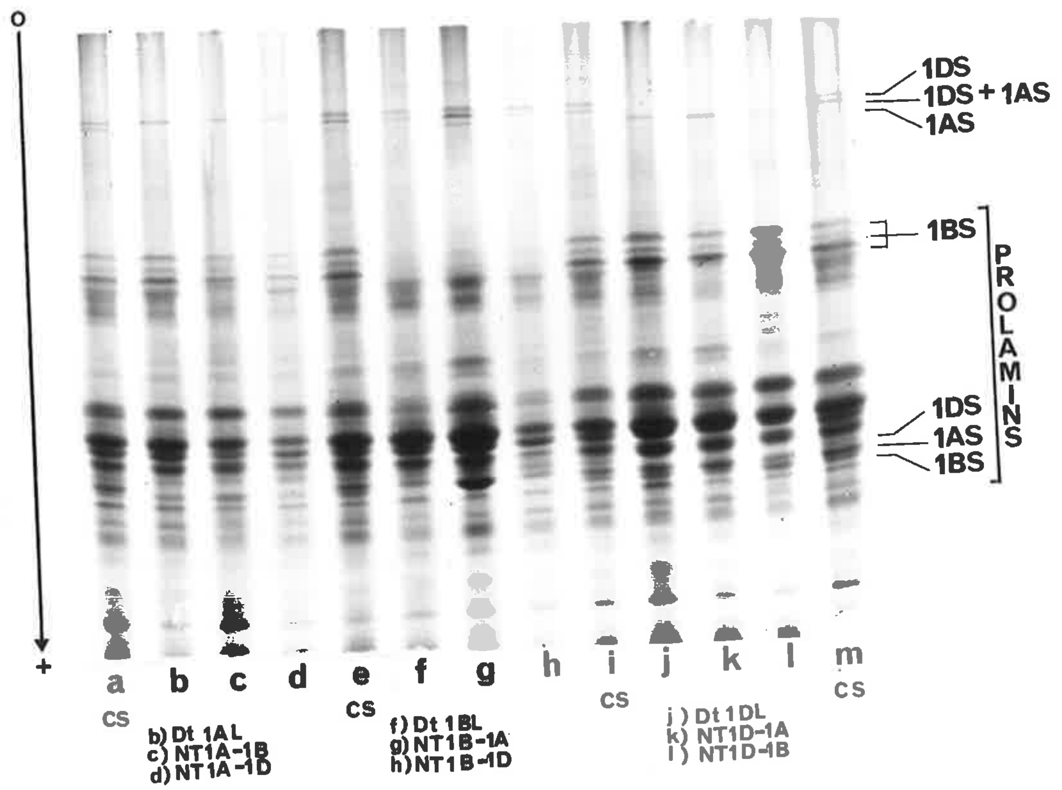
Fig. 3.2.

1-D SDS-PAGE patterns of unreduced total seed protein extracts from euploid Chinese Spring (CS) and ditelocentric (Dt) and nullisomic-tetrasomic (NT) stocks. Chromosomal control of triplet bands and some of the prolamin bands is indicated. The numbering of triplet bands in the text corresponds with the labelling in the triplet region of the gel as follows:

Tri-1 = **1DS**

Tri-2 = **1DS+1AS**

Tri-3 = **1AS**


o
+
1DS
1DS + 1AS
1AS
1BS
PROLAMINS
1DS
1AS
1BS
a b c d e f g h i j k l m
CS
CS
CS
CS
b) Dt 1AL
c) NT1A-1B
d) NT1A-1D
f) Dt 1BL
g) NT1B-1A
h) NT1B-1D
j) Dt 1DL
k) NT1D-1A
l) NT1D-1B

obtained with SDS-PAGE are consistent with these findings. These bands controlled by the short arms of these chromosomes are labelled prolamins in Fig. 3.2. As depicted in Fig. 3.2, these prolamins can be divided into two main groups. The first group, representing ω-gliadins as inferred from their slower electrophoretic mobility on SDS-PAGE (Payne *et al.,* 1982a), consists of four major bands three of which are controlled by chromosome arm 1BS (bands labelled in Fig. 3.2). The fourth band was controlled by 1DS but is not labelled due to its poor resolution in the gel depicted. The second group of prolamins includes a number of faster moving components representing α-, β- and γ-gliadins. These bands are less well resolved compared to the ω-gliadin bands, however, three of these bands could be assigned to chromosome arms 1AS, 1BS and 1DS (bands labelled in Fig. 3.2). In general, the number of prolamin bands observed in the present non-reducing SDS-PAGE system is less than the 1-D acid PAGE and starch gel systems, but the present system is simple to apply and could be useful in separating some of the prolamins which otherwise overlap in the acid pH gels. The best resolution is obtained for ω-prolamins, particularly ω-secalins, as described later in Chapter 4.

3.3.1.2 Variation of triplet band patterns in wheat cultivars

In a survey of unreduced protein extracts from seeds of 137 hexaploid wheat cultivars from a world collection, all cultivars except five possessed three electrophoretically slow moving bands in the cathodal portion of the gel. However, as in Chinese Spring, the fastest moving band (Tri-3) was always faint and difficult to score. The other two bands (Tri-1 and Tri-2) occurred in four distinct patterns referred to as 'broad, slow-moving', 'broad, fast-moving', 'narrow, slow-moving' and 'narrow, fast-moving' (Fig. 3.3, a, b, c, e, respectively) . Five cultivars showed a single diffuse band (Fig. 3.3, f) posssibly because Tri-1 and Tri-2 bands in these cultivars had similar electrophoretic mobilty. The nine durum wheats analysed possessed only one band with similar mobility to Tri-3 in Chinese Spring (Fig. 3.3, h). The triplet protein banding patterns of these 146 wheat cultivars along with the countries of their origin are listed in Appendix A (page 126)

Fig. 3.3

Variation of triplet band patterns in wheat cultivars (Only the first two bands of the triplet were considered in this classification).

a, d, g, i broad, slow-moving (e.g. Chinese Spring);

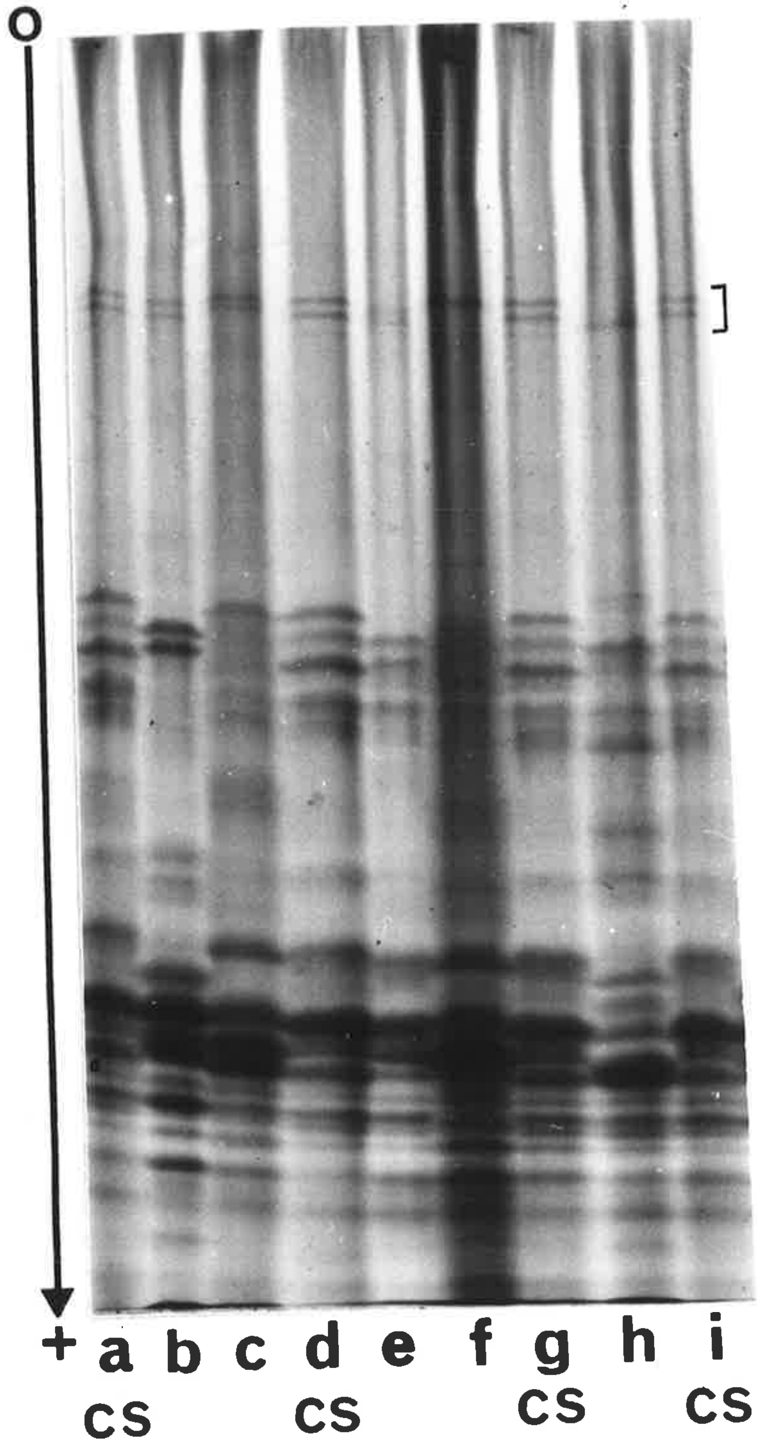
b broad, fast-moving (e.g. India 115);

c narrow, slow-moving (e.g. Hope);

e narrow, fast-moving (e.g. Sonalika);

f single diffuse band (e.g. Tainui) ;

h single band (e.g. Durati, a durum wheat cultivar).



O
+
a
b
c
d
e
f
g
h
i
cs
cs
cs
cs

3.3.1.3 Two-dimensional separation of wheat endosperm proteins

To resolve the structure of the triplet bands it was necessary to identify the subunits released from these bands after reduction, and a 2-D method of separation, with unreduced proteins in the first dimension and reduced proteins in the second (Wang and Richards 1974), was used for this purpose. The breakdown products of the triplet bands could be identified by their position vertically below the location of the triplet bands after the first phase of this separation. Chinese Spring T1A, in which all three triplet bands are clearly visible, was used for this purpose. Unreduced gel rods when run in the second dimension, gave the expected 45° line and the triplet bands were recognizable as dark dots approximately 2 cm from the origin in the streaky region of the gel (Fig. 3.4, A). The reduced rods gave a very different electrophoretic pattern (Fig. 3.4, B). The triplet bands separated into four spots; consisting of two slower moving components (M.W. 58,000 and 52,000) and two very fast moving components (M.W. 23,000 and 22,000). The dark streak present in the unreduced gels was much less prominent after reduction and at least 20 different subunits (including four HMW glutenin subunits and four subunits of triplet bands) appeared as continuous bands starting from the origin of the rod gel and extending almost to the 45° line. The group of proteins which did not change their mobility upon reduction, remaining as spots on the 45° line, were thought to be monomeric (α, β, γ and ω gliadins). However, a few of the faster moving gliadin spots (α, β and γ groups) were displaced from the 45° line after reduction, indicating slightly lower mobility in the second dimension (Fig. 3.4, B, c). This may be due to the reduction of the intramolecular disulphide bonds in gliadin molecules, resulting in more complete unfolding of the polypeptide chains. It has been shown earlier that electrophoretic mobility of α-, β- and γ-gliadins was significantly reduced after the reduction of disulphide bonds (c.f. Woychik *et al.* 1964; Beckwith *et al.* 1965; Nielsen *et al.* 1968). This reduction in mobility was explained by the theory that in the unreduced state gliadin molecules have folded conformations stabilized by intramolecular disulphide bonds. After reduction of these

Fig. 3.4

2-dimensional (2-D) SDS-PAGE patterns of total seed protein extracts from Chinese Spring tetrasomic 1A.

A Non-reducing conditions in both dimensions (-ME × -ME). Only 25 µl of protein extract was loaded for the better resolution of triplet dots;

B Non-reducing conditions in the first dimension and reducing conditions in the second dimension (-ME × +ME);

a, b, c reference samples:

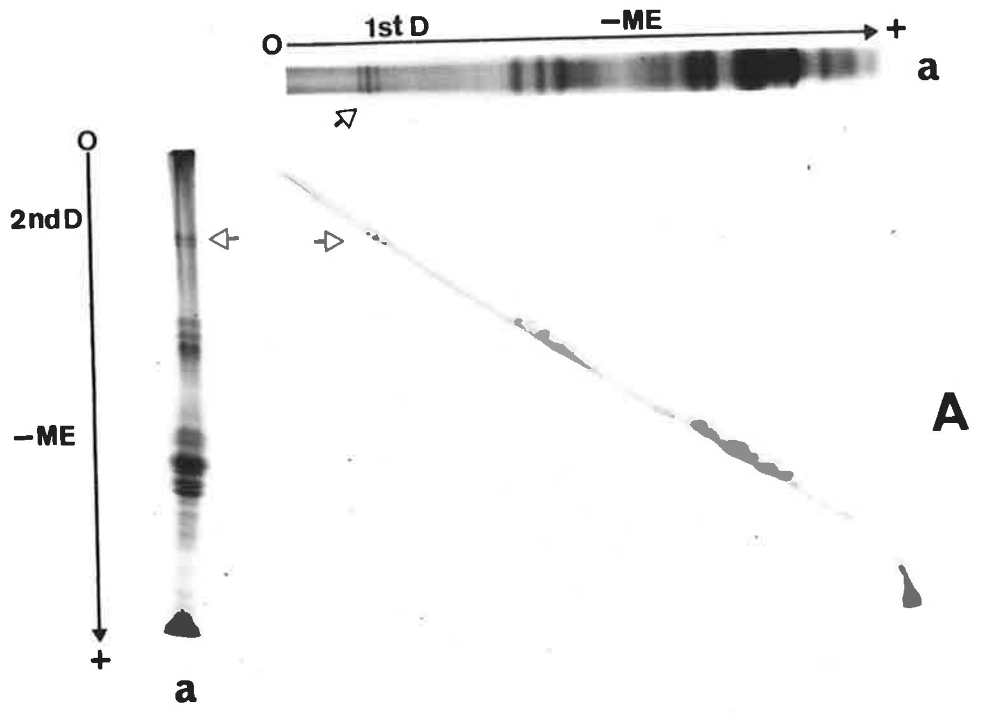
a 1-D separations of unreduced extracts

b molecular weight standards (Pharmacia):

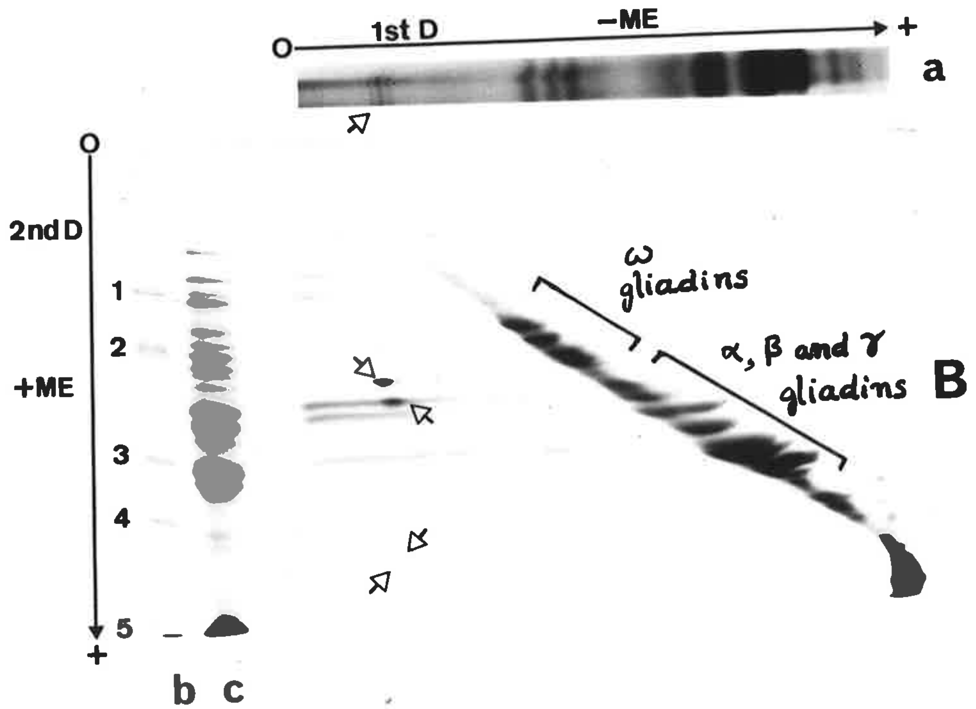
1 phosphorylase b (94,000), **2** bovine albumin (67,000), **3** ovalbumin (43,000), **4** carbonic anhydrase (30,000), **5** trypsin inhibitor (21,000)

c 1-D separation of reduced extracts.

Arrows indicate the positions of bands and spots related to triplet proteins.



O
1st D
−ME
+
a
O
2nd D
−ME
+
a
A



O
1st D
−ME
+
a
O
2nd D
+ME
+
1
2
3
4
5
b
c
ω gliadins
α, β and γ gliadins
B

bonds, the gliadin molecules become more linear and due to the sieving effect of the gel medium , show reduced electrophoretic mobility in the gel.

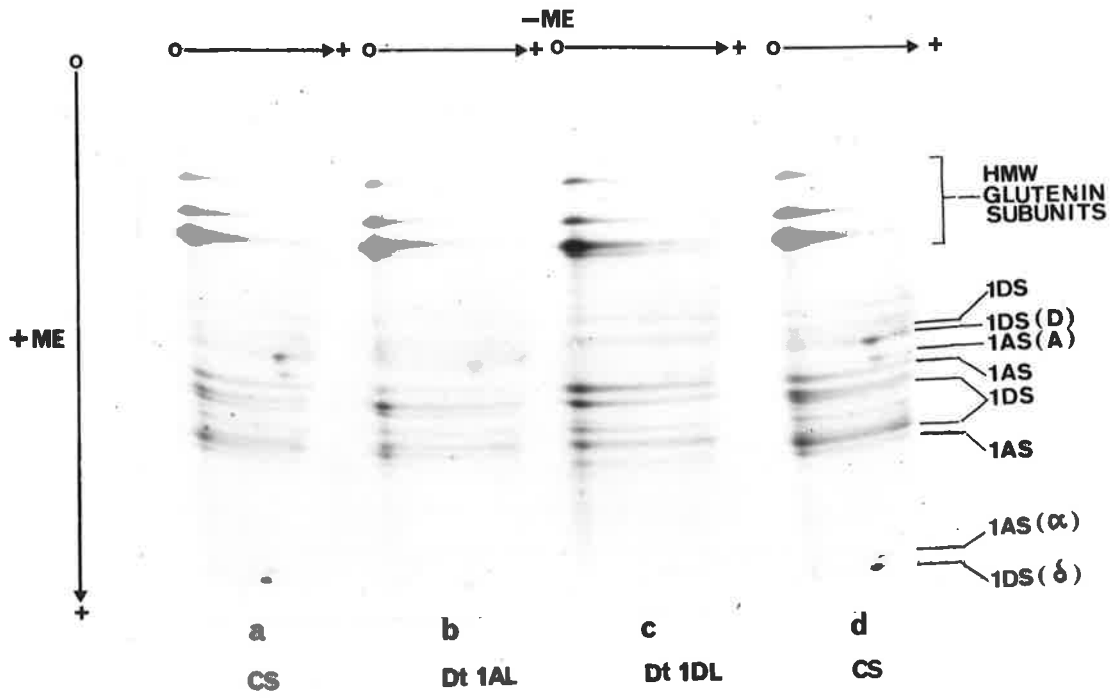
3.3.1.4 Genetic control of the triplet and LMW glutenin subunits

Since the unreduced triplet bands occurred in the first 20 mm of the separating gel in the tubes, it was possible to cut out this section from four separate tube gels and after reduction, include all four pieces, representing four different samples, on a slab gel in the second dimension of electrophoresis. In this way, the subunit composition of the triplet bands present in euploid Chinese Spring could be compared directly with those present in ditelocentric lines 1AL and 1DL (Fig. 3.5). The slowest moving spot (designated as D) and the fastest moving spot (designated as δ) were absent when chromosome arm 1DS was missing (Fig. 3.5, c) and therefore these are controlled by gene(s) on chromosome arm 1DS. Similarly, the other two spots (A, α) were absent in the stock lacking chromosome arm 1AS and are controlled by gene(s) on 1AS (Fig. 3.5, b). Thus, chromosome arms1DS and 1AS each code for one slow moving and one fast moving subunit of the triplet bands. A lighter 'shadow' spot can be seen below the D spot (Fig. 3.5, a, b, d) but its significance is not known.

Removal of these chromosome arms was also associated with the loss of some of the LMW subunits which appeared as continuous bands in the second dimension of electrophoretic separation. Chromosome arm 1DS appeared to control two of these bands which, together with the subunits of Tri-1, are labelled 1DS in Fig. 3.5. When chromosome arm 1AS was absent, there was a marked decrease in the intensity of two other bands which, together with the subunits of Tri-3, are labelled 1AS in Fig. 3.5. The removal of chromosome arm 1BS , although not affecting the triplet pattern, resulted in the loss of one of the LMW subunits (data not shown). Not all of the protein bands could be assigned to particular chromosomes and it is thought that these bands may represent a mixture of two or more polypeptides with the same electrophoretic mobility but controlled by more than one chromosome.

Fig. 3.5

Modified 2-D SDS-PAGE patterns of total seed protein extracts from euploid Chinese Spring (CS) and its ditelocentrics (Dt 1AL and Dt 1DL). This gel was obtained by loading small pieces (expected to contain triplet bands and some streak) of four different rod gels onto a single slab gel. Rod gel pieces were reduced with ME before running in the second dimension. The chromosome arms controlling some of the LMW subunits of glutenin and triplet band subunits (**D**, **A**, **α**, **δ**) are indicated. Note the darker staining intensities of **D** and **δ** subunits compared to **A** and **α** subunits in tracks **a** and **d**.



−ME
+ME
HMW
GLUTENIN
SUBUNITS
1DS
1DS(D)
1AS(A)
1AS
1DS
1AS
1AS(α)
1DS(δ)
a
CS
b
Dt 1AL
c
Dt 1DL
d
CS

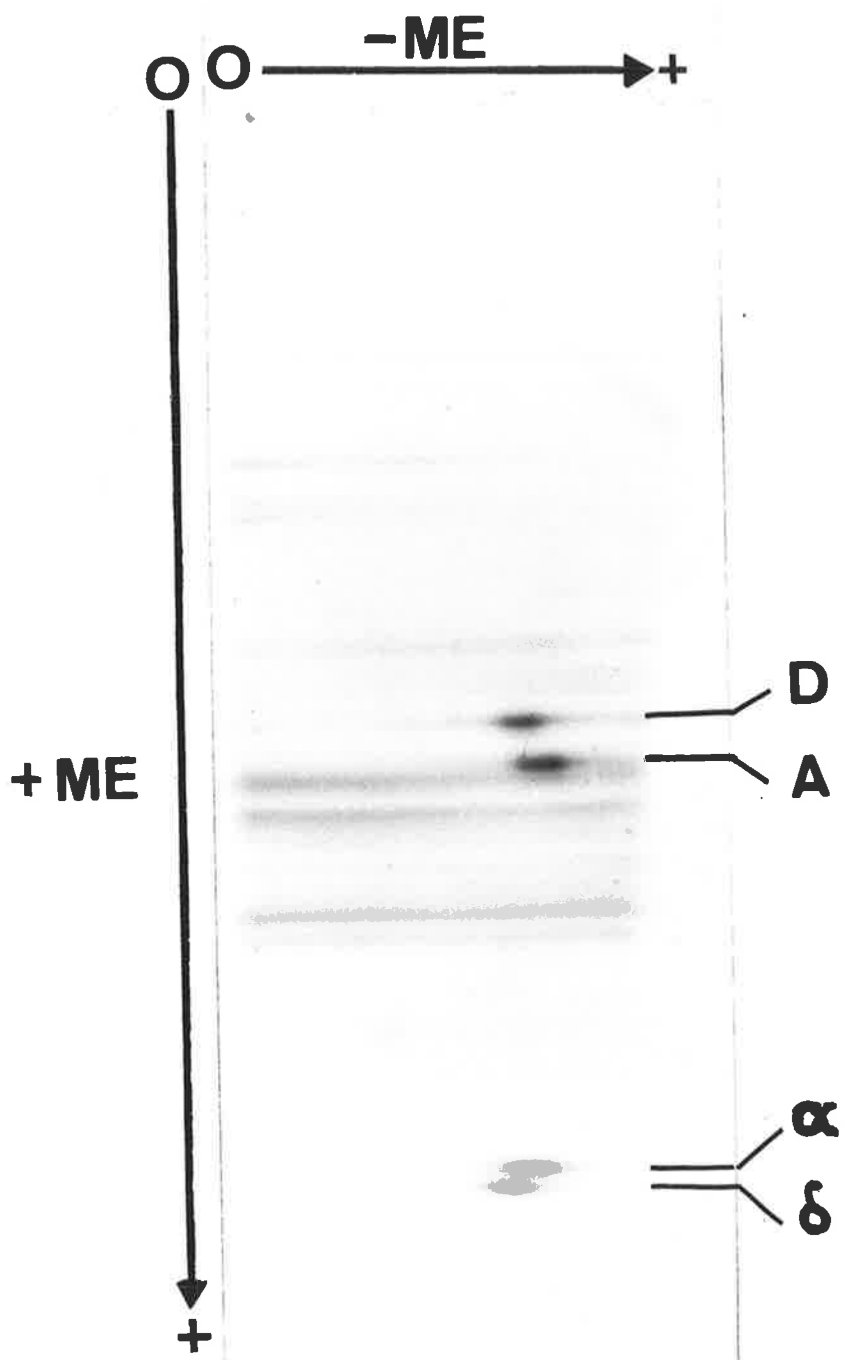
3.3.1.5 Subunit composition of triplet bands

From the behaviour of triplet bands displayed in Figs. 3.1, 3.2 and 3.5 it is concluded that Tri-1 is a disulphide-linked aggregate of subunits D and δ controlled by chromosome arm 1DS, Tri-3 is an aggregate of subunits A and α controlled by 1AS, and that Tri-2 is a hybrid aggregate involving protein subunits controlled by both 1AS and 1DS. Furthermore, the structure of the subunit spots observed in the second dimension of separation after reduction of the triplet bands of Chinese Spring T1A, clearly shows two different subunits coming from each of the Tri-1 and Tri-3 band positions (D, δ and A, α, respectively) and all four subunits (D, A, δ and α) coming from the Tri-2 band position (Fig. 3.6). This shows that Tri-2 must be at least a tetramer, and it follows that Tri-1 and Tri-3 must have a similar composition because of their similar molecular size.

Additional evidence for this conclusion came from the detection of two more spots with intermediate electrophoretic mobility in the gels with partially reduced proteins (Fig. 3.7, b, c, d). To obtain this gel, unreduced extracts of Chinese Spring T1A were electrophoresed in 12 rod gels and 1 cm sections were cut from each gel in the region expected to contain the triplet bands. These gel sections were reduced for two hours in separate equilibration tubes containing 12 different ME concentrations ranging from 0 to 2.0% (see legend, Fig. 3.7, for details), before loading them onto a slab gel for electrophoresis in the second dimension. Fig. 3.7 clearly shows that in the presence of trace amounts of ME the triplet bands are reduced to two intermediate-sized products, and in two of these cases (Fig. 3.7, c, d) the four monomeric subunits appear as well. At higher concentrations of ME, these intermediate products disappear and only the four subunits are present. These intermediate-sized spots are interpreted to be dimeric products Dδ and Aα, coming from the partial reduction of tetrameric triplet bands DδDδ (Tri-1), DδAα (Tri-2) and AαAα (Tri-3). One of the gel sections in Fig. 3.7 did not contain the triplet bands (track e) and four of them were mistakenly loaded with reversed polarity (tracks c, i, k, l) i.e. during loading, the origin side of these gel sections was reversed to

Fig. 3.6

2-D SDS-PAGE (-ME × +ME) pattern of total seed protein extracts from Chinese Spring tetrasomic 1A. Note the overlap of four triplet subunit spots (D, A, α and δ) in the central position of these bands and overlap of only two subunits on left (D, δ) and right (A, α) corresponding to breakdown products of Trp-2, Trp-1 and Trp-3 bands, respectively.



O
O
−ME
+
+ME
+
D
A
α
δ

Fig. 3.7

Modified 2-D SDS-PAGE patterns of total seed protein extracts from Chinese Spring tetrasomic 1A. The twelve rod gel pieces (a—l), expected to contain triplet bands, were loaded on to the slab gel after equilibration with a range of ME concentrations [% (v/v)] as follows:

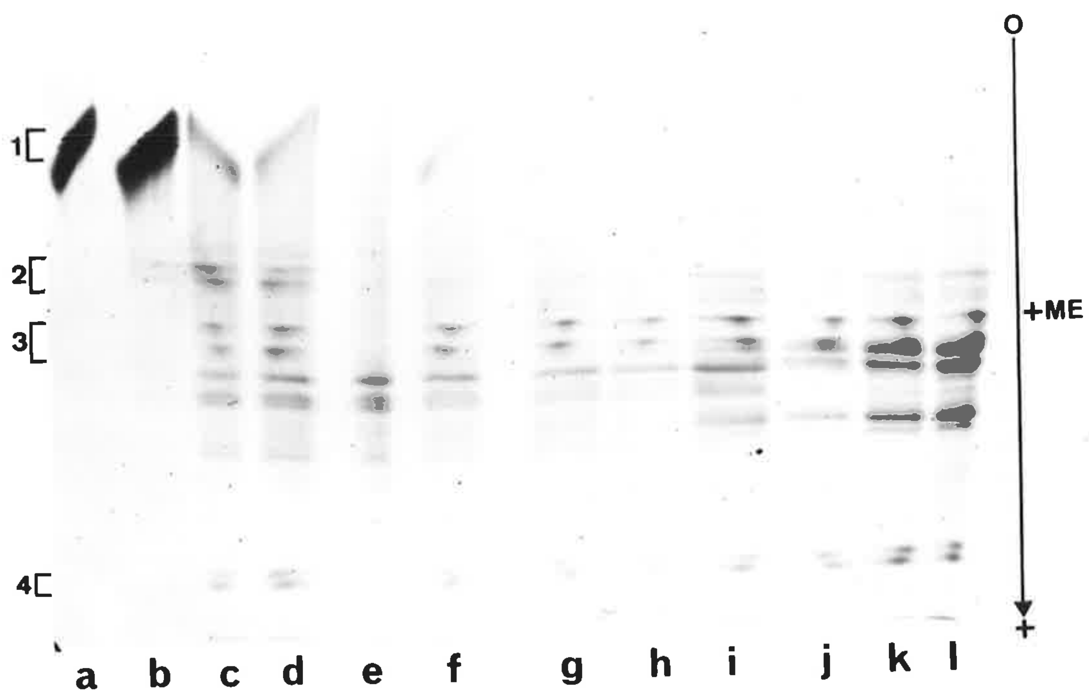
a 0; **b** 0.001; **c** 0.01; **d** 0.0125; **e** 0.015; **f** 0.0175; **g** 0.02; **h** 0.05; **i** 0.1; **j** 0.5; **k** 1.0; **l** 2.0

1 triplet bands (DδDδ, DδAα and AαAα tetramers are not well resolved due to heavy loading);

2 intermediate breakdown products (Dδ and Aα dimers);

3 large subunits (D, A monomers);

4 small subunits (α, δ monomers).


1
2
3
4
O
+ME
+
a
b
c
d
e
f
g
h
i
j
k
l

the right hand side on the gel rather than the left hand side as done with the remaining gel sections. These discrepancies in the loading of the gel sections are evident in Fig. 3.7 by the absence of major spots associated with triplet bands in track e and by the change in the orientation of the diagonal in tracks c i, j, and k. However, these anomalies had no bearing on the conclusions drawn from this experiment.

There are two other features of interest in Fig. 3.7. First, the LMW glutenin subunits tend to show decreased mobility with increasing concentrations of ME up to a threshold of 0.1% (Fig. 3.7, i), and it is thought that this could be due to the disruption of intramolecular disulphide bonds with higher concentrations of ME resulting in greater unfolding of these subunits, just as was found with some of the classical gliadins (Fig. 3.4). Second, the shadow bands of the D subunits described earlier (Fig. 3.5) are more pronounced in this gel and there appear to be shadow bands under the A subunits as well.

A model of the structure of the triplet bands based on the above observations is given in Fig. 3.8. The molecular weights of subunits D, δ, A and α are estimates based on comparison with the mobilities of standard protein markers (Fig. 3.4, B, b). However, the values given for the triplet bands and the partial breakdown products are deduced values only, based on the assumed tetrameric and dimeric structure of these bands. However, in several gels (e.g. Fig. 3.11, B, ii and Fig. 3.7, b, c, d) the electrophoretic mobility of the partial breakdown products, was observed to be similar to that of ω-gliadins which have a molecular weight range of 65 to 80 kd (see Booth and Ewart, 1969; Bietz and Wall 1972; Charbonnier, 1974 and Fig. 3.4, B, b in section 3.3.1.3 of this thesis), indicating that their actual molecular weights were consistent with the deduced values.

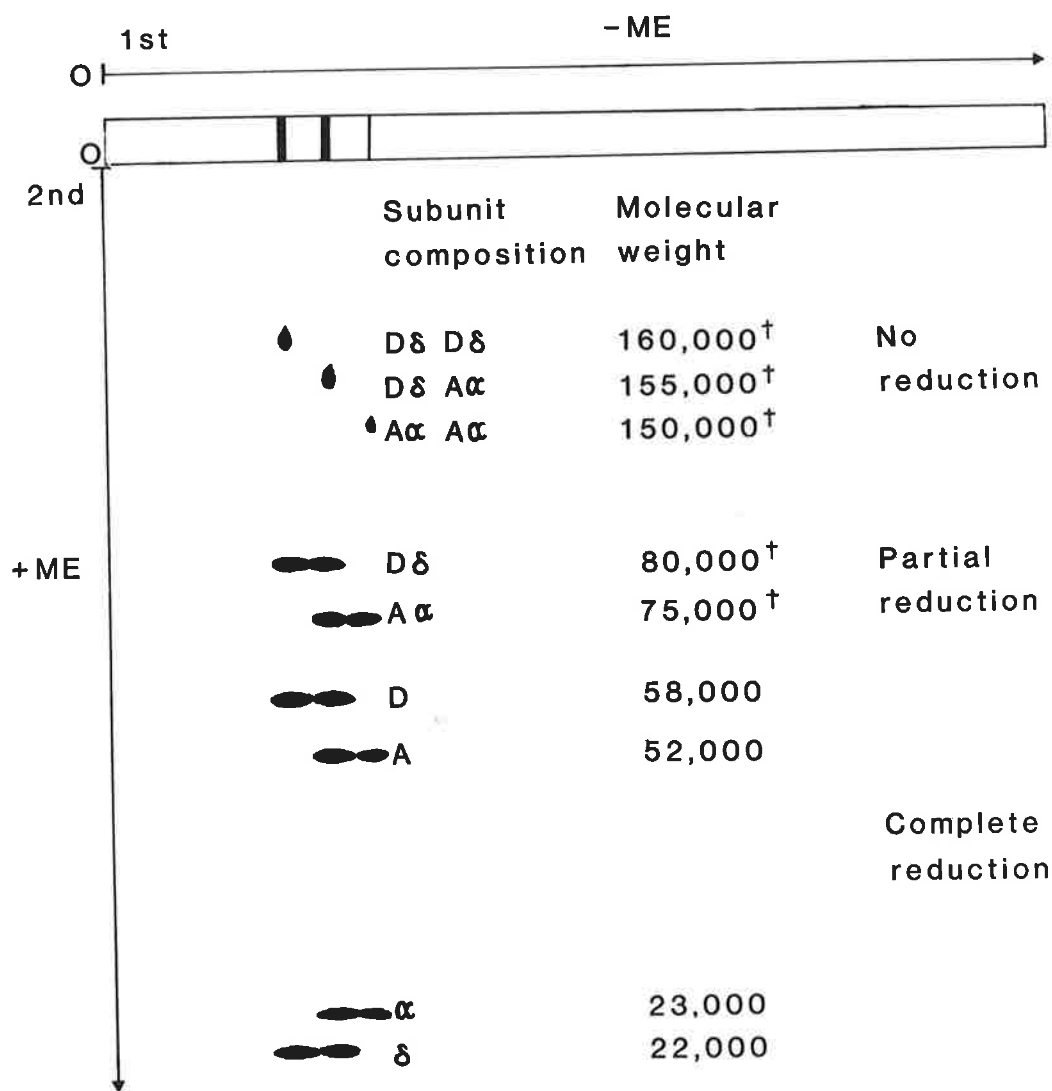
3.3.1.6 Origin of the streak and parallel lines

Two striking features of this 2-D electrophoretic procedure applied to total protein extracts of wheat endosperm are: (i) the pronounced streaking in the cathodal half of the gel in the first dimension and (ii) after reduction with ME, the appearance of many parallel lines

Fig. 3.8

Model for the structure of triplet bands. Molecular weights of D, A, α and δ subunits were estimated by comparing their electrophoretic mobility with the standard proteins after SDS-PAGE under reducing conditions. † Deduced values based on the assumed dimeric and tetrameric structure of intermediate breakdown products and triplet proteins, respectively.

TRIPLET BAND STRUCTURE



extending from the origin in the first run to a point close to the position of the diagonal line in the second dimension (Fig. 3.4). The streaking in the first dimension is not due to continuous and prolonged entry of one or a few proteins into the gel, because it is maintained as a well defined narrow diagonal line in the second dimension of separation of unreduced extracts (Fig. 3.4, A). Thus the position of each component of the streak in the first dimension reflects a particular electrophoretic mobility which is maintained in the second dimension . The streak is thought to represent the electrophoretic separation of a complex mixture of wheat proteins having a continuous array of sizes, which is consistent with the conclusions drawn earlier from gel filtration studies (Crow and Rothfus, 1968; Danno *et al.*, 1974; Huebner and Wall ,1976; Payne and Corfield, 1979; Bottomley *et al.*, 1982).

In seeking an explanation for the parallel lines, it was necessary first to eliminate the possibility that protein subunits had diffused along the rod during the equilibration procedure or during the second dimension electrophoresis. This was considered unlikely since the lines were restricted to the origin side of the gel and did not extend beyond the 45° line. However, proof that diffusion was not involved was obtained by cutting the rod gel into seven ordered pieces after the first dimension run and equilibrating each piece separately in the presence of ME. When these gel pieces were loaded onto a slab gel, end-to-end so as to reconstitute the original rod gel, and run in the second dimension, it was observed that the adjacent pieces had the same bands appearing as parallel lines, but the bands did not extend beyond the cut ends of the gel pieces (Fig. 3.9).

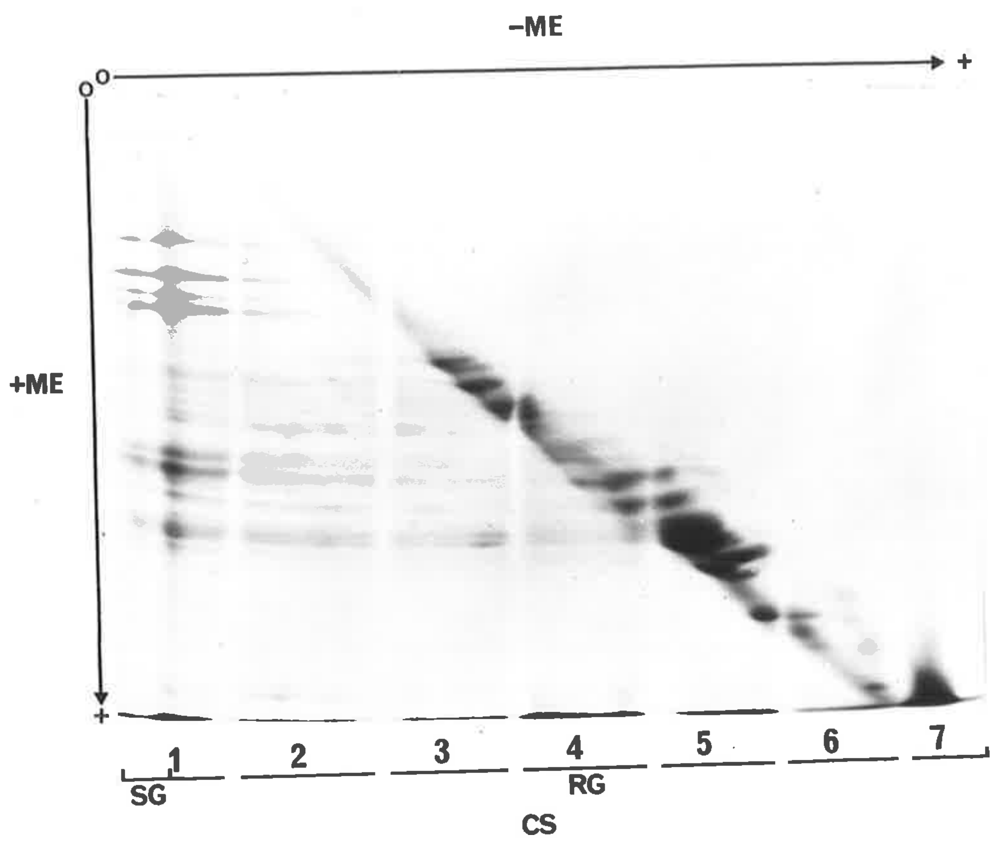
Thus, it is concluded that the bands occurring as parallel lines represent the array of subunits which make up the different-sized protein oligomers forming the cathodal streak in unreduced rod gels. The four slowest moving of these bands correspond in size to the HMW glutenin subunits of Chinese Spring (Bietz and Wall, 1972). The other bands, because of their size and control by genes on the short arm of group 1 chromosomes, are thought to be the LMW glutenin subunits (c.f. Jackson *et al.*, 1983) and the four triplet subunits. As shown earlier, some proteins remain as spots on the diagonal line, and these are thought to be the classical α, β, γ and ω gliadins. Thus this simple 2-D procedure

Fig. 3.9

2-D SDS-PAGE (-ME × +ME) patterns of Chinese Spring (CS). The unreduced protein extract (-ME) was first electrophoresed in a rod gel which was then cut into seven pieces and each piece was equilibrated separately with ME before running in the second dimension.

1—7 refer to the sequential pieces of the cut disc gel, which were loaded end to end so as to recreate their order in the original roa gel.

SG= stacking gel portion of the rod gel; **RG**= running gel portion of rod gel.



–ME
O
O
+
+ME
+
1
2
3
4
5
6
7
SG
RG
CS

separates wheat endosperm proteins into those that occur as projections below the diagonal line and those which remain as spots on this line, corresponding with protein subunits forming disulphide-linked aggregates and those which remain as monomers, respectively. Both the streak and the parallel lines were absent from the 2-D gels when protein extracts were reduced prior to the first dimension of electrophoresis (Fig. 3.10, a, b) and this gave additional evidence for the parallel lines being due to disulphide bonding.

3.3.1.7 Importance of triplet proteins in the aggregation of glutenin

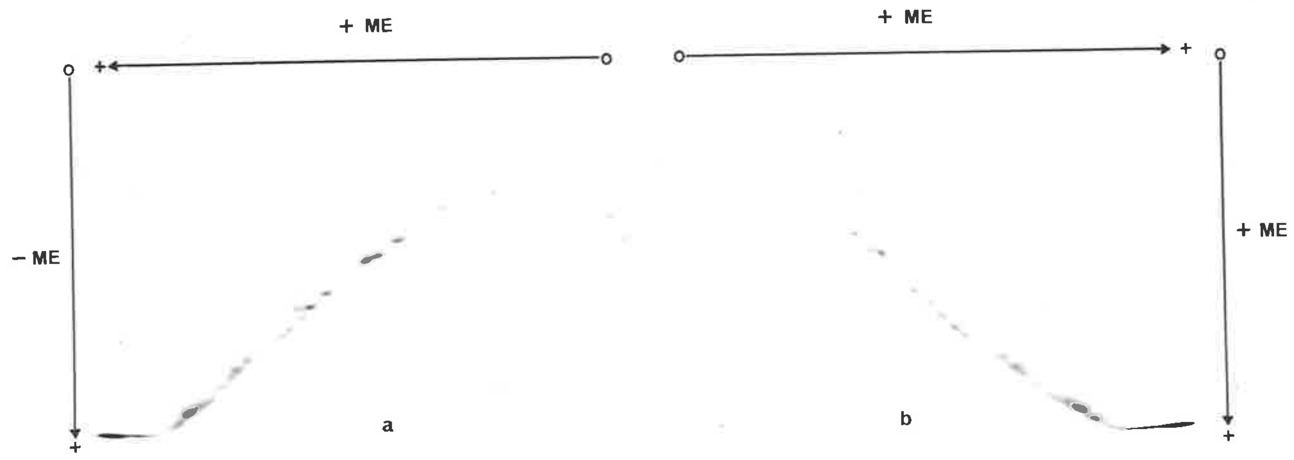
The continuous nature of the bands forming the parallel lines is thought to result from two main sources. The main contribution to continuity is expected to come from the formation, in the endosperm, of protein aggregates of different sizes due to disulphide bonding among the many different HMW and LMW subunits of glutenin and the four triplet protein subunits. In this model a given glutenin subunit may be present in a wide array of different-sized aggregates ranging from very large multimers to a small dimer involving just this subunit and the smallest interacting subunit, possibly the α and δ subunits. The parallel lines corresponding to the A and D triplet subunits can be seen in Figs. 3.4, B, 3.9 and 3.11, A, but the lines corresponding to the α and δ subunits, although clearly present in the original slab gels, are too faint to show up in the photographs of Coomassie Blue stained gels. Several of these 2-D gels were stained by using the silver staining procedure of Morrissey (1981) and it was observed consistently that parallel lines corresponding to the α and δ subunits do not extend completely to the diagonal but terminate as well defined spots at the positions expected for reduced αα and δδ dimers, respectively (Fig. 3.11, B, a, vi). A close perusal of these spots at the 'vi' position reveals that the spot on the α line is projected towards the left and that on δ line is projected towards the right hand side. Also there is some overlap between the position of α and δ spots which would correspond to the breakdown products of αδ dimers. These observations are consistent with the α subunit being larger than the δ subunit and therefore the αα dimer being larger than the δδ dimer with the αδ dimer having an

Fig. 3.10

2-D SDS-PAGE patterns of total seed protein extracts from Chinese Spring.

a reduced (+ME) in the first dimension and
unreduced (-ME) in the second dimension;

b reduced (+ME) in both first and second dimensions.


+ ME
o
+
+
− ME
o
a
+ ME
o
+
o
+ ME
b
+

intermediate size. Because of the slower electrophoretic mobility of the larger aggregates in the first dimension run, their breakdown products would be situated at the left hand side in the 2-D gels and vice versa.

This projection of the α subunit towards the left hand side and that of the δ subunit towards the right hand side at the position 'vi' in Fig. 3.11 is reversed at position 'i' which corresponds to the breakdown products of the tetrameric triplet bands (see also Fig. 3.6). However, this is expected because in triplet bands, α subunit is present in a smaller aggregate (Tri-3) than the δ subunits (Tri-1) [see Fig. 4.8 in section 3.3.1.5]. Apart from these terminating spots, the α and δ lines contain five other prominent spots superimposed on them and these are labelled i, ii, iii, iv and v in Fig. 3.11, B, a. It is clear from earlier observations that spots, labelled i and ii correspond to the breakdown products of tetrameric and dimeric states of triplet proteins, respectively, but the significance of the remaining three spots is not clear. However, it is reasonable to assume that some of these spots and much of the continuous lines associated with the α and δ subunits are the result of interaction between the subunits of triplet and glutenin proteins. This hypothesis is supported by the observation that all of the parallel lines corresponding to HMW and LMW glutenin subunits extend well beyond the expected homodimer position for these subunits, and usually very close to the diagonal. That is, it is thought that the α and δ subunits of the triplet proteins combine with glutenin monomer subunits, giving them only slightly lower electrophoretic mobility than the glutenin monomer subunit, and therefore, after reduction of disulphide bonds in the present 2-D system, the breakdown products of these glutenin/α or glutenin/δ heterodimers will be situated very close to the diagonal.

Another factor contributing to the continuity of these lines could be the occurrence of conformational isomers among the protein aggregates, due to differences in the number and sites of disulphide linkages between two or more interacting subunits. Thus, an aggregate involving a particular number and type of subunits, may have two or more different three dimensional shapes. Different shapes may result in varying level of SDS-protein binding thus giving a range of charge densities to the SDS-protein complexes, and/or differential frictional drag during electrophoresis in the gel. In this

Fig. 3.11

2-D SDS-PAGE (-ME × +ME) patterns of total seed protein extracts of Chinese Spring tetrasomic 1A. For first dimension separation 25 µl of unreduced proteins were loaded in sample wells of a slab gel and then a 10 mm wide (width of the sample slots) gel strip was cut out and reduced before loading on to another slab gel (11% acrylamide) for second dimensional electrophoresis.

A gel stained with Coomassie Blue R stain.

a molecular weight standards (SIGMA):

1 myosin (205,000), **2** β-galactosidase (116,000), **3** phosphorylase b (97,400), **4** bovin albumin (66,000), **5** egg albumin (45,000), **6** carbonic anhydrase (29,000).

b 2-D separation of wheat protiens.

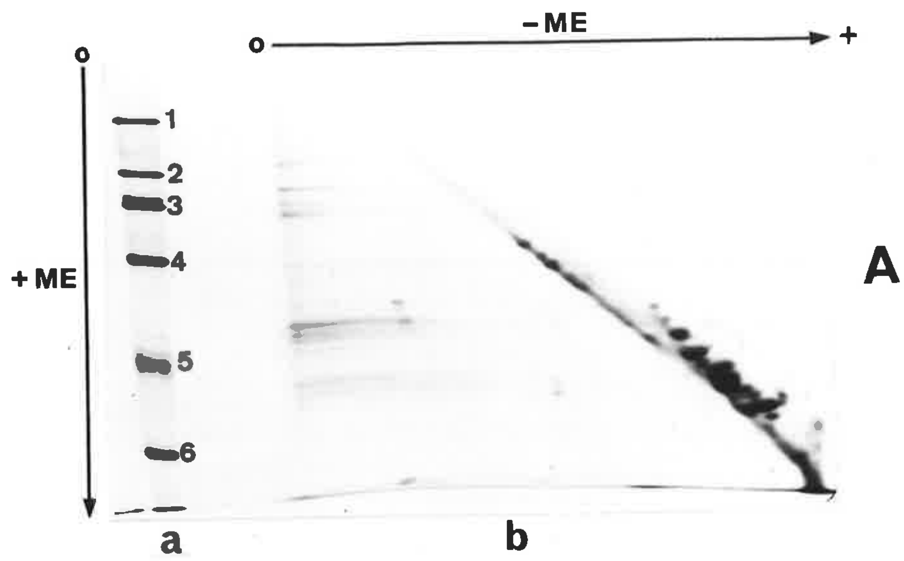
B gel stained with silver stain.

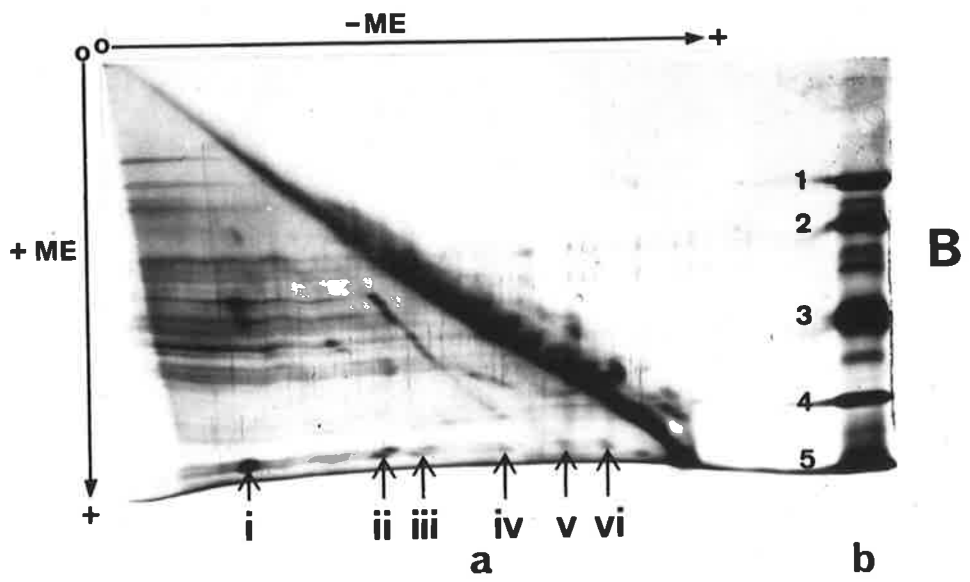
a 2-D separation of wheat proteins.

b molecular weight standards (Bio-Rad):

1 phosphorylase b (92,500), **2** bovin serum albumin (66,200), **3** carbonic anhydrase (31,000), **4** soybean trypsin inhibitor (21,500), **5** lysozyme (14,000).

Arrows (**i** to **vi**) indicate the concentrated spots on the parallel lines corresponding to α and δ subunits of triplet. Spots **i**, **ii** and **vi** are thought to come from the reduction of heterotetrameric (DδDδ, DdAα, AαAα) heterodimeric (Dδ, Aα) and homodimeric/ heterodimeric (αα, δδ and αδ) aggregation products, respectively.


-ME
o
+
o
+ME
1
2
3
4
5
6
a
b
A


-ME
o
+
o
+ME
+
1
2
3
4
5
i
ii
iii
iv
v
vi
a
b
B

way, conformational isomerism could cause a particular aggregated protein molecule to have a range of electrophoretic mobilities in the gel. However, the large differences in the electrophoretic mobilities can not be explained by this phenomenon and these are best explained by intermolecular disulphide bonding between the various protein subunits.

3.3.1.8 Interaction between HMW glutenin subunits and LMW subunits

This 2-D method was used to analyse the aggregation behaviour of the glutenin subunits from a special stock of wheat developed by Dr. G. J. Lawrence and apparently having a single major HMW glutenin subunit (Lawrence and Shepherd, 1981 b). It was intended to use this stock to investigate whether HMW subunits of glutenin can combine directly with the LMW subunits to form higher aggregates, as proposed by Bietz and Wall (1980). It is clear that the HMW glutenin subunit, present as a single major band of MW ≈ 100,000 in 1-D gel (Fig. 3.12, b), forms a strongly stained continuous band reaching from the origin to the 45° line in the 2-D gel (Fig. 3.12, c), although there is a marked tailing off of the band towards the diagonal.

To explain this observation it is postulated that the HMW subunit combines with one or more of the LMW glutenin subunits and/or triplet protein subunits by disulphide bonding to give a wide array of different-sized aggregates of glutenin as originally proposed by Bietz and Wall (1980). There is no evidence to suggest that the HMW glutenin subunit preferentially forms homopolymers analogous to the oligomers of glutenin subunits detected by Lawrence and Payne (1983). However, in their work they employed very mild reducing conditions and added cystamine to the gels. If the HMW subunit did show preferential binding into homopolymers, distinct spots should be present in the 2-D separation at points corresponding to the expected positions for the dimers, trimers etc after the first run. As shown in Fig. 3.12, c there was no evidence of such spots. Furthermore, if the HMW subunit did not combine with the LMW subunits, the line representing this subunit should not extend closer to the diagonal than the indicated homodimer position (labelled 'd' in Fig. 3.12).

Fig. 3.12

SDS-PAGE patterns of total seed protein extracts from 'Single glutenin' line.

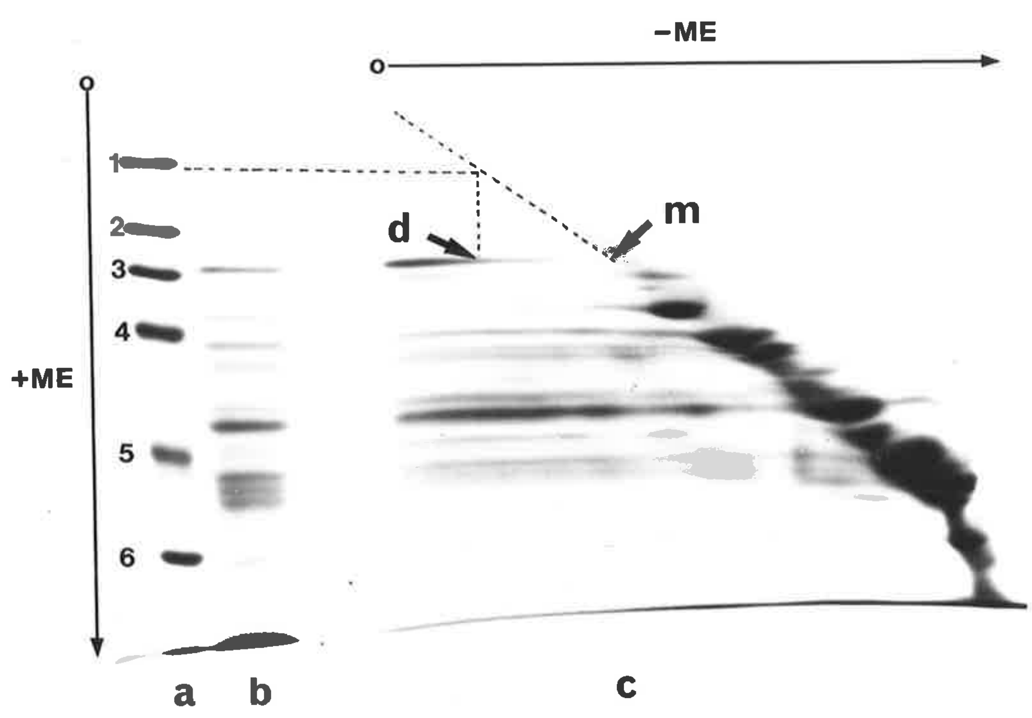
a molecular weight standards (SIGMA):

1 myosin (205,000), **2** β-galactosidase (116,000), **3** phosphorylase b (97,400), **4** bovin albumin (66,000), **5** egg albumin (45,000), **6** carbonic anhydrase (29,000)

b 1-D separation of reduced (+ME) protein extracts;

c 2-D separation (-ME × +ME).

Arrows indicate the monomer (**m**) and expected homodimer (**d**) position for the HMW glutenin subunit.



−ME
o
o
1
2
3
4
5
6
+ME
d
m
a
b
c

3.3.1.9 Analysis of 'pure' gliadin and glutenin

This new 2-D method, which allows proteins that are present as disulphide-linked aggregates to be easily separated from the monomeric proteins, was used to determine the composition of gliadin and glutenin fractions prepared by the classical method of differential solubility (Osborne, 1907). Mr. J. A. Bietz, U.S.D.A., Peoria, Illinois, kindly donated samples of gliadin and glutenin which had been prepared by sequential extraction of flour with 0.04M NaCl, 70% ethanol, and 0.7% acetic acid. Wheat flour was first suspended in 0.04M NaCl and shaken for 30 min with sufficient vigour to keep it suspended. After centrifugation of this suspension, the supernatant was discarded and the pellet was resuspended in 0.04M NaCl and centrifuged to remove the remaining albumins and globulins. After this, the pellet was suspended in 70% ethanol to extract gliadins and the remaining pellet was then suspended in 7% acetic acid to extract glutenins. The glutenins solubilized in acetic acid were finally precipitated by adding ethanol to 70% concentration and adjusting the pH to 6.6-8.0 with 2N NaOH in order to remove traces of gliadins not removed by the earlier treatments (see Bietz *et al.* 1975).

The gliadin and glutenin fractions prepared in this way were dissolved separately in SDS-Tri-HCl buffer at pH 6.8 and subjected to 2-D electrophoresis using both unreduced and reduced proteins in the second dimension of electrophoresis. The rod gels containing unreduced gliadins (Fig. 3.13, b) and unreduced glutenin (Fig. 3.13, f), both gave pronounced streaking along the 45° line in the second dimension of separation, but whereas, there were many darkly stained protein spots on the diagonal line from the gliadin sample, there were only a few lightly stained spots on the diagonal for the glutenin sample. In the 2-D gels run after the reduction of proteins, the streaky portion of the diagonal line became much fainter in both of these samples. The reduced gliadin sample (Fig. 3.13, d) showed a trace of HMW glutenin subunits, a few LMW glutenin subunits appearing as parallel lines, and many dense spots on the diagonal, which had mobilities identical to the α, β, γ and ω-gliadins (compare Fig. 3.13, c and d). In contrast, the glutenin sample

Fig. 3.13

SDS-PAGE patterns of 'pure' gliadin and glutenin samples:

a-d gliadin:

a 1-D separation of unreduced (-ME) proteins included for reference;

b 2-D separation (-ME × -ME);

c 1-D separation of reduced (+ME) proteins included for reference;

d 2-D separation (-ME × +ME).

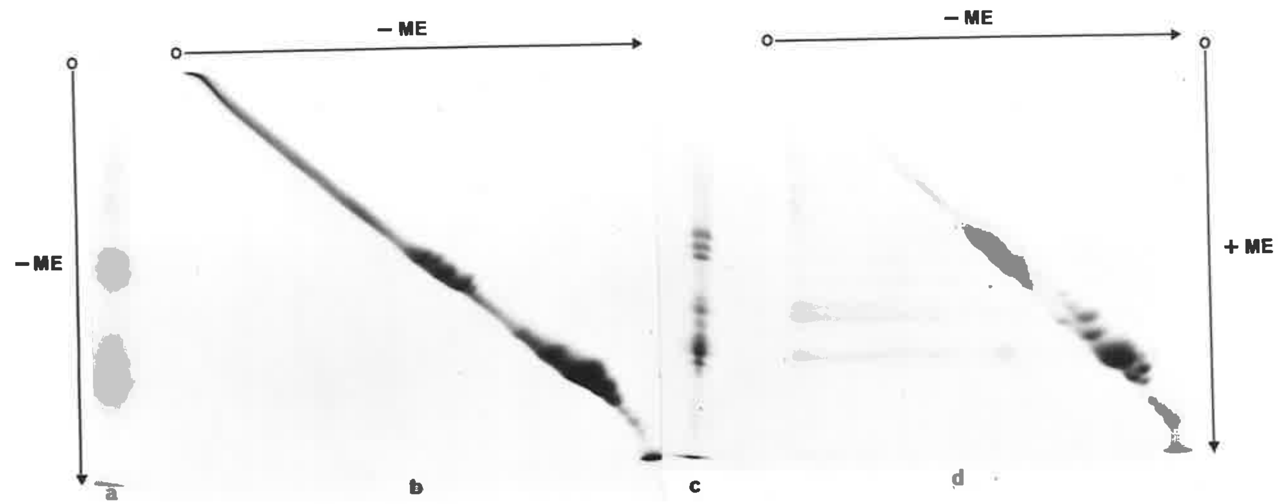
e-h glutenin:

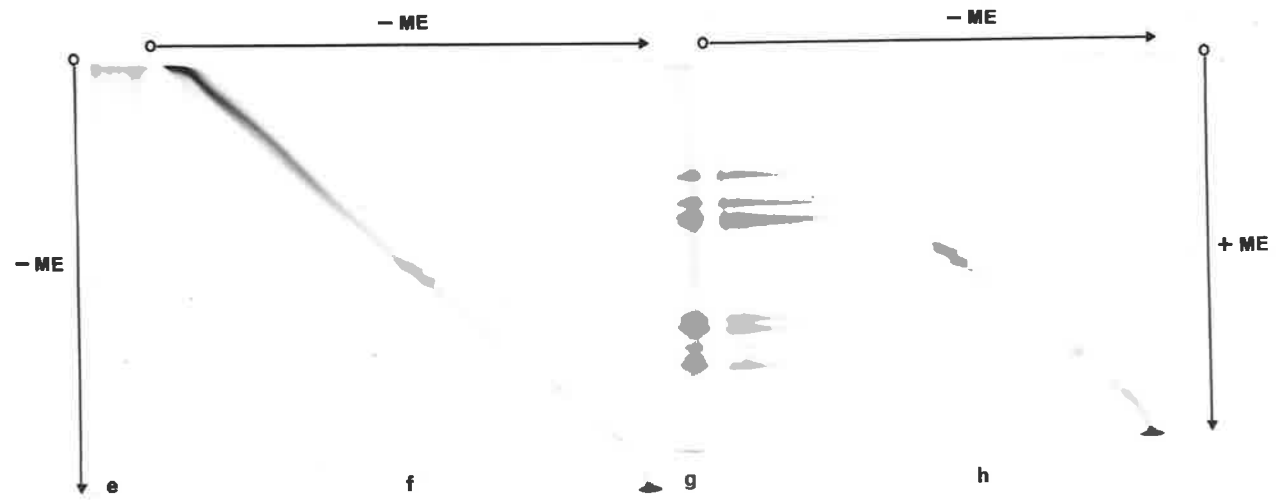
e 1-D separation of unreduced (-ME) proteins included for reference;

f 2-D separation (-ME × -ME);

g 1-D separation of reduced proteins (+ME) included for reference;

h 2-D separation (-ME × +ME).


− ME
− ME
− ME
+ ME
a
b
c
d


− ME
− ME
− ME
+ ME
e
f
g
h

mainly contained the full spectrum of HMW and LMW glutenin subunits appearing as parallel lines (Fig. 3.13, h) . The LMW subunits present in the glutenin sample had similar electrophoretic mobilities to those in the gliadin sample, except one of the prominent LMW subunits in the glutenin appeared to be absent from the reduced gliadin sample. Three prominent spots in the region of ω-gliadins remained on the diagonal line of the reduced glutenin sample, and these showed no indication of being involved in disulphide bonding (Fig. 3.13, h).

These results indicate that the fractions obtained by differential solubility are not pure, since there is some overlap of components in each fraction as also observed in gel filtration studies (Payne and Corfield 1979; Bietz and Wall 1980). However, the present approach is much simpler to apply and moreover, the contaminating components can be visualized in the gels. Finally, it should be noted that there was no indication of triplet bands in either of these gliadin and glutenin samples prepared by sequential extraction of flour with 0.04M NaCl, 70% ethanol and 0.7% acetic acid.

3.3.1.10 Characterization of triplet proteins

(i) Solubility characteristics:

Since triplet proteins could not be detected in the classical gliadin and glutenin fractions, the major storage proteins of the wheat endosperm, their solubility properties were investigated in some detail. Triplet proteins were not present in 0.04M NaCl solution, 70% aqueous ethanol, 0.1M acetic acid or 2M urea extracts of wheat flour at room temperature (Fig. 3.14, a, b, c, d, respectively) These proteins were best extracted when strong dissociating agents such as SDS or sodium dodecanoate were present in the extractant (Fig. 3.14, f, g, respectively). A small amount of triplet proteins was extracted with 6M urea at room temperature or treatment with 1M NaCl solution for 15 h at 60°C but bands were too faint to show up in the photographs (Fig. 3.14, e, h). The most significant difference between the gel patterns of 6M urea and 1M NaCl extracted proteins was the presence of heavy background streaking with the urea extracts. This indicated that the 6M

Fig. 3.14

1-D SDS-PAGE patterns of proteins extracted from the seeds of Chinese Spring tetrasomic 1A using various solvents.

a 0.04M sodium chloride;

b 70% (v/v) aqueous ethanol;

c 0.1M acetic acid;

d 2M urea;

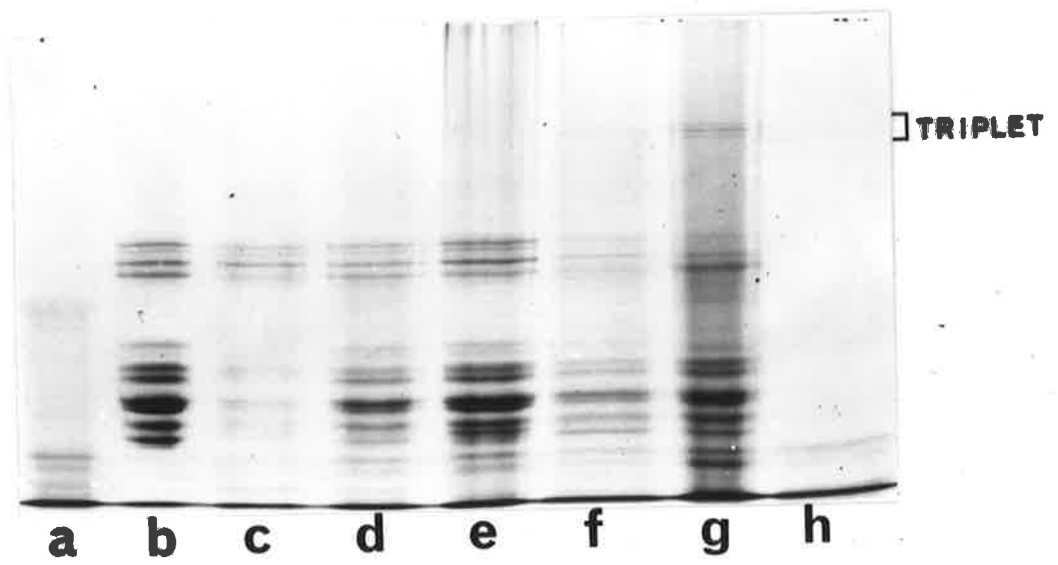
e 6M urea;

f 4% (w/v) SDS in Tris-HCl buffer pH 6.8;

g 1% (w/v) sodium dodecanoate;

h 1M Sodium chloride

Proteins were extracted from endosperm halves of single grains by treatment with 0.1 ml of the above solvents for about 16 h at room temperature except for SDS and sodium dodecanoate (37°C) and 1M NaCl (60°C).


TRIPLET
a b c d e f g h

urea solution extracted gliadins and glutenins along with triplet proteins whereas, salt solution differentially extracted triplet proteins and other globulin type proteins. The triplet proteins were present in gluten balls prepared by washing wheat dough according to the method described by Kent-Jones and Amos (1967) and also in the residue proteins after the sequential extraction of albumins, globulins, gliadins and glutenins according to Osborne's (1907) procedure. Therefore, it is suggested that they occur in the wheat endosperm as HMW aggregates held together by hydrophobic interactions and hydrogen bonds, so that they are not extracted in the normal Osborne fractions. Strong dissociating agents such as SDS are thought to disrupt these forces and to release the triplet proteins for solubilization.

(ii) Subcellular localization in the protein bodies:

Homogenates of developing endosperm of Chinese Spring wheat were separated on a sucrose density gradient [16 to 60 % (w/w)] by ultracentrifugation at 25,000 rpm for 2.5 h. The bottom half of the centrifuge tube showing two clearly visible bands is depicted in Fig. 3.15, A. The cloudy band which corresponds in appearance and position with one previously shown to contain protein bodies (Miflin *et al.*, 1981; Field *et al.*, 1983b), is indicated. The dark region at the bottom of the tube was a starch pellet and the unlabelled band which had a greenish colour has been shown by others to contain endoplasmic reticulum, mitochondria and microbodies (Field *et al.*, 1983b). Fifteen 1.2 ml fractions (numbered 1 to 15) were collected by piercing the bottom of the tube and after dilution with equal volume of distilled water they were pelleted by centrifugation at 40,000 g for 10 min. These pellets were then dissolved in the SDS-Tris-HCl extraction buffer without the addition of ME and the electrophoretic patterns obtained with each of the fifteen fractions are shown in Fig. 3.15, B, b-p. Track 'a' is a control pattern of the total proteins extracted from mature seed of Chinese Spring wheat, with the position of triplet bands and gliadins indicated. Clearly, the triplet proteins are mainly concentrated in the 3rd, 4th and 5th fractions (Fig. 3.15, B, d ,e, f). The average density of these fractions, as indicated in this figure, is comparable with those obtained in earlier studies using same methods, viz 1.268

Fig. 3.15

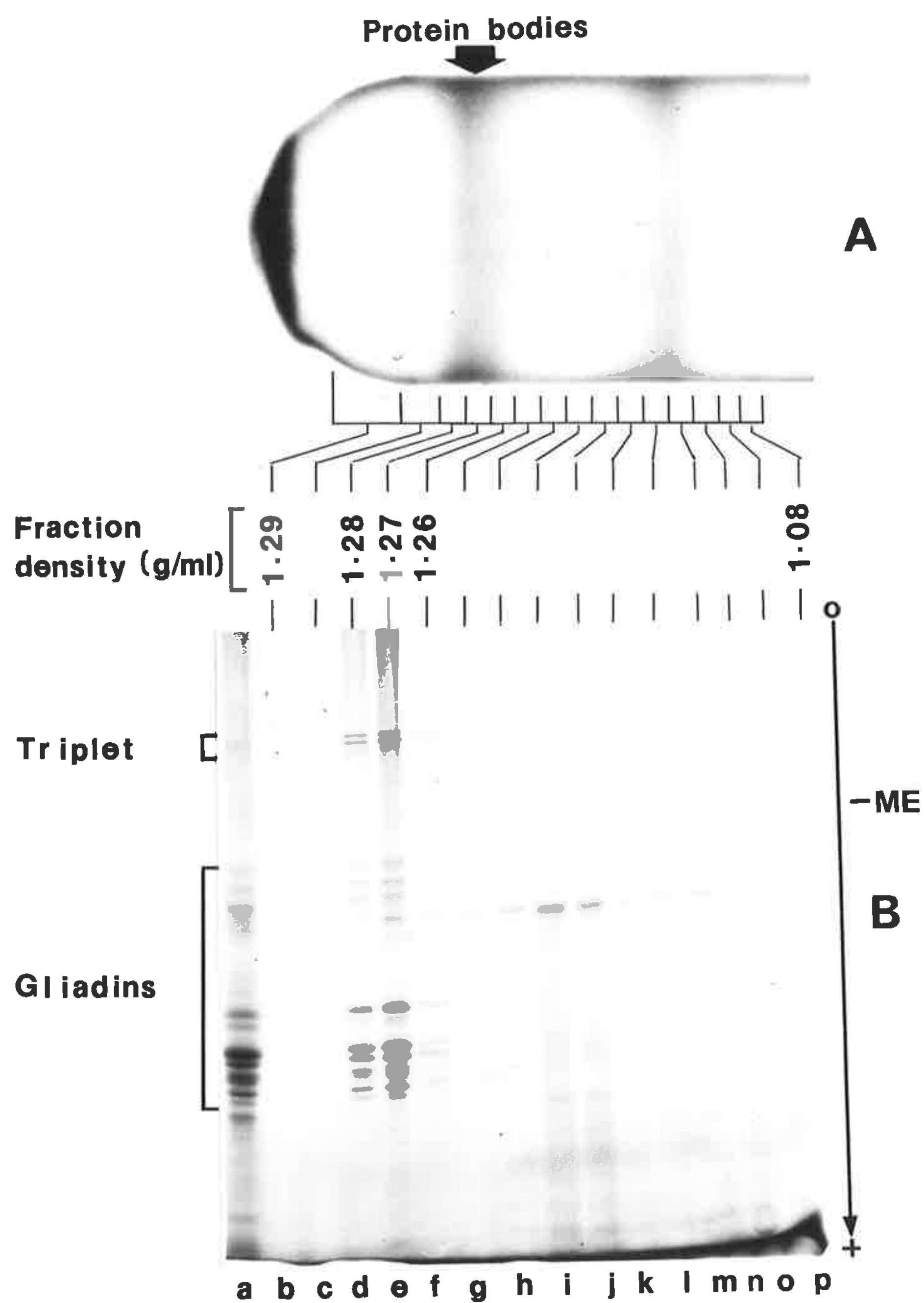
Sucrose density gradient ultracentrifugation of homogenates of the developing endosperms of Chinese Spring wheat and SDS-PAGE patterns of separate 1.2 ml fractions.

A Part of the centrifuge tube showing a portion of the sucrose gradient including the protein body band. The density of the critical fractions of the gradient is indicated.

B 1-D SDS-PAGE patterns of proteins extracted from individual fractions

a reference sample of proteins extracted from mature seeds of Chinese Spring;

b—p proteins extracted from fractions 1—15 of the sucrose gradient.



Protein bodies
A
Fraction density (g/ml)
1·29
1·28
1·27
1·26
1·08
Triplet
Gliadins
-ME
B
a b c d e f g h i j k l m n o p

g/ml (Miflin *et al.*, 1981) and 1.27 g/ml (Field *et al.*, 1983b). Examination of fractions in this region of the gradient by electron microscopy (Miflin *et al.*, 1981) demonstrated conclusively that protein bodies were in these fractions. Furthermore, the electrophoretic studies demonstrated the presence of all the gluten protein subunits in these fractions (Field *et al.*, 1983b). The presence of gliadin storage proteins in these fractions in the present examination, also shows that these are protein body fractions. Electrophoresis of the reduced fractions (data not shown) confirmed that the HMW glutenin subunits are also specifically associated with these three fractions as expected from the heavy background streak in tracks d, e and f in Fig. 3.15, B. The remaining fractions show nil or only trace amount of gliadin and triplet bands. One intermediate-sized protein band is present in all of the fractions, but is most concentrated in the region including and surrounding the unlabelled band in the centrifuge tube (Fig. 3.15, A), and it is thought to be a non-storage protein. From these results, it is concluded that the triplet proteins, like gliadins and glutenins, are deposited in the protein bodies during the endosperm development as storage proteins.

3.3.2 Rye Endosperm Proteins

After the discovery of triplet proteins in the SDS-PAGE patterns of unreduced proteins of wheat endosperm, it was decided to analyse rye proteins using the same methods, to investigate the possible presence of triplet-like proteins in rye. Furthermore, the non-reducing/reducing form of electrophoresis was also employed to investigate the nature of disulphide bonding in rye proteins. The results of the 1-D and 2-D electrophoretic studies on rye proteins are described below.

3.3.2.1 One-dimensional separation of rye endosperm proteins

The banding pattern of reduced protein extracts from Chinese Spring wheat, Imperial rye, their amphiploid (2n=54) which was deficient for a pair of rye chromosomes, and addition

lines of chromosomes 1R and 2R are shown in Fig. 3.16, a-f. The chromosomal location of individal rye bands was similar to that found by Lawrence and Shepherd (1981a). Two HMW glutelin subunits of rye (Fig. 3.16, b, e, labelled as 1R) were present in the 1R addition line and in the amphiploid, although only one of these two subunits matched with the rye parent cultivar (Fig. 3.16, d). Furthermore, the rye parent cultivar showed three very dark staining bands in the region of the gel where bands controlled by chromosome 2R are normally located (Fig. 3.16, labelled as 2R) . However, only the fastest moving of these three bands, was distinguishable in the 2R addition line and in the amphiploid (Fig. 3.16, c, e). The two slower moving 2R bands in these two tracks overlap with wheat band as it was confirmed later that all of the three bands are controlled by 2R (section 3.3.2.3). Because of very slight difference among the mobility of the first two 2R subunits and one wheat subunit this variable overlapping is possible due to slight difference in electrophoretic conditions. The difference between the HMW subunits present in the rye parent cultivar and those present in the amphiploid suggests that the rye plant used in the original cross, to produce the amphiploid (Driscoll and Sears, 1971), possibly had at least one different allele for this protein compared to the seeds from Imperial rye analysed in Fig. 3.16.

The banding patterns of unreduced endosperm protein extracts from the same rye and wheat genotypes in Fig. 3.16, a-f, are shown in Fig. 3.16, g-l in the same order. In the unreduced protein extracts from diploid rye, there was no indication of any protein band corresponding to the triplet bands of wheat (Fig. 3.16, j). The main feature of the unreduced rye proteins was the presence of many discrete slow-moving bands in the cathodal region of the gel, which disappeared after the reduction of disulphide bonds (Fig. 3.16, d). However, these bands were not present in the unreduced extracts of the addition lines and the amphiploid (Fig. 3.16, h, i, k). That is, the aggregation behaviour of these subunits seems to be very different in the wheat background.The only discrete bands in the wheat-rye amphiploid and the addition lines were the normal wheat triplet and a very faint pair of bands in the amphiploid and in the 2R addition line (Fig. 3.16, i, k, band labelled 2R'). Also lower down in the gel there was a group of bands corresponding in mobility

Fig. 3.16

1-D SDS-PAGE patterns of total seed protein extracts from wheat, rye and wheat-rye derivatives.

+ME = reduced proteins; -ME = unreduced proteins.

a, f, g, l Chinese Spring wheat (CS);

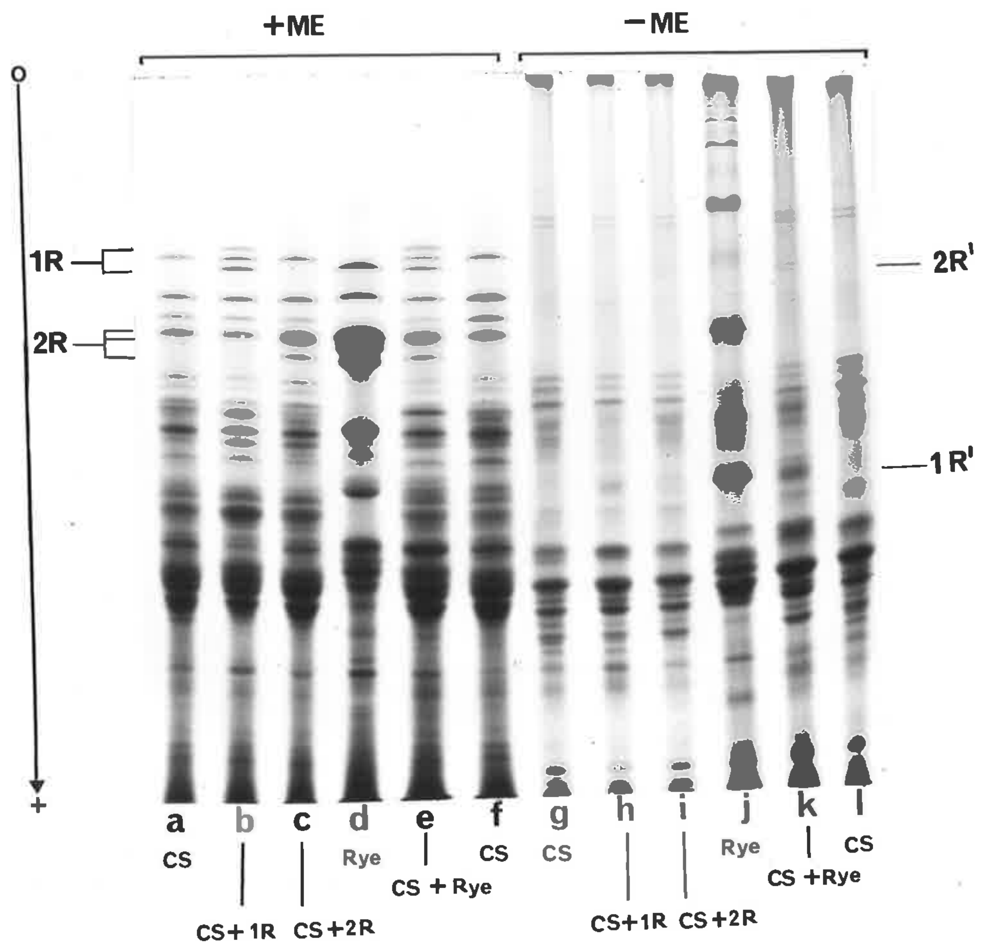
b, h Disomic wheat-rye addition line 1R (Chinese Spring+Imperial rye 1R);

c, i Disomic wheat-rye addition line 2R (Chinese Spring+Imperial rye 2R);

d, j Imperial rye;

e, k Chines Spring+Imperial rye amphiploid (2n=54).

1R = HMW glutelin subunits; **2R** = 75kd γ-secalin subunits; **2R'** = Aggregates controlled by chromosome 2R; **1R'** = ω-secalins.


+ME
−ME
O
1R
2R
2R'
1R'
+
a
b
c
d
e
f
g
h
i
j
k
l
CS
Rye
CS
CS
Rye
CS
CS + Rye
CS +Rye
CS+ 1R
CS +2R
CS+1R
CS +2R

to ω-secalins present in the amphiploid and 1R addition line (Fig. 3. 16, h, k , band labelled 1R') as expected from earlier observations (Lawrence and Shepherd, 1981a; Singh and Shepherd, 1984a).

3.3.2.2 Two-dimensional separation of rye endosperm proteins

The most striking effect of reduction on the proteins from Imperial rye is the disappearance of the series of discrete aggregates of high molecular weight and a marked increase in the staining intensity of three 2R subunits (Fig. 3.16, compare 'd' with 'j'). Two other subunits, thought to be HMW glutelin subunits, also appeared in the reduced rye sample. Thus, it was inferred that the oligomer-like aggregates in the unreduced samples are composed mainly of these 5 subunits linked together by disulphide bonds as suggested by Field *et al.* (1983a,b). The alternative suggestion of Preston and Woodbury (1975) was that these bands may be conformational isomers due to different sites of disulphide bonding within the same polypeptide chains resulting in differential SDS-protein binding which in turn changes the electrophoretic mobility of the SDS-protein complex. However, this is considered to be an unlikely explanation because these discrete HMW bands were also observed when aqueous ethanol or 2M urea extracts were electrophoresed in a SDS-free acid PAGE system in aluminium lactate buffer at pH 3.1 using the method of Bushuk and Zillman (1978), indicating that the presence of these bands was not due to the differential SDS-protein binding. Furthermore, the electrophoretic separation in acid PAGE is based mainly on the intrinsic charge differences and the size of protein molecules and it is very unlikely that the conformational changes would result in such big differences in electrophoretic mobility.

In a one-dimensional separation, however, it was not possible to determine which subunits were involved in the formation of oligomers. This problem was solved by using the non-reducing/reducing form of 2-D electrophoresis so that the breakdown products of the oligomers could be identified by their position vertically below their position in the rod gel after the first separation, analogous to the system used to analyse the composition of

triplet bands. The unreduced rod gels, when run in the second dimension, showed the expected 45° line on which HMW-aggregates were recognizable as dark staining spots in the electrophoretically slow-moving region of the gel (Fig. 3.17, a). Upon reduction, all of these aggregates separated into two faintly stained HMW subunits and three 75kd γ-secalin subunits as parallel lines with superimposed spots(Fig. 3.17, b). Also, unlike in wheat, there was no evidence of any major LMW subunit involved in the intermolecular disulphide bonding. The two HMW subunits of rye, which are homoeologous to HMW glutenin subunits of wheat and D-hordein of barley (Field *et al.*, 1982), were mainly concentrated near the origin of the gel after the first run under non reducing conditions. Most of the aggregates were due to the 75 kd γ-secalin (2R) subunits as inferred by the comparative staining intensity and the distribution of these subunits in the 2-D gel. Two pairs of spots occurred on the continuous lines corresponding to the two slower moving 2R subunits. The third (fastest moving) 2R subunit appeared as a continuous line without any major spot on it, indicating that it has different aggregation behaviour to the other two. Two dark staining spots were also observed on the 45° line where 2R bands joined the diagonal in the reduced sample (Fig. 3.17, b) and these spots were also present in the unreduced sample (Fig. 3.17, a) indicating that in diploid rye some of the 75kd γ-secalins remain free as monomers, or at least are not linked into aggregates by interchain disulphide bonds.

Almost all of the intermediate-sized and LMW proteins in rye, which correspond to ω-secalins and 45kd γ-secalins, showed similar mobility in both unreduced and reduced separations indicating that these are monomeric proteins which do not aggregate by interchain disulphide bonds (Fig. 3.17, a, b). However, one subunit with an electrophoretic mobility between 75 kd γ- and ω-secalins, showed a very faint continuous line (not clear in photograph) suggesting that some other protein subunits may also be involved in disulphide bonding to a limited extent. The decreased mobility of some of the very fast moving spots near the diagonal in Fig. 3.17, b after reduction with ME may reflect the reduction of intrachain disulphide bonds, resulting in the complete unfolding of the polypeptide chains, giving them lower mobility in the gel as already observed in rye

Fig. 3.17

2-D SDS-PAGE patterns of total seed protein extracts from Imperial rye.

a unreduced in both first and second dimensions (-ME × -ME);

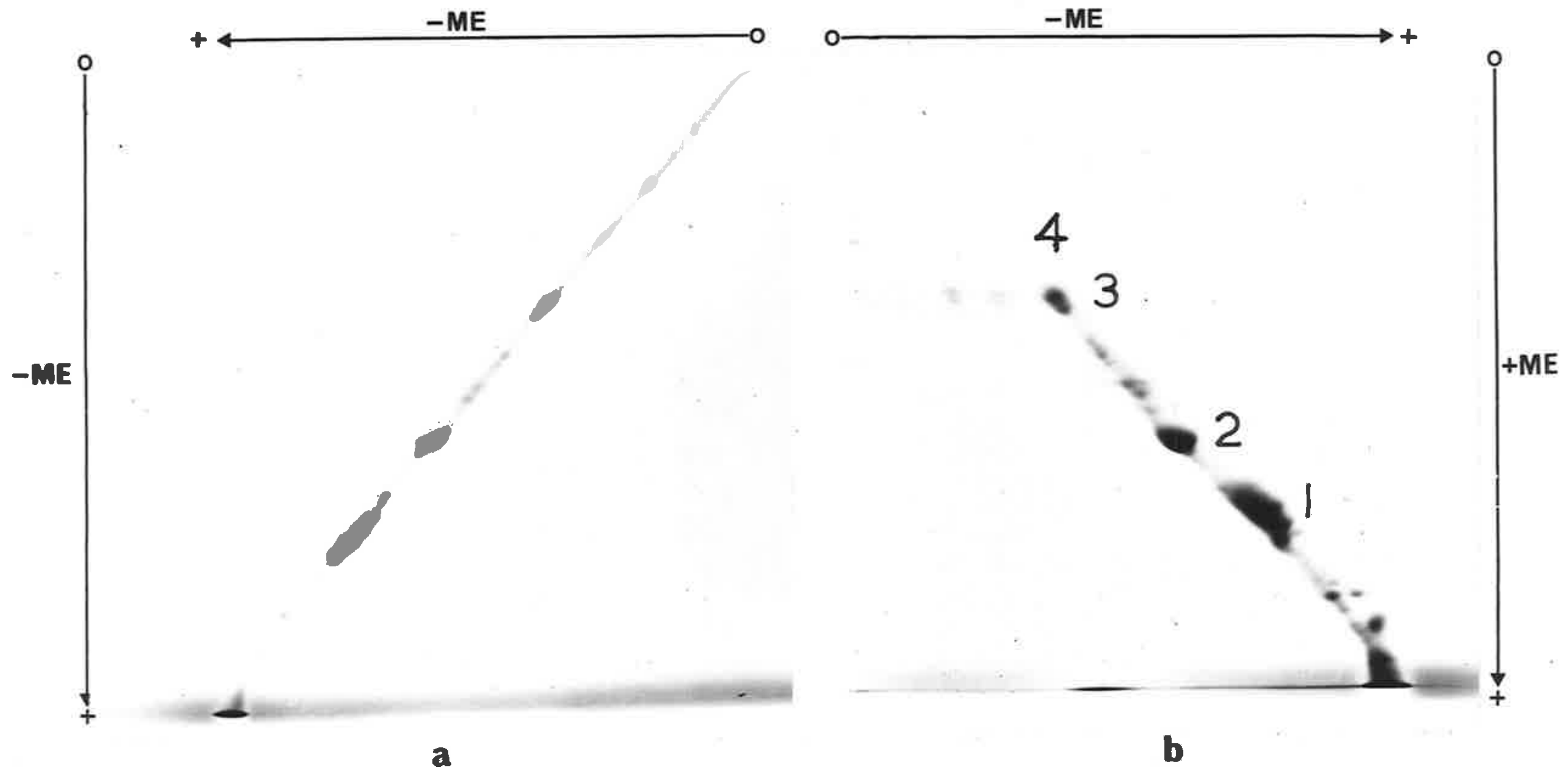
b unreduced in first dimension and reduced in second dimension (-ME × +ME):

1 = 40kd g-secalins;

2 = w-secalins;

3 = 75kd g-secalins;

4 = HMW glutelin subunits.



Imperial Rye

by Caldwell (1983a,b) and in wheat by Woychik *et al.* (1964), Beckwith *et al.* (1965), Nielsen *et al.* (1968) and Singh and Shepherd (1985).

As stated earlier, the discrete oligomer-like bands were also seen in the acid PAGE patterns of rye proteins extracted with 2M urea. A non-reducing /reducing form of 2-D electrophoresis combining acid PAGE (pH 3.1) in the first dimension and SDS-PAGE in the second dimension was employed to determine whether the composition of these bands was similar to the oligomers observed in SDS-PAGE system. Fig. 3.18, A, B depicts two gels, obtained by this 2-D method, showing the electrophoretic patterns of endosperm proteins from Imperial rye. Discrete oligomeric bands can be seen in Fig. 3.18, A, near the origin of the gel. The lack of true diagonal separation of these proteins in the gel is because some proteins have different electrophoretic mobilities in acid PAGE and SDS-PAGE systems. Nevertheless, the relative mobility of the different protein components is comparable in both the systems except for the most prominent protein spot, representing ω-secalin which has much higher mobility in the SDS-PAGE system, thus moving further down the diagonal than expected. The earlier results with non-reducing/reducing 2-D SDS-PAGE are confirmed again here in Fig. 3.18, B, where all of the oligomers were reduced into two HMW subunits and three 75 kd γ-secalin subunits forming continuous lines in the gel. Furthermore, there was no significant change in the electrophoretic mobility of ω- and 45kd γ-secalins after reduction.

3.3.2.3 Aggregation of rye protein subunits when present in wheat endosperm

A comparison of the banding patterns of the unreduced endosperm proteins from rye, wheat-rye amphiploid and wheat (Fig. 3.16, j, k, l) indicated that the aggregating protein subunits of rye do not show discrete oligomeric bands when present in a wheat background. To investigate the way in which they aggregate in wheat, the 2-D SDS-PAGE procedure was used to separate the proteins of the 2R adddition line of Imperial rye into Chinese Spring wheat. This line possesses three 75kd γ-secalin subunits

Fig. 3.18

2-D separations of seed proteins of Imperial rye extracted in 2M urea .

A Non-reducing acid polyacrylamide gel electrophoresis (APAGE) in the first dimension and non-reducing SDS-PAGE in the second dimension:

1 40kd γ-secalin;

2 ω-secalin;

3 HMW oligomers.

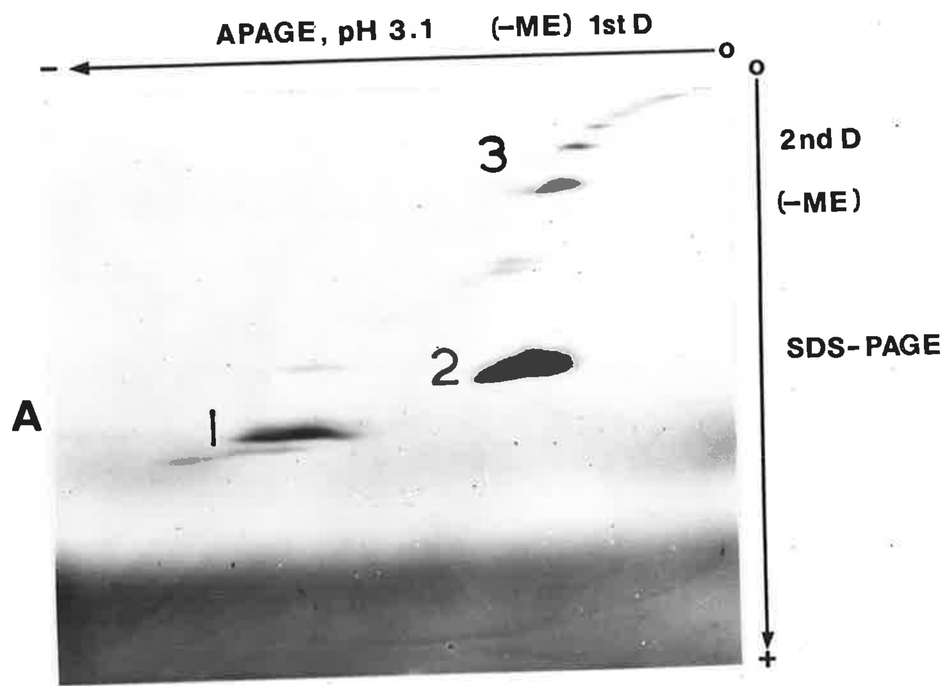
B Non-reducing APAGE in the first dimension and reducing SDS-PAGE in the second dimension:

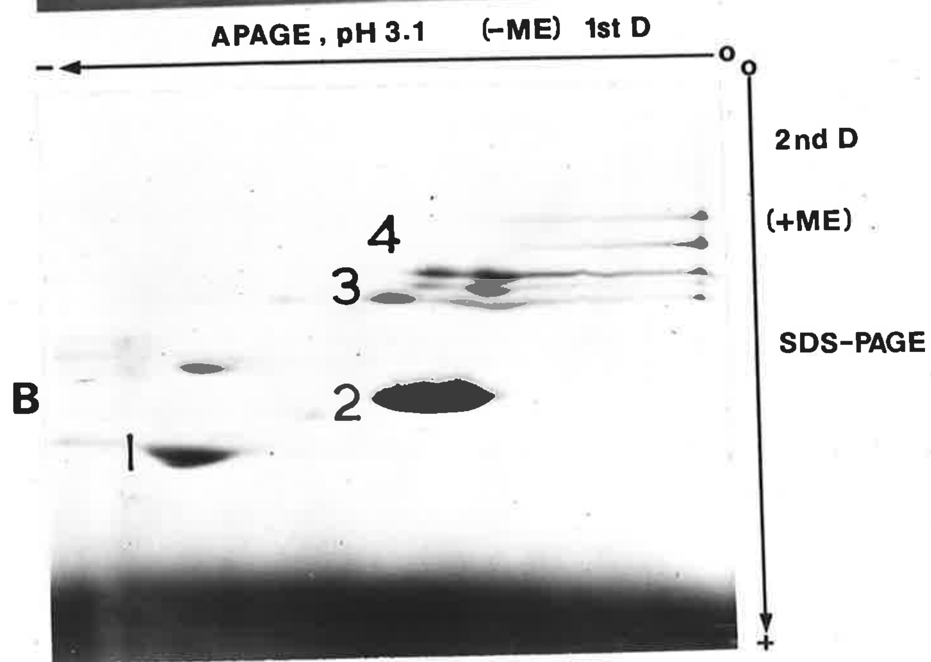
1 40kd γ-secalin;

2 ω-secalin;

3 75kd γ-secalin subunits;

4 HMW glutelin subunits.


APAGE, pH 3.1 (-ME) 1st D
2nd D
(-ME)
SDS-PAGE
A
1
2
3


APAGE, pH 3.1 (-ME) 1st D
2nd D
(+ME)
SDS-PAGE
B
1
2
3
4

(Fig. 3.19, a, labelled as 2R) in addition to Chinese Spring protein subunits (Fig. 3.19, b). However, their aggregation in wheat background appears to be different from that in diploid rye in two ways. Firstly, there are no dark spots on the diagonal at the monomer position of these subunits, and secondly, the spots on the continuous lines are less prominent, a sharp contrast from the banding pattern of these subunits in diploid rye (Fig. 3.17, b). A likely explanation for this is that the rye subunits, when present in a wheat background, combine with wheat subunits, presumably by the formation of disulphide bridges, and hence there are no residual monomers. In diploid rye, where the number of covalently interacting subunits is small, the possible combinations in which a subunit can interact is also limited, resulting in discrete oligomeric bands. In wheat and in the wheat-rye amphiploid and 2R addition line, the number of such subunits is much larger, giving a continuous size-range of aggregates which are not resolved as discrete bands. Another reason why many discrete oligomers could be observed in rye and not in wheat may be the occurrence of a very large amount of 75kd γ-secalin subunits in rye seeds. According to Shewry *et al.* (1983b), these subunits comprise 56±0.6% of the total secalins in mature rye seeds whereas, HMW subunits comprise only 7±0.4%. Thus, because of the limited supply of HMW glutenin subunits and lack of any major covalently interacting LMW subunits, 75kd γ-secalins not only tend to aggregate with themselves but also some of these subunits are left unaggregated as monomers. On the other hand, in a wheat background not only is the proportion of 75kd γ-secalins is relatively small, but also there is a wide range of HMW and LMW subunits available to interact with, therefore, giving a more continuous range of aggregate size.

3.3.2.4 Location of the genes controlling 75kd γ-secalins on rye chromosome arm 2RS

One-dimensional SDS-PAGE patterns of various derivatives of chromosome B (=2R) of Imperial rye, in a wheat background, are shown in Fig. 3.20. Two bands labelled as 2RS have similar electrophoretic mobility to those located on chromosome 2R by Lawrence and

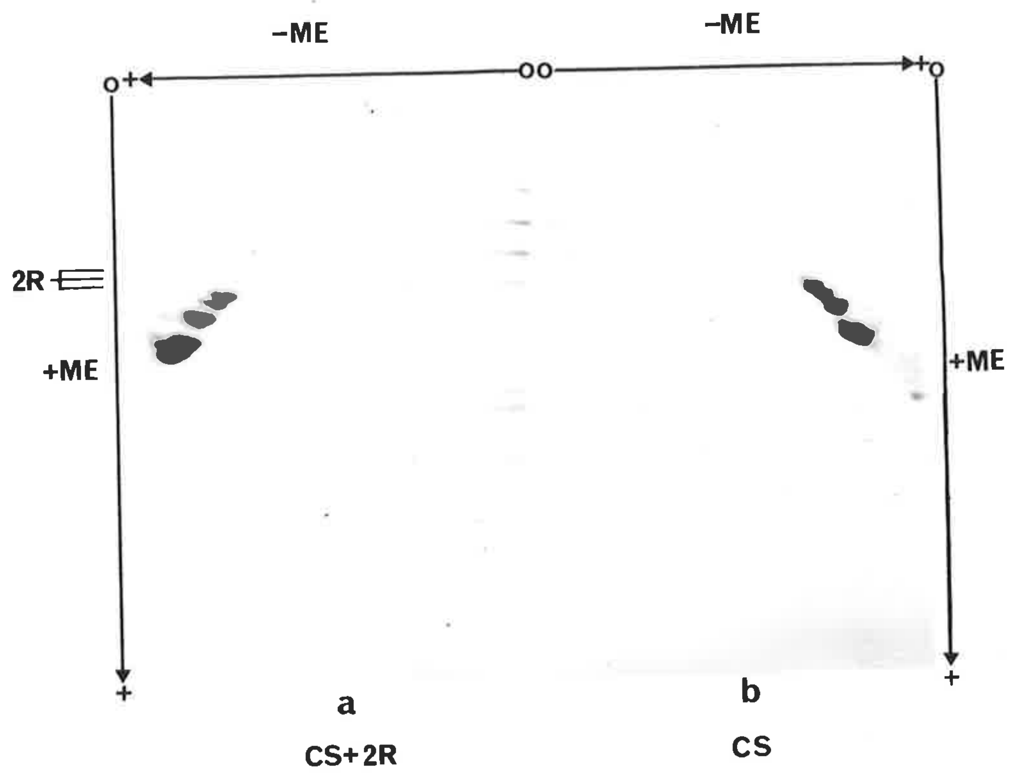
Fig. 3.19

2-D SDS-PAGE (-ME × +ME) patterns of total seed protein extracts from.

a Chinese Spring+ 2R addition line of Imperial rye;

b Chinese Spring wheat.

2R = three subunits of 75kd γ-secalins controlled by chromosome 2R



−ME
−ME
o+
oo
+o
2R
+ME
+ME
+
+
a
CS+2R
b
CS

Shepherd (1980) and later characterized as 75kd γ-secalins by Shewry *et al.* (1982, 1984a) These bands were absent from a Chinese Spring 2BS-2RL translocation stock (Fig. 3.20, a) but could be detected in a stock where complete 2R has been substituted for 2B of Chinese Spring (Fig. 3.20, b). Since complete 2RL arm is present in the former stock (May and Appels, 1980), it was inferred that the genes controlling these subunits are located on the short arm. However, direct evidence was derived by analysing F_2 seeds segregating for a 2BL-2RS translocation (Fig. 3.20, c-f). One of the wheat parents involved in this heterozygote was not Chinese Spring, but the absence of any wheat band in this parent in the gel region having 2R bands (Fig. 3.20, g), facilitated the scoring of the 75kd γ-secalins. Detection of these 75kd γ-secalins in one of the F_2 seeds (Fig. 3.20, d) confirmed that the genes controlling them must be located on chromosome arm 2RS.

Fig. 3.20

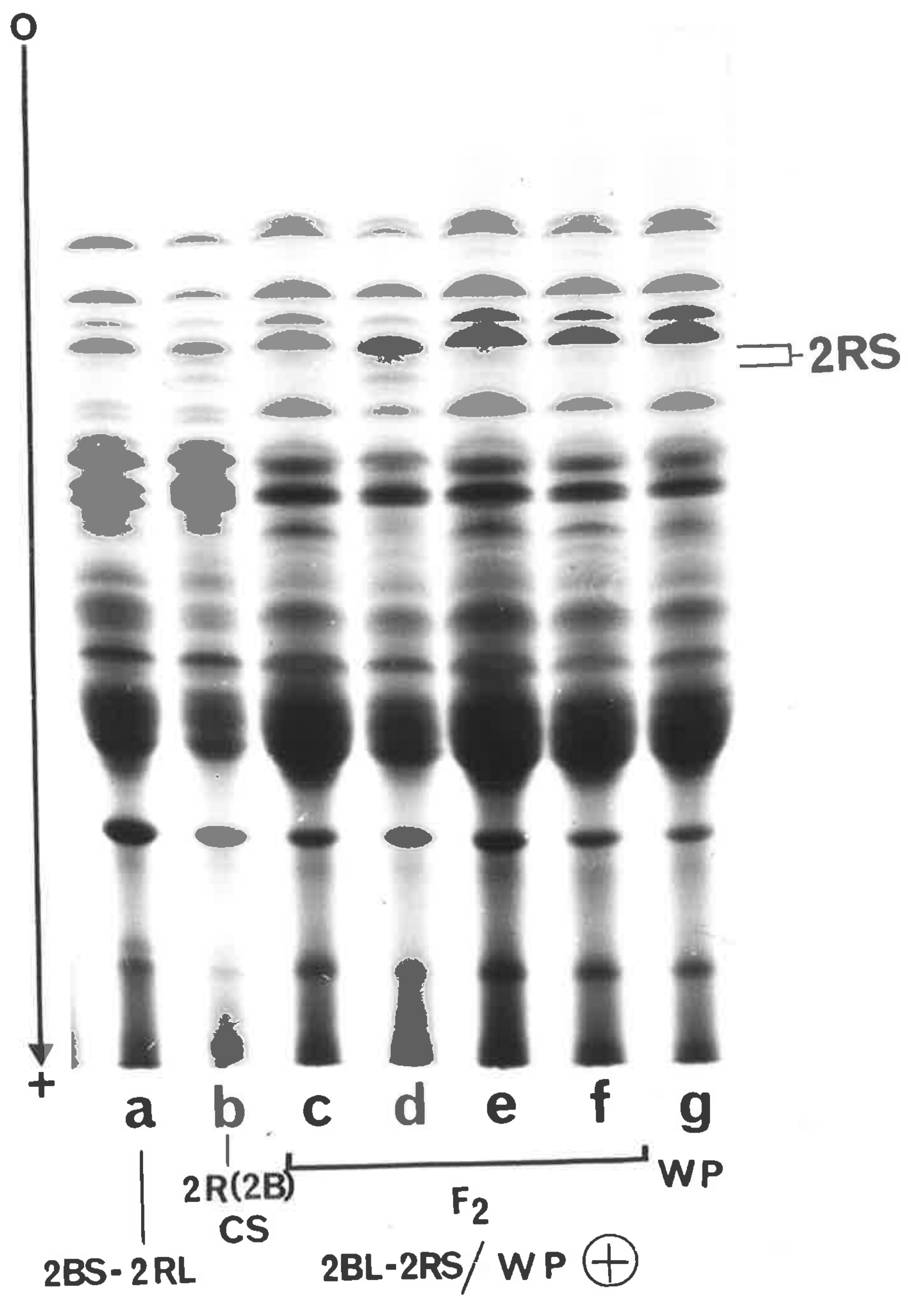
1-D SDS-PAGE patterns of reduced total seed proteinextracts from wheat and wheat-rye (chromosome 2R) derivatives.

a Chinese Spring (CS) translocation 2BS-2RL;

b CS substitution 2R (2B);

c—f F_2 seeds from the cross CS 2BL-2RS × euploid wheat (W P) with sample **'d'** possessing the critical rye bands;

g wheat parent (W P) used in the cross, lacking HMW glutenin bands in the 2RS region.


O
+
2RS
a
b
c
d
e
f
g
2BS-2RL
2R(2B)
CS
F2
2BL-2RS/WP ⊕
WP

3.4 DISCUSSION

The electrophoretic mobility of the triplet bands is different from that of the other known wheat endosperm proteins and therefore they are considered to be a new class of endosperm proteins. In this study attempts were made to characterize these proteins by determining their structure, genetic control and their relationship to the other endosperm storage proteins in wheat and rye. Their behaviour, after reduction with ME, indicates that they are composed of subunits held together by intermolecular disulphide bonds just as proposed for the glutenin protein complex (see review by Lásztity, 1984). However, in contrast to the models suggesting more or less random associations of many different subunits forming native glutenin, the triplet proteins seem to have a preferential tetrameric structure, composed of two large (A=52,000 MW, D=58,000) and two small (α=23,000, δ=22,000) subunits. The simplest model consistent with the available evidence depicts that the large and small subunits associating by disulphide linkage into Aα and Dδ subunit pairs which combine in all possible pairwise combinations, and again by disulphide linkage, to give tetrameric triplet bands Tri-1 (DδDδ), Tri-2 (DδAα) and Tri-3 (AαAα). Since there is no evidence of α subunits in Tri-1 nor δ ubunits in Tri-3 (see Fig. 6), it is concluded that subunit pairs Aδ and Dα are not involved in the formation of triplet bands.

The much stronger staining intensity of the Tri-1 band compared to the Tri-3 band observed in the euploid Chinese Spring, also occurs commonly in other wheat cultivars, even when the equivalent bands have slightly different electrophoretic mobilities to those in Chinese Spring. The observed variations in staining intensity of the triplet bands in aneuploid stocks of Chinese Spring, suggested that the subunits (D, δ) controlled by genes on chromosome 1D are produced in approximately twice the quantity of those (A,α) controlled by chromosome 1A. One possibility is that the genes for triplet subunits are duplicated on chromosome 1D but present as only one copy on chromosome 1A. However, if duplicate loci were present on 1D, then following mutation at one of these two genes, we would expect to find more complex pattern than just three bands, due to the presence of two different alleles at the duplicate loci. In the cultivar survey allelic variants

of Tri-1 were observed but no cultivar showed more than three bands. Therefore it is believed that the difference in staining intensity of Tri-1 and Tri-3 may reflect a difference in the expression efficiency of the triplet genes on chromosomes 1D and 1A, rather than the occurrence of duplicate genes on 1D. However, if the mutational event causing allelic variation occurred before the presumed duplication on chromosome 1D, then we would not expect more than three bands in the cultivars showing allelic variation.

The presence of parallel lines with the same mobility as all four triplet protein subunit spots in the 2-D gels suggests that these subunits also occur in other states of aggregation than tetrameric, particularly in the heterodimeric (Aα, Dδ) and homodimeric (αα, δδ) states. However, no discrete bands or spots having the electrophoretic mobility expected for hexameric or octomeric associations of the triplet subunits were observed. Although at present there is no conclusive evidence, there is a good indication from the behaviour of triplet proteins that they might play a key role in the aggregation of glutenin and hence they might also influence the functional properties of dough. The two main observations that support this theory are: (i) the occurrence of parallel lines corresponding to each of the four subunits of triplet proteins, indicating their presence in the aggregates covering a wide range of sizes (ii) the presence of triplet proteins in the residue fraction of the Osborne solubility classes, and in washed gluten ball, indicating the difficulty in solubilization of triplet proteins because of their possible interaction with other gluten proteins.

The genes controlling the subunits of triplet proteins have been located on chromosome arms 1AS and 1DS, each of which also carries a complex of tightly linked genes controlling gliadins and LMW glutenin subunits (Payne *et al.*, 1984d; Singh and Shepherd, 1984b). The genes controlling the large subunits showed 40.1±2.9% and 40.3±2.6% recombination with the ω-gliadin genes present on chromosome arms 1AS and 1DS, respectively, and were closely linked (11.0 ±1.8% and 14.9± 2.0%) with their respective centromeres (see Chapter 4 for details), but except for their chromosome arm location, there is no precise information on the location of the genes controlling the small subunits of the triplet bands. However, Koebner (1985), in his wheat-rye recombination experiments, has shown that the genes controlling large and small subunits of triplet on

chromosome arm 1DS are not separated. Furthermore, if triplet proteins are indeed equivalent to the 11-12S globulins, the large and small subunits should be due to the post-translational processing of the single gene product (see Chapter 5)

Although the genes on chromosome arms 1AS and 1DS controlling the large subunits of the triplet bands are clearly homoeoallelic, no equivalent locus could be detected on chromosome arm 1BS. Recently, Galili and Feldman (1984) detected a protein band, in the total protein extracts from the endosperm of Chinese Spring—Thatcher 1B substitution line, which is controlled by a gene located between the centromere and the ω-gliadin locus on the short arm of chromosome 1B, showing 25.5% recombination with the ω-gliadin genes and 23.5% recombination with genes controlling HMW glutenin subunits on long arm of chromosome 1B. Although this location is not directly comparable with the position of genes controlling triplet subunits on1AS and 1DS, its intermediate position suggested that it could possibly be a homoeoallele of the triplet genes. Examination of the 2-D pattern of the Chinese Spring—Thatcher1B substitution line in the present study indicated that the protein band detected by Galili and Feldman (1984) normally occurs in an aggregated state until reduced by ME, but it does not seem to be invoved in the formation of triplet bands.

The localization of triplet proteins, along with gliadins and glutenin subunits, in the protein bodies of the developing wheat endosperm indicated that they are also storage proteins. Solubility tests revealed that the triplet proteins are not present in distilled water, 0.04M salt solution, 70% aqueous ethanol or 0.1M acetic acid extracts from wheat endosperm at room temperature. These proteins were recovered only in the presence of strong dissociating agents such as SDS and to a much lesser extent 6M urea . To account for the difficulties in extracting triplet proteins it is postulated that they occur in the wheat endosperm as high molecular weight aggregates in the glutenin complex, held together by hydrophobic or hydrogen bonds, so that they are not extracted in the normal Osborne (1907) fractions. Strong dissociating agents such as SDS are thought to disrupt this hydrophobic or hydrogen bonding and thus release the triplet proteins for extraction. However, a significant amount of triplet proteins could be differentially extracted by

treatment with 1M NaCl solution at 60°C overnight, indicating some similarity to globulins. The solubility of the triplet proteins when present in a free state, is likely to be quite different from that of the postulated aggregates, but there is no information on this.

Thus on solubility criteria the triplet proteins do not correspond to either glutelins or prolamins. Instead, because of their partial solubilty in 1M NaCl and a striking common feature in the subunit structure of the triplet proteins and that of the subunit pairs making up the globulin storage proteins of several diverse plant species, it is considerd that the triplet proteins might be part of the globulin storage proteins of wheat. It has been shown that the subunit pairs of the 11-12S globulins of *Glycine max* (Moreira *et al.*, 1979), *Pisum sativum* (Gatehouse *et al.*, 1980),*Vicia faba* (Matta *et al.*, 1981) and *Avena sativa* (Peterson, 1978) each consist of a large and a small subunit joined by disulphide linkage. The size of these subunits is variable but of the same order as the large and small subunits of the triplet bands. It has been shown that there is much amino acid sequence homology between the small subunits of 11-12S globulins present in such diverse plant species as legumes (Casey *et al.*, 1981) and oats (Walburg and Larkins, 1983), and it has been suggested that the genes controlling the small subunits of oats and legume globulins may have been derived from the same ancestral gene. Clearly additional work needs to be done to characterize more fully the triplet proteins of wheat. Determination of their amino acid content, and particularly their amino acid sequence, would provide the most definitive evidence for deciding whether they also might be globulin storage proteins. The possible relationship of triplet proteins with 11-12S globulins will be discussed latter in Chapter 5.

The present 2-D method utilizing a combination of unreduced and reduced protein samples has demonstrated that the streak observed on SDS-PAGE of unreduced endosperm proteins is due to the presence of a complex mixture of disulphide-linked aggregates of glutenin and triplet protein subunits. Furthermore, this method has provided evidence, in the form of well-defined parallel lines in 2-D separations, that a given glutenin or triplet subunit is present in a wide range of different-sized aggregates. These results support the earlier conclusions of Payne and Corfield (1979) and Bietz and Wall (1980) that the HMW and LMW subunits of glutenin combine at random to give the

continuous array of different-sized aggregates which make up glutenin. As an extension of this model, it is postulated that LMW native glutenin molecules (soluble in 70% ethanol) result when, by chance, only LMW subunits are included in an aggregate, whereas HMW native glutenins (insoluble in 70% ethanol) include both types of subunits. In general, these results parallel those obtained with combined gel filtration and electrophoresis of subfractions (Crow and Rothfus, 1968; Danno *et al.*, 1974; Huebner and Wall, 1976; Payne and Corfield, 1979; Bottomley *et al.*, 1982). However, the present 2-D approach has the clear advantage that it can be applied simply and quickly to protein extracts from single kernels. The disadvantage is that this analysis is restricted to only those aggregates, up to a molecular weight of approximately one million, which will enter a 10% acrylamide gel. This problem can be solved partly by reducing the acrylamide concentration in the disc gels, but since glutenin is thought to contain aggregates with molecular weights of several million, a large proportion will still be excluded from the 2-D separation. However, this is not a problem if these higher molecular weight aggregates of glutenin are composed of the same subunits as those which enter the 10% acrylamide gels, as seems likely from the results of gel filtration studies. This 2-D method has proven particularly useful for separating the LMW subunits of glutenin from classical gliadins, which have similar electrophoretic mobilities on SDS-PAGE. Based on this approach, a two-step 1-D procedure was developed which allows the LMW glutenin subunit composition of 20 samples to be analysed in a single slab gel. This procedure has greatly facilitated the analysis of large numbers of progeny for mapping the genes controlling LMW glutenin subunits (see Chapter 4 for details).

When unreduced protein extracts from the seeds of cereal rye *cv.* Imperial were separated by SDS-PAGE, they did not produce the zone of heavy streaking obtained with wheat samples. Instead, only light background streaking was observed but the most noticeable effect was a series of discrete bands in the cathodal half of the gel. Most of these bands were found to be a series of oligomers involving 75kd γ-secalins controlled by genes on chromosome 2R (Lawrence and Shepherd, 1981a; Shewry *et al.*, 1984a). The only other aggregating subunits detected in rye were the HMW glutelin subunits

controlled by chromosome arm 1RL (Lawrence and Shepherd, 1981a; Singh and Shepherd, 1984a) and aggregates involving these subunits remained near the origin of the separating gel. In Imperial rye, no major LMW aggregating subunits with similar mobility to the LMW glutenins of wheat were detected. However, one such subunit was detected in King II rye. Thus, in contrast to bread wheat, it seems that cereal rye possesses few if any major LMW aggregating subunits. Most of the LMW proteins in rye remained on the diagonal after 2-D electrophoresis, and it is concluded that these represent ω- and 40kd γ-secalins which are related to the monomeric (α, β, γ and ω) gliadins of wheat (Shewry *et al.*, 1984 b) .

Although the tendency of the oligomeric aggregates to form a streak increases with an increase in the number of interacting subunits, the association between the subunits does not seem to be completely random. It is clear from the presence of discrete triplet bands in the unreduced protein extracts of wheat and wheat-rye derivatives that there is some preferential association between the triplet protein subunits. This may be due to one or both of the following factors : (i) variation in the aggregating ability of the polypeptides due to the number and position of cysteine residues or other amino acid residues influencing the secondary and tertiary structures of protein molecules; (ii) Non-random availability of different subunits after translation at a particular time and place in the cytoplasm during endosperm development. Jones and Carnegie (1971) have suggested that the disulphide linked gluten polymers are only formed as a result of the dehydration and rehydration that occurs during grain maturation and dough formation. But recent studies by Field *et al.* (1983b) indicate that aggregates are possibly formed shortly after protein synthesis or during their deposition in the protein bodies and that the rough endoplasmic reticulum is the probable site of aggregation through disulphide bonds. Isolation of aggregated triplet proteins and glutenins from the 21-day old endosperms of wheat in the present study also suggests that the disulphide bonding occurs before the seed maturation phase. The possibility that these aggregates could have been formed *in vitro* during protein extraction was ruled out because under similar protein extraction conditions, a physical mixture of the flour from two wheat stocks, one containing only Tri-1 band and

the other only Tri-3 band of the triplet proteins, did not produce the hybrid band Tri-2 indicating that the disulphide bonds were not formed during protein extraction. If this is correct, then the synchronized synthesis of different subunits in a particular location in the cytoplasm will be important for their preferential aggregation.

The correlation between certain HMW glutenin subunits of wheat and bread-making quality (Payne *et al.*, 1979, 1981b; Burnouf and Bouriquet, 1980; Moonen *et al.*, 1982, 1983) is ascribed to the superior aggregating ability of these subunits. No similar work has been reported so far with the LMW-glutenin subunits to evaluate the relative ability of these subunits to alter bread-making quality. It is still to be determined which of these two groups is more important. Since LMW glutenin subunits comprise about 30% of the total wheat endosperm storage proteins, as compared to the HMW glutenin subunit which comprise only about 10% of the total, their effect on protein quality is expected to be quite significant. Wrigley *et al.* (1982b) have found that the flour quality-protein associations were stronger with gliadin than with HMW glutenin subunits and since genes controlling gliadins are very tightly linked with those controlling LMW glutenin subunits, it may be that quality associations observed by Wrigley *et al.* (1982b) were due to the LMW glutenin subunits. Lack of a suitable electrophoretic technique to fractionate the LMW glutenin subunits has prevented the comprehensive studies on the association of these subunits with flour quality. It is shown later (see Chapter 4) that the two-step 1-D procedure, developed in the present work, is very efficient for separating and analysing these subunits.

Another aspect requiring further study is to find the influence of the 75kd γ-secalins on the bread-making quality of wheat flour. Since these subunits exhibit good aggregating ability they may improve the strength of the dough derived from the lines possessing these subunits.

Chapter 4

LINKAGE MAPPING OF THE GENES CONTROLLING ENDOSPERM STORAGE PROTEINS ON GROUP 1 CHROMOSOMES IN WHEAT AND RYE

4.1 INTRODUCTION

As reviewed in Chapter 2, most of the gliadins are monomeric proteins which have been classsified into α, β, γ and ω components by electrophoresis in starch (Woychik *et al.*, 1961) or polyacrylamide gels (Bushuk and Zillman, 1978), at acid pH. Genes controlling all of the ω-gliadins, most of the γ-gliadins and a few of the β-gliadins have been located on the short arms of chromosomes 1A, 1B and 1D (Shepherd, 1968; Wrigley and Shepherd, 1973). In addition, the genes controlling the synthesis of LMW glutenin subunits (Jackson *et al.*, 1983) and triplet proteins (Singh and Shepherd, 1985 and Chapter 3 of this thesis) have been located on these same chromosome arms. The genes controlling the monomeric gliadins on chromosomes 1A,1B and 1D, have been respectively designated *Gli-1A, Gli-1B* and *Gli-1D* by Sozinov and Poperelya (1980) and *Gli-A1, Gli-B1* and *Gli-D1* by Payne *et al.* (1982b). The linkage relationship between the gliadin genes at each of these loci has been studied by several research workers but most extensively by Sozinov and Poperelya (1980) and Metakovsky *et al.* (1984). The gliadin proteins controlled by genes on each of these chromosome arms were shown to be inherited as a unit showing no recombination, or very rare recombination. Recent studies on the linkage relationship between the genes controlling monomeric gliadins and LMW glutenin subunits on chromosome arms 1AS and 1BS, have shown that these genes are also very closely linked with no recombination being observed except in one of the experiments involving the loci on chromosome 1B (Payne *et al.*, 1984d). Thus these genes coding for different groups of monomeric gliadins and LMW glutenin subunits on the short arms of group 1 chromosomes are thought to be part of a complex locus made up

of a cluster of very tightly linked genes.

The long arms of chromosomes 1A, 1B and 1D carry the genes controlling synthesis of HMW glutenin subunits (Orth and Bushuk, 1974; Bietz *et al.*, 1975; Brown *et al.*, 1979,1981; Lawrence and Shepherd, 1980, 1981a; Payne *et al.*, 1980, 1981a; Galili and Feldman, 1983a, b) and these genes have been designated as *Glu-A1, Glu-B1* and *Glu-D1*, respectively (Payne *et al.*, 1982b). At least two of these loci (*Glu-B1* and Glu-D1) are complex and code for more than one protein subunit. However, once again the genes controlling these must be very tightly linked because they do not recombine (Payne *et al.*, 1980; Lawrence and Shepherd, 1981b). The *Gli-1* and *Glu-1* loci, located on the short and long arms of homoeologous group 1 chromosomes, respectively, have been shown to segregate independently (Lawrence and Shepherd, 1981b; Chojecki *et al.*, 1983) or, sometimes to exhibit loose linkage (Payne *et al.*, 1982b). More accurate information on the location of these two loci has come from mapping experiments which have included an additional locus, or the centromere, as a reference point between them. The *Gpi-D1* locus, coding for an isozyme of glucosephosphate isomerase has been located proximal to the *Gli-D1* locus on chromosome arm 1DS and it showed 34.5 % recombination with *Gli-D1* and 36.2 % recombination with *Glu-D1* gene on the long arm (Chojecki *et al.*, 1983). Also, Galili and Feldman (1984) showed that the gene controlling a particular gliadin band (B-30, according to their nomenclature) mapped proximally to the *Gli-B1* locus and showed 25.5 % recombination with it, and 23.5 % recombination with the *Glu-B1* locus on the long arm. Furthermore, the *Gli-B1* locus has been located cytologically in the satellited region of chromosome 1B (Payne *et al.*, 1984a).

Comparable results have been obtained with cereal rye where genes controlling secalins are situated on the short arm of chromosome 1R (Shepherd, 1968; Shepherd and Jennings, 1971) and those controlling HMW glutelin subunits are located on the long arm (Lawrence and Shepherd, 1981a). As in wheat, these two loci on chromosome 1R are widely separated (Shewry *et al.*, 1984a) and the locus *Glu-R1* on the long arm, is closely linked to the centromere (Singh and Shepherd, 1984a). The locus controlling ω-secalins has been designated *Sec-1* by Shewry *et al.* (1984a), and that controlling HMW glutelin

subunits, *Sec-3* (c.f. *Glu-R1* of Singh and Shepherd, 1984a). The 40kd γ-secalins have not yet been assigned a gene symbol. The locus on chromosome 2R, controlling a group of 75kd γ-secalins has been designated *Sec-2* by Shewry *et al.* (1984a).

Although considerable progress has been made in the last few years towards mapping of endosperm protein genes in wheat, none of these findings was published before the present project was initiated in 1981. Furthermore, some important genetic linkages are still unknown, for example none of the seed protein genes located on the short arms of group 1 chromosomes of wheat and rye has been mapped directly with respect to the relevant centromere except the one gliadin gene on 1BS which showed 42% recombination with the centromere (Rybalka and Sozinov, 1979). Also there is no published report on the mapping of the genes controlling triplet proteins on chromosome arms 1AS and 1DS and the genes controlling LMW glutenin subunits on 1DS. However, using the telocentric mapping method of Sears (1962, 1966b), Payne *et al.* (1982b) have shown 7.6%, 9.2% and 10.1% recombination between the *Glu-A1, Glu-B1* and *Gli-D1* loci and the respective centromeres on the long arms of group 1 chromosomes of wheat. It seems very likely that these recombination values are underestimated as it has been shown in cotton (Endrizzi and Kohel, 1966) and in wheat (Sears, 1972) that for proximal loci, closely linked to the centromere, there is a drastic reduction in the recombination values when telocentric mapping is used as compared with mapping the complete chromosome.

In this chapter an alternative translocation mapping method is described which was used to map the centromere-gene distances for the genes controlling HMW glutenin subunits, monomeric gliadins, triplet proteins and LMW glutenin subunits on homoeologous group 1 chromosomes of wheat and the genes controlling HMW glutelin subunits, secalins and stem rust resistance on chromosome 1R of rye. Some of these results are compared with the telocentric mapping results obtained from parallel experiments carried out in the present study and also with those obtained elsewhere by other researchers.

4.2 MATERIALS AND METHODS

4.2.1 Genetic Stocks Analysed

4.2.1.1 Wheat cultivars and breeding lines

Seeds of all these materials were maintained at the Waite Agricultural Research Institute and were kindly provided by either Dr. A.J. Rathjen or Dr. K.W. Shepherd. These included cultivars Chinese Spring, Hope, Gabo, India 115, Kite, Halberd and Heron and breeding lines MKR/211/7 and WR/17.

4.2.1.2 Intervarietal chromosomal substitution lines

These included disomic substitution lines Chinese Spring—Hope 1A and Chinese Spring —Hope 1B which, respectively, possess a pair of 1A and 1B chromosomes of Hope substituted for their counterparts of Chinese Spring. These two lines were initially produced by Sears *et al.* (1957) and seeds were obtained from Dr. R.A. Boyd, University of Western Australia.

4.2.1.3 Ditelocentric lines

Seeds of the Chinese Spring ditelocentric (Dt) lines Dt 1AL, Dt 1BL, Dt 1DL and Dt 1BS, initially produced by Dr. E.R. Sears (Sears and Sears, 1978) were obtained from the stocks maintained at the Waite Agricultural Research Institute.

4.2.1.4 Wheat-rye translocation lines

All six combinations of wheat-rye translocation lines, involving homoeologous replacement of each arm of the group 1 chromosomes of wheat, with one or other arm of

chromosome 1R of rye were available. These translocations are thought to have arisen by the fusion of wheat and rye telocentrics produced after simultaneous misdivision of wheat and rye univalents in double monosomics. Thus it is assumed that the break point in these translocation chromosomes is at the centromere giving a chromosome made up of one complete arm of rye chromosome 1R joined to a non-homoeologous complete arm of a group 1 wheat chromosome. The origin and genetic background of these lines are described below.

(i) Rye short arm translocations:

1DL-1RS: This line was isolated by Shepherd (1973) from among the progeny of a Chinese Spring wheat plant double monosomic for chromosomes 1D and 1R from Imperial rye ($20^{II} + 1D^{I} + 1R^{I}$). Seed prolamin markers were used to select progeny having the 1RS prolamins and lacking the 1DS prolamins. Most of the progeny carried a complete 1R chromosome substituted for chromosome 1D, but in three cases it was deduced from chromosome pairing that 1RS must be joined to 1DL. The chromosome arm 1RS was derived from the Chinese Spring-Imperial rye addition line 'E' (=1R) produced by Driscoll and Sears (1971), and it carries a gene(s) for stem rust resistance.

1BL-1RS: This line was isolated by Shepherd (unpublished) while attempting to transfer the complete 1R substitution of Imerial rye for 1B in Chinese Spring to a Gabo background by backcrossing. During the 4th backcross to Gabo a line was isolated which, from chromosome pairing studies, possessed a spontaneous 1BL-1RS translocation instead of the complete 1R chromosome.

1AL-1RS: This line was produced in a Chinese Spring wheat background during the course of the present study, using the method of Shepherd (1973). However, since chromosome arm 1AL of Chinese Spring does not carry any seed protein marker, the double monosomic line ($20^{II} + 1A^{I} + 1R^{I}$), required as a starting point, was produced by crossing Chinese Spring-Hope 1A substitution line to Chinese Spring-Imperial rye 1R(1A) substitution line. Thus chromosome arm 1AL present in the selected translocation line is derived from the cultivar Hope.

(ii) Rye long arm translocations:

Translocation lines 1AS-1RL (Singh and Shepherd, unpublished, see Driscoll, 1983), 1BS-1RL and 1DS-1RL (Lawrence and Shepherd, 1981a) were all selected in a Chinese Spring background and chromosome arm 1RL in each of these lines was derived from Imperial rye. These lines were isolated using the same procedure of selecting progeny from double monosomics by their protein phenotypes as originally used by Shepherd (1973) for the isolation of 1DL-1RS translocation.

4.2.1.5 Wheat-rye substitution lines

The 1R(1B) and 1R(1D) substitution lines were produced by Lawrence (1969) using the relevant monosomics of Chinese Spring wheat crossed to the Holdfast wheat + King II rye chromosome V(=1R) addition line (Riley and Chapman, 1958). Hence these lines possessed 1R of King II rye present in a mixed wheat background of Holdfast and Chinese Spring.

4.2.1.6 Special parents for test-crosses

Special lines were chosen as male parents in test-cross programs to minimize the overlap of the protein bands coming from the male parents with the segregating bands to be scored in the test-cross progeny.

(i) Single glutenin line:

This line was isolated in a mixed background from intercross between Chinese Spring × Gabo × NapHal (see Lawrence and Shephed, 1981b). It possesses a single HMW glutenin subunit controlled by chromosome 1B of Gabo combined with null alleles for HMW glutenin subunits controlled by chromosomes 1A of Chinese Spring and 1D of NapHal.

(ii) Null Glu-D1/null Gli-D1 line:

This line, which lacks HMW glutenin subunits and ω-gliadins normally controlled by chromosome 1D, was isolated in a mixed wheat background from amongst the selfed progeny of a F_1 hybrid between the single glutenin line and cultivar India 115 which is null for the 1D ω-gliadin bands.

4.2.2 Crossing Procedures

4.2.2.1 Translocation mapping

The test-cross procedures used for mapping individual loci controlling the synthesis of endosperm proteins are described in detail in the Results section. In outline, each procedure was based on producing F_1 plants which were heterozygous for electrophoretically different alleles at the locus to be mapped on group 1 chromosomes, as well as heterozygous for a translocation chromosome involving one arm of rye chromosome 1R. The F_1 plants produced 20 normal pairs of wheat chromosomes plus one regular rod bivalent where the non-homologous wheat and rye arms remained unpaired. In this way the translocation chromosome could be used to mark the position of the centromere in the mapping experiments. The parents were chosen so that each of the four arms of the bivalent containing the translocation had recognizably different endosperm protein markers. To complete the test-cross the F_1 heterozygotes were crossed as female to a third parent which had no, or only a few, endosperm protein bands overlapping those produced by the genes on the critical rod bivalent in the F_1. With this procedure the parental, recombinant and aneuploid protein phenotypes of the test-cross seeds could be scored in the gels without difficulty. Whenever there was any doubt about the classification, the embryo halves of the test-cross seeds were grown and then progeny tested for confirmation.

In one experiment F_2 mapping was used in addition to test-cross mapping to map the genes controlling HMW glutenin subunits on chromosome arm 1AL.

4.2.2.2 Telocentric mapping

The telocentric mapping method developed by Sears (1962, 1966b) was also used to map the genes controlling HMW glutenin subunits on chromosome arm 1DL and those controlling the ω-gliadins on chromosome arm 1BS. The parent lines were the same as used for the translocation mapping of these genes, except Chinese Spring Dt 1DL and Dt 1BS were used in place of Chinese Spring translocation lines 1DL-1RS and 1BS-1RL, respectively. These experiments were conducted in similar environments to those employing the translocation mapping procedure, so that these two procedures could be compared.

4.2.3 Cytological Analysis

Pollen mother cells (PMCs) from the F_1 heterozygotes were analyzed to find the degree of pairing between the wheat-rye translocation chromosomes and their normal group 1 wheat chromosome homologues. When the long arms were present as homologues, pairing at metaphase I of meiosis was usually much higher than that when the short arms were homologous. In the latter examples, PMCs were also analysed at diakinesis to find whether the low level of metaphase I pairing observed might be due to desynapsis (precocious terminalisation of chiasmata). The following staining procedures were used.

4.2.3.1 C-banding

The long arm of chromosome 1R possesses a well-defined telomeric heterochromatic band and PMCs from all F_1 heterozygotes containing this chromosome arm were stained with a C-banding procedure, modified from Vosa and Marchi (1972), as follows:

Individual anthers at the required stage of meiosis (metaphase I or diakinesis) were selected and fixed in a freshly prepared solution of 3 absolute ethanol : 1 glacial acetic acid. After fixation for 30 min to 2 h at room temperature, PMCs were dissected out into 45%

(v/v) glacial acetic acid on a slide and squashed. The coverslips were removed using liquid nitrogen and the slides were then dried on a hot plate at low setting for about 20 min and then left at room temperature in a desiccator for one to three days. The dried slides were treated with a filtered solution of saturated barium hydroxide [$Ba(OH)_2$] at room temperature for 5-10 min. The exact treatment time varied with the particular batch of $Ba(OH)_2$ used and it had to be standardised in each experiment. After $Ba(OH)_2$ treatment the slides were rinsed in distilled water and treated with SSC (saline sodium citrate) solution, containing 17.43% (w/v) sodium chloride and 8.82% (w/v) tri-sodium citrate, at 60°C for 30-40 min. After rinsing in distilled water, the slides were stained in 7% (v/v) Gurrs Improved R66 Giemsa stain in 0.03M Sorensen's phosphate buffer (pH 6.8) for 10-30 min. The slides were then rinsed in distilled water, air dried and mounted in euparal.

4.2.3.2 Feulgen staining

PMCs from F_1 heterozygotes that did not have any rye chromosome with pronounced heterochromatic banding (those involving telocentric chromosomes or 1RS from Imperial rye), were stained using the Feulgen procedure. Individual anthers at the required stage of meiosis, were selected and fixed in 3 absolute ethanol : 1 glacial acetic acid for 12-24 h at 4°C. The anthers were hydrolysed in 1N HCl at 60°C for 14 min and then stained with Feulgen stain for approximately 2 h at room temperature, before being squashed in 45% acetic acid for microscopic examination.

4.2.4 Reaction to Stem Rust

In the experiment designed to map the genes on chromosome arm 1RS controlling ω-secalins, opportunity was taken to map the gene(s) controlling resistance to stem rust (*Puccinia graminis tritici*). The reaction of the progeny to stem rust was determined as follows.

Test-cross seedlings were grown, along with the parent lines as controls, in 15 cm pots (9 seedlings per pot) to the two leaf stage. They were then inoculated with the spores of stem rust (strain 343 - ANZ 1, 2, 3, 5, 6) and kept in a highly humid environment in plastic bins for 24 h. The plants were then removed from the bins and left in the glass-house for another 14 days when their reaction to stem rust was recorded. The plants were classified into resistant and susceptible classes based on the relative size of pustules and the presence or absence of necrosis in the infected regions of the leaves.

4.2.5 Extraction of Seed Proteins and Electrophoretic Separation

Total seed proteins were extracted from the endosperm halves of the single kernels as described earlier (Chapter 3.2.2). Except where reduced proteins could be used to detect all the relevant protein bands, unreduced proteins were extracted first with SDS in Tris-HCl buffer (pH 6.8) without mercaptoethanol and separated by SDS-PAGE to score for triplet proteins and prolamins. These extracts were then reduced with a drop of mercaptoethanol added to each sample tube and were subjected to further 1D SDS-PAGE to allow the HMW glutenin subunits to be scored. In some experiments, especially with mapping genes controlling secalins on 1RS, better resolution of prolamins was obtained using an ethanol [30% (v/v)]—sucrose [20% (w/v)] mixture as the extractant but the triplet bands were not present in these extracts. SDS-Tris-HCl buffer containing ME was added to the sample tubes for the subsequent analysis of HMW glutelin subunits.

The LMW glutenin subunits were separated either by the modified 2-D electrophoresis already described in Chapter 3.2 (see Figs. 3.5 and 3.7) or by a two-step (2-S) electrophoresis which is described below. In the first step, 30 µl of unreduced total protein extract from each of 20 test-cross seeds is loaded onto the usual 1.4 mm thick slab gel having a separating gel containing 10% (w/v) acrylamide, 0.08% (w/v) bisacrylamide and a stacking gel with 3% (w/v) acrylamide, 0.08% (w/v) bisacrylamide . After electrophoresis for about 1 h, the tracking dye front migrates approximately 5 cm into the separating gel, and then the first 1 cm of the separating gel from the origin is cut out and

removed to an equilibration solution containing 1% (v/v) freshly added ME, 10.3% (w/v) glycerol, 0.07M Tris, 2.4% (w/v) SDS and made to pH 6.8 with HCl, for reduction of proteins. In the second step of this procedure, this gel strip now containing 20 samples of reduced proteins is loaded directly onto the top of the stacking gel of a new slab gel with similar composition to the first gel by forcing the gel strip between the two glass side plates. The second step slab gel is made slightly thicker (1.8 mm) to facilitate the loading of the gel strip from the first run. The second stage of electrophoresis is then carried out at a constant current of 40 mA/gel until the brown front reached the bottom of the gel.

The electrophoregrams obtained using either the modified 2-D procedure or the 2-S procedure could be used to score both HMW and LMW glutenin subunits. However, when only HMW glutenin subunits were to be analysed, the simple 1-D SDS-PAGE was used.

4.2.6 Genetic Analysis

In the test-cross mapping experiments, recombination fractions (p) were calculated directly by dividing the observed number of recombinants (R) with the total number of progeny analysed (n) excluding the aneuploid progeny.

The standard error (S_p) of the recombination fraction was calculated using the formula for the binomial distribution (c.f. Mather, 1951)

$S_p = \sqrt{[\ p(1-p) \div n\ ]}$ ....(i)

Where no recombinants were detected between two protein markers in the test-cross progeny, the upper limit (at the 95% confidence level) for the recombination fraction (p) between the genes controlling them was calculated using the method of Hanson (1959).

$p = [\ 1-(0.05)^{-n}\ ]$ ....(ii)

where, n = number of euploid progeny analysed

In the F_2 mapping experiment, the recombination fraction and its standard error was calculated using the method of maximum likelihood (Haldane, 1919; Mather, 1951). Because endosperm protein phenotypes are codominant, the F_2 progeny could be

classified into 9 out of the 10 possible genotypic classes in a completely classified F_2 segregating for two factors. The two classes that could not be distinguished from each other comprised individuals involving all four alleles of the two genes to be mapped. These individuals combined either both parental types or both recombinant gametes. It was assumed that the recombination frequencies were the same in both male and female gametes and using the maximum likelihood method, the following cubic equation was derived for calculating recombination fraction (p) from a nine class F_2 data.

$$(b+2d) - 2p(a+2b+c+3d) + 2p^2(2a+3b+3c+4d) - 4p^3(a+b+c+d) = 0 \quad \text{.......(iii)}$$

where, a = combined observed frequency of two homozygous parental classes;

b = combined observed frequency of four classes involving one parental and one recombinant gamete;

c = observed frequency of the double heterozygote class showing all four alleles and;

d = combined observed frequency of two homozygous recombinant classes

The equation used to calculate the standard error (S_p) of the recombination fraction was as follows:

$$\frac{1}{Vp} = 2n\left[1 + \frac{(1-2p+2p^2)}{p(1-p)} - \frac{2(1-3p-3p^2)}{(1-2p+2p^2)}\right] \quad \text{..............................(iv)}$$

where, $V_p = (S_p)^2$

Map distances (cM) and their standard errors were calculated from recombination frequencies, using the Kosambi function (Kosambi, 1944) as applied earlier in linkage mapping of wheat proteins by Payne *et al.* (1982b).

$$cM = 25 \times \ln[(100+2R) \div (100-2R)] \pm 2500\, S_R \div (2500-R^2) \quad \text{..............(v)}$$

where, R = recombination percentage; S_R = standard deviation of R

4.3 RESULTS

4.3.1 Assignment of Symbols to Loci and their Alleles Encoding Endosperm Proteins in Wheat and Rye

The symbols for the genes, located on homoeologous group 1 chromosomes of wheat and rye, that control the synthesis of various types of endosperm storage proteins, are listed in Table-4.1. The symbols *Glu-A1, Glu-B1* and *Glu-D1* have been assigned to the genes controlling HMW glutenin subunits in wheat (Payne *et al.*, 1982b) and these have been adopted in the present study. The genes controlling analogous HMW glutelin subunits in cereal rye have been assigned the symbol *Glu-R1* (Singh and Shepherd, 1984a) to conform with the standard system of nomenclature for wheat (McIntosh, 1973). However, Shewry *et al.* (1984a) have assigned the symbol *Sec-3* to the genes controlling these subunits in rye, based on the analogy with the *Hor-3* locus in barley.

The gene symbols *Gli-A1, Gli-B1* and *Gli-D1* of Payne *et al.* (1982b) for gliadins will be followed because these conform more closely with the standard system of gene nomenclature in wheat (McIntosh, 1973) than the symbols first assigned by Sozinov and Poperelya (1980). The symbol *Sec-1* (Shewry *et al.*, 1984a) will be used for denoting genes controlling ω-secalins because their exact relationship with wheat loci is not known as yet.

The genes located on the short arms of chromosomes 1A, 1B and 1D that control the synthesis of LMW glutenin subunits and which are closely linked with the *Gli-1* locus have not been assigned any symbol so far in the literature and they will be denoted by the symbols *Glu-A3, Glu-B3* and *Glu-D3*, respectively, in this thesis. The symbol *Glu-2* was not chosen because this has already been reserved by Dr. P. I. Payne (pers. comm.) for genes controlling some acidic protein subunits in wheat, which recombine freely with the *Gli-1* locus.

The choice of symbols *Tri-A1* and *Tri-D1* for the genes controlling triplet proteins, is based on the same nomenclature system used for the genes controllig glutelins and

Table 4.1 Symbols for the loci encoding endosperm storage proteins in wheat and rye

Protein class	Chromosome arm location	Gene symbol	Allele symbol (ϕ)
HMW glutelin subunits	1AL	*Glu-A1* †	*a* (Hope); *b* (Gabo); *c* (Chinese Spring)
	1BL	*Glu-B1* †	*b* (Chinese Spring); *d* (Hope); *e* (Halberd, MKR/211/7, WR/17); *i* (Gabo)
	1DL	*Glu-D1* †	*a* (Chinese Spring, India 115, Kite); *d* (Halberd, Heron); null (Single glutenin line)
	1RL	*Glu-R1* **	*i* (Imperial); *k* (King II)
Prolamins	1AS	*Gli-A1* ††	*cs* (Chinese Spring); *g* (Gabo); *h* (Hope)
	1BS	*Gli-B1* ††	*cs* (Chinese Spring); *h* (Halberd) *g* (Gabo, Hope, MKR/211/7, WR/17)
	1DS	*Gli-D1* ††	*cs* (Chinese Spring); *i* (India 115); *h* (Halberd, Single glutenin line)
	1RS	*Sec-1* *	*i* (Imperial); *k* (King II)
LMW glutenin subunits	1AS	*Glu-A3*	*cs* (Chinese Spring); *h* (Hope)
	1BS	*Glu-B3*	*cs* (Chinese Spring); *h1* and *h2* (Hope)
	1DS	*Glu-D3*	*cs* (Chinese Spring); *i* (India 115)
Triplet proteins	1AS	*Tri-A1*	*cs* (Chinese Spring); *h* (Hope)
	1DS	*Tri-D1*	*cs* (Chinese Spring); *i* (India 115)

†gene and allele symbols of Payne and Lawrence (1983); ††gene symbols of Payne *et al.*, (1982b); *gene symbol of Shewry *et al.* (1984a); **gene and allele symbols of Singh and Shepherd (1984a); ϕnames in parentheses refer to the the cultivar/breeding line used in test-crosses

gliadins. However, the characterization of triplet proteins is not complete as yet and therefore these gene symbols are tentative only.

The different alleles controlling HMW glutenin subunits of wheat have been catalogued by Payne and Lawrence (1983) and these symbols will be used in the present study. In the absence of any standard system for cataloguing the alleles of the genes

controlling the HMW glutelin subunits in rye, prolamins in wheat and rye and LMW glutenin subunits and triplet proteins in wheat, the alleles for these genes have been designated by the initial letter(s) of the cultivars possessing the different alleles (Table-4.1). Although allele symbols (*1, 2, 3,...*) have been assigned to the wheat gliadins by Sozinov and Poperelya (1980) this system is not being followed here because different techniques were used for separating gliadins in the present study, and it is very difficult to match up the equivalent bands in the two studies.

4.3.2 Mapping Genes Controlling Endosperm Proteins on the Short Arms of Group 1 Chromosomes in Wheat

4.3.2.1 Crossing procedures

The test-cross procedures for the translocation and telocentric mapping of the genes controlling endosperm proteins (*Tri-1, Gli-1* and *Glu-3*) on the short arm of group 1 homoeologues of wheat are depicted in Fig. 4.1.

(i) Translocation mapping

The crossing procedures were based on the method described by Singh and Shepherd (1984a). To illustrate this method, the procedure used for mapping *Tri-A1, Gli-A1* and *Glu-A3* genes on chromosome arm 1AS, is described below:

To generate a suitable F_1 hybrid heterozygous for alleles at the *Tri-A1, Gli-A1* and *Glu-A3* loci on chromosome arm 1AS, the translocation line 1AS-1RL in Chinese Spring was first crossed to intervarietal chromosome substitution line Chinese Spring-Hope 1A. The protein phenotypes produced by the presumed alleles at these loci were electrophoretically distinguishable from each other. The other (long) arms of these two chromosomes were marked with different glutelin genes (*Glu-R1i* and *Glu-A1a*) in the F_1 heterozygotes. The protein bands corresponding to all of these different genes could be easily recognized in the test-cross progeny because the male parent (Chinese Spring Dt

Figure 4.1

Test-cross combinations employed in mapping experiments.

Translocation mapping:

1A [(CS 1AS-1RL × CS—Hope 1A)] × CS Dt 1AL;

1B [(CS 1BS-1RL × Gabo)] × Halberd;

1D [(CS 1DS-1RL × India 115)] × Warigal 1DL-1RS.

Telocentric mapping:

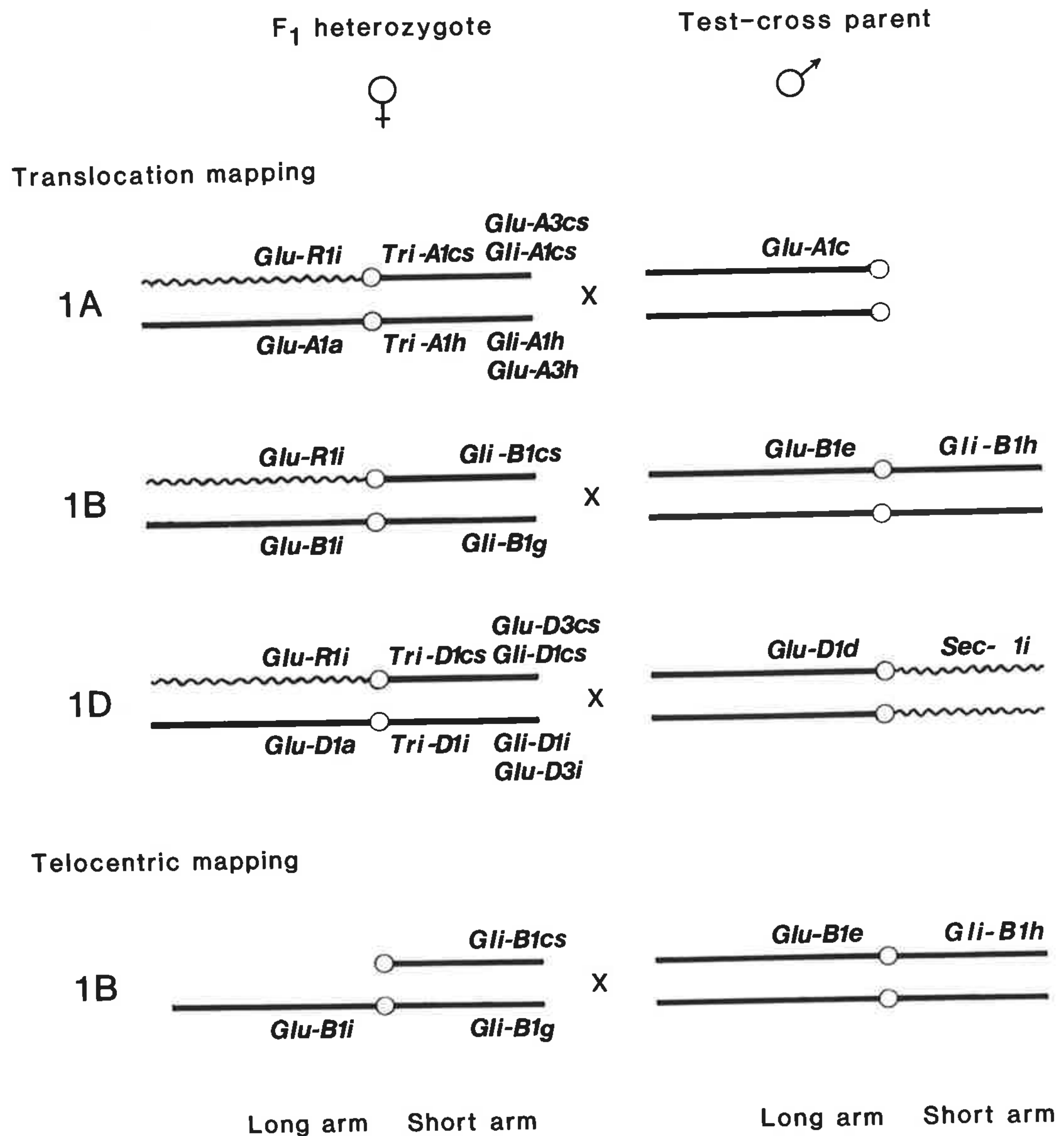
1B [(CS Dt 1BS × Gabo)] × Halberd.

CS = Chinese Spring

Gene symbols used:

General symbol		Gene product
Glu-1	=	HMW glutelin subunit
Glu-3	=	LMW glutelin subunit
Tri-1	=	Triplet protein
Gli-1	=	Prolamin of wheat
Sec-1	=	Prolamin of rye
Specific symbol		
Glu-R1i	=	Allele *i* of *Glu-1* locus in R genome

PROCEDURES FOR MAPPING GENES CONTROLLING ENDOSPERM PROTEINS ON THE SHORT ARMS OF GROUP 1 CHROMOSOMES



1AL) chosen for the test-cross was deficient for all genes controlled by chromosome arm 1AS and also it had a null allele (*Glu-A1c*) for glutelin bands. Thus the presence of recognizably different protein markers on each of the four arms of the 1A/1AS-1RL bivalent allowed test-cross progeny to be scored in gels for parental and recombinant types and also for aneuploid variants.

Providing that the translocation arose from centric fusion between 1AS and 1RL, normal pairing and crossing over is expected to occur between the *homologous* short arms of chromosome 1A but no pairing is expected between the *homoeologous* chromosome arms 1AL and 1RL because of the inhibitory effect of the *Ph-1b* gene located on chromosome 5B of wheat. In this way the F_1 heterozygote can be used to map the position of any locus on the short arm of chromosome 1A with respect to the centromere in a way analogous to the telocentric mapping method developed by Sears (1962, 1966b) for mapping gene—centromere distances in wheat.

Similar procedures were followed in the translocation mapping of the related genes on chromosome arms 1BS and 1DS. The test-cross combinations used are listed below and the different alleles present are shown in Fig. 4.1,1B, 1D.

Parents used for mapping the *Gli-B1* locus on 1BS:

[(Chinese Spring 1BS-1RL × Gabo) × Halberd]

Parents used for mapping the *Tri-D1, Gli-D1* and *Glu-D3* loci on 1DS:

[(Chinese Spring 1DS-1RL × India115) × Chinese Spring 1DL-1RS]

In each case the male parent for the test-cross was chosen so that it did not contribute any electrophoretically similar band which would mask the segregation of the critical protein bands in the F_1 heterozygote.

(ii) Telocentric mapping

The telocentric mapping experiment with the *Gli-B1* locus was carried out concurrently with one of the translocation mapping experiments, designed to map the same locus, in order to compare these two methods of mapping gene—centromere distances. The crossing procedure used was similar to the translocation mapping procedure except

Chinese Spring Dt 1BS was used in place of Chinese Spring translocation 1BS-1RL (see Fig. 4.1).

(iii) Intrachromosomal mapping

The recombination frequency occurring between the *Gli-B1* and *Glu-B3* loci on chromosome arm 1BS was determined using the following triparental cross:

[(Chinese Spring × Chinese Spring-Hope 1B) × Chinese Spring Dt 1BL]

Since the 1B chromosomes from cultivars Chinese Spring and Hope have different alleles at the*Gli-B1* and*Glu-B3* loci, and the male parent was deficient for all the genes present on chromosome arm 1BS, any recombination between the *Gli-B1* and *Glu-B3* loci could be easily scored in the test-cross progeny. Furthermore, these two loci could be mapped with respect to the *Glu-B1* locus present on the long arm of these chromosomes because cultivar Hope had a different allele to Chinese Spring at this locus. However, gene—centromere distances could not be determined by this method.

4.3.2.2 Mapping the *Tri-A1, Gli-A1* and *Glu-A3* loci on chromosome arm 1AS

(i) Recombination frequencies

The protein phenotypes produced by alleles *Tri-A1cs* and *Tri-A1h* (triplet proteins), *Gli-A1h* (gliadin), *Glu-A3cs* (LMW glutenin subunit) and homoeoalleles *Glu-R1i* and *Glu-A1a* (HMW glutelin subunits) are quite distinctive in the parents as shown in Fig. 4.2, A, B. However there was no unique protein band produced by alleles *Gli-A1cs* and *Glu-A3h* that could be recognized in the gels and therefore the presence of these alleles could only be inferred by the absence of the alternative allele in the test-cross progeny. However, the *Gli-A1cs* allele produced a band (Fig. 4.2.A, a) which had the same electrophcretic mobility as a band produced by *Gli-A1h*, hence this band was absent only in test-cross progeny which lacked both of these alleles.

The triplet and gliadin protein phenotypes (Tri-A1 and Gli-A1) were determined by

Figure 4.2

SDS-PAGE patterns of total seed protein extracts from the parental lines (P_1, P_2, P_3) used to map the *Tri-A1*, *Gli-A1* and *Glu-A3* loci. The distinctive bands for each allele are labelled.

A 1-D SDS-PAGE of unreduced proteins;

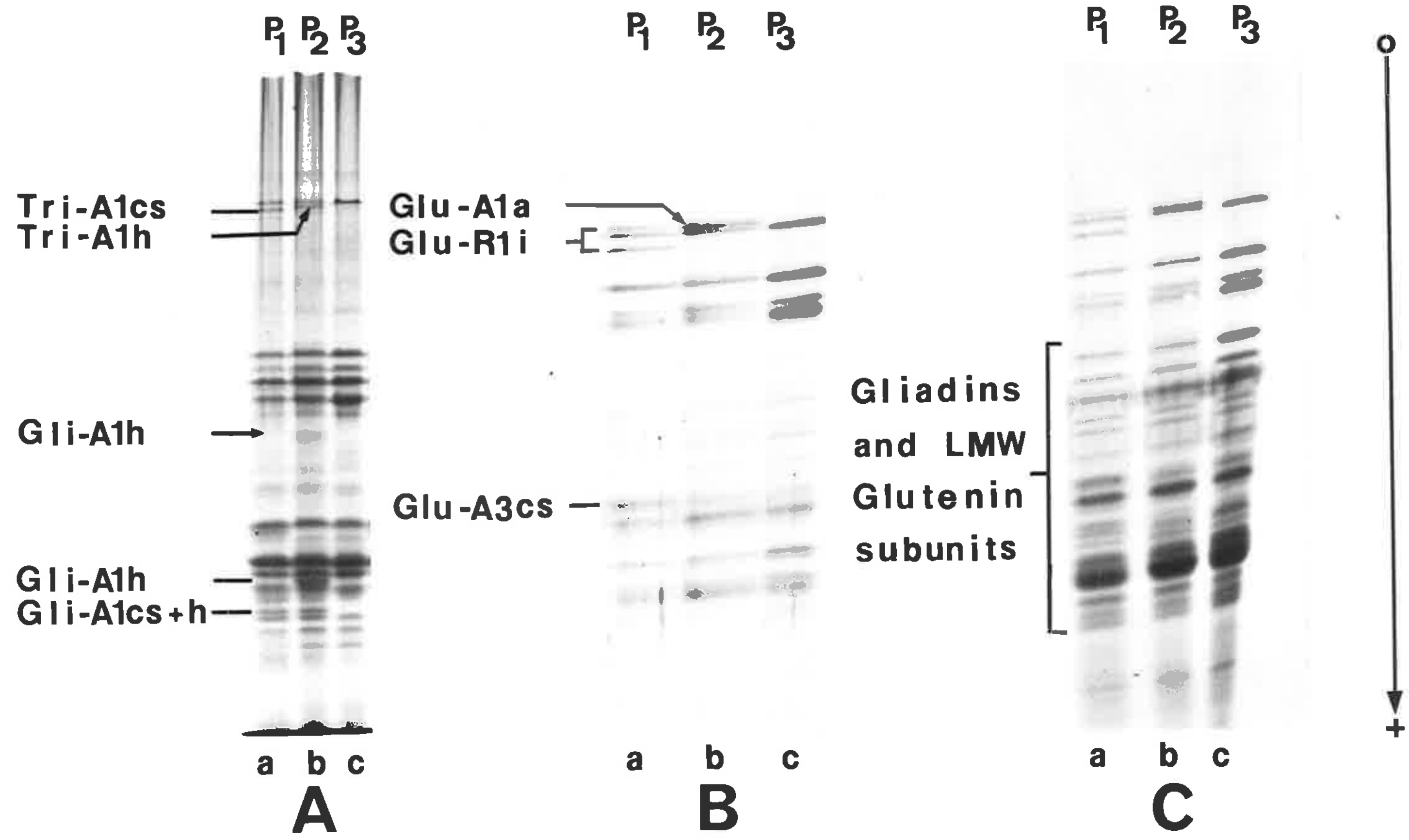
B 2-step (2-S) electrophoresis of reduced proteins (see section 4.2.5 for details);

C 1-D SDS-PAGE of reduced proteins.

a Chinese Spring 1AS-1RL translocation;

b Chinese Spring-Hope 1A substitution;

c Chinese Spring Dt 1AL.



P1 P2 P3
Tri-A1cs
Tri-A1h
Gli-A1h
Gli-A1h
Gli-A1cs+h
a b c
A
Glu-A1a
Glu-R1i
Glu-A3cs
a b c
B
Gliadins and LMW Glutenin subunits
a b c
C
o
+

1-D SDS-PAGE of unreduced protein extracts (Fig. 4.2.A) whereas, the Glu-A1, Glu-R1 and Glu-A3 phenotypes were scored in the electrophoregrams (Fig. 4.2.B) obtained by 2-S electrophoresis as described earlier (section 4.2.5). Single-kernel analysis was feasible with both of these techniques. As shown in Fig. 4.2.C, the normal 1-D SDS-PAGE of reduced proteins allowed the HMW glutelin subunits to be scored but it could not be used for scoring triplet bands, gliadins or LMW glutenin subunits.

Since the male parent Chinese Spring Dt 1AL (P_3 in Fig. 4.2, A, B) lacked all of these protein markers controlled by chromosomes 1A and 1R, all the protein phenotypes could be reliably determined in the test-cross progeny and the parental (P), recombinant (R) and aneuploid (A) phenotypes were easily distinguished (Fig. 4.3, A, B). Recombinant phenotypes resulting from crossing over between *Tri-A1* and *Gli-A1* genes are labelled 'R' in Fig. 4.3,A, and those resulting from crossing over between the *Glu-A3* gene and the centromere, marked here by Glu-A1 and Glu-R1 proteins, are labelled 'R' in Fig.4.3,B. The two gels were run using extracts from the same individual test-cross seeds and therefore the complete linkage analysis was done by combining the data from the two gels. Since the Glu-A1a band has the same electrophoretic mobilty as one of the Glu-R1i bands, it was not possible to be certain, without further progeny testing, whether test-cross seeds showing Glu-R1i bands possessed the Glu-A1a band also. However, because the genes controlling these two glutelin patterns are homoeoallelic, it is expected that they will normally segregate as alternatives in the test-cross progeny.

Altogether 296 test-cross seeds were analysed in this way. Data obtained from two different F_1 plants were homogeneous ($\chi^2_{[6]}$ =5.6, P>0.3) and thus only pooled data are shown in Table-4.2. A small proportion (4.7%) of the test-cross progeny were aneuploids possibly resulting from irregular disjunction of the unpaired 1A and 1AS-1RL chromosomes at anaphase I of meiosis. However, their effect on estimates of the recombination frequencies, although complex to determine, will not be large and for simplicity these aneuploid progeny have been ignored in the initial analysis. As shown in Table-4.2, there is close agreement with the expected 1:1 segregation ratio for presumed homoeoalleles *Glu-A1a* and *Glu-R1i* and presumed allele pairs *Tri-A1cs/Tri-A1h*,

Figure 4.3

SDS-PAGE patterns of total seed protein extracts from some test-cross progeny used to map the *Tri-A1*, *Gli-A1* and *Glu-A3* loci.

A 1-D SDS-PAGE of unreduced proteins showing recombination between the *Tri-A1* and *Gli-A1* loci;

B 2-S electrophoresis of reduced proteins obtained from the same individual seeds analysed in part **A**, showing recombination between the centromere (marked with *Glu-1*) and the *Glu-A3* locus.

P = parental; **R** = recombinant; **A** = aneuploid

P P P R R P R A R P P
Tri-A1cs
Tri-A1h
Gli-A1h
Gli-A1h
Gli-A1h+cs
a b c d e f g h i j k
A

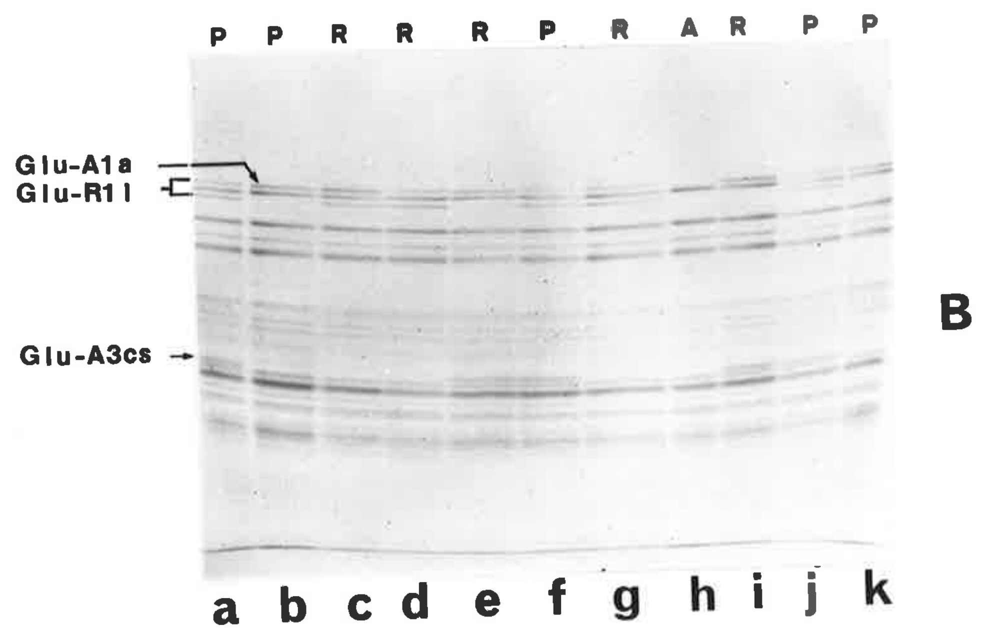

P P R R R P R A R P P
Glu-A1a
Glu-R1i
Glu-A3cs
a b c d e f g h i j k
B

Table 4.2 Observed frequencies of protein phenotypes in test-cross progeny used to map the *Tri-A1*, *Gli-A1* and *Glu-A3* loci, grouped into parental, recombinant and aneuploid classes

Progeny class	Protein phenotypes: Glu-1	Tri-A1	Gli-A1 Glu-A3	No. of progeny	p (%)	Map distance (cM)
Parentals:						
	A1a	h	h	64		
	R1i	cs	cs	77		
Recombinants:						
c.o. region 1	A1a	cs	cs	8		
	R1i	h	h	20	11.0	11.2 ± 2.0
c.o. region 2	A1a	h	cs	55		
	R1i	cs	h	55	40.1	55.2 ± 8.2
double c.o.	A1a	cs	h	1		
	R1i	h	cs	2		
				282		
Aneuploids:						
hypoploid	-	-	-	11		
hyperploid?	R1i+A1a?	h+cs?	h+cs	2		
misdivision	R1i	-	-	1		
				14 (4.7%)		

χ^2 Table:

	Glu-A1a/ Glu-R1i	Tri-A1cs/ Tri-A1h	Gli-A1cs/ Gli-A1h	P: R (region 1)	P: R (region 2)	P: R (region 1or 2)
Expected ratio	1: 1	1: 1	1: 1	1: 1	1: 1	1: 1
Observed No.	128:154	141:141	140:142	217:65	169:113	144:138
χ^2 values (P, d.f.= 1)	2.4 (P>0.1)	0.0 (P=1.0)	0.01 (P>0.7)	171.6 (P<0.001)	11.12 (P<0.001)	0.13 (P>0.95)

'–' = absent

c.o. region 1 = Between centromere and *Tri-A1*
c.o. region 2 = Between *Tri-A1* and *Gli-A1*

Gli-A1cs/Gli-A1h and *Glu-A3cs/Glu-A3h*. From a consideration of the frequency of parental and recombinant types there is evidence of strong linkage between the centromere and the *Tri-A1* locus and a weak linkage between the *Tri-A1* and *Gli-A1* loci. However, there is no evidence of linkage between the centromere and the *Gli-A1* locus. No recombination was observed between the Gli-A1 and Glu-A3 loci and the upper limit of

recombination (95% confidence limit) between these two loci was calculated to be 1.1%, according to the method of Hanson (1959) as described in Material and Methods. The recombination frequencies and the map distances for the individual intervals are shown in Table-4.2.

(ii) Chromosome pairing and the occurrence of aneuploidy

The validity of this method of gene mapping also depends on the chromosome arm 1AS of the translocation pairing with the complete 1A chromosome with the same frequency as would occur between the two complete homologues of 1A and furthermore, the absence of pairing between chromosome arms 1AL and 1RL. If the presence of the translocation reduces pairing between the 1AS arms of these two chromosomes, the map distance will be underestimated, whereas any homoeologous pairing between 1AL and 1RL would lead to an overestimate. To check on these possible sources of error the pairing between 1AS-1RL translocation and complete 1A chromosomes was investigated at metaphase I and diakinesis stages of meiosis in the PMCs from F_1 plants.

The translocation chromosome was easily identified in C-banded preparations since the chromosome arm 1RL had a prominent telomeric heterochromatin band and this made it easier to study the pairing of this chromosome with the complete 1A chromosome (Fig. 4.4, a, b, c). Altogether 209 PMCs from two of the F_1 plants, used to produce some of the test-cross seeds analysed above, were examined at metaphase I and no pairing was detected between 1AL and 1RL, whereas the short arms of these two chromosomes were paired in an average of 90.4% PMCs (Table-4.3).

Table 4.3. Observed frequency of pairing between short arms of 1AS-1RL translocation and complete 1A chromosomes at two stages of meiosis

F_1 combination	Meiotic stage	No. of PMCs	No. paired	% pairing
CS 1AS-1RL × CS (Hope 1A)	Metaphase I	209	189	90.4
	Diakinesis	80	79	98.8

This pairing frequency was homogeneous between the two families ($\chi^2_{[1]}$=0.72, P>0.3).

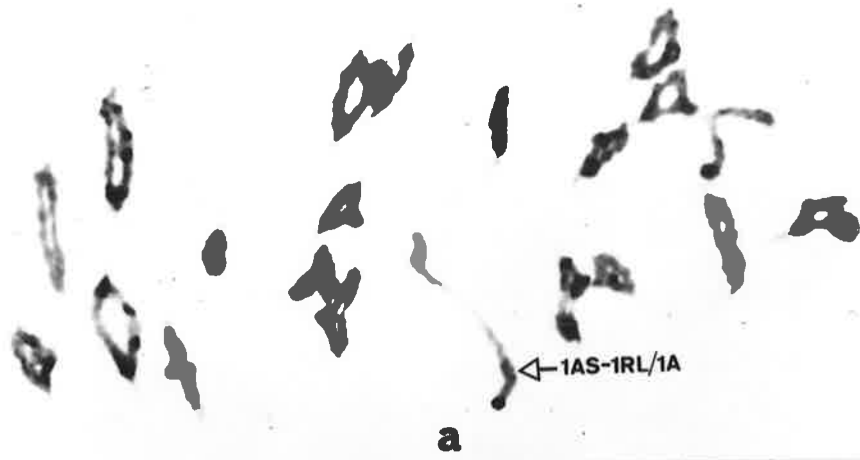
Figure 4.4

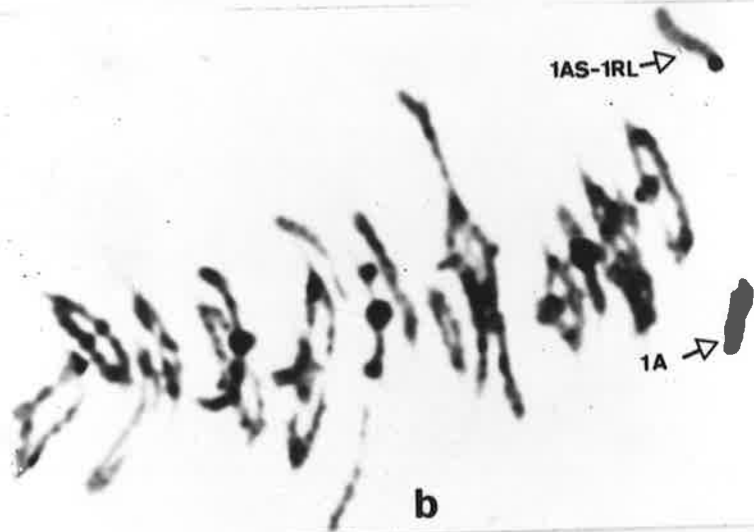
C-banded squash preparations of PMCs, at two stages of meiosis, from the F_1 heterozygotes used to map the genes on the short arm of chromosome 1A.

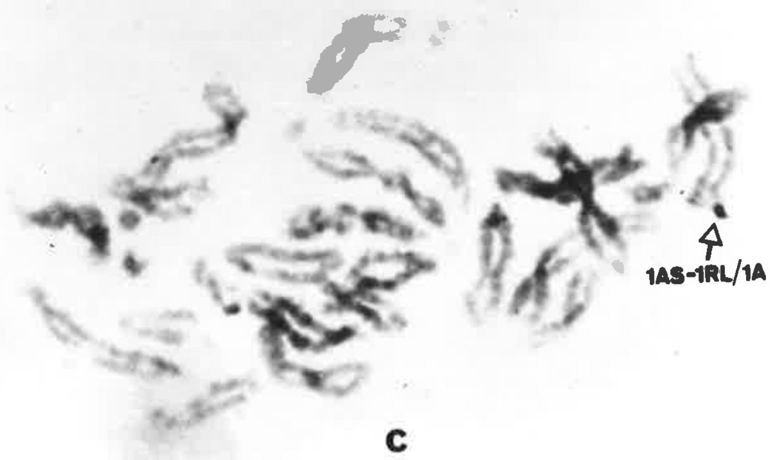
a metaphase I cell showing 1A/1AS-1RL bivalent;

b metaphase I cell showing 1A and 1AS-1RL univalents;

c diakinesis cell showing 1A/1AS-1RL bivalent.


1AS-1RL/1A
a


1AS-1RL
1A
b


1AS-1RL/1A
c

A similar frequency of univalents at metaphase I would be expected to occur in the megaspore mother cells and these will be the likely source of the 4.7% aneuploids detected among the test-cross progeny. However, it is clear from the high degree of pairing (98.8%) observed at diakinesis in the PMCs that the univalents are due mainly to desynapsis (precocious terminalization of chiasmata) rather than asynapsis (total lack of pairing). The possible influence of this lack of metaphase pairing on the estimation of recombination freqeuncies will be considered later in the Discussion.

4.3.2.3 Mapping the *Gli-B1* locus on chromosome arm 1BS

(i) Recombination frequency

Both the translocation and telocentric mapping procedures were used to produce test-cross seeds to map the locus *Gli-B1* , as described earlier in sections 4.2.2 and 4.3.2.1. The translocation mapping experiment involving Chinese Spring × Gabo F_1 heterozygotes, was repeated at three times of the year viz summer 1981, winter 1982 and spring 1982. The spring 1982 experiment was carried out at the same time as the telocentric mapping experiment for comparison. In all of these experiments except one, Halberd was used as the male parent and no overlap of relevant bands occurred (see Fig. 4.5, A, B). In one case, the substitution line having 1R of King II rye substituted for 1B in a mixed Chinese Spring and Holdfast wheat background was used and once again there was no overlap of critical protein bands (not shown in Fig.). The protein phenotypes of the presumed homoeoalleles *Glu-R1i* and *Glu-B1i* (HMW glutelin subunits) and the presumed alleles *Gli-B1cs* and *Gli-B1g* (gliadins) could be classified without difficulty in the parents (Fig. 4.5.A, a, b, c) following 1-D SDS-PAGE of reduced proteins. Similarly all these protein phenotypes could be reliably determined among the test-cross progeny and the parental (Fig. 4.5.A, d, e), recombinant (Fig. 4.5.A,f, g) and several aneuploid types (Fig. 4.5.A, h-m) were distinguished.

Altogether 606 test-cross seeds were produced in three translocation mapping experiments and analysed by SDS-PAGE. The data, including the frequency of

Figure 4.5

1-D SDS-PAGE patterns of reduced total protein extracts from the parental lines (P_1, P_2, P_3) and some test-cross progeny used to map the *Gli-B1* locus.

A Translocation mapping:

a Chinese Spring 1BS-1RL translocation;

b Gabo;

c Halberd;

d-m test-cross progeny.

B Telocentric mapping:

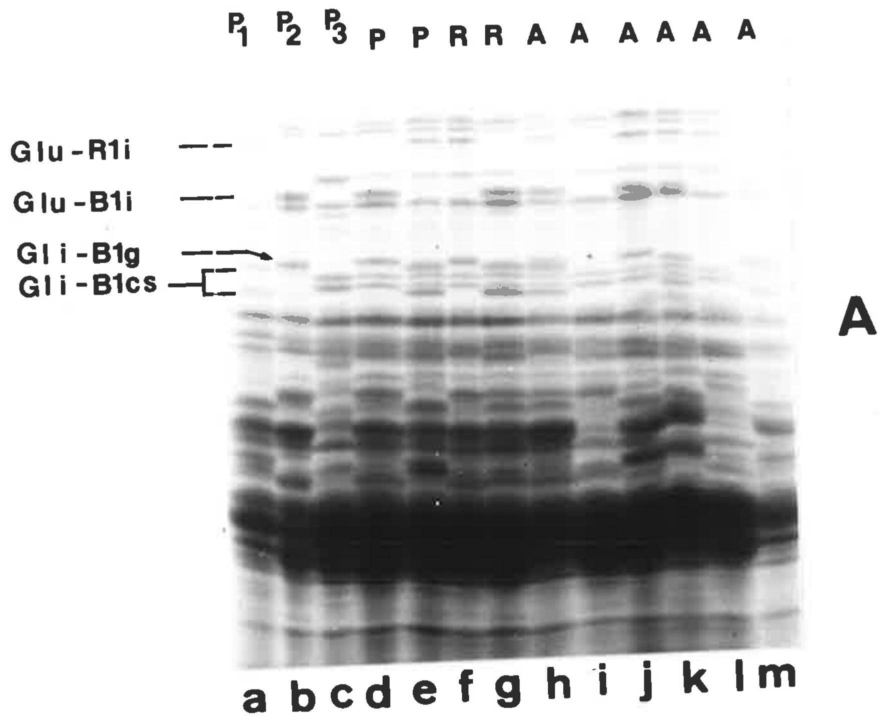
a Chinese Spring Dt 1BS;

b Gabo;

c Halberd;

d-k test-cross progeny.

P = parental; **R** = recombinant; **A** = aneuploid


P1 P2 P3 P P R R A A A A A A
Glu-R1i
Glu-B1i
Gli-B1g
Gli-B1cs
A
a b c d e f g h i j k l m

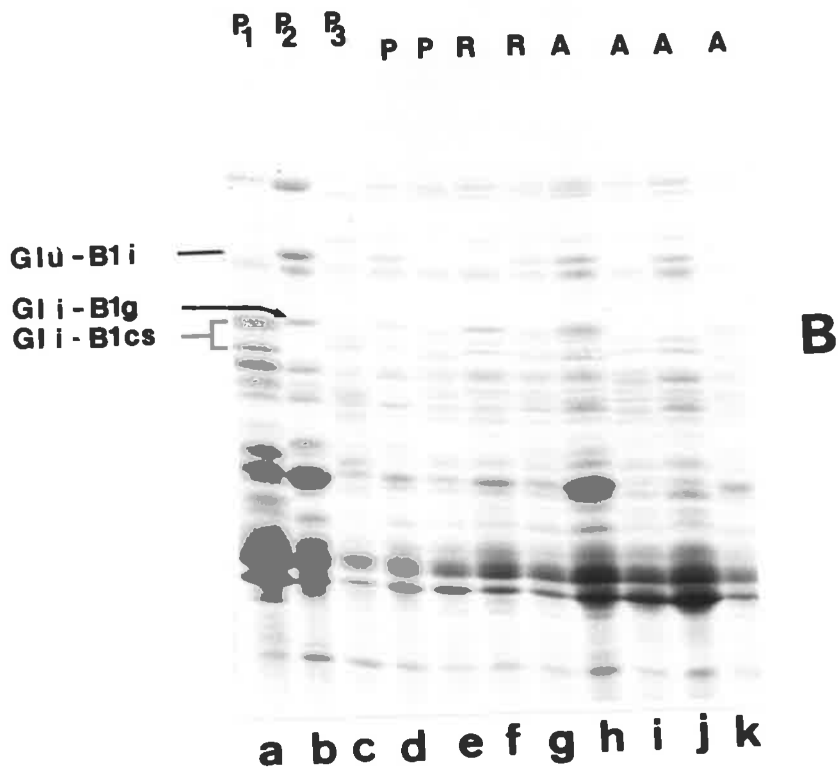

P1 P2 P3 P P R R A A A A
Glu-B1i
Gli-B1g
Gli-B1cs
B
a b c d e f g h i j k

Table 4.4. Observed frequency of protein phenotypes in test-cross progeny used to map the *Gli-B1* locus, grouped into parental, recombinant and aneuploid classes

Test-cross	*Parentals* Glu-B1i Gli-B1g	(Glu-R1i)† Gli-B1cs	*Recombinants* Glu-B1i Gli-B1cs	(Glu-R1i)† Gli-B1g	Total progeny	p (%)	Map distance (cM)
(a) Translocation mapping							
	156	133	113	93	495	41.6	59.7 ± 7.1
(b) Telocentric mapping							
	63	49	44	32*	188	40.4	56.1 ± 10.3

Aneuploids:

Aneuploid Class	Protein phenotype Glu-B1/R1†	Gli-B1	Translocation mapping	Telocentric mapping
hypoploid	-	-	69	31
hyperploid	B1i+R1i	g+cs	22	-
	B1i	g+cs	-	10
misdivision products	B1i	-	7	3
	R1i	-	1	-
	B1i	g+cs	1	-
	-	g+cs	0	2
	B1i+R1i	g	9	-
	B1i+R1i	cs	2	-
Total			111 (18.3%)	46 (19.7%)

χ^2 Table:

Expected ratio	Glu-B1i/ Glu-R1i† 1: 1	Gli-B1g/ Gli-B1cs 1: 1	P/R 1:1
(a)Translocation mapping			
Observed No.	269: 226	249: 246	289: 206
χ^2 values	3.73	0.02	16.56
(P, d.f.=1)	(P>0.1)	(P>0.7)	(P<0.01)
(b)Telocentric mapping			
Observed No.	107: 81	95: 93	112: 76
χ^2 values	3.60	0.02	7.28
(P, d.f.=1)	(P>0.05)	(P>0.8)	(P<0.01)

†Not present in the telocentric mapping ; *Assumed to be recombinant rather than misdivision products
'-' = absent

aneuploids, obtained from the three families were on the borderline of homogeneity ($\chi^2_{[8]}$=16.71, 0.05>P>0.02) even though the three experiments had been carried out at different times of the year. When aneuploid progeny were excluded from the analysis the families were more homogeneous ($\chi^2_{[6]}$=8.43, P>0.2) and the data have been pooled in Table-4.4. Although a large proportion (18.3%) of the test-cross progeny in the translocation mapping experiments were aneuploids, these have been excluded in the initial estimation of map distance. Their possible influence on this estimate will be considered later. As shown in the χ^2 section of Table-4.4, the segregation for the presumed homoeoalleles *Glu-B1i/Glu-R1i* and presumed alleles *Gli-B1cs/Gli-B1g* is in close agreement with the expected 1:1 ratio. However, because of the significantly greater proportion of parental to recombinants there is an indication of weak linkage between the *Gli-B1* locus and the centromere, marked here with *Glu-1* genes. Overall the data showed 41.6% recombination between the centromere and this locus, which converts to a map distance of 59.7±7.1 cM.

The telocentric mapping experiment was carried out concurrently with one of the translocation mapping experiments. The parental, recombinant and aneuploid protein phenotypes were distinguished in a way similar to that used for translocation mapping except for the lack of Glu-R1i protein bands in these progeny (Fig. 4.5.B, a-k). The recombination frequncy obtained with the telocentric mapping experiment (40.4%) was similar to that obtained with the translocation mapping experiments (41.6%) as was the aneuploid frequency (19.7% and 18.3%, respectively). However, a more valid comparison involves the data obtained from the concurrent mapping experiments. Once again there was no significant difference between the two values for recombination (46.3% vs 40.4%) and aneuploid frequencies (19.7% vs 16.1%) for telocentric and translocation mapping, respectively.

(ii) Chromosome pairing and the occurrence of aneuploidy

As shown before with the 1AS-1RL translocation, the 1BS-1RL chromosome was easily identified in C-banded preparations of PMCs at metaphase I by the presence of a

prominent telomeric band. However, at diakinesis, the 1B/1BS-1RL bivalent was identified in Feulgen preparations by its pronounced heteromorphic appearance (Fig. 4.6, a, b, c). The telocentric chromosome 1BS was easily recognized by its smaller size and the clear heteromorphic conformation of the bivalent containing it (Fig. 4.7, a, b, c).

Altogether 424 PMCs from the translocation heterozygotes were analysed at metaphase I and a further 153 PMCs were analysed at diakinesis and no pairing was observed between chromosome arms 1BL and 1RL. However, on an average there was 59.4% pairing at metaphase I and 88.5% pairing at diakinesis between the homologous 1BS arms in summer 1981, and a higher frequency of 81.6% at metaphase I and 93.1% at diakinesis in the spring of 1982 (Table-4.5). Thus it seems that the chromosome pairing frequency is reduced at the higher glass-house temperatures of summer. These results are consistent with the findings of Fu and Sears (1973) who also observed reduction in pairing and recombination frequencies at higher temperature. However, the difference in pairing frequency due to high temperature was less pronounced at diakinesis. Thus, it appears that the difference in pairing frequency at metaphase I between summer 1981 and spring 1982 was partly due to more desynapsis at the higher temperature (19.1% in summer vs 11.5% in spring).

When the pairing of translocation chromosome 1BS-1RL and telocentric chromosome 1BS with a normal 1B chromosome was compared in the same genetic background and identical environmental conditions, the telocentric showed significantly less pairing than the translocation at both the metaphase I and diakinesis stages of meiosis (Table-4.5). Thus the presence of rye arm 1RL does not interfere with the pairing of the 1BS arm on the other side of the centromere, rather it appears to promote the pairing. However, data were not available for the degree of pairing shown between the short arms of two complete 1B homologues in the same environment and genetic background.

Because of the irregular distribution of univalents at anaphase I of meiosis in the female parent, lack of metaphase pairing is expected to contribute to the aneuploidy observed in the test-cross progeny (Table-4.4). These aneuploid gametes can be divided

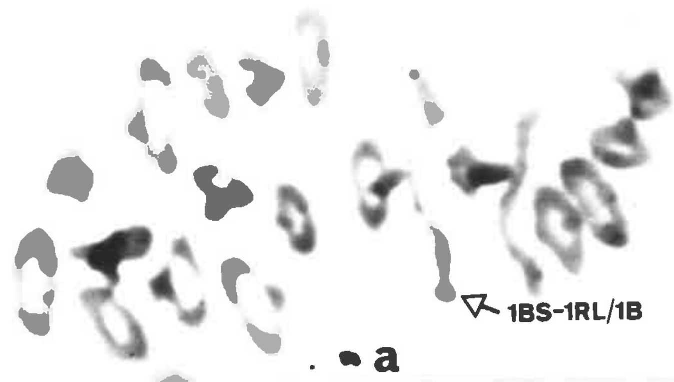
Figure 4.6

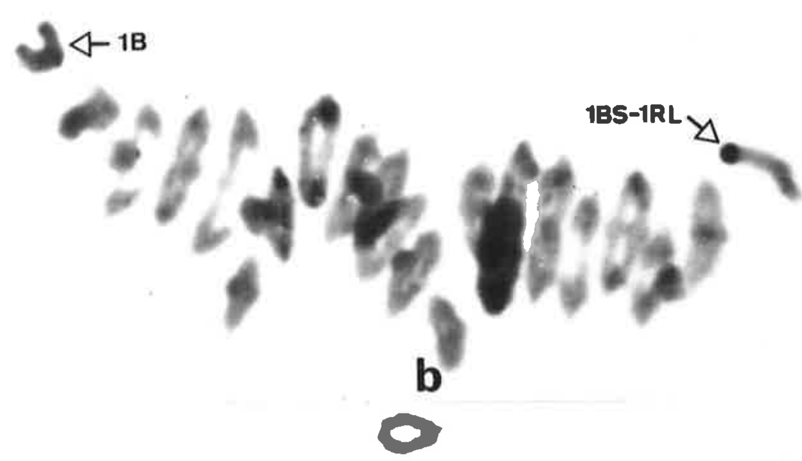
Squash preparations of PMCs, at two stages of meiosis, from the F_1 heterozygotes used to map the genes on the short arm of chromosome 1B.

a C-banded metaphase I cell showing 1B/1BS-1RL bivalent;

b C-banded metaphase I cell showing 1B and 1BS-1RL univalents;

c Feulgen stained diakinesis cell showing 1B/1BS-1RL bivalent.


1BS-1RL/1B
a


1B
1BS-1RL
b

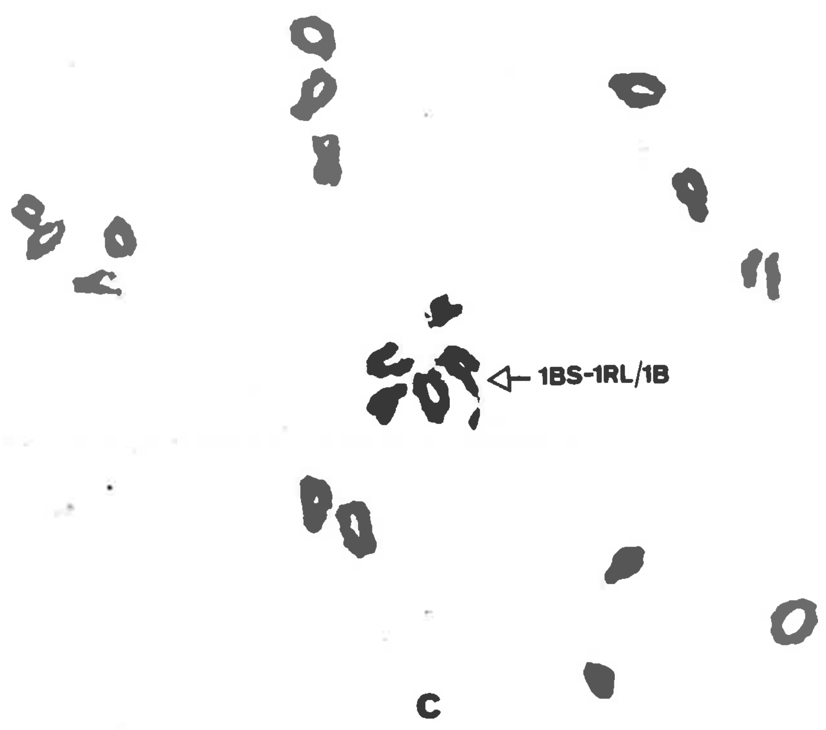

1BS-1RL/1B
c

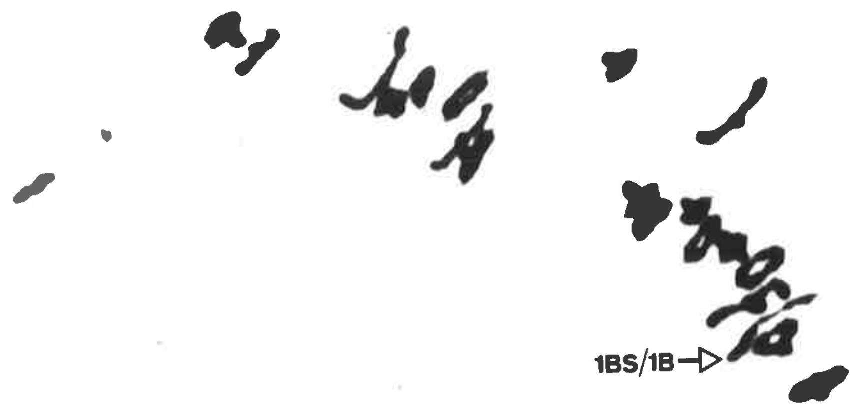
Figure 4.7

Feulgen squash preparations of PMCs, at two stages of meiosis, from the F_1 heterozygotes used to map the genes on the short arm of chromosome 1B.

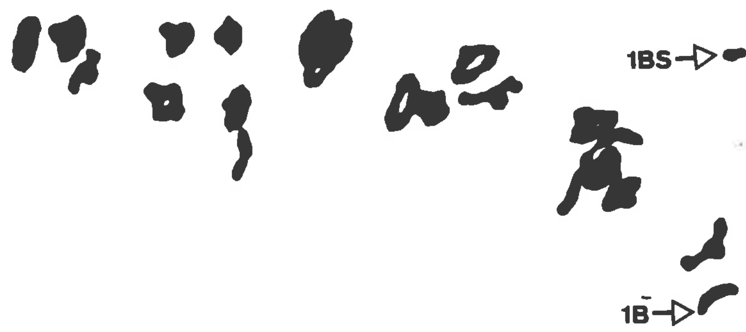
a metaphase I cell showing 1B/1BS heteromorphic bivalent;

b metaphase I cell showing 1B and 1BS univalents;

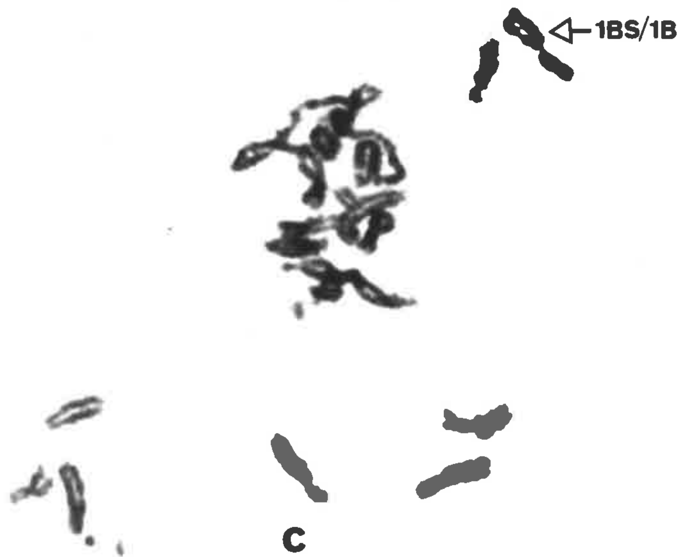
c diakinesis cell showing 1B/1BS heteromorphic bivalent.



a



b



c

Table 4.5. Observed frequency of pairing between the short arm of complete 1B chromosome and 1BS-1RL translocation and 1BS telocentric chromosomes at two stages of meiosis

F_1 combiantion	season grown	Meiotic stage	No. of PMCs	No. paired	% pairing
CS 1BS-1RL x Gabo	Summer (1981)	Metaphase I	207	123	59.4
		Diakinesis	52	46	88.5
	Spring (1982)	Metaphase I	217	177	81.6
		Diakinesis	101	94	93.1
CS Dt 1BS x Gabo	Spring (1982)	Metaphase I	249	122	49.0
		Diakinesis	63	45	71.4

into three main groups as follows:

Hypoploids- These progeny are the most frequent and are derived from chromosome deficient eggs (n=20) which lack both univalents present at metaphase I. The average probability of non-inclusion of a univalent into the female gametes is 3/4 (Sears, 1954), therefore the probability that neither of the two univalents will be included is $3/4 \times 3/4 = 9/16$. Hypoploid progeny are recognized by the lack of all protein markers controlled by both of the reference chromosomes i.e. 1B and 1BS-1RL of the F_1 heterozygote (see Fig. 4.5.A, i and B, i).

Hyperploids- These progeny are derived from aneuploid gametes resulting from the inclusion of both univalents into the same egg cell. This is a smaller class , as expected, because of the low transmission of univalents through the female gametes. If the average transmission rate is 1/4 (Sears, 1954) the probabilitiy of inclusion of both univalents in the same egg cell will be $1/4 \times 1/4 = 1/16$. Hyperploid progeny were recognized by the presence of all protein markers controlled by the two reference chromosomes (Fig.4.5.A, h and B, h).

Misdivision products- These progeny are derived from egg cells containg at least one freshly produced telocentric chromosome. The frequency of occurrence of misdivision and the transmission frequency of telocentrics for different chromosomes is quite variable and unpredictable. Furthermore, individuals having a telocentric for the long arm may have different viability to those having telocentrics for the short arms. The number of

progeny containing misdivision products was similar to that of hyperploids in the translocation mapping experiments but this class was much less frequent than the hyperploids in the telocentric mapping experiment. However, in the latter experiment it has been assumed that the telocentric with a Gabo allele (*Gli-B1ig*) was produced by recombination between the centromere and this locus, but some of these could also have been derived from the misdivision of a complete 1B chromosome from Gabo. Therefore it is likely that the number of misdivision products in this experiment is slightly underestimated. The misdivision products (Fig. 4.5.A, j, k, l, m) were recognized by the presence of only one or three of the four protein markers representing the four arms of the 1B/1BS-1RL bivalent in the translocation heterozygote. In the telocentric mapping experiment, progeny showing only a long arm protein marker (Glu-B1i) or those showing both short arm markers only (Gli-B1g+Gli-B1cs) were considered to be misdivision products.

4.3.2.4 Mapping the *Gli-B1* and *Glu-B3* loci on chromosome arm 1BS

Since the *Glu-B3* gene(s) controlling the synthesis of LMW glutenin subunits on chromosome arm 1BS could not be mapped in the crosses used for mapping the centromere—*Gli-B1* distance described above, a new triparental test-cross was produced to map *Glu-B3* with respect to *Gli-B1* and *Glu-B1*, and the procedure is described in section 4.3.2.1. The F_1 in this test-cross combination [Chinese Spring × Chinese Spring-Hope 1B) × Chinese Spring Dt 1BL] was heterozygous for alleles at the *Glu-B1*, *Gli-B1* and *Glu-B3* loci and the protein phenotypes produced by these alleles are shown in Fig. 4.8, A, B. All these bands could be scored in the gels after 1-D SDS-PAGE of reduced proteins (Fig. 4.8.B, a, b). However, it was necessary first to determine which particular protein bands were gliadins (Gli-B1cs, Gli-B1g) or LMW glutenin subunits (Glu-B3cs, Glu-B3h1 and Glu-B3h2). This was achieved by comparing the SDS-PAGE patterns of unreduced and reduced proteins from the parental lines in Fig. 4.8, A, B. The 1-D SDS-PAGE patterns of unreduced proteins showed that the two parents differ only in

Figure 4.8

1-D SDS-PAGE patterns of seed protein extracts from the parental lines (P_1, P_2, P_3) and some test-cross progeny used to map the *Glu-B1, Gli-B1* and *Glu-B3* loci.

A unreduced gliadins extracted with an ethanol-sucrose mixture from the parental lines:

a Chinese Spring;

b Chinese Spring-Hope 1B substitution;

c Chinese Spring Dt 1BL.

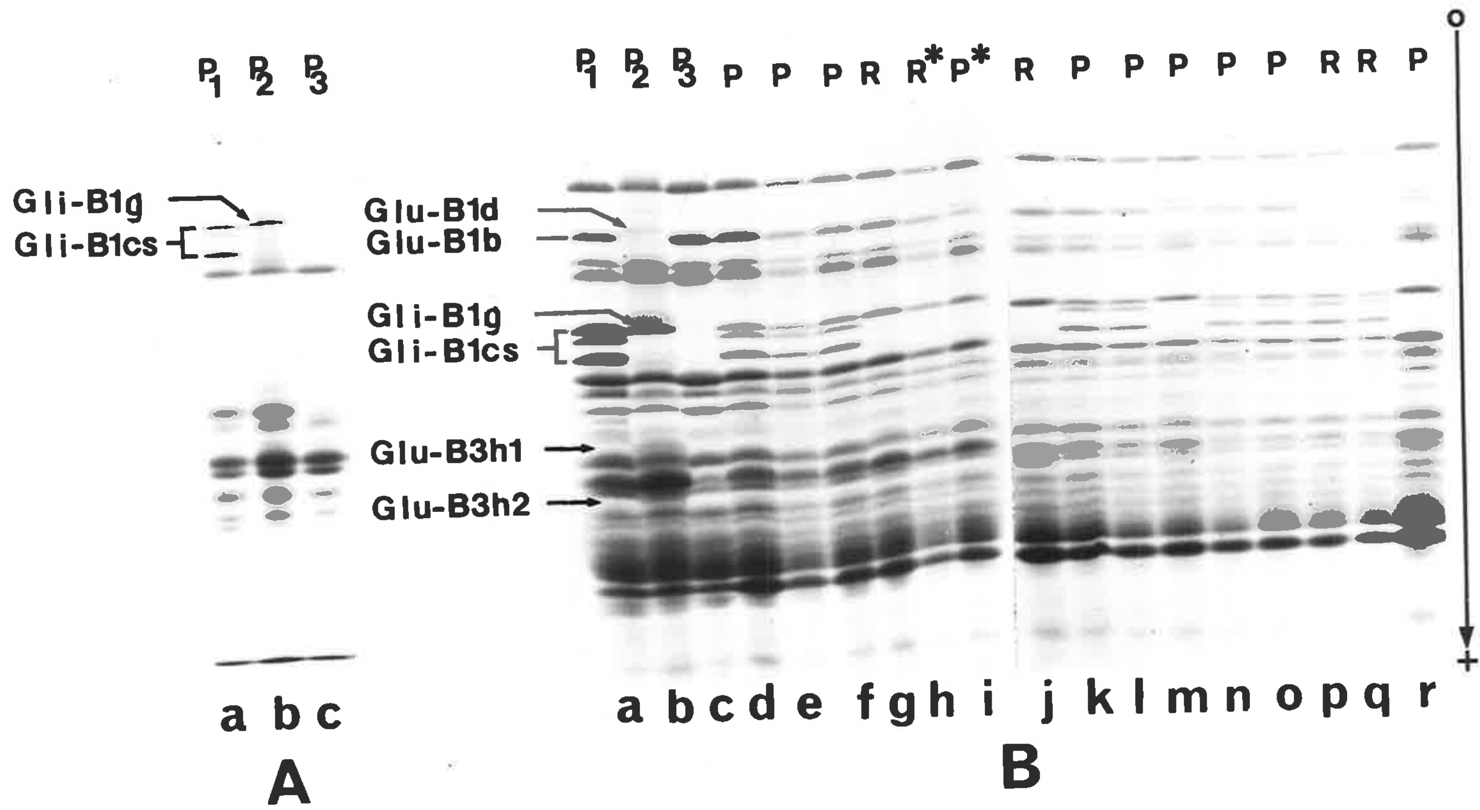
B reuduced total seed protein extracts from parental lines and test-cross progeny:

a, b and **c** see **A** above;

d-r test-cross progeny .

P = parental progeny; **R** = recombinant progeny resulting from a cross-over between *Glu-B1* and *Gli-B1* loci.

* progeny showing rare recombination within the *Glu-B3* locus.



P1 P2 P3
Gli-B1g
Gli-B1cs
a b c
A
P1 P2 P3 P P P R R* P* R P P P P P R R P
Glu-B1d
Glu-B1b
Gli-B1g
Gli-B1cs
Glu-B3h1
Glu-B3h2
a b c d e f g h i j k l m n o p q r
B
o
+

the ω-gliadin region of the gel (bands labelled as Gli-B1cs, Gli-B1g). Thus the additional difference in the banding patterns of these two parents in the gels with reduced proteins, must be due to HMW or LMW subunits of glutenin as shown in Fig. 4.8.B, a, b. This conclusion was confirmed by comparing the differences in banding patterns of these two parents after 2-S SDS-PAGE (Fig. 4.9, B, a, b), where only the subunits of glutenin are present.

After confirming the identity of the bands by the two-step procedure, the test-cross progeny were analysed by the more simple to apply 1-D SDS-PAGE of reduced proteins (Fig. 4.8, B). Since the test-cross parent Chinese Spring Dt 1BL lacked the bands controlled by *Gli-B1* and *Glu-B3* genes, the segregation of these protein bands in the progeny was scored in the gels without much difficulty. HMW glutenin subunit coded by allele *Glu-B1d* was easily identified but because of overlap with proteins from the male parent, Glu-B1b was scored on the basis of its staining intensity in the gels [scored as present in dark bands (three doses) and absent in light bands (one dose from male gametes)]. However, this scoring was facilitated because these bands occurred as strict alternatives to band Glu-B1d. Thus, the 234 test-cross progeny could be classified into parental and recombinant types, with only one aneuploid progeny (Table-4.6). The χ^2 table shows that the alleles for the three loci *Glu-B1, Gli-B1* and *Glu-B3* all segregated in a 1:1 ratio, but the ratio of the parental and recombinant types was significantly different from the 1:1 expected for independent genes, in all three regions compared. The estimate of 31.3% recombination between the *Glu-B1* and *Gli-B1* loci is significantly less than the values obtained for the centromere—*Gli-B1* interval in translocation and telocentric mapping experiments (41.6% and 40.4%, respectively). Possible reasons for this discrepancy are: (i) More frequent double cross overs between the *Glu-B1* and *Gli-B1* loci because of possible negative interference across the centromere, (ii) the influence of the different genetic backgrounds used in these experiments and (iii) different environmental conditions. The second reason seems to be the most plausible because Payne *et al.* (1982b) have observed two different recombination values (39% and 47%) for the same interval in different cultivar crosses.

Table 4.6. Observed frequency of protein phenotypes in test-cross progeny used to map the *Glu-B1*, *Gli-B1* and *Glu-B3* loci, grouped into parental, recombinant and aneuploid classes

Class	Protein phenotypes Glu-B1	Gli-B1	Glu-B3	Total progeny	p (%)	Map distance (cM)
Parentals:						
	d	g	h	83		
	b	cs	cs	76		
Recombinants:						
c.o. region 1	b	g	h	40		
	d	cs	cs	30	31.3	36.7±5.0
c.o. region 2	d	g	h1+cs	1		
	b	cs	h	0	1.7	1.7±0.8
double c.o.	b	g	h1+cs	3		
	d	cs	h	0		
Aneuploids:						
misdivision product	b	-	-	1		
Total				234		

χ^2 Table:

	Glu-B1d/ Glu-B1b	Gli-B1g/ Gli-B1cs	Glu-B3h1/ Glu-B3cs	P: R (region 1)	P: R (region 2)	P: R (region 1or 2)
Expected ratio	1: 1	1: 1	1: 1	1: 1	1: 1	1: 1
Observed No.	114: 116	127: 106	123: 110	160: 73	229: 4	162: 71
χ^2 values (P, d.f.= 1)	0.009 (P>0.9)	1.55 (P>0.2)	0.96 (P>0.3)	32.48 (P<0.001)	217.27 (P<0.001)	31.00 (P<0.001)

'-' = absent c.o. region 1 = Between *Glu-B1* and *Gli-B1*
h = h1+h2 c.o. region 2 = Between *Gli-B1* and *Glu-B3*

In contrast to the absence of observed recombination between the *Gli-A1* and *Glu-A3* loci, four progeny were detected having non-parental combinations of Gli-B1g and Glu-B3h2. However, subunit Glu-B3h1 did not show recombination with the gliadin bands, which indicates that like *Gli-B1* , the *Glu-B3* locus is also complex. Because these recombinants were very rare, attempts were made to confirm their identity in progeny tests. Three of these plants were germinated but two of them died as seedlings and the protein phenotypes of F_2 seeds obtained from the one surviving plant are shown in Fig. 4.9, A,

Figure 4.9

1-D SDS-PAGE patterns of total seed protein extracts from the original test-cross parents and the progeny of a test-cross plant showing rare recombination within *Glu-B3* locus.

A unreduced proteins;

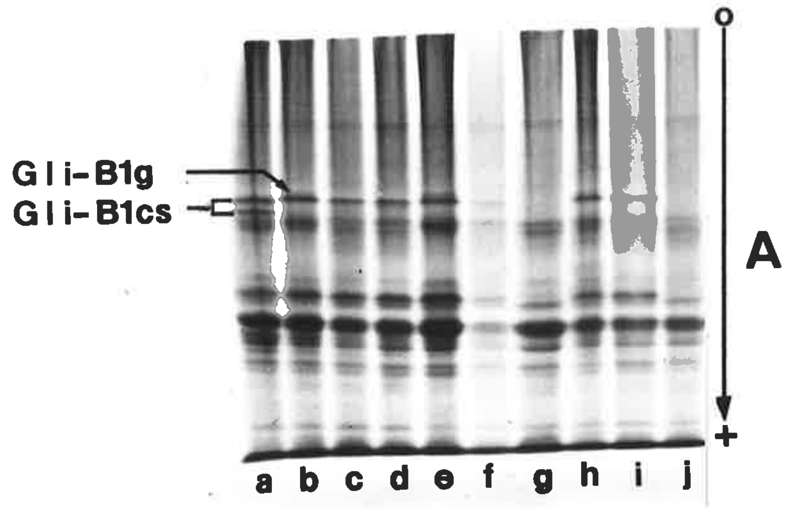
a Chinese Spring;

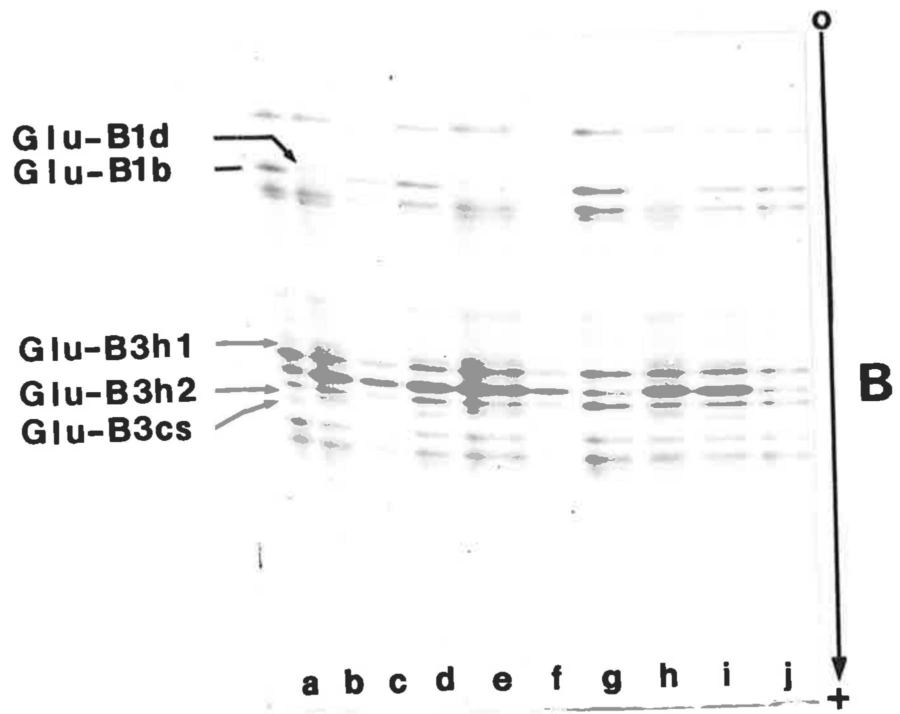
b Chinese Spring-Hope 1B substitution;

c—j selfed seeds of the recombinant test-cross plant.

B reduced proteins from the same samples as shown in part **A.**

Note: samples with recombinant phenotype **Gli-B1g**$^{+}$ **Glu-B1b**$^{+}$ **Glu-B3h1**$^{+}$ **Glu-B3cs**$^{+}$ (e.g.tracks **d** and **i**).


Gli-B1g
Gli-B1cs
A
o
+
a b c d e f g h i j


Glu-B1d
Glu-B1b
Glu-B3h1
Glu-B3h2
Glu-B3cs
B
o
+
a b c d e f g h i j

B. Part 'A' of this figure shows the gliadin phenotypes whereas, part 'B' shows the HMW and LMW glutenin subunits extracted from the same seeds. After comparing the electrophoretic patterns of the two parents [Chinese Spring (track a) and Chinese Spring-Hope1B (tracks b, e, h)] and F_2 progeny of the recombinant test-cross plant (tracks c, d, f, g, i, j) it can be concluded that two of these progeny (tracks g, j) were aneuploids (ditelocentric) because they lacked all of the 1BS protein markers and the remaining progeny (tracks c, d, f, i) were either monosomic or disomic for chromosome 1B which has recombined Gli-B1g from Hope with Glu-B3cs from Chinese Spring whilst retaining the Glu-B3h1 from Hope.

4.3.2.5 Mapping the *Tri-D1*, *Gli-D1* and *Glu-D3* loci on chromosome arm 1DS

(i) Recombination frequencies

The translocation mapping experiment carried out to estimate the map distance between the centromere and the loci on the short arm of chromosome 1D was similar to that used for mapping the homoeoloci on 1AS (sections 4.2.2 and 4.3.2.1). The protein phenotypes produced by the presumed homoeoalleles *Glu-R1i* and *Glu-D1a* controlling HMW glutelin subunits and the alleles *Tri-D1cs* and *Tri-D1i* controlling triplet proteins, *Gli-D1cs* controlling gliadins and *Glu-D3cs* controlling LMW glutenin subunits were classified without difficulty in the parents (Fig. 4.10, A, B, tracks a and b). However, there were no recognizable protein products corresponding to the supposed alleles Gli-D1i and Glu-D3i in the gels. Segreagtion for triplet and gliadin bands was analysed in the test-cross progeny after 1-D SDS-PAGE of unreduced proteins and segregation for HMW and LMW glutenin subunits was analysed by 2-S electrophoresis in a way similar to that used for analysing the analogous proteins on 1AS (section 4.3.2.2). Individual test-cross seeds were classified into parental (P), recombinant (R) and aneuploid phenotypes (Fig. 4.11, A, B). Recombinant phenotypes resulting from crossing over between the *Tri-D1* and *Gli-D1* loci are shown in Fig. 4.11.A and those resulting from crossing over between

Figure 4.10

SDS-PAGE patterns of total seed protein extracts from the parental lines (P_1, P_2, P_3) used to map the *Tri-D1*, *Gli-D1* and *Glu-D3* loci.

A 1-D SDS-PAGE of unreduced proteins;

B 2-S electrophoresis of reduced proteins;

C 1-D SDS-PAGE of reduced proteins.

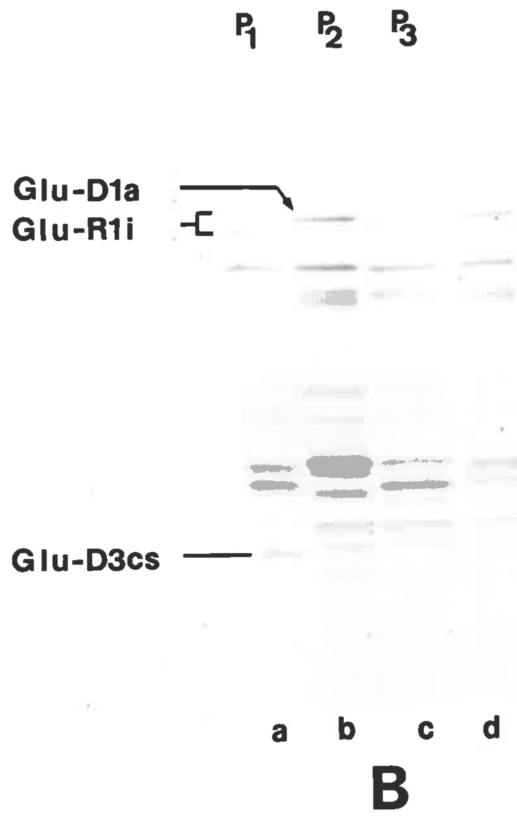
a Chinese Spring 1DS-1RL translocation;

b India 115;

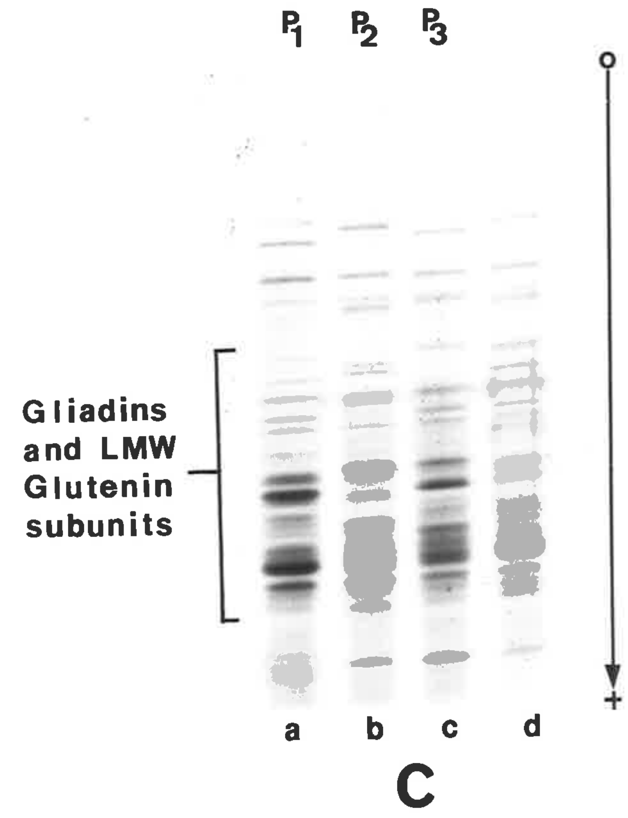
c Warigal 1DL-1RS translocation;

d Chinese Spring Dt 1DL.

P1 P2 P3
Tri-D1i
Tri-D1cs
Gli-D1cs
a b c d
A



P1 P2 P3
Glu-D1a
Glu-R1i
Glu-D3cs
a b c d
B



P1 P2 P3
Gliadins
and LMW
Glutenin
subunits
a b c d
C

Figure 4.11

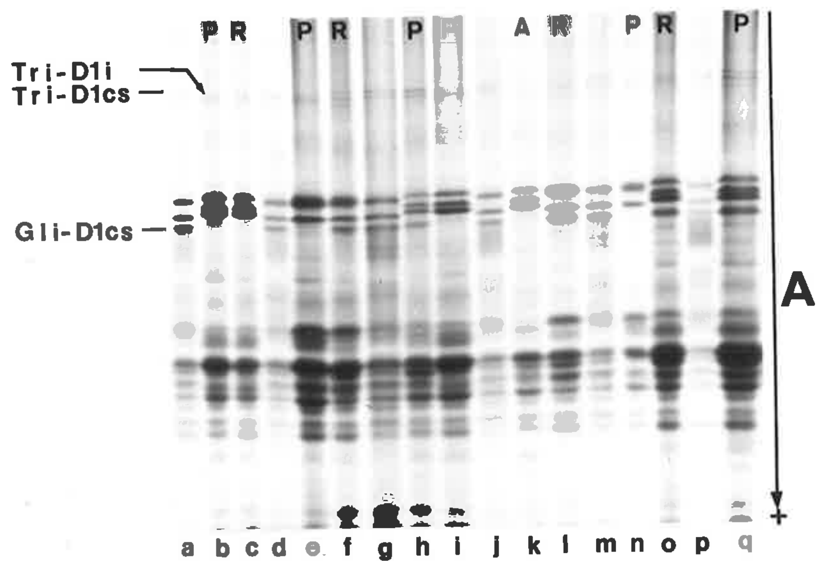
SDS-PAGE patterns of total seed protein extracts from some test-cross progeny used to map the *Tri-D1, Gli-D1* and *Glu-D3* loci:

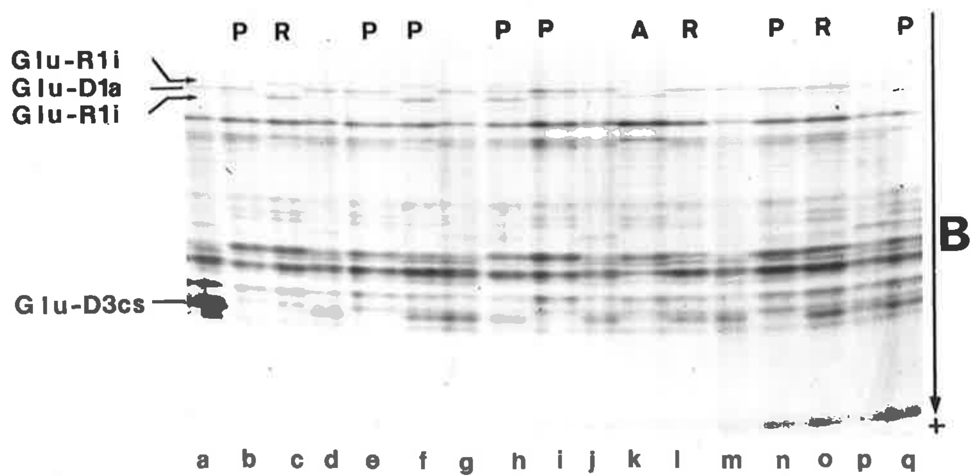
A 1-D SDS-PAGE of unreduced proteins showing recombinantion between the *Tri-D1* and *Gli-D1* loci:

a, d, g, j, m, p Chinese Spring controls.

B 2-S electrophoresis of reduced proteins obtained from the same seeds as used in part A, showing recombination between the centromere (marked with *Glu-1*) and the *Glu-D3* locus.

P = parental; **R** = recombinant; **A** = aneuploid


P R P R P P A R P R P
Tri-D1i
Tri-D1cs
Gli-D1cs
A
a b c d e f g h i j k l m n o p q


P R P P P P A R P R P
Glu-R1i
Glu-D1a
Glu-R1i
B
Glu-D3cs
a b c d e f g h i j k l m n o p q

Glu-1 (centromere) and *Glu-D3* are shown in Fig. 4.11.B. The two gels were obtained using extracts from the same seeds and for the full linkage analysis, data from both gels were combined.

Table 4.7 Observed frequency of protein phenotypes in test-cross progeny used to map the *Tri-D1*, *Gli-D1* and *Glu-D3* loci, grouped into parental, recombinant and aneuploid classes

Progeny class	Protein phenotypes Glu-1	Tri-D1	Gli-D1 Glu-D3	No. of progeny	p (%)	Map distance (cM)
Parentals:						
	D1a	i	i	83		
	R1i	cs	cs	101		
Recombinants:						
c.o. region 1	D1a	cs	cs	15		
	R1i	i	i	17	14.9	15.4± 2.1
c.o. region 2	D1a	i	cs	65		
	R1i	cs	i	59	40.3	55.8± 7.4
double c.o.	D1a	cs	i	8		
	R1i	i	cs	14		
				362		
Aneuploids:						
hypoploid:	-	-	-	61		
hyperploid?	R1i+D1a	cs+i?	cs+i?	8		
misdivision products:						
	D1a	-	-	3		
	R1i	-	-	3		
	-	i	i	1		
	-	cs+i?	cs+i?	2		
				78 (17.9%)		

χ^2 Table:

	Glu-D1a/ Glu-R1i	Tri-D1cs/ Tri-D1i	Gli-D1cs/ Gli-D1i	P: R (region 1)	P: R (region 2)	P: R (region 1 or 2)
Expected ratio	1: 1	1: 1	1: 1	1: 1	1: 1	1: 1
Observed No.	171: 191	183: 179	195: 167	308: 54	216: 146	206: 256
χ^2 values	1.10	0.09	2.17	178.22	13.53	6.91
(P, d.f.= 1)	(P>0.2)	(P>0.7)	(P>0.1)	(P<0.001)	(P<0.001)	(P>0.01)

'-' = absent

c.o. region 1 = Between centromere and *Tri-D1*
c.o. region 2 = Between *Tri-D1* and *Gli-D1*

The experiment was conducted at two different times of the year (spring 1982 and winter 1982) and altogether 441 test-cross seeds were produced for electrophoretic analysis. Except for aneuploidy and the single factor segregation of alleles at the *Gli-D1* locus, the data obtained from the two families were homogeneous ($\chi^2_{[5]}$= 9.07, P>0.1) and they have been pooled in Table-4.7. Overall, 17.9% of the progeny were aneuploids and these were ignored in the initial estimation of the recombination frequencies. However, their influence on the estimation of these values is considered later in the Discussion. As shown in Table-4.7, all of the presumed homoeoalleles and alleles followed a 1:1 segregation ratio. There was strong linkage between the centromere and *Tri-D1* (14.9% recombination) and weak linkage between *Tri-D1* and *Gli-D1* (40.3%) and between the centromere and *Gli-D1* (43.1%). Furthermore, there was complete linkage between the *Gli-D1* and*Glu-D3* loci with the upper limit for recombination calculated to be 1.1% (95% level of probability) using the method of Hanson (1959).

(ii) Chromosome pairing and the occurrence of aneuploidy

The 1DS-1RL translocation was easily identified in C-banded preparations (Fig. 4.12, a, b). Altogether 265 PMCs from a plant grown in summer of 1981 and a further 273 PMCs from a plant grown in spring 1982 were analysed at metaphase I of meiosis and no pairing was detected between the 1DL and 1RL chromosome arms. However, the short arms of these two chromosomes were paired in an average of 59.6% PMCs in summer and in 70% PMCs in spring. Clearly metaphase pairing was reduced at the higher temperature (Table-4.8). However, this reduction in pairing due to high temperature was much smaller at diakinesis. These results parallel those observed with the 1BS-1RL translocation (section 4.3.2.3). From these data it is evident that desynapsis is an important source of the univalents observed at metaphase I.

The phenotypes and frequencies of the aneuploids detected in these crosses are listed in Table-4.7. As before, the hypoploids were the most frequent class. Although hyperploids were difficult to classify because of the lack of positively identifiable protein

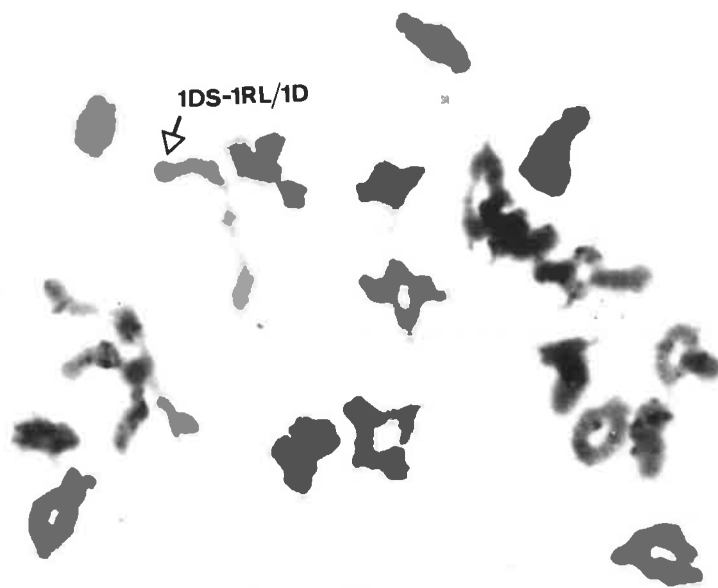
Figure 4.12

Squash preparations of PMCs, at two stages of meiosis, from the F_1 heterozygotes used to map the genes on the short arm of chromosome 1D.

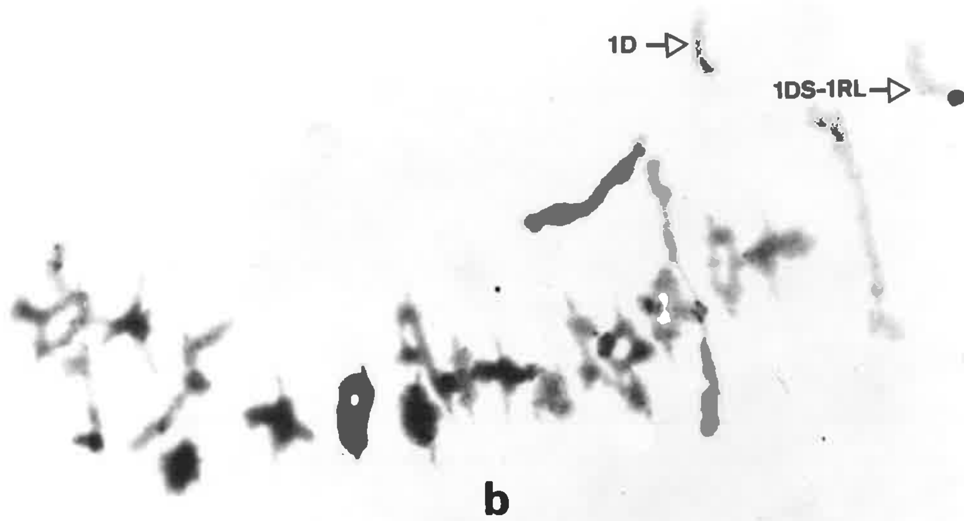
a C-banded metaphase I cell showing 1D/1DS-1RL bivalent;

b C-banded metaphase I cell showing 1D and 1DS-1RL univalents;

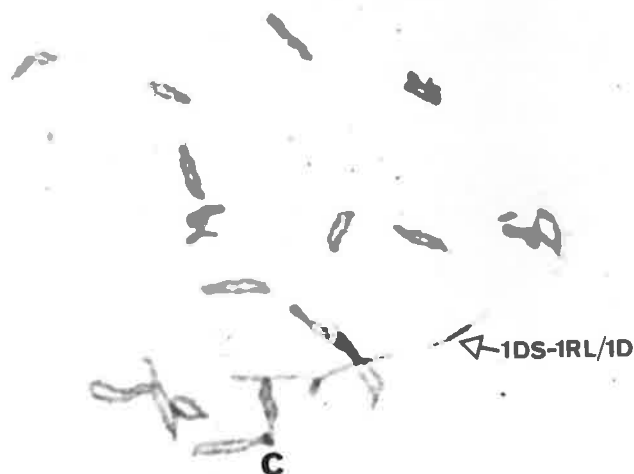
c Feulgen stained diakinesis cell showing 1D/1DS-1RL bivalent.



1DS-1RL/1D
a



1D
1DS-1RL
b



1DS-1RL/1D
c

Table 4.8. **Observed frequency of pairing between short arms of 1DS-1RL translocation and complete 1D chromosomes at two stages of meiosis**

F_1 combination	Season grown	Meiotic stage	No.of PMCs	No. paired	% pairing
CS 1DS-1RL x India 115	Summer (1982)	Metaphase I	265	158	59.6
		Diakinesis	45	41	91.1
	Spring (1982)	Metaphase I	273	191	70.1
		Diakinesis	38	36	94.7

markers on chromosome arm 1DS of cv. India 115 when present with Chinese Spring 1DS, test-cross progeny showing all of the identifiable protein bands have been classified as hyperploids. Their frequency, and that of the misdivision products, was much less than the hypoploids.

4.3.3 Mapping Genes Controlling HMW Glutenin Subunits on the Long Arms of Group 1 Chromosomes in Wheat

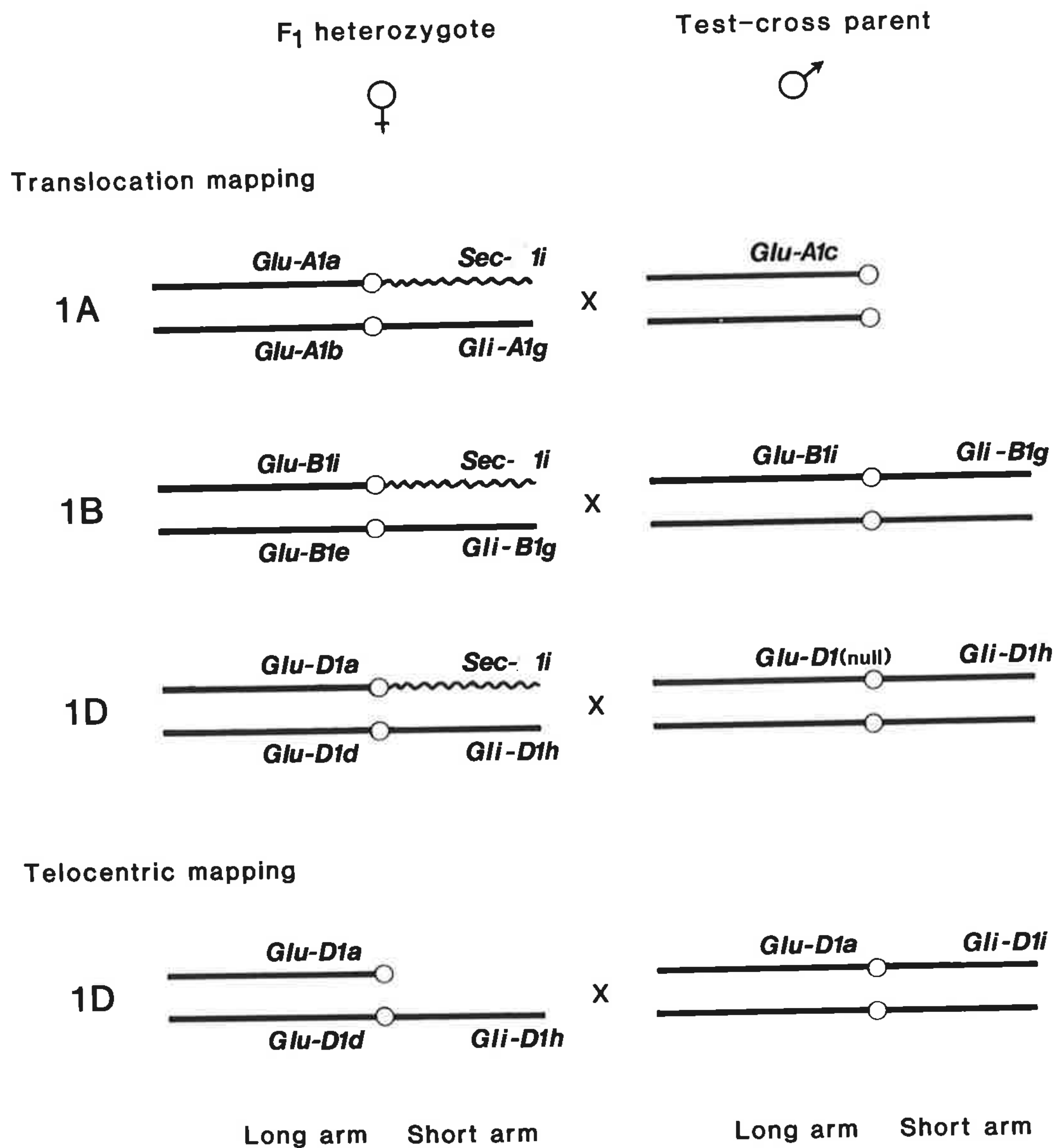
4.4.3.1 Crossing procedures

The genes controlling the synthesis of HMW glutenin subunits in wheat are located on chromosome arms 1AL, 1BL and 1DL and the loci have been designated *Glu-A1*, *Glu-B1* and *Glu-D1*, respectively. Whereas there are at least three different loci coding for storage proteins on the short arms of group 1 chromosomes, *Glu-1* is the only known locus on the long arm. The typical procedures used for mapping the *Glu-A1*, *Glu-B1* and *Glu-D1*, loci employing a translocation and for mapping the *Glu-D1* locus using a telocentric are depicted in Fig. 4.13.

(i) Translocation mapping

The crossing procedures were based on the method described by Singh and Shepherd

PROCEDURES FOR MAPPING GENES CONTROLLING HMW GLUTENIN SUBUNITS



(1984a) and outlined in the Material and Methods section. The main difference from the procedures used for mapping the genes on the short arm of wheat chromosomes was the use of translocation chromosomes involving the short arm of 1R (1AL-1RS, 1BL-1RS and 1DL-1RS) instead of the long arm. Thus the F_1 hybrids possessed *homologous* (wheat/wheat) chromatin on the long arms heterozygous for different *Glu-1* alleles and the short arms of these chromosomes were *homoeologous* (wheat/rye). The male parents were chosen so that as far as possible there was a minimum overlap of the proteins from the male parent with the critical protein bands segregating in the test-cross progeny.

(ii) Telocentric mapping

The telocentric mapping experiments were carried out in parallel with the translocation mapping in order to compare the recombination frequencies obtained from these two methods. However, this comparison was made wit!. .he *Glu-D1* locus only.

4.3.3.2 Mapping the *Glu-A1* locus on chromosome arm 1AL

(i) Recombination frequency

The protein phenotypes produced by alleles *Glu-A1a* and *Glu-A1b* for the genes controlling HMW glutenin subunits and presumed homoeoalleles *Sec-1i* and *Gli-A1cs* controlling prolamins were classified without much difficulty in the parents as shown in Fig. 4.14, A, B (tracks a and b). The electrophoretic mobility of Glu-A1a and Glu-A1b was only slightly different from the slowest moving band ('Glu-D1a') in the male parent (Fig. 4.14.A, c band not labelled) and this sometimes resulted in some overlap of protein bands. In slow runs (50 mA/ gel) Glu-A1a was clearly resolved because of its distinctly slower mobility but Glu-A1b overlapped Glu-D1a whereas, in fast runs (100 mA/gel), Glu-A1a overlapped with Glu-D1a and Glu-A1b had distinctly faster mobilty. Usually each sample was electrophoresed under both slow and fast conditions as described above. However, the gel photographs presented here (Fig. 4.14.A and Fig. 4.14.A) were obtained by running at medium current (80 mA/gel) which resolved both of the 1A alleles,

Figure 4.14

1-D SDS-PAGE patterns of total seed protein extracts from the parental lines (P_1, P_2, P_3) and some test-cross progeny used to map the *Glu-A1* locus.

A reduced proteins;

P_1 Chinese Spring—Hope 1AL-1RS;

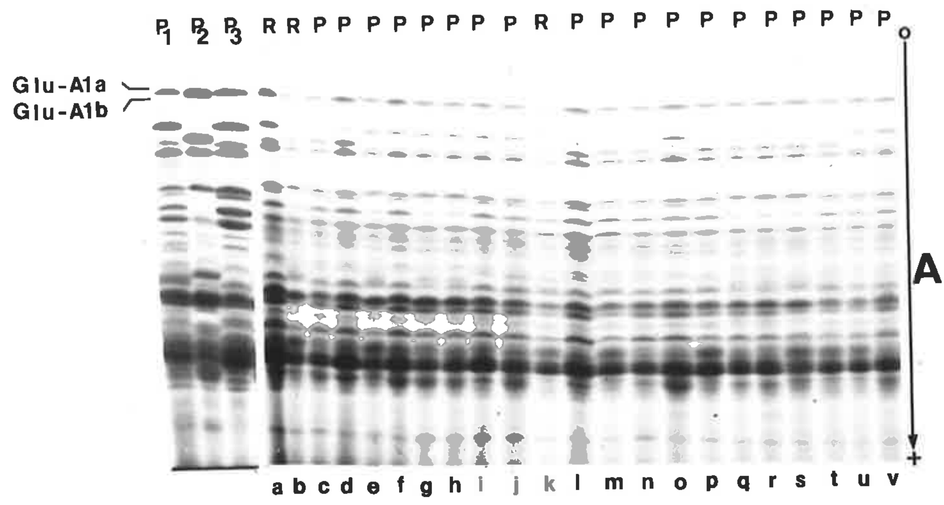
P_2 Gabo;

P_3 Chinese Spring Dt 1AL;

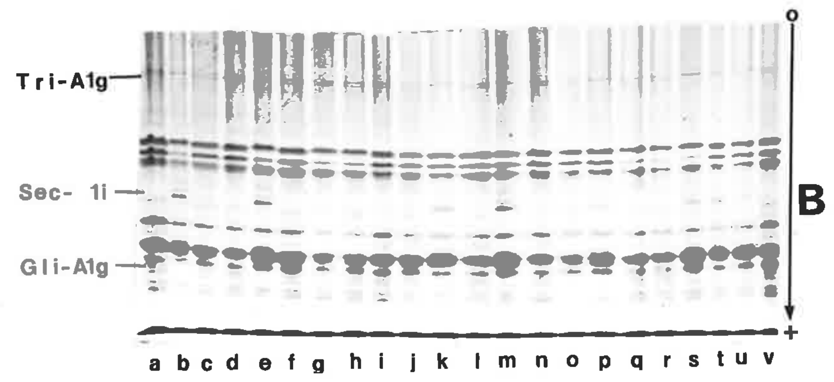
a—v test-cross seeds.

B unreduced proteins obtained from the same seeds used in part **A.**

P = parental; R = recombinant



P1 P2 P3 R R P P P P P P P P R P P P P P P P P P P P
Glu-A1a
Glu-A1b
A
a b c d e f g h i j k l m n o p q r s t u v



Tri-A1g
Sec- 1i
Gli-A1g
B
a b c d e f g h i j k l m n o p q r s t u v

although their separation is not ideal. In addition to Gli-A1cs, chromosome arm 1AS also possessed Tri-A1 protein which was responsible for the presence of the hybrid triplet band (Fig. 4.14, B). Thus each of the four arms of the 1A/1AL-1RS bivalent was marked with at least one recognizable protein marker. Since the male test-cross parent Chinese Spring Dt 1AL lacked all of these critical protein markers, the protein phenotypes of the test-cross progeny were unambiguously classified into parental and recombinant types (Fig. 4.14, A, B). HMW glutenin subunits were scored in the gels after 1-D SDS-PAGE of reduced proteins (Fig. 4.14, A) whereas the triplet proteins and prolamins were scored following 1-D SDS-PAGE of unreduced proteins (Fig. 4.14, B). A total of 79 test-cross seeds were produced from two different F_1 plants and since the data were homogeneous ($\chi^2_{[3]}$= 2.67, P> 0.2), only pooled values are given in Table-4.9. None of the test-cross progeny were aneuploid and the average recombination frequency was 10.1%.

Table 4.9. Observed frequency of protein phenotypes in test-cross progeny used to map the *Glu-A1* locus, grouped into parental and recombinant classes

Protein phenotypes						
Parentals		***Recombinants***				
Glu-A1a Sec-1i	Glu-A1b Tri-A1h	Glu-A1a Tri-A1h	Glu-A1b Sec-1i	Total	p (%)	Map distance (cM)
31	40	3	5	79	10.1	10.2±3.5

χ^2 Table:

	Glu-A1a/ Glu-A1b	Tri-A1h/ Sec-1i	P/R
Expected ratio	1:1	1:1	1:1
Observed No.	34: 45	43: 36	71: 8
χ^2 values	1.53	0.63	50.2
(P, d.f.=1)	(P>0.2)	(P>0.3)	(P<0.001)

In addition to these test-cross progeny, 158 F_2 seeds from 3 different F_1 plants were also analysed electrophoretically (Fig. 4.15, A, B). Because of the codominant expression of these protein phenotypes the F_2 progeny could be classified into nine different classes out of the ten possible phenotypic classes, one class could not be scored because the

Figure 4.15

1-D SDS-PAGE patterns of total seed protein extracts from F_2 progeny used to map the *Glu-A1* locus.

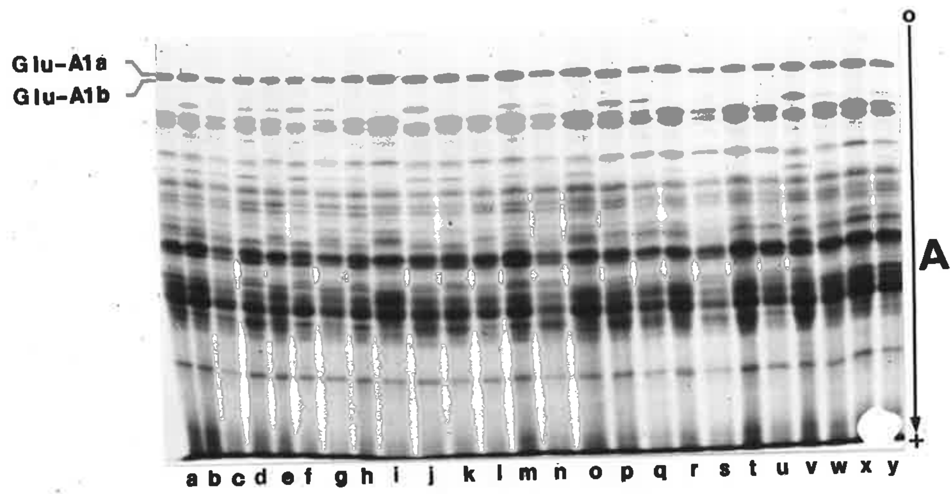
A reduced proteins;

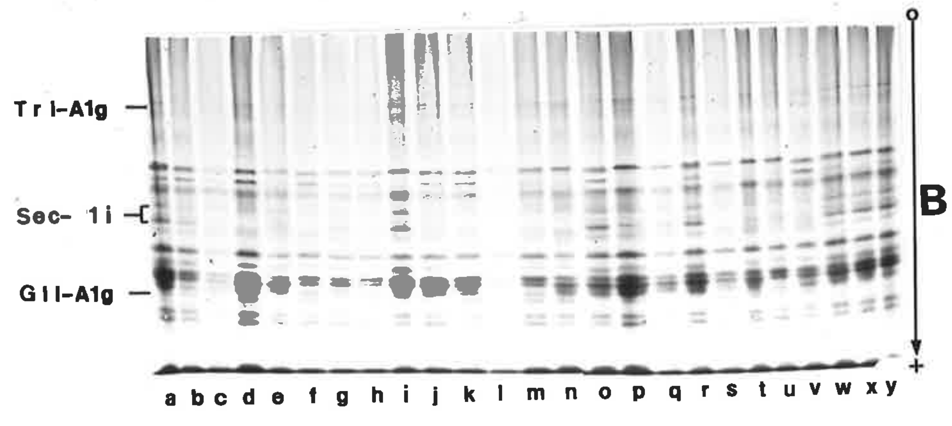
B unreduced proteins obtained from the same seeds used in part **A.**

The F_2 progeny (a—y) were scored as follows:

	Glu-A1a	Glu-A1b	Sec-1i	Tri-A1g
a	+	+	+	+
b	+	+	+	+
c	+	+	-	+
d	+	+	-	+
e	+	+	+	+
f	+	+	-	+
g	+	+	+	+
h	+	-	-	+
i	+	+	+	-
j	+	+	+	+
k	+	+	-	+
l	-	+	-	+
m	+	+	-	+
n	+	-	-	+
o	+	-	+	-
p	-	+	+	+
q	+	-	+	+
r	+	-	+	-
s	-	+	-	+
t	-	+	+	+
u	+	+	+	+
v	+	+	+	+
w	+	+	+	-
x	+	-	+	-
y	+	-	+	-

- = absent + = present


Glu-A1a
Glu-A1b
A
a b c d e f g h i j k l m n o p q r s t u v w x y


Tri-A1g
Sec- 1i
Gli-A1g
B
a b c d e f g h i j k l m n o p q r s t u v w x y

double heterozygote parental was indistinguishable from the double heterozygote recombinant without further progeny testing. However,with incompletely dominant genes allowing the F_2 progeny to be classified into these nine classes, F_2 data are always more efficient than test-cross data for the measurement of linkage and are as good as test-cross data for the detection of linkage (Mather,1936). The data obtained from three different plants were homogeneous ($\chi^2_{[6]}$=9.76, P>0.1) for the four phenotypic classes (nine classes were grouped into four classes as shown in Table-4.10), and are therefore pooled.

Table 4.10 Observed frequency of protein phenotypes in F_2 progeny used to map the *Glu-A1* locus

Protein phenotypes Glu-A1a	Glu-A1b	Sec-1i	Tri-A1h	No.of Progeny	Total	Expected frequency	p (%)	Map distance (cM)
+	-	+	-	16			29.5	33.9±5.4
-	+	-	+	15	31	$0.5\ (1-p)^2$		
+	+	+	-	12				
+	+	-	+	27				
+	-	+	+	15				
-	+	+	+	8	62	$2p\ (1-p)$		
+	+	+	+	58	58	$0.5p^2 + 0.5\ (1-p)^2$		
+	-	-	+	5				
-	+	+	-	2	7	$0.5\ p^2$		
Total				158	1			

p= porportion of recombinant gametes
1-p= proportion of parental gametes

χ^2 Table:

	Glu-A1a/ Glu-A1b	Tri-A1h/ Sec-1i	P:R
Expected ratio	1:1	1:1	1:1
Obseved No.*	154: 139	148: 129	**
χ^2 values (P, d.f.=1)	o.76 (P>0.5)	1.30 (P>0.1)	24.88 (P<0.001)

* number of gametes; **cannot be determined, χ^2 value calculated indirectly;
– = absent; + = present

Using the maximum likelihood method, the recombination frequency was calculated to be 29.5%. This value is almost three times more than the one obtained in the test-cross experiment. Since the F_2 seeds were obtained from the identical plants which were used to produce test-cross seeds, this difference could not be due to environment or genotype. It is possible that there is a much higher recombination frequency in the PMCs compared to the megaspore mother cells (MMCs). Further experimentation is needed to resolve this question.

In both test-cross and F_2 mapping experiments the presumed alleles *Glu-A1a* and *Glu-A1b* and presumed homoeoalleles *Sec-1i* and *Gli-A1g* followed the expected 1:1 segregation ratio and the linkage between *Glu-A1* and the centromere was significant.

(ii) Chromosome pairing and the occurrence of aneuploidy

Unlike the translocation chromosomes involving chromosome arm 1RL, translocation chromosomes with 1RS derived from Imperial rye could not be recognized in C-banded preparations and therefore Feulgen preparations were used for the analysis of pairing between the 1AL-1RS translocation and complete 1A chromosomes. These chromosomes were not easily identifiable except for the slight heteromorphy of the bivalent and a poorly differentiated satellite on chromosomse arm 1RS (Fig. 4.16, a, b). Therefore, these two chromosomes were considered to be definitely paired in only those PMCs showing 21 bivalents. This practice will lead to an underestimate of the pairing frequency because in some PMCs the univalents will be chromosomes other than 1A or 1AL-1RS. A total of 190 PMCs were analysed at metaphase I and 89.5% of these showed 21 bivalents. There was at least one rod bivalent in each of these PMCs suggesting that chromosome arms 1AS and 1RS did not pair. As evident from the lack of aneuploids amongst the test cross progeny, asynapsis must have been insignificant and the observed 89.5% pairing is probably an underestimate. Because of the good pairing at metaphase, PMCs were not analysed at diakinesis. Thus, it is considered unlikely that asynapsis had any significant influence on the estimates of recombination values.

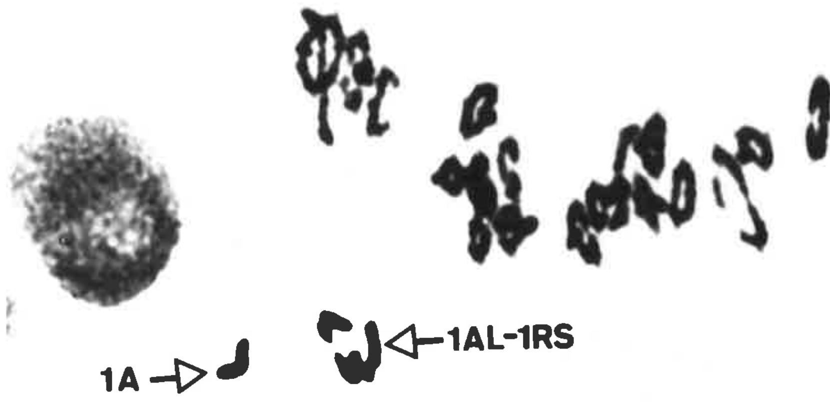
Figure 4.16

Squash preparations of Feulgen-stained PMCs from the F_1 heterozygotes used to map the genes on the long arm of chromosome 1A.

a metaphase I cell showing 1AL-1RS/1A bivalent;

b metaphase I cell showing 1AL-1RS and 1A univalents.

a



b

4.3.3.3 Mapping the *Glu-B1* locus on chromosome arm 1BL

(i) Recombination frequency

The three different test-cross combinations listed below were used to produce a total of 430 test-cross seeds.

1† [Gabo 1BL-1RS x (MKR) 211/7] × Single glutenin line

2* [Gabo 1BL-1RS x (MKR) 211/7] × Gabo 1BL-1RS

3† [Gabo 1BL-1RS x WR/17] × Single glutenin line

The protein phenotypes produced by the presumed alleles *Glu-B1i* and *Glu-B1e* for the genes controlling HMW glutenin subunits and presumed homoeoalleles *Gli-B1g* and *Sec-1i* for the genes controlling prolamins could be classified without difficulty in the parents (Fig. 4.17, A, B, tracks a and b). The prolamins and the HMW glutenin subunits were scored in the gels after 1-D SDS-PAGE of the unreduced and reduced proteins, respectively. The unreduced proteins shown in Fig. 4.17, B were extracted with an ethanol/sucrose mixture as described in Materials and Methods. Among the test-cross progeny, Sec-1i and Glu-B1e were classified without difficulty, but since Glu-B1i and Gli-B1g proteins were also produced by the male parent, these bands could only be detected by differences in their staining intensity. That is, the strong bands were inferred to have 3 doses of these alleles and the weak bands 1 dose, corresponding to the presence or absence, respectively, of the alleles from the female parent. However, this classification was facilitated because each of these bands segregated as an alternative to the easily identifiable protein bands Glu-B1e and Sec-1i, respectively (Fig. 4.17, A, B, tracks d-p).

The data obtained from the three test-cross families, including the aneuploid frequency, were homogeneous ($\chi2_{[8]}$=8.55, P>0.3) and they were pooled as shown in Table-4.11. The presumed alleles *Glu-B1i* and *Glu-B1e* and presumed homoeoalleles *Gli-B1g* and *Sec-1i* gave the expected 1:1 segregation ratio. However, the χ^2 value for an equal number of parentals and recombinants was significant, indicating linkage

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† grown in summer 1982; *grown in spring 1982

Figure 4.17

1-D SDS-PAGE patterns of seed protein extracts from the parental lines (P_1, P_2, P_3) and some test-cross progeny used to map the *Glu-B1* locus.

A reduced total seed protein extracts;

B unreduced gliadins (ethanol-sucrose extracts) from the same seeds used in part A.

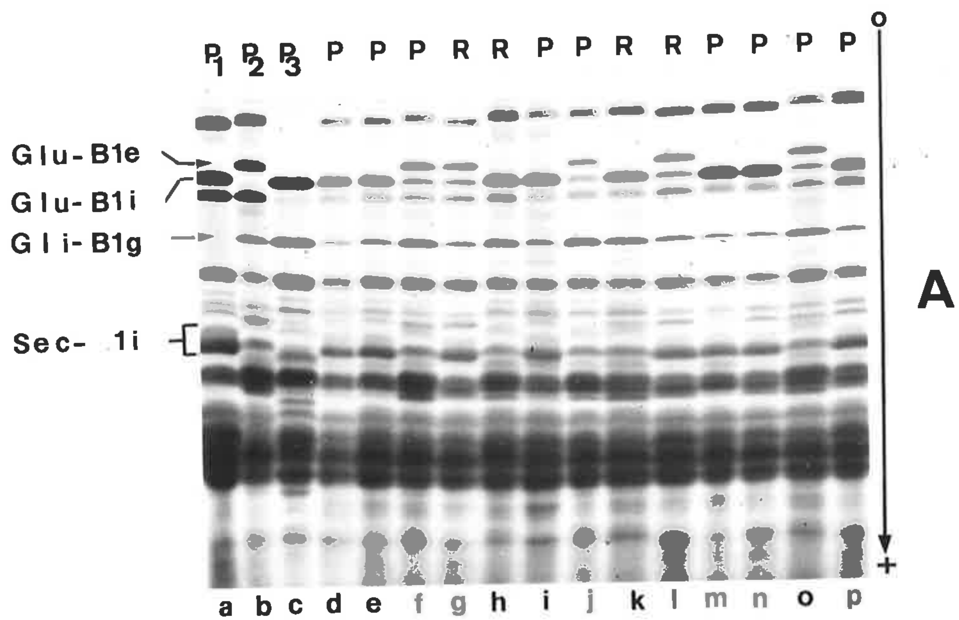
a Gabo 1BL-1RS;

b MKR/211/7;

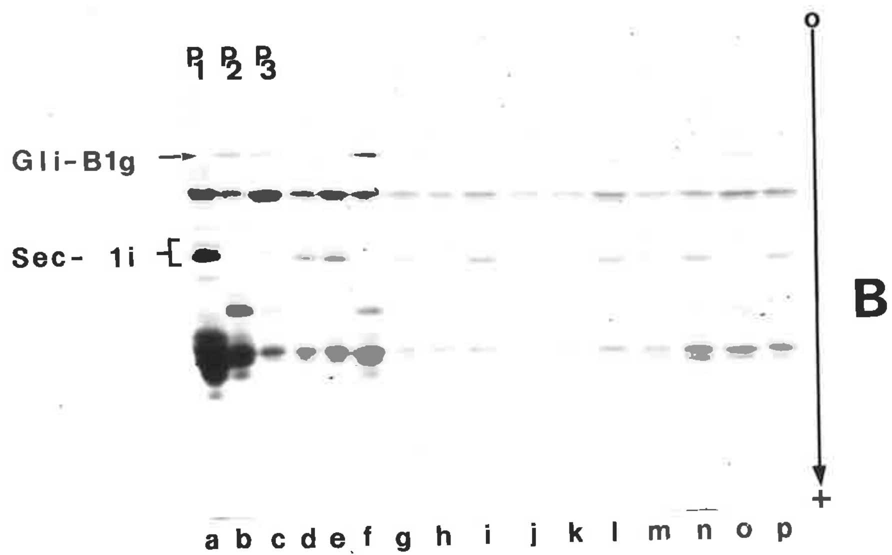
c Single glutenin line;

d-p test-cross progeny.

P = parental; **R** = recombinant



P1 P2 P3 P P P R R P P R R P P P P
o
Glu-B1e
Glu-B1i
Gli-B1g
Sec- 1i
A
+
a b c d e f g h i j k l m n o p



P1 P2 P3
o
Gli-B1g
Sec- 1i
B
+
a b c d e f g h i j k l m n o p

between the centromere and the Glu-B1 locus, with an average recombination frequency of 25.5%.

Table 4.11. Observed frequency of protein phenotypes in test-cross progeny used to map the *Glu-B1* locus, grouped into parental recombinant and aneuploid classes

	Protein phenotypes							
	Parentals		*Recombinants*		*Aneuploids*			
	Glu-B1i Sec-1i	Glu-B1e Gli-B1g	Glu-B1i Gli-B1g	Glu-B1e Sec-1i	(hypoploid)	Total	p (%)	Map distance (cM)
Total	150	168	57	52	3	430	25.5	28.1±2.8

χ^2 Table:

	Glu-B1i/Glu-B1e	Gli-B1g/ Sec-1i	P/R
Expected ratio	1:1	1:1	1:1
Observed No.	207: 220	202: 225	318: 109
χ^2 values (P, d.f.=1)	0.39 (P>0.5)	1.23 (P>0.2)	102.3 (P<0.001)

(ii) Chromosome pairing and the occurrence of aneuploidy

The 1BL-1RS and 1B chromosomes were identified in the Feulgen preparations of PMCs at metaphase I by the presence of the satellite on 1BS and the heteromorphy of the bivalent involving these chromosomes (Fig. 4.18, a, b). However, this identification was not completely reliable and therefore only the PMCs showing 21 bivalents were taken as evidence for pairing between the two reference chromosomes. Of the total 303 PMCs examined, 96.4% showed 21 bivalents at metaphase I, therefore asynapsis was insignificant as reflected in the low level of aneuploidy (0.7%) among the test-cross progeny. Thus, asynapsis had little influence on the estimate of recombination frequency. Each PMC showed at least one rod bivalent suggesting that 1BS and 1RS arms always remain unpaired.

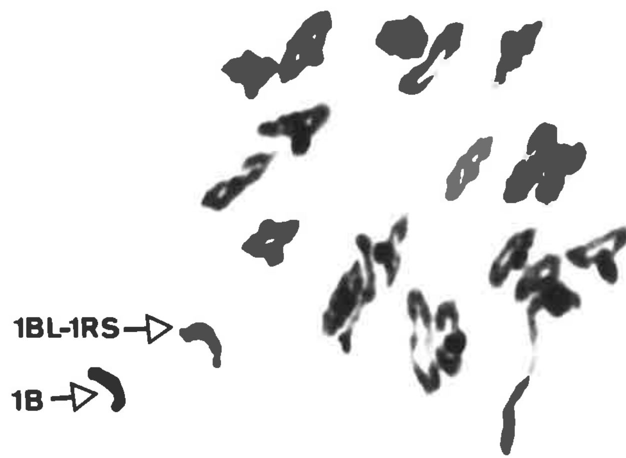
Figure 4.18

Squash preparations of Feulgen-stained PMCs from the F_1 heterozygotes used to map the genes on the long arm of chromosome 1B.

a metaphase I cell showing 1BL-1RS/1B bivalent;

b metaphase I cell showing 1BL-1RS and 1B univalents.

a



b

4.3.3.4 Mapping the *Glu-D1* locus on chromosome arm 1DL

(i) Recombination frequency

Four different test-cross combinations for translocation mapping and two test-cross combinations for telocentric mapping were used to produce 720 test-cross seeds as shown below:

1† [CS 1DL-1RS × Halberd] × Single glutenin line

2* [CS 1DL-1RS × Halberd] × Null Glu-D1/null Gli-D1 line

3† [CS 1DL-1RS × Heron] × Single glutenin line

4† [Halberd 1DL-1RS × Kite] × Single glutenin line

5† [CS Dt 1DL × Halberd] × India 115

6* [CS Dt 1DL × Halberd] × Warigal 1DL-1RS

The translocation mapping experiment involving Chinese Spring 1DL-1RS x Halberd F_1 was repeated twice at different times of the year (summer 1982 and spring,1982). In one of these experiments, a specially bred null Glu-D1/null Gli-D1 line was used as the male parent since it had no band that overlapped the critical protein bands from the F_1 heterozygote. When the single glutenin line was used as the male parent one of its bands overlapped Gli-D1h. The telocentric mapping experiment was repeated in two seasons using two different male parents, each of which provided one band (Glu-D1a in India 115 and Glu-D1d in Warigal 1DL-RS) which overlapped a critical band in the F_1 heterozygote.

As usual, HMW glutenin subunits were scored in the gels with reduced proteins and prolamins were scored in the gels with unreduced proteins. The protein phenotypes manifested by the alleles *Glu-D1a* and *Glu-D1d* for the genes controlling HMW glutenin subunits, and the presumed homoeoalleles *Gli-D1h* and *Sec-1i* for the genes controlling prolamins, were easily classified in the parents (Fig. 4.19, A, b, c and B, b, c, respectively). Similarly all protein phenotypes could be reliably determined in the test-cross progeny even though the presence and absence of Gli-D1h protein was

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†grown in summer 1982; * grown in spring 1982

Figure 4.19

1-D SDS-PAGE patterns of seed protein extracts from the parental lines (P_1, P_2, P_3) and some test-cross progeny used to map the *Glu-D1* locus.

A reduced total seed protein extracts;

B unreduced gliadins (ethanol-sucrose extracts) obtained from the same seeds used in part **A**.

a Chinese Spring control;

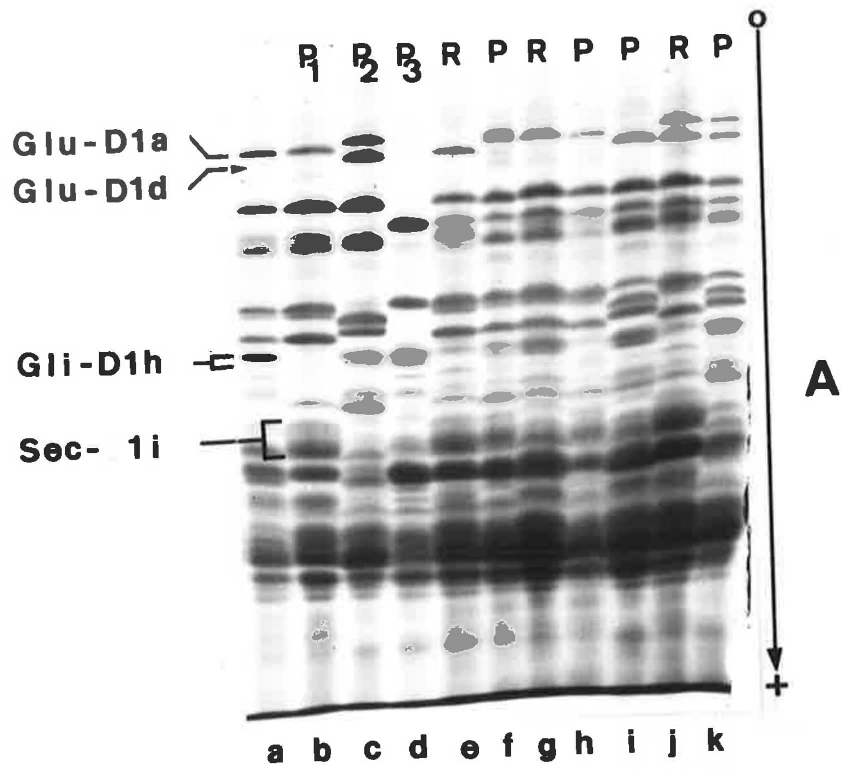
b Chinese Spring 1DL-1RS;

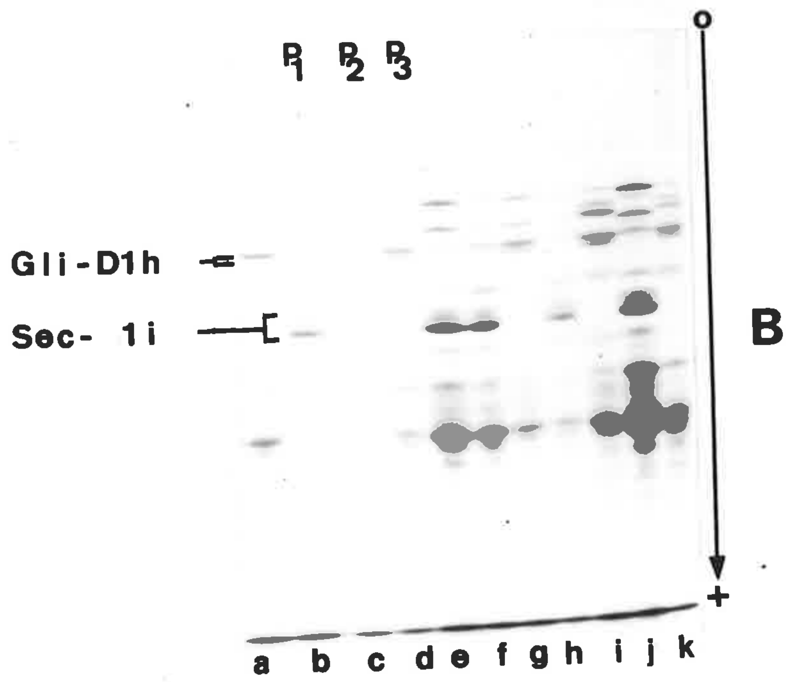
c Halberd;

d Single glutenin line;

e-k test-cross progeny.

P = parental; **R** = recombinant


P1 P2 P3 R P R P P R P
Glu-D1a
Glu-D1d
Gli-D1h
Sec- 1i
A
a b c d e f g h i j k


P1 P2 P3
Gli-D1h
Sec- 1i
B
a b c d e f g h i j k

determined on the basis of band staining intensity (Fig. 4.19, A, B tracks e to k). Parental, recombinant and aneuploid types were distinguished in both the translocation and telocentric mapping experiments (Table 4.12).

The data obtained from the four translocation mapping experiments and the two telocentric mapping experiments, except for the aneuploid frequency, were all homogeneous ($\chi^2_{[15]} = 17.35$, P>.2). However, for the purpose of comparison they are pooled separately for each type of mapping experiment and listed separately in Table-4.12.

Table 4.12. Observed frequency of protein phenotypes in test-cross progeny used to map the *Glu-D1* locus, grouped into parental, recombinant and aneuploid classes

Protein phenotypes									
Parentals		***Recombinants***		***Aneuploids***					
Glu-D1a (Sec-1i)†	Glu-D1d Gli-D1h	Glu-D1a Gli-D1h	Glu-D1d (Sec-1i)†	hypo-ploid	hyper-ploid	mis-division	Total	p (%)	Map distance (cM)
(a) Translocation mapping:									
186	185	58	83	13	1	3	529	27.5	30.9±2.7
(b) Telocentric mapping:									
71	74	11	27	6	1	1	191	20.7	22.0±3.5

χ^2 Table:

	Glu-D1a/ Glu-D1d	Gli-D1h/(Sec-R1i)*	P/R
Expected ratio	1:1	1:1	1:1
(a) Translocation mapping:			
Observed No.	244: 268	243: 249	371: 141
χ^2 values	1.125	0.07	103.3
(P, d.f.=1)	(P>0.2)	(P>0.7)	(P<0.001)
(b) Telocentric mapping:			
Observed No.	82: 101	85: 98	145: 48
χ^2 values	1.97	0.92	62.5
(P, d.f.=1)	(P>0.1)	(P>0.3)	(P<0.001)

†not present in telocentric mapping

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It is clear from the χ^2 table that each of the presumed allele and homoeoallele pairs followed the expected 1:1 segregation ratio, whereas the joint segregation showed significant linkage. The estimate of the recombination frequency obtained from the translocation mapping experiments (27.5 %) was much higher than that obtained with telocentric mapping (20.7 %). Although the overall level of aneuploidy was low (3.5%), it occurred with higher frequency in the telocentric mapping experiments than in the translocation mapping procedure.

This comparison of the results from translocation and telocentric mapping procedures parallels that observed when mapping the centromere-*Glu-B1* distance (section, 4.3.2.3) except the difference is more extreme in the present experiments. These results are consistent with the findings of Sears (1972) in wheat, and of Endrizzi and Kohel (1966) in cotton, where recombination frequencies for proximal loci were markedly reduced (up to four times) when telocentric mapping was used instead of full chromosome mapping . However, both of these authors found that this reduction in recombination frequency is not significant with distal loci . The proximal loci analysed in their experiments were very close to the centromere (less than 4.5% recombination) whereas the *Glu-D1* locus in the present study is much less closely linked with the centromere. Thus it may be concluded that loci more distant from the centromere will show less of a reduction in recombination frequency with the telocentric mapping procedure.

(ii) Chromosome pairing and the occurrence of aneuploidy

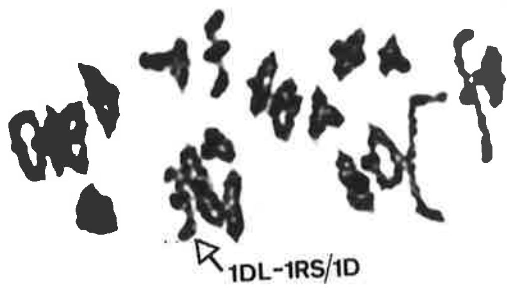
The translocation chromosome 1DL-1RS was significantly larger than chromosome 1D and these two chromosomes were easily identified in Feulgen preparations of PMCs at metaphase I of meiosis (Fig. 4.20, a, b) and the telocentric chromosome was even easier to recognise (Fig. 4.21, a, b). Altogether 128 PMCs from the monotelodisomic 1DL and 151 PMCs from 1DL-1RS translocation heterozygotes were examined. No pairing was observed between 1DS and 1RS in the latter PMCs. However, there was 90.7% and 83.6% pairing between the long arm of the complete chromosome 1D with the 1DL arm of the translocation and telocentric 1DL, respectively. No observations were made at

Figure 4.20

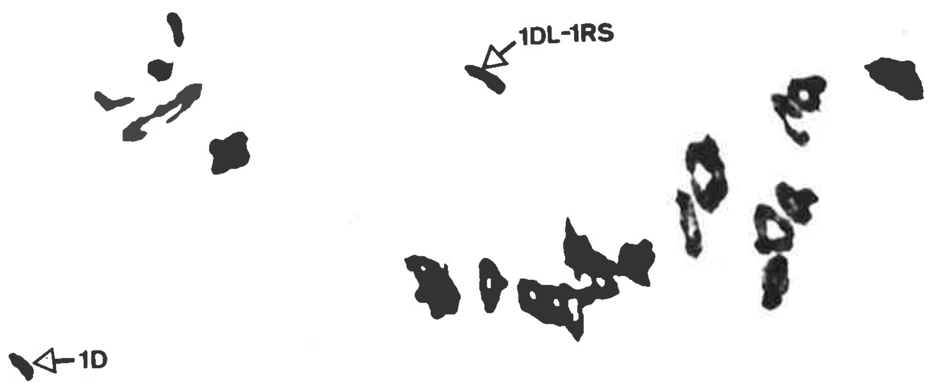
Squash preparations of Feulgen-stained PMCs from the F_1 heterozygotes involving translocation 1DL-1RS used to map genes on the long arm of chromosome 1D.

a metaphase I cell showing 1DL-1RS/1D bivalent;

b metaphase I cell showing 1DL-1RS and 1D univalents.



a



b

Figure 4.21

Squash preparations of Feulgen-stained PMCs from the F_1 heterozygotes involving telocentric 1DL used to map genes on the long arm of chromosome 1D.

a metaphase I cell showing 1DL/1D heteromorphic bivalent;

b metaphase I cell showing 1DL and 1D univalents.

1DL/1D
a

1D
1DL
b

diakinesis,but some desynapsis is expected similar to that observed in each of the short arm mapping experiments.

The proportion of aneuploids detected in the test-cross progeny was small with 3.2% and 4.2% observed in the translocation and telocentric mapping experiments, respectively. Therefore, the influence of asynapsis and aneuploidy on the estimation of recombination values should not be very great.

(iii) Rare recombination within the Glu-D1 locus

There are two protein bands controlled by allele *Glu-D1a* (subunits 2 and 12) and similarly two bands (subunits 5 + 10) controlled by the allele *Glu-D1d.* Each of these two pairs of bands are normally inherited as a unit and no recombinant type (2+10 or 5+12) was detected in 479 test-cross seeds analysed by Lawrence and Shepherd (1981b) and a further 138 F_2 seeds analysed by Payne *et al.* (1980). However, in the present study a 2+10 recombinant was observed among the 720 test-cross progeny analysed. Unfortunately, this plant died as a seedling, but the gel photograph clearly shows the evidence for such recombination (Fig. 4.22, c). This rare recombinant was detected among the test-cross progeny from the cross [(Chinese Spring 1DL-1RS × Heron) × Single glutenin line]. It is of interest that the frequency of recombination between the centromere and the *Glu-D1* locus was highest (35.2 %) in this cross.

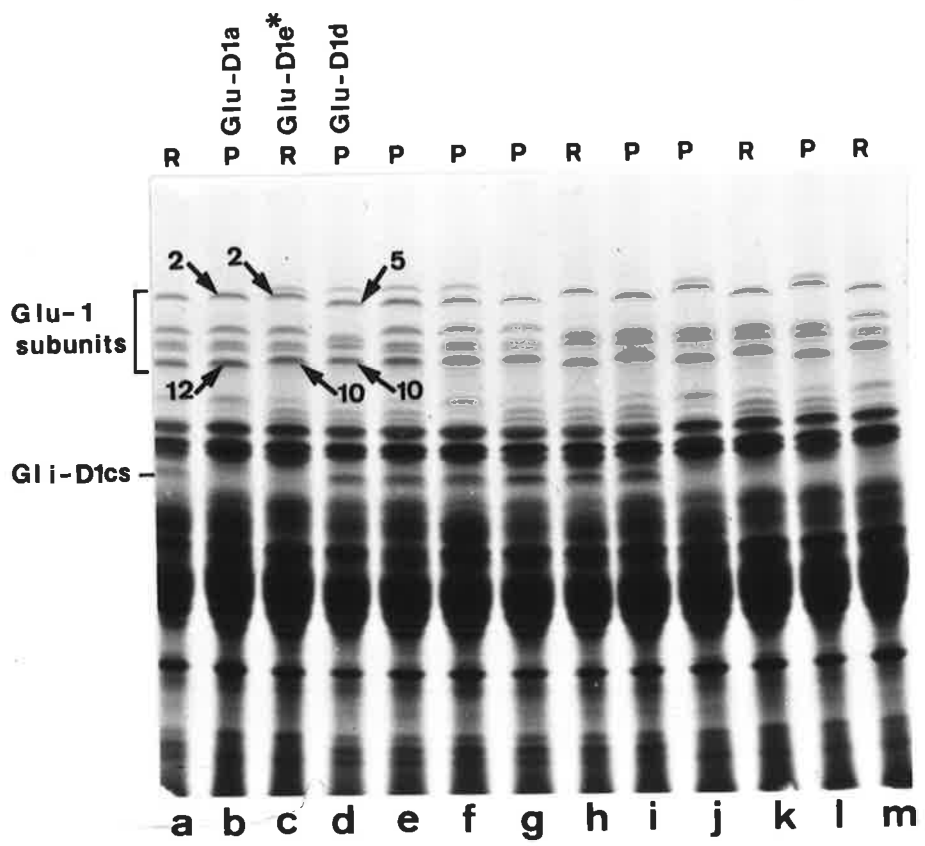
This rare recombination event may explain the origin of some of the naturally occurring rare patterns which combine two subunits from the two different frequently-occurring alleles for this locus e.g. the French cultivar Flinore has subunits 2+10 and this is designated as allele '*Glu-D1e* ' (Payne and Lawrence, 1983). The recovery of this recombinant 'allele' from the parents having alleles *Glu-D1a* and *Glu-D1d* suggests that the bands which are considered part of an allele are in fact the products of two very tightly linked genes forming part of a complex locus.

Figure 4.22

1-D SDS-PAGE patterns of reduced total seed protein extracts from test-cross progeny showing recombination between Gli-D1 subunits.

P = Parental and **R** = Recombinant with respect to centromere marker (**Gli-D1cs**) and the *Glu-D1* locus.

*rare recombinant combining bands 2 and 12 resulting from cross over within the *Glu-D1* locus.



Glu-D1a
Glu-D1e*
Glu-D1d
R P R P P P P R P P R P R
2
2
5
Glu-1
subunits
12
10
10
Gli-D1cs
a b c d e f g h i j k l m

4.3.4 Mapping Genes Controlling Endosperm Proteins and Stem Rust Resistance on Chromosome 1R of Rye

Conventional inheritance and linkage mapping studies in cereal rye (*Secale sereale* L.) have been restricted mainly because of the difficulty of obtaining homozygous parents due to the cross-pollinating habit of the species which, in turn, is promoted by self-incompatibility alleles (Lundquist, 1954, 1956). However, with the advent of cytogenetic techniques allowing the addition or substitution of individual rye chromosomes to the wheat genome (O'Mara, 1940; Riley, 1965), it has become relatively easy to locate certain rye genes on particular rye chromosomes. Thus genes controlling rye prolamins (secalins) have been located on chromosome arm 1RS (Shepherd, 1968; Shepherd and Jennings, 1971) , rye glutelins on 1RL (Lawrence and Shepherd, 1981a) and various isozymes have been assigned to 6 of the 7 rye chromosomes (Tang and Hart, 1975). However, none of these genes in rye has been accurately mapped along the rye chromosomes. In this section, translocation mapping procedures similar to that used for mapping wheat genes, have been used for mapping the genes controlling HMW glutelin subunits on the long arm of rye chromosome 1R, and genes controlling secalins and stem rust resistance on the short arm of this chromosome.

4.3.4.1 Crossing procedures

The translocation mapping procedures used for mapping rye genes were similar to those described earlier for wheat (sections 4.2.2 and 4.3.2.1) except with rye genes the translocation chromosomes in the F_1 heterozygotes had homologous rye arms to pair with instead of homologous wheat arms. Thus normal pairing and crossing over took place between the rye arms on one side of the centromere and on the other side the wheat and rye arms remained unpaired. Consequently these F_1s could be used for estimating recombination frequencies between the rye loci and the centromere. Genes controlling the HMW glutelin subunits (*Glu-R1*) on the long arm of 1R were mapped using the CS

1DS-1RL translocation line, whereas those controlling secalins (*Sec-1*) and stem rust resistance (*Sr*) on the short arm of 1R were mapped using the Gabo 1BL-1RS translocation. The rye chromatin in each of these translocations was derived from Imperial rye. The F_1 heterozygotes were produced by crossing these translocations to the wheat-rye substitution lines 1R(1D) and 1R(1B), respectively, involving chromosome 1R of *cv* King II rye which possesses different alleles than *cv* Imperial for all three loci to be mapped. The test-cross procedures are shown in Fig. 4.23.

4.3.4.2 Mapping the *Glu-R1* locus on chromosome arm 1RL

(i) Recombination frequency

Eight F_1 plants were used to produce 479 test-cross seeds for electrophoretic analysis. The HMW glutelin subunits controlled by alleles *Glu-R1i* and *Glu-R1k* and the prolamins controlled by the *Gli-D1* and *Sec-1* genes, could be classified without difficulty in the parents (Fig. 4.24, a, b). Since the test-cross parent India 115 did not have any protein band overlapping these proteins, all of the relevant bands could be reliably scored in the test-cross progeny and parental (Fig. 4.24, tracks d, e), recombinant (tracks f, g) and aneuploid (tracks h-o) types were distinguished. The parent India 115 in Fig. 4.24, c has a band with electrophoretic mobility similar to that of Gli-D1cs band and Gli-D1cs could be scored reliably when the gels were only partly destained. After long destaing the intensity of Gli-D1cs bands is reduced compared to the overlapping band from India 115. The data obtained from the separate F_1 hybrids were homogeneous ($\chi^2_{[28]}$=24.02, P>0.5) and consequently they have been pooled in Table-4.13. As shown in the table , there is close agreement with the expected 1:1 segregation ratio for alleles *Glu-R1i* and *Glu-R1k* and for the presumed homoeoalleles *Gli-D1* and *Sec-1*. However, strong linkage is evident between the glutelin locus on the long arm and the centromere (marked by the prolamin loci). For simplicity, the 14.6 % aneuploid progeny were ignored in the initial analysis of recombination frequency. The recombination frequency between *Glu-R1* locus and the

Figure 4.23

Test-cross combinations employed in mapping experiments.

HMW glutelin subunits:

[CS+Holdfast 1R(1D) × CS 1DS-1RL] × India 115.

Secalins and stem rust resistance:

[CS+Holdfast 1R (1B) × Gabo 1BL-1RS] × CS 1BS.

CS = Chinese Spring

Gene symbols:

Sr = stem rust resistant;

sr = stem rust susceptible;

others—see Table-4.1.

PROCEDURES FOR MAPPING GENES CONTROLLING ENDOSPERM PROTEINS AND STEM RUST RESISTANCE ON CHROMOSOME 1R OF RYE

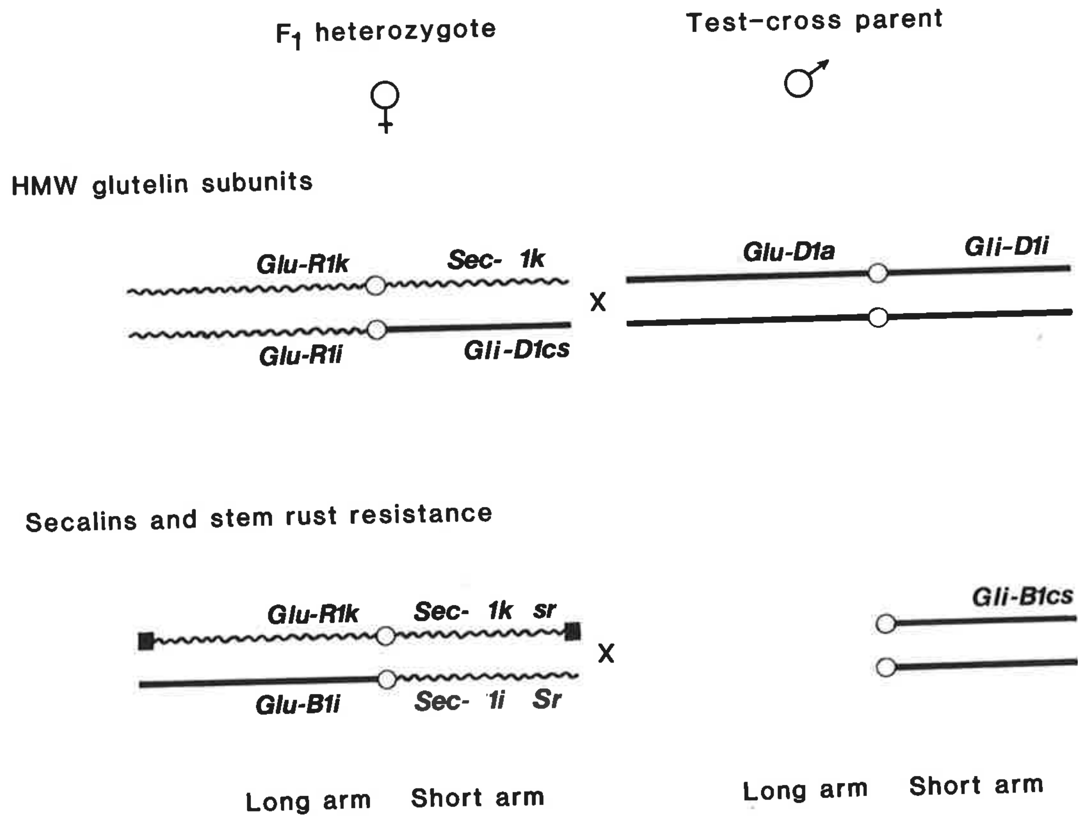


Figure 4.24

1-D SDS-PAGE patterns of reduced total protein extracts from the parental lines (P_1, P_2, P_3) and some test-cross progeny used to map the *Glu-R1* locus.

a Chinese Spring 1DS-1RL translocation;

b Chinese Spring+Holdfast 1R(1D) substitution;

c India 115;

d-o test-cross progeny.

P = parental; **R** = recombinant; **A** = aneuploid

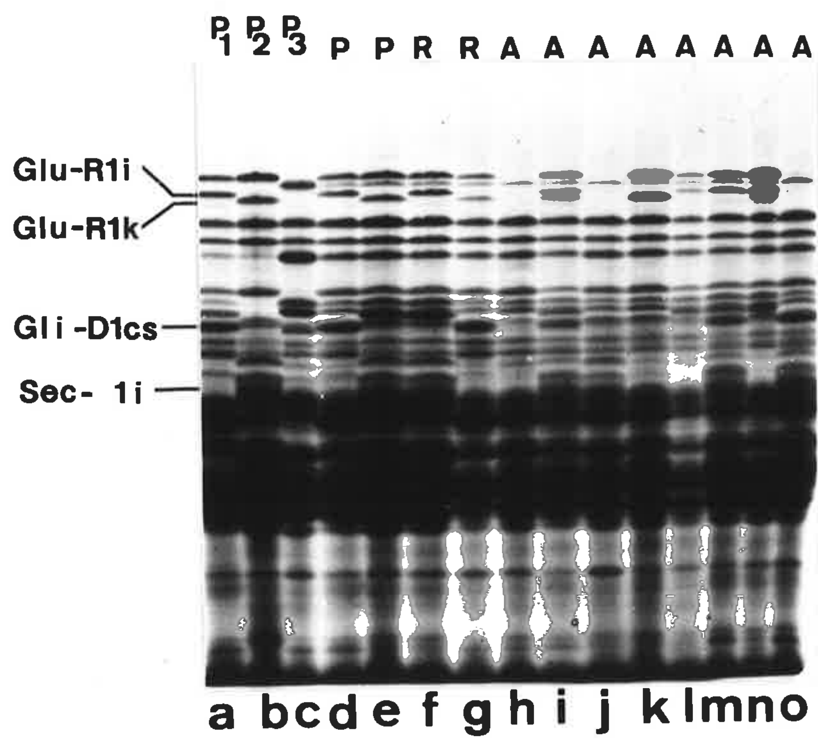

P 1
P 2
P 3
P
P
R
R
A
A
A
A
A
A
A
A
Glu-R1i
Glu-R1k
Gli -D1cs
Sec- 1i
a b c d e f g h i j k l m n o

Table 4.13. Observed frequency of protein phenotypes in test-cross progeny used to map the Glu-R1 locus, grouped into parental, recombinant and aneuploid classes

Protein phenotypes							
Parental		***Recombinants***		***Aneuploids***			
Glu-R1i Gli-D1cs	Glu-R1k Sec-1k	Glu-R1i Sec-1k	Glu-R1k Gli-D1cs		Total	p (%)	Map distance (cM)
196	194	10	9	70	479	4.6	4.6±1.0

Aneuploids

Aneuploid class	Gli-D1cs	Sec-1k	Glu-R1i	Glu-R1k	No. of progeny	% of population
Hypoploid	-	-	-	-	49	10.2
Hyperploid	+	+	+	+	2	0.4
Misdivision products:	-	+	-	-	3	
	-	-	+	-	3	
	-	-	-	+	9	
	+	+	+	-	2	
	+	+	-	-	1	
	-	-	+	+	1	4.0
Total					70	14.6

- = absent; + = present

χ^2 Table:

	Glu-R1i/ Glu-R1k	Gli-D1cs/ Sec-1k	P/R
Expected ratio	1:1	1:1	1:1
Observed No.	206: 203	205: 204	390: 19
χ^2 values (P, d.f.=1)	0.02 (P>0.8)	0.002 (P>0.9)	336.53 (P<0.001)

centromere was calculated to be 4.6±1.0 %, assuming that the translocation arose from a centric fusion event.

(ii) Chromosome pairing and the occurrence of aneuploidy

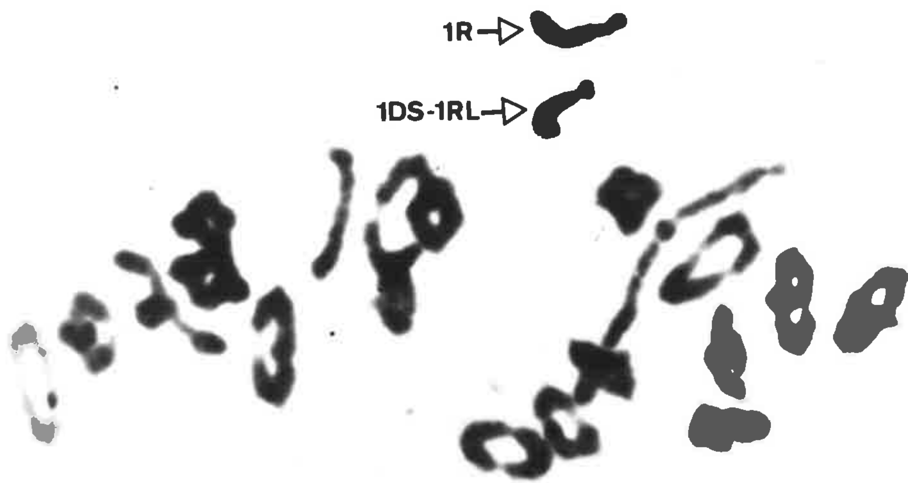
Both the complete 1R chromosome and the 1DS-1RL translocation, could be easily recognized in C-banded preparations since the three rye chromosome arms had prominent telomeric bands, whereas 1DS was unbanded (Fig.4.25, a, b). Altogether 607 PMCs

Figure 4.25

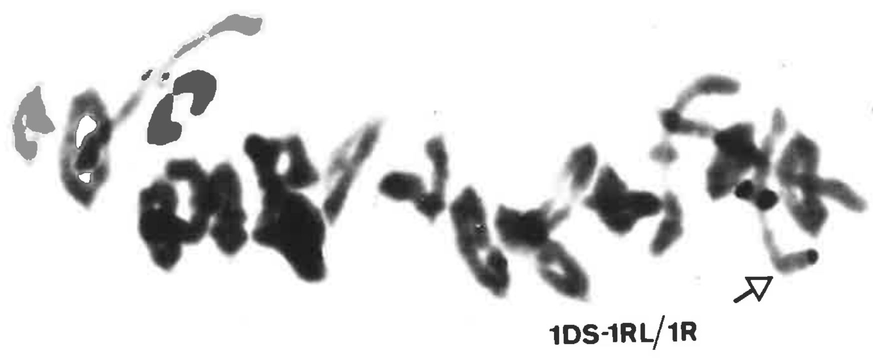
C-banded squash preparations of the PMCs from the F_1 heterozygotes used to map the genes on the long arm of chromosome 1R.

a metaphase I cell showing 1DS-1RL and 1R univalents;

b metaphase I cell showing 1DS-1RL/1R bivalent.



a



b

were examined from the seven F_1 plants and no pairing was observed between 1DS and 1RS, whereas there was an average of 62.2 % pairing between the long arms of these chromosomes but this pairing frequency was heterogeneous ($\chi^2_{[6]}$ = 34.45, P<.01) between the plants. This heterogeneity of chromosome pairing contrasts with the homogeneity of recombination and aneuploid frequencies observed with the same F_1 plants. It is believed that the heterogeneity of pairing frequency reflects different degrees of desynapsis in these plants, resulting from fluctuations in glass-house temperatures, and the selection of anthers at different stages of metaphase I (c.f. Fu and Sears, 1973).

4.3.4.3 Mapping the *Sec-1* and *Sr* loci on chromosome arm 1RS

(i) Recombination frequency

The protein phenotypes of the presumed alleles *Sec-1i* and *Sec-1k* (Fig. 4.26, A, a, b) and presumed homoeoalleles *Glu-B1i* and *Glu-R1k* (Fig. 4.26, B, a, b) were easily classified in the parents. Furthermore, the chromosome 1R pair in the disomic substitution line 1R(1B) had four prominent C-bands, one on each of the four telomeres, which can be seen as prominent dots in the C-banded preparations of interphase nuclei (Koebner and Shepherd, 1985). In contrast the 1BL-1RS translocation line showed no major dots in the interphase nuclei because the telomere of 1RS of Imperial rye does not exhibit good C-banding (Fig. 4.27, a, b). Besides these protein and C-band differences, the Gabo 1BL-1RS parent line was resistant to stem-rust (*P. graminis tritici*), with the gene(s) being located on chromosome arm 1RS from Imperial rye, whereas the other parent line which possessed chromosome 1R of King II rye substituted for chromosome 1B, was susceptible to stem rust(Fig. 4.28, a, b). Since the test-cross parent Chinese Spring Dt 1BS lacked all of the critical protein bands present in the F_1 heterozygote, was susceptible to stem rust and had no prominent heterochromatic dot in the C-banded interphase nuclei, all of the phenotypes listed above could be distinguished in the test-cross progeny. The electrophoretic mobility of the major Sec-1i band was similar to one of the two Sec-1k bands so in the presence of Sec-1k bands it was not possible to be certain whether the

Figure 4.26

1-D SDS-PAGE patterns of seed protein extracts from the parental lines (P_1, P_2, P_3) and some test-cross progeny used to map the *Sec-1* and *Sr* loci.

A unreduced gliadins extracted with ethanol-sucrose mixture:

a Gabo 1BL-1RS;

b Chinese Spring+Holdfast 1R(1B) substitution;

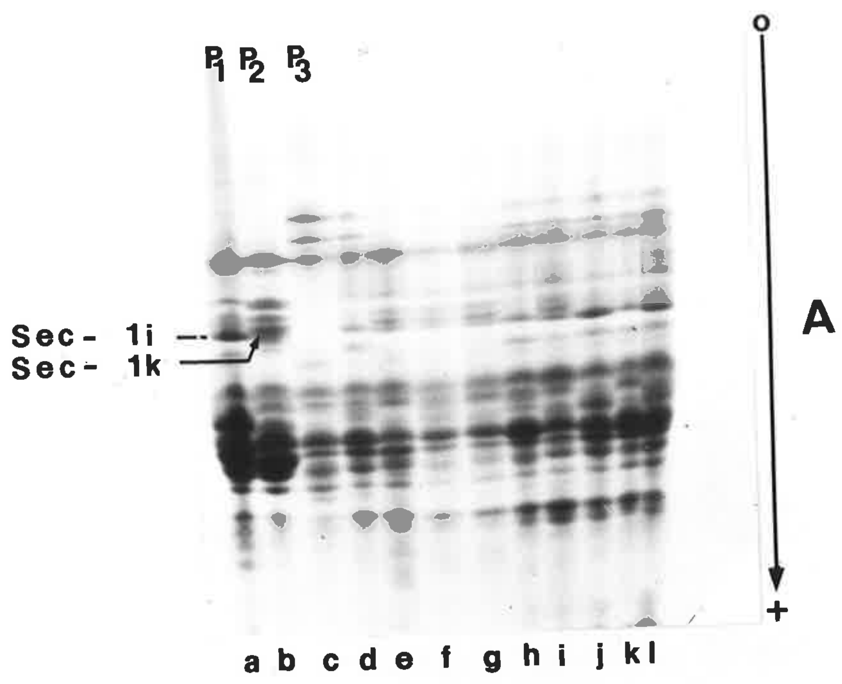
c Chinese Spring Dt 1BS;

d-l test-cross progeny, scored **Sec-1i** positive [**d, h, j, k, l** (one band)] and **Sec-ik** positive [**e, f, g, i** (two bands)].

B reduced total seed protein extracts:

a, b, c as in part **A**;

d-n test-cross progeny, scored **Glu-R1k** positive (**d, f, g, h, k**) and **Glu-B1i** positive (**e, k, l, m, n**).


P1 P2 P3
Sec- 1i
Sec- 1k
o
A
+
a b c d e f g h i j k l

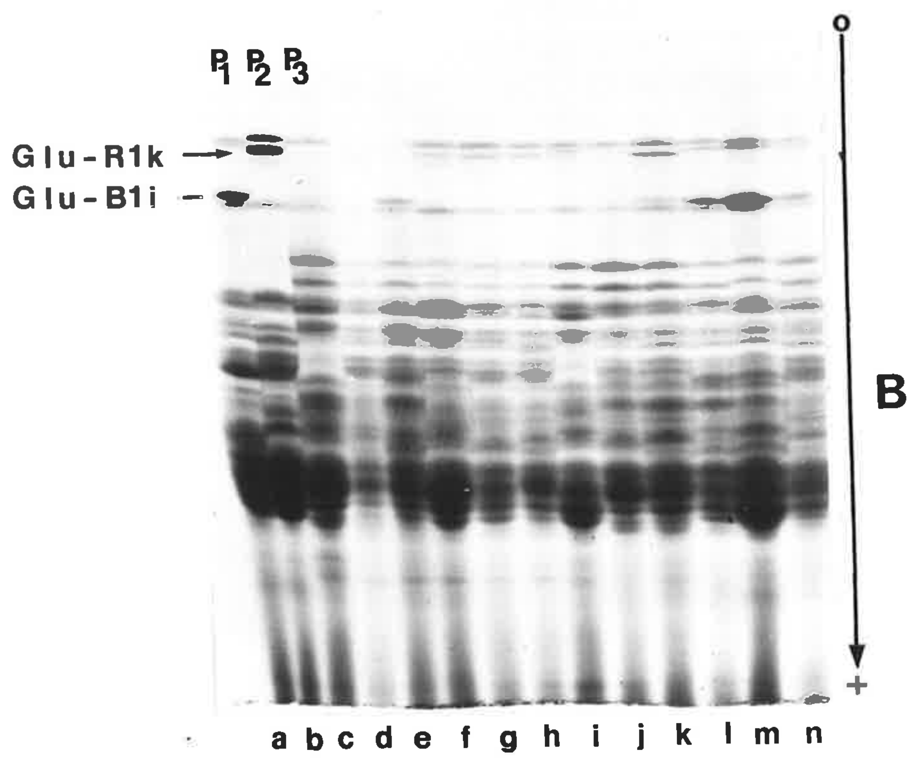

P1 P2 P3
Glu-R1k
Glu-B1i
o
B
+
a b c d e f g h i j k l m n

Figure 4.27

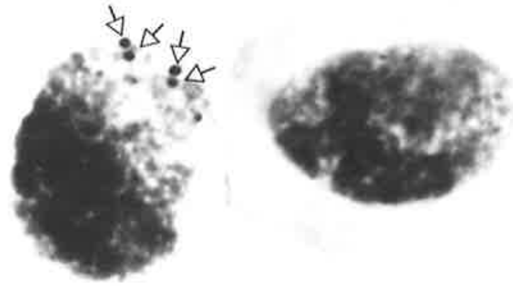
C-banded squash preparations of interphase nuclei in root tip cells from the parental lines and some test-cross progeny used to map the genes on the short arm of chromosome 1R. Each major dot represents a telomere of chromosome 1R of King II rye.

a complete 1R (King II rye) disomic substitution for 1B in Chinese Spring+ Holdfast (4 dots);

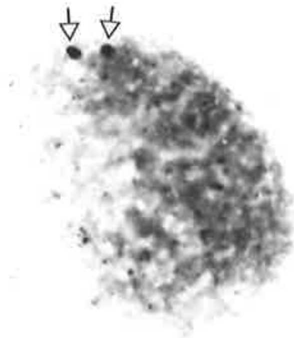
b 1BL-1RS (Imperial rye) translocation in Gabo (0 dot);

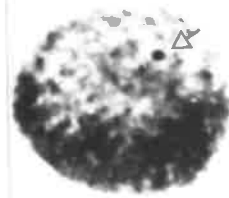
c, d and **e** test-cross progeny showing 2, 0 and 1 dots.

Arrows indicate the position of the major heterochromatic dots.



a b





c d e

Figure 4.28

Reaction to stem rust (*Puccinia graminis tritici*) of parental lines used to map the *Sec-1* and *Sr* loci on the short arm of chromosme 1R.

a Resistant (R), Gabo 1BL-1RS translocation;

b Susceptible (S), Chinese Spring+Holdfast 1R(B) substitution and Chinese Spring Dt 1BS.

a b

Sec-1i band was present also. However, among the test-cross progreny these two alleles are expected to segregate as alternatives, and furthermore, the phenotypic identity of test-cross progeny was confirmed later in progeny tests. The segregation of the protein phenotypes among test-cross progeny is shown in Fig. 4.26, A, B.

Because the two C-bands observed in the interphase nuclei were located at both telomeres of the complete 1R chromosome, any cross over involving 1RS from Imperial rye and King II rye should lead to two recombinant type gametes each of which will produce only one dot (long or short arm of 1R of King II), in contrast to the parental gametes which will produce two or no dots. Three types of interphase nuclei with 2, 0 and 1 dots are shown in Fig. 4.27, c, d, e, respectively. C-banded preparations of root tip meristems from test-cross seedlings usually contain 50 or more interphase cells and the classification of their phenotypes was made only after observing a consistent number of dots in several cells.

Altogether 141 test-cross seeds were produced for the analysis of endosperm proteins, dots in the interphase nuclei and reaction to stem rust. However, after the initial electrophoretic analysis of these seeds 59 of them were found to be aneuploids and therefore no further tests were made on these. The remaining 82 seeds with euploid type protein phenotypes were placed in Petri dishes for germination but only 72 of them germinated. One root tip from each seedling was used for C-banding and then seedlings were planted in pots for stem rust testing and progeny testing. The results of these tests are summarized in Table-4.14. One of the supposed euploids proved to be aneuploid in the progeny test giving 71 euploids and 60 aneuploids. As shown in the χ^2 part of the table, all the presumed alleles and homoeoalleles followed the expected 1:1 segregation ratio in the euploid progeny but all of the linkage values were significant. It is important to note that the total recombination frequency between the centromere and the telomere on 1RS was calculated to be only 28.2% instead of the expected 50% over the whole arm . This difference from expectation has been attributed to the low amount of pairing that occurred between the 1RS arms of the two chromosomes (see next section). Thus it is likely that all of the recombination values in the table are underestimated.

Table 4.14. Observed frequency of protein phenotypes in test-cross progeny used to map the*Sec-1* and *Sr* loci, grouped into parental, recombinant and aneuploid classes

Progeny class	Protein phenotypes Glu-1	Sec-1	Reaction to stem rust	No. of telomeric dots	No. of progeny	p (%)	Map distance (cM)
Parentals:							
	B1i	i	R	0	28		
	R1k	k	S	2	21		
Recombinants:							
c.o. region 1							
	B1i	k	S	1	4		
	R1k	i	R	1	4	11.3	11.5±4.0
c.o. region 2							
	B1i	i	S	1	1		
	R1k	k	R	1	2	7.0	7.0±3.1
c.o. region 3							
	B1i	i	R	1	7		
	R1k	k	S	1	2	15.5	16.0±4.8
double c.o. (region 2+3)							
	B1i	i	S	0	1		
	R1k	k	R	2	1		
					71		
Aneuploids:							
hypoploid	-	-			23		
*hyperploid	B1i+R1k	k or k+i			11		
misdivision	B1i+R1k	-			1		
products	B1i	i+k (from progeny test)		1			
	B1i	-			3		
	R1k	-			10		
	-	k or k+i			3		
	-	k			8		
Total no. of aneuploids					60		

c.o. region 1 = Between centromere and *Sec-1* ; c.o. region 2 = Between *Sec-1* and *Sr*
c.o. region 3 = Between *Sr* and telomeric heterochromatin;

* may include misdivision products; – = absent

χ^2 Table for segregation amongst euploid progeny

	Glu-B1i/ Glu-R1k	Sec-1i/ Sec-1k	R/S	telomere i vs k**	P/R region 1	P/R region 2	P/R region 3
Observed No.	41: 30	41: 30	42: 29	37: 34	63: 8	66: 5	60: 11
χ^2 values (P, d.f.=1)	1.70 (P>0.2)	1.70 (P>0.2)	2.38 (P>0.05)	1.26 (P>0.1)	42.6 (P<0.001)	52.4 (P<0.001	35.2 (P<0.001)

** 1RS telomere of Imperial (i) vs King II (k) rye

However, the gene order centromere— *Sec-1*— *Sr*— telomere, and their relative distance from each other, should be correct.

(ii) Chromosome pairing and the occurrence of aneuploidy

Complete chromosome 1R was easily recognized in the C-banded preparations of PMCs and its pairing with the unbanded 1BL-1RS translocation chromosome at metaphase I of meiosis (Fig. 4.29, a, b) was examined in 381 PMCs. Only 10.2 % of these PMCs showed pairing between the short arms of the two chromosomes and there was no pairing between the homoeologous long arms 1BL and 1RL. However, 68.4 % pairing was observed at diakinesis. Since the telomeric banding at diakinesis was not vey clear, only those cells with 21 recognizable bivalents, and no univalents, were counted as evidence for the pairing between the reference chromosomes. This 68.4% pairing at diakinesis is comparable to the value (56.4%) predicted from the observed recombination frequency (28.2%) between the centromere and the telomere on chromosome arm 1RS. The predicted value is obtained by multiplying centromere recombination frequency by two because each cross over results in two parental and two recombinant chromatids.

It is obvious that the very high frequency of univalents at metaphase I has contributed to the large number of aneuploids in the test-cross progeny (Table-4.14). As indicated above, in the presence of the Sec-1k protein band, it was difficult to tell without progeny testing, whether Sec-1i was also present and therefore the number of misdivision products in the table is most likely an overestimate because some of these will be hyperploids. However, there was no doubt about the classification of the hypoploids and some of the misdivision products. The 72 completely classified test-cross seeds with euploid protein phenotypes, were progeny tested and 71 were found to be correct and 1 was shown to be aneuploid.

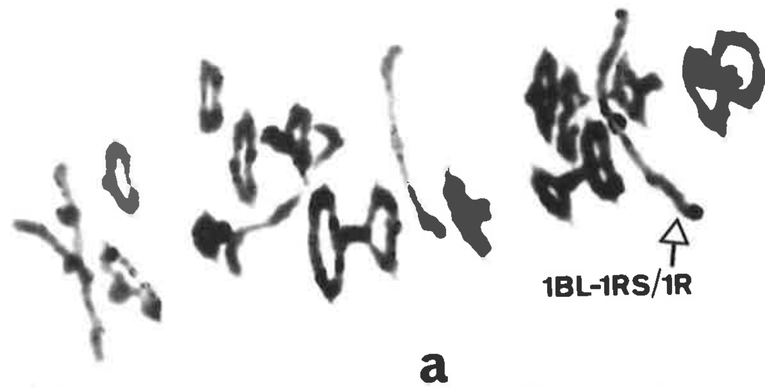
Figure 4.29

C-banded squash preparations of PMCs, at two stages of meiosis, from the F_1 heterozygotes used to map the *Sec-1* and *Sr* loci on the short arm of chromosome 1R.

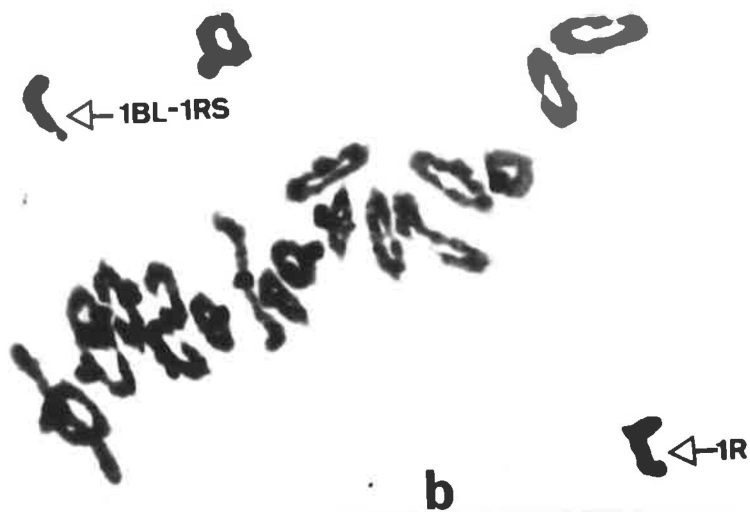
a metaphase I cell showing 1BL-1RS/1R bivalent;

b metaphase I cell showing 1BL-1RS and 1R univalents;

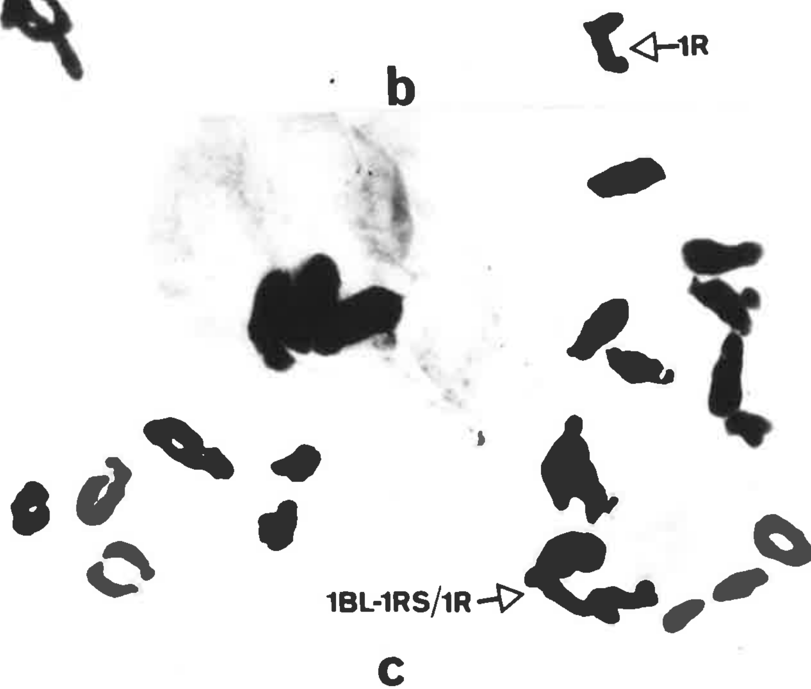
c diakinesis cell showing 1BL-1RS/1R bivalent.



a



b



c

4.4 DISCUSSION

4.4.1. Effect of Asynapsis and Aneuploidy on the Estimation of Map Distance

The validity of the method of mapping gene—centromere distances using translocation lines described in the Results section, depends on the *homologous* (wheat/wheat or rye/rye) arms of the translocation chromosomes pairing with complete chromosomes with the same frequency as would occur between the two complete homologues, and the absence of pairing between the *homoeologous* (wheat/rye) arms. If the translocation chromosome reduces the pairing between homologous arms, map distance will be underestimated, whereas any homoeologous pairing between wheat and rye arms would lead to an overestimate. From the observations made at metaphase I and diakinesis stages of meiosis, there was no evidence of homoeologous pairing between the wheat and rye chromosome arms. However a variable amount of pairing was observed between the homologous arms of the translocation chromosome and the complete chromosome. Usually the pairing at metaphase I was lower than that observed at diakinesis. The variable number of univalents involving the reference chromosomes at metaphase I in PMCs of different F_1 heterozygotes was reflected in the number of aneuploid progeny observed in the test cross progeny and these results are summarized in Table-4.15.

As shown in Table-4.15, there was a high level of metaphase pairing between the homologous long arms of group 1 wheat chromosomes involved in translocations.. Because of the low number of univalents at metaphase I, the number of aneuploids in the test-cross progeny was also very low (≤3.2%). Similarly ,there was a high level of pairing between the 1DL telocentric and complete 1D chromosomes at metaphase I and few aneuploids in test-cross progeny in telocentric mapping. Considering the amount of fluctuations in the recombination frequency due to environmental factors, it is concluded that the lack of chromosome pairing had little efect on the estimates of recombination frequency.

Table 4.15. Summary of chromosome pairing in F_1 heterozygotes and the observed frequencies of recombinants and aneuploids among the test-cross progeny used to map the loci controlling endosperm proteins and some other markers on group 1 chromosomes of wheat and rye

Reference chromosomes in F_1 heterozygote	Pairing (%) Metaphase I	Pairing (%) Diakinesis	Aneuploid frequency (%)	Test-cross progeny	Recombination freqency * Interval	Recombination freqency * (%)
Mapping long arms of 1A, 1B and 1D						
1AL————o~~~ 1RS 1AL————o——— 1AS	≥89.5	n.d.	0	(158)† 79	Cent.—*Glu-A1*	(29.5)† 10.1
1BL————o~~~ 1RS 1BL————o——— 1BS	96.4	n.d.	0.7	430	Cent.—*Glu-B1*	25.5
1DL————o~~~ 1RS 1DL————o——— 1DS	90.7	n.d.	3.2	529	Cent.—*Glu-D1*	27.5
1DL————o 1DL————o——— 1DS	83.6	n.d.	4.2	191	Cent. —*Glu-D1*	20.7
Mapping short arms of 1A, 1B and 1D						
1RL~~~~o——— 1AS 1AL————o——— 1AS	90.4	98.8	4.7	296	Cent. —*Tri-A1* *Tri-A1*—*Gli-A1*	11.0 40.1
1RL~~~~o——— 1BS 1BL————o——— 1BS	70.8	90.8	18.3	606	Cent. —*Gli-B1*	41.6
o——— 1BS 1BL————o——— 1BS	49.0	71.4	19.7	234	Cent. —*Gli-B1i*	40.4
1RL~~~~o——— 1DS 1DL————o——— 1DS	64.9	92.8	17.9	440	Cent. —*Tri-D1* *Tri-D1*—*Gli-D1*	14.9 40.3
Mapping long and short arms of 1R						
1RL~~~~o——— 1DS 1RL~~~~o~~~ 1RL	62.2	n.d.	14.6	479	Cent. —*Glu-R1*	4.6
1BL————o~~~ 1RS 1RL~~~~o~~~ 1RS	10.2	68.5	31.2	141	Cent. —*Sec-1* *Sec-1*— *Sr* *Sr*—Telomere	11.3 7.0 15.5

* Aneuploids excluded; † F_2 mapping; n.d.= not determined; Cent. = Centromere

However, because of the considerable reduction in metaphase pairing between the homologous short arms of all group 1 chromosomes, except 1A, in the mapping experiments and the high frequency of aneuploids (Table-4.15), it is necessary to consider the likely influence of these factors on the estimates of map distance.

To resolve this problem fully it would be necessary to know the total number of testcross progeny which had been derived from chromosomes which had not paired during meiosis (asynapsis). Such chromosomes would have had no opportunity to recombine, and if included in the estimates of map distance would lead to an underestimate of true map distance. Although the exact number of these progeny could not be determined in the present work, it is possible to obtain an estimate provided certain assumptions are made (Singh and Shepherd, 1984a). The first assumption is that all aneuploid progeny are derived from irregular separation of univalent chromosomes at metaphase I. Since not all univalents at metaphase I give rise to aneuploid gametes, we need to obtain an estimate of the number of euploid gametes which came from this source. This number was calculated by using the frequency of observed hyperploid and hypoploid progeny to calculate the probability of a univalent to be included or not included in the egg cell, respectively.

The second assumption is that both univalents are transmitted independently of each other, and this seems valid since there is no evidence of selection against any of these chromosomes in the test-cross progeny. It follows that in the F_1 heterozygotes, the two critical univalents will have an equal probability ('*i*') of inclusion in a functional egg. If the number of gametes arising from the univalent chromosomes, excluding the number of misdivision products ('*m*'), is '*a*' then the expected number of hyperploid, euploid and hypoploid gametes due to these univalents will be $a\,(i^2)$, $a\,[2i\,(1-i)]$ and $a\,(1-i)^2$, respectively. Since the frequency of two of these classes (hyperploid and hypoploid) are observed directly, the value of a can be calculated. Then $(a+m)$ gives the total number of test-cross progeny (gametes) derived from megaspore mother cells which had the two reference chromosomes as univalents. Thus the number of gametes derived from univalents could be estimated for each of the experiments designed to map genes on

chromosome arms 1BS, 1DS and 1RL.

The experiment involving chromosome arm 1RS was not considered because the level of aneuploidy in this experiment was much too high to provide accurate recombination frequencies. However, the linkage data obtained from this test-cross were useful in determining the gene order and gave an indication of their relative distance from each other.

4.4.1.1 Chromosome arm 1BS

The pooled data from the translocation mapping experiment provide the following equations: $a\,(i^2) = 22$; $a\,(1\text{-}i)^2 = 69$ and $m = 20$. Solving the first two equations gives $i = 0.36$ and $a = 169$. Thus the estimate of the total number of gametes derived from univalents will be $a{+}m = 189$. Similarly in the telocentric mapping experiment, $a\,(i^2) = 10$; $a\,(1\text{-}i)^2 = 31$ and $m = 5$, which gives $i = 0.36$ and $a = 77$ and the total number of gametes involving univalents equals 83.

Another estimate of the number of gametes containing chromosomes which did not pair during meiosis can be derived from the observed number of PMCs showing univalents at first meiotic division, but this calculation is complicated by the phenomenon of desynapsis (precocious terminalisation of chiasmata). In the translocation heterozygotes the two reference chromosomes were paired in an average of 90.8% PMCs at diakinesis (Table-4.15), therefore the maximum amount of asynapsis is only 9.2% and among 606 test-cross progeny 56 would be expected to be derived from PMCs showing asynapsis. On the other hand, if we consider the level of non-pairing at metaphase I (29.2%) then the expected number of test-cross progeny containing either asynaptic or desynaptic products will be 177, which is very close to the number (189) estimated by the formula derived from the frequency of hypoploid, hyperploid and progenies with misdivision products. Thus it is clear that aneuploidy in the test-cross progeny is due to the occurrence of univalents at metaphase I and univalents resulting from desynapsis also contribute to the aneuploidy. A similar trend was apparent with the telocentric mapping experiment, but the agreement between the two approaches was not so good. On the basis of the observed

number of univalents at diakinesis and metaphase I, the number of gametes expected to come from asynapsis was 69 with 119 expected to come from the sum of both desynapsis and asynapsis. Using the frequency of aneuploids approach, the expected number was 83. However, once again it is clear that univalents arising from desynapsis also contributed to the aneuploidy observed.

Since the exact amount of asynapsis is not known we can not apply an exact correction to the estimates of recombination values to account for the asynapsis and desynapsis. However it is possible to define the limits based on the two extreme assumptions of either maximum asynapsis or no asynapsis.

(i) Maximum asynapsis:

From the observations at diakinesis the maximum limit for asynapsis is 9.2% because pairing was observed in 90.8% of PMCs in the translocation F_1 heterozygotes. Thus the gametes resulting from these 9.2% PMCs should be excluded from the analysis because chromosomes in these cells would not have had a chance to cross over and their inclusion will lead to an underestimation of the true recombination frequency. Thus the maximum number of gametes and hence progeny coming from asynapsis is only 56 out of the total of 606 test-cross progeny. Clearly this will only have a small effect on the estimate of recombination frequency. In the initial estimate of the recombination frequency, 111 aneuploid progeny have already been ignored which is much greater than the expected number for maximum asynapsis and therefore the values could not have been underestimated.

(ii) No asynapsis:

If there was no asynapsis, all univalents observed at metaphase I will have paired at prophase I and have had a chance to cross over so that the frequency of recombinant gametes derived from metaphase univalents should be the same as in the gametes derived from metaphase bivalents, providing that the probability of desynapsis is independent of the position of chiasmata along the chromosome. However, it is thought that

chromosomes with distal chiasmata are more likely to desynapse than those with proximal ones. If we make the extreme assumption that all the univalents resulted from desynapsis of chiasmata that were distal to the *Gli-B1* locus, then all of the aneuploid gametes should be added to the parental class of gametes. This gives the minimum limit for the recombination value between *Gli-B1* and the centromere to be 34.0±1.9%, which is equivalent to loose linkage. However, since it is unlikely that all desynapsed chiasmata occurred beyond *Gli-B1*, the true value is likely to be higher than 34%.

The equivalent recombination values obtained for the telocentric mapping experiment making the same extreme assumptions of maximum asynapsis and no asynapsis are 46.1±3.9% and 32.5±3.1%, respectively.

4.4.1.2 Chromosome arm 1DS

The maximum and minimum limits for the estimates of recombination between *Glu-D1* and the centromere were calculated using the same arguments developed above for mapping the *Gli-B1* locus. The maximum possible asynapsis is 7.2% since these chromosomes were paired at diakinesis in 92.8% of PMCs examined (Table-4.15). Thus the number of gametes that should be excluded from the analysis is 31to give the maximum limit of the recombination frequency. Inclusion of these gametes could result in an underestimation of the recombination frequency between the loci. However, 79 aneuploid gametes were ignored in the intial estimate. Thus it is concluded that the initial estimates of 14.9% and 40.3% recombination frequencies in the intervals centromere—*Tri-D1* and *Tri-D1*—*Gli-D1* , respectively, are not underestimated.

At the other extreme, assuming that there was no asynapsis of the reference chromosomes but all observed univalents were due to desynapsis, then the minimum limit of recombination frequency between centromere and *Tri-D1* locus is calculated to be 12.2±1.6%, using a procedure similar to that described for chromosome arm 1BS. Similarly, the minimum limit for recombination between the *Tri-D1* and *Gli-D1* loci is calculated to be 32.9±2.2. Thus these minimum values are not distinctly different from the

values obtained initially by ignoring the aneuploids.

4.4.1.3 Chromosome arm 1RL

The upper and lower limits for the recombination occurring between the *Glu-R1* locus and the centromere could be set to allow for chromosome pairing failure and aneuploidy similar to the method described above for chromosome arms 1BS and 1DS. Although no data were available on pairing at diakinesis in this experiment, the amount of asynapsis could not have been more than the 37.8% pairing failure observed at metaphase I. Thus the maximum number of gametes, including all the aneuploids, that could be excluded from the analysis will be 479×0.378=181 which gives a maximum limit to the recombination frequency equal to 6.4±1.4%. To set the minimum limit, we assume that desynapsis rather than asynapsis was involved in the observed pairing failure, and then all the aneuploids should be added to the pool of parental gametes giving a minimum limit to the recombination frequency of 4.0±0.9%.

4.4.2. Linkage Relationships in the 1A, 1B, 1D and 1R Chromosomes

The translocation mapping results described in this chapter show that the *Tri-A1* and *Tri-D1* genes controlling the synthesis of triplet proteins are closely linked with the centromere on the short arms of chromosomes 1A and 1D, respectively. The map distances for these intervals are similar for both chromosomes (Fig. 4.30, 1A, 1D) the difference being within the range of standard error. The present study did not provide any evidence for the existence of a similar locus on chromosome 1B controlling triplet proteins. However, recently Galili and Feldman (1984) mapped a gene on chromosome 1B between the *Glu-B1* and *Gli-B1* loci and they assigned the symbol *Gld-B6* for this locus. Although located in the same region of the chromosome as the triplet loci on chromosomes 1A and 1D, it is yet to be proven that *Gld-B6* is analogous to *Tri-1* genes.

The genes controlling the triplet proteins (*Tri-1*) have been shown to be loosely

Figure 4.30

Linkage maps of the group 1 chromosomes of wheat and rye showing location of genes controlling endosperm proteins and stem rust resistance. Map distances are shown in cM (Kosambi, 1944).

* Results of F_2 data (all other values were obtained using test-cross data);

** Maximum limit of the recombination frequency (95% confidence limit);

p = recombination percentage .

LOCATION OF GENES CONTROLLING ENDOSPERM PROTEINS ON GROUP 1 CHROMOSOMES OF WHEAT AND RYE

Long arm Short arm

1A

Glu-A1 *Tri-A1* *Glu-A3* *Gli-A1*

33·9* ± 5·4
10·2 ± 3·5
11·2±2·0
55·2 ± 8·2
0
1·1**

1B

Glu-B1 *Glu-B3* *Gli-B1*

28·1 ± 2·8
59·7 ±7·1
1·7±0·8

1D

Glu-D1 *Tri-D1* *Glu-D3* *Gli-D1*

30·9 ± 2·7
15·4±2·1
55·8 ± 7·4
0
1·1**

1R

Glu-R1 *Sec- 1* *Sr* Telomeric heterochromatin

4·6±1·0
11·5±4·0
7·0±3·1
16·0±4·8
(p = 28·2%)

linked with those controlling gliadins (*Gli-1*) and the map distances between them on chromosomes 1A and 1D are very similar (55.2±8.2 and 55.8±7.4, repectively). The *Gli-A1* locus on chromosome 1A was independent of the centromere but the *Gli-B1* and *Gli-D1* loci showed loose but significant linkage with their respective centromeres. However, the average map distances between the centromere and *Glu-1* loci on chromosomes 1A, 1B and 1D were similar (Fig 4.30, 1A, 1B, 1D). The observation that the *Glu-A3*, *Glu-B3* and *Glu-D3* loci controlling the synthesis of LMW glutenin subunits are very tightly linked to the respective *Gli-1* genes, is in agreement with the recent findings of Payne *et al.* (1984d) with 1A and 1B chromosomes. However, the detection of recombination between the *Gli-B1* and *Glu-B3* genes (or within the *Glu-B3* locus) on chromosome arm 1BS indicates that these are separate loci which can recombine with a very low frequency. This finding also justifies assigning a different gene symbol (*Glu-B3*) to the genes controlling LMW glutenin subunits. Although, the map distances between the *Glu-1* and *Gli-1* loci and those between the *Glu-1* locus and the centromere, have been reported before (Payne *et el.*, 1982b), this is the first occasion where *Tri-1*, *Gli-1* and *Glu-3* genes have been mapped with respect to the centromere.

The genes controlling the HMW glutenin subunits in wheat were found to be closely linked to the centromere on the long arms of chromosomes 1A, 1B and 1D. The map distances between these intervals are summarized in Fig. 4.30. Although two different map distances, 33.9% from F_2 data and 10.2% from test-cross data, were obtained for the *Glu-A1* —centromere interval, the first value is considered to be more reliable because it is based on a much larger population size than the second value. However, further work is required to explain this observed difference in map distances obtained with test-cross and F_2 data. The map distances between the *Glu-B1* and *Glu-D1* loci and their respective centromeres (28.1±2.8 and 30.9±2.7, respectively) were comparable to the F_2 mapping results obtained with the *Glu-A1* locus. However, these map distances are approximately three times higher than the average value (9.0±1.2 cM) reported by Payne *et al.* (1982b) for the same gene intervals using the telocentric mapping procedure.

The telocentric mapping procedure was also used in the present study but only for

mapping the *Glu-D1* locus. Although the estimated map distance (22.7±3.5) was still higher than the value (10.2±2.4) obtained by Payne *et. al.*(1982b), it was much lower than that obtained with translocation mapping (30.9±2.7). The difference in the telocentric mapping results of Payne *et al.* (1982b) and the current studies may be due to one or more of the following factors: (i) a difference in the environmental conditions particularly the temperature at which the plants were grown, (ii) a difference in the genetic backgounds of the cultivars used and (iii) underestimation of recombination values in the study of Payne *et al.* (loc cit) because aneuploids were not separated from the total population.

However, the consistently lower map distance obtained with telocentric mapping compared to translocation mapping is most probably due to a reduction in the frequency of crossing over near the centromere when one member of the bivalent concerned is telocentric. For example, Endrizzi and Kohel (1966) found only 1.0 % and 4.4% exchange with the centromere for two genes located on opposite arms of chromosome 6 of cotton, using the respective telocentrics, but obtained 22.1% recombination between the same genes when complete chromosomes were involved. Although the translocation chromosomes used in the present mapping experiments involved alien chromosome segments from rye, nevertheless, they were complete chromosomes with two arms. In fact, Sears (1972) has shown that even where most of the chromosome arm consisted of alien chromatin*, the recombination frequency in the region between the centromere and an awn inhibitor gene, *B*, was increased almost four fold as compared to the values obtained in telocentric mapping experiments (3.5% vs 0.87%). However, this reduction in recombination frequency observed with proximal loci was not significant for loci which were distal to the centromere (Endrizzi and Kohel, 1966; Sears,1972) . Parallel results were obtained in the present work since similar map distances between *Gli-B1* and the

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*For example, Translocation 40 (Sears, 1956) involves the replacement of nearly all of the short arm of chromosome 6B by a chromosome segment from *T. umbellulatum* (Zhunk.) Bowden (*Aegilops umbellulata*)

centromere (41.6% and 40.4%) were obtained with both translocation and telocentric mapping. The proximal loci studied by Endrizzi and Kohel (1966) and Sears (1972) were much closer to the centromere than *Glu-1* and this may explain why the reduction in recombination frequency is less pronounced in the present experiment. This diffrence will be accentuated if the reduction in crossing over caused by a terminal centromere is greatest near the centromere and becomes progressively less towards the telomere.

Although the translocation mapping procedure has consistently given higher map distances compared to telocentric mapping, further work is required to find whether these values are similar to those occurring with complete homologoues. The higher chromosome pairing frequencies obtained between homologous arms of complete chromosomes and translocation chromosomes, compared to the pairing obtained between a telocentric chromosome and a complete chromosome, indicates that the rye chromosome arm did not interfere with the pairing on the other wheat arm. However, it is not known how these pairing frequencies compare with those occurring between pairs of homologous complete chromosomes.

Chromosome 1R was similar to group 1 wheat chromosomes in having a *Glu-1* locus closely linked to the centromere on the long arm and a *Sec-1* locus loosely linked to the centromere on the short arm, both coding for analogous proteins to those in wheat. Chromosome 1R is similar to 1B of wheat in not possessing a *Tri-1* locus. Although the estimated map distance between *Glu-R1* and the centromere of rye chromosome 1R is much smaller than the analogous map distances in wheat, this may not be real difference in an evolutionary sense. Thus the rye chromosomes were mapped in wheat cytoplasm and a wheat genetic background and this may give a different result from the measurements made in rye background. Similarly, the *Sec-1* locus was found to be much more tightly linked to the centromere than the supposed analogous *Gli-1* loci in wheat. Although these map distances on 1RS were clearly underestimated by upto 50% due to poor chromosome pairing, if the rye recombination values were doubled they would still show stronger linkage than that obtained in wheat. Recently, Lawrence and Appels (1985) have also shown that *Sec-1*, locus is closely linked to the centromere (32.2 cM) in linkage mapping

in rye itself and these authors suggest that in heterozygous condition rye chromosomes recombine less frequently than their wheat counterparts. Nevertheless, the overall data indicate a great deal of similarity between the gene order and their relative distances apart on chromosomes 1A, 1B, 1D and 1R and they support the theory that the ancestral genome of the tribe Triticeae had a *Glu-1* locus closely linked to the centromere on the long arm , and *Gli-1* and *Glu-3* loci loosely linked to the centromere and perhaps a *Tri-1* locus closely linked to the centromere, on the short arm. The apparent lack of a *Tri-1* locus on chromosomes 1B and 1R and a *Glu-3* locus on 1R may be due to the differentiation of this ancestral chromosome during speciation, or alternatively it could be due to the biphyletic origin of these species.

Chapter 5
GENERAL DISCUSSION

The current investigations on the nature of triplet proteins and their genetic control indicate that they are different from any previously described wheat proteins. They do not correspond closely to any of Osborne's (1907) four classical solubility fractions viz albumins, globulins, gliadins or glutenins. In fact, after differential extraction of flour with distilled water, 0.04M sodium chloride, 70% aqueous ethanol and 0.1M acetic acid at room temperature, the triplet proteins remain as part of the unextracted residue proteins. However, they could be solubilized relatively easily by using strong dissociating agents such as SDS or sodium dodecanoate which dissolve almost all the protein components of flour. Because of their strong interactions with gluten proteins, triplet proteins are very difficult to purify by differential solubility procedures. Therefore techniques which combine solubility differences with electrophoresis or gel filtration, will be required to achieve purification. However, in recent studies it was found that triplet proteins could be differentially extracted from flour with 1M sodium chloride solution at 60°C and this indicates that they might be globulin type proteins.

Triplet proteins are different from 'C-M proteins' characterized by Redman and Ewart (1973) because the molecular weights of all triplet protein subunits are higher than C-M proteins (13,000-15,000). These proteins are are also different from 'Ligolins' described by Frazier *et al.* (1981). The ligolin protein has been implicated in the lipid-mediated aggregation of gluten proteins (Frazier *et al.*, 1984; Bushuk *et al.*, 1984). Unlike ligolins, native triplet proteins are insoluble in water and acetic acid. Furthermore, the molecular weights of the small subunits (α and δ) of triplet proteins are almost double that of the ligolins (10,000-12,000).

An important feature of the structure of triplet proteins which resembles that of globulins, present as storage proteins in other species such as oats and legumes, is the occurrence of one large polypeptide linked to a small polypeptide by disulphide bond(s)giving a dimeric unit which subsequently aggregates into tetramers and perhaps

even higher aggregates. The globulin proteins such as legumin from pea (*Pisum sativum*) or broad bean (*Vicia faba*) and glycinin from soybean (*Glycine max*) are the major storage proteins in legumes. These proteins in their native state have a molecular weight of 300,000 to 360,000 and a sedimentation coefficient of 11-12S and consist of six subunit-pairs each with a molecular weight of 60,000 to 65,000. Each of these subunit pairs contains one large (M.W.=38,000 to 44,000) and one small (M.W.=17,000 to 20,000) polypeptide held together by a single disulphide bond (Derbyshire *et al.*, 1976). It has been demonstrated that legumin is synthesized as a precursor with molecular weight of approximately 60,000 to 65,000, which is later converted to mature legumin with subunits of molecular weight of about 40,000 and 19,000 by post-translational processing of the precursor (Croy *et al.*, 1980; Spencer and Higgins, 1980). It has been shown that the processing of the legumin precursor in pea is completed within 2 h after it has been synthesized (Chrispeels *et al.*, 1982; Chrispeels, 1984). Similar post translational processing has also been shown to occur in glycinin (Tumer *et al.*, 1981).

In contrast to the legumes, the major storage proteins of the cereals are the alcohol-soluble prolamins, and glutelins. However, oats is an exception to this where the globulins are present as the major storage proteins and make up more than 50% of the total seed proteins. Peterson (1978) has shown that oat seed globulins are multimeric proteins containing large and small subunits with molecular weights of 35,000-40,000 and 20,000-25,000. As with legumin, the native protein with a molecular wight of 320,000 and a sedimentaion coefficient of 12S, was found to contain six of the large and six of the small subunits. Furthemore, like legumin and glycinin, oat globulins are also synthesized as precursors which are processed after translation to produce large and small polypeptides (Brinegar and Peterson, 1982; Walburg and Larkins, 1983).

Isoelectric focusing studies on 11-12S globulins of *Glycine max* (Moreira *et al*, 1979), *Pisum sativum* (Gatehouse *et al.*, 1980), *Vicia faba* (Matta *et al.*, 1981), and *Avena sativa* (Walburg and Larkins, 1983) have revealed that the large polypeptides of the globulin subunit pair are acidic whereas the small polypeptides are basic. Generally the large polypeptides have isolelectric points ranging from pH 4 to 6 and the small subunits

from pH 7.5 to 9. A comparison of the N-terminal amino acid sequences of the basic polypeptides of the 11-12S globulins from the above four species has revealed considerable conservation of the sequence in this region across such a wide range of plant species (Casey *et al.*, 1981; Walburg and Larkins, 1983). Similar 11-12S globulin-like proteins have been reported in many other plant species but these have not been studied in much detail (e.g. sunflower, cucurbits, rice).

So far the available data indicate some strong resemblance between triplet proteins and the 11-12S globulins of other species. However, there was no indication of specific hexameric holoproteins in wheat, as reported in legumes and oats. Instead, the heterotetramer triplet bands (DδDδ, DδAα and AαAα) seems to be the most prevalent form of triplet aggregates along with some Aα and Dδ dimers. Furthermore, the subunits of the triplet proteins seem to associate extensively with other proteins, particularly with glutenin, through intermolecular disulphide bonds. This finding suggests that triplet proteins may have an important role in the formation of the visco-elastic complex of gluten proteins.

Further investigations are needed to confirm whether triplet proteins are indeed the wheat equivalent of 11-12S globulins of legumes and oats. These studies would include:

(i). Determination of isoelectric points of the large (A and D) and small (α and δ) polypeptides of triplet proteins.

(ii) *In-vitro* translation of mRNA encoding triplet proteins to determine whether they are synthesiszed as precursor molecules.

(iii) Analysis of amino acid composition and sequence of the small subunits of triplet proteins to determine whether they show homology with the 11-12S globulins.

If further work proves that the triplet proteins are 11-12S globulins, their aminoacid sequence would provide interesting information on the evolutionary relationship of seed storage proteins in general and of wheat in particular. The conservation of the N-terminal amino acid sequence of the small subunits of these globulins (Walburg and Larkins, 1983) in species that diverged millions of years ago, may indicate that they have important functional properties, apart from any function as reserve nutrients for the germinating seed.

The intervarietal variation in triplet proteins and the difference in the staining intensity of the triplet bands controlled by 1A and 1D chromosomes is an indication of the possible complexity of these proteins. The intervarietal variation may have resulted from mutation at the triplet loci on chromosomes 1A and 1D (*Tri-A1* and *Tri-D1* , respectively). The *Tri-D1* locus appears to produce twice as much protein as produced by the *Tri-A1* locus, as judged by staining intensity of the bands. There are two hypotheses to account for this. Either there has been a duplication of the *Tri-D1* locus hence twice the number of genes are present giving twice the amount of product, compared to *Tri-A1*. Alternatively, it is possible that there has been a change in the control mechanism of *Tri-1* making the *Tri-D1* locus more efficient for protein synthesis than *TriA1*. Heterogeneity among the large and small polypeptides of the 11-12S globulins has been observed in soybean (Nielsen, 1984) and oats (Walburg and Larkins, 1983). The latter authors have suggested that part of this heterogeneity may be due to the post-translational modifications of the gene products.

The linkage mapping results presented in Chapter 4 of this thesis clearly reflect the homoeologous relationship of group 1 chromosomes in wheat and rye (see Fig. 4.30, Chapter 4). However, triplet-like genes have not been detected on chromosomes 1B and 1R, except for the location of an intermediate locus between the *Gli-1* and the *Glu-1* loci on chromosome 1B. (Galili and Feldman ,1984). The separate location of the genes controlling triplet proteins is a further indication, in addition to their electrophoretic mobility and solubility properties, that they represent a new class of proteins in wheat. The close linkage between the loci controlling LMW glutenins (*Glu-3*) and gliadins (*Gli-1*) suggests that these two groups of genes may have evolved from the same ancestral gene by duplication and mutation. This hypothesis is further supported by the similar amino acid composition of these two groups of proteins except the LMW glutenin subunits have the propensity to aggregate by intermolecular disulphide bonds. The exact mechanism for this difference in the aggregating properties of the LMW glutenin subunits and gliadins is not known as yet.

Besides their evolutionary significance these linkage data have already proven useful in experiments designed to recombine the group 1 chromosomes of wheat and rye by

induction of homoeologous pairing (Koebner and Shepherd, 1985; Koebner, 1985). In these experiments, glutelin, triplet and prolamin markers, in combination with isozymes and other morphological and cytological markers, were used to mark the particular chromosome segments. The 7.0% recombination between the *Sec-1* and *Sr* loci on chromosome arm 1RS is important to plant breeders because it demonstrates that the desirable rust resistance gene can be separated from the rye secalin locus (Koebner, 1985). If the observed quality defect in certain wheat-rye translocation lines possessing chromosome arm 1RS is due to the proteins produced by this locus, or a related wheat locus, these data indicate that they could be separated from each other in recombination experiments.

APPENDIX A : Variation in tripet protein banding patterns

Serial No.	Cultivar / Breeding line	No. of seeds tested	Country of origin
	1 Hexaploid wheats		
	a. Broad slow-moving triplet		
1	Chinese Spring ('cs' standard pattern , Fig. 3.3, a, d, g, i)	>100	China
2	Thatcher	6	Canada
3	Halberd	10	Australia
4	Heron	10	Australia
5	Kite	4	Australia
6	Condor	20	Australia
7	Zenith	1	Australia
8	Cheyenne	2	USA
9	Marquis	2	Canada
10	Gala	1	Australia
11	Festiguay	1	Australia
12	Emblem	1	Australia
13	Aroona	2	Australia
14	Gluclub	1	Australia
15	Kewell	1	Australia
16	Hopps	1	Australia
17	Sherpa	3	Australia
18	Mersey	4	Australia
19	Wongoondy	1	Australia
20	Kalkee	1	Australia
21	Jabiru	1	Australia
22	Mendos	4	Australia
23	Mengavi	1	Australia
24	Darius	2	Japan
25	Drott	1	Japan
26	Gluyas	1	Australia
27	Justin	1	USA
28	Orca	1	The Netherlands
29	Free Gallipoli	1	Australia
30	C-518	1	India
31	C-273	1	India
32	C-306	1	India
33	BT-2296	1	Tunisia
34	Crim	1	USA
35	Currawa	1	Australia
36	Xelaju-66	1	Guatemala
37	Nayab-70	1	Pakistan
38	BT-2288	1	Tunisia
39	Toquifen*S*	1	Chile
40	Bencubbin	4	Australia
41	(MH3* (M*K*R)/111/8)	1	Australia
42	Inia-66	1	Mexico
43	Mexicano 1481	1	Portugal
44	Son-64*Kl Rend	1	Argentina
45	Buckmantial	1	Argentina
46	Teal	1	Australia
47	IAS-20 Iassul	1	Brazil
48	Purple Straw	1	Australia
49	(MH3*(M*M*C)/17/16) MH 17	1	Australia
50	Bajio	1	Mexico

51	Wards Prolific	1	Australia
52	Haruhikari	1	Japan
53	Jufy-1	1	Belgium
54	Insignia	1	Australia
55	Gaboto	1	Argentina
56	Opal	1	The Netherlands
57	Kenya Leopard	1	Kenya
58	Pato Argentino	1	Argentina
59	Huelquen	1	Chile
60	Calipad	1	Mexico
61	Svenno (A-11854)	1	Sweden
62	Manitou	1	Canada
63	Chris	1	USA
64	Federation	1	Australia
65	Nabawa	1	Australia
66	Lerma Rojo 64-A	1	Mexico
67	Giza-150	1	Egypt
68	Rescue	10	Canada
69	Rieti	2	Japan
	b. Broad fast-moving triplet		
1	India 115 ('i' standard pattern, Fig. 3.3, b)	>50	India
2	Gabo	>50	Australia
3	Warigal	>20	Australia
4	Gatcher	4	Australia
5	NapHal	2	Nepal
6	Tarsa	3	Australia
7	Eka	3	Japan
8	Norin 20	1	Japan
9	Choti Lerma	1	India
10	Lundi	2	Rhodesia
11	(MH3*(C*P)/8/4) MH 14	2	Australia
12	Giza 155	2	Egypt
13	(MH3 x Warimba) MH 11	1	Australia
14	C-271	1	India
15	Hira	1	India
16	Kalyansona	1	India
17	UP-302	1	India
18	(MH3 x Warimba) MH 9	1	Australia
19	Reg Egyptian	4	USA
20	Glaive	2	Australia
21	BB Inia	1	Mexico
22	Dirk	4	Australia
23	Ariana-66	1	Tunisia
24	Selkirk	1	Canada
25	Sonora-64A	3	Mexico
26	Klein Rendidor	1	Argentina
27	Potam-70	1	Mexico
28	Chanab-70	1	Pakistan
29	Victor-1	1	Italy
30	Vicam-71	3	Mexico
31	Gasser	2	Alaska
32	Jaral*S*/LEE-Sk-MNANA	2	Zimbabwe
33	Tobari-60	3	Mexico
34	Carazinho	1	Brazil
35	Magnif	1	Argentina
36	Roque-73	1	Mexico

37	Bonza-55	1		Colombia
38	Eagle A	5		Australia
39	Eagle B	5		Australia
40	Falcon	2		Australia
41	Madden	2		Australia
42	CSP-44	2		Australia
43	Sirashaya-1	2		Japan
	c. Narrow slow-moving triplet			
1	Hope ('h' standard pattern, Fig. 3.3, c)	>50		USA
	d. Narrow fast-moving triplet			
1	Sonalika ('s' standard pattern, Fig. 3.3, e)	12		India
2	Karamu	10		New Zealand
3	Egret	5		Australia
4	Gamut	3		Australia
5	Tamori-71	2		New Zealand
6	Penjamo-62	2		Mexico
7	Roque Reseln	3		Mexico
	e. Single diffuse band			
1	Tainui ('t' standard pattern, (Fig. 3.3, f)	15		New Zealand
2	Isis	5		Australia
3	Zambezi	1		Zimbabwe
4	T-64-2W	1		Tunisia
5	Crespo	3		Colombia
	f. Lines showing mixed patterns for triplet			
1	Turpin-7	2	(cs, i)	South Africa
2	Nainari-60	2	(cs, i)	Mexico
3	Era	2	(cs, i)	U.S.A.
4	Lerma Rojo	3	cs, cs, s)	Mexico
5	Oxley	2	(cs, i)	Australia
6	Hezera 2152	3	(cs, i, i)	Israel
7	Gamenya A	3	(i, i, cs)	Australia
8	Gamenya B	3	(i, i, cs)	Australia
9	Gambee	3	(i, i, cs)	Australia
10	Kopara	3	(cs, cs, i)	New Zealand
11	Zipa-68	4	(cs, cs, cs, i)	Colombia
12	Mara-Sepremo-Mentana-MCM	3	(cs, cs, i)	Chile
137				
	2 Durum wheats (Single band, Fig. 3.3, h)			
1	Durati	5		Australia
2	*Duramba A	5		Australia
3	*Duramba B	5		Australia
4	Duramba C	5		Australia
5	Duramba D	5		Australia
6	Dural	1		Australia
7	Langdon	3		USA
8	Kharkof	3		USSR
9	Edmore	3		USA

*slightly faster moving band

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