GENETIC VARIABILITY OF STARCH IN *Triticum* SPECIES

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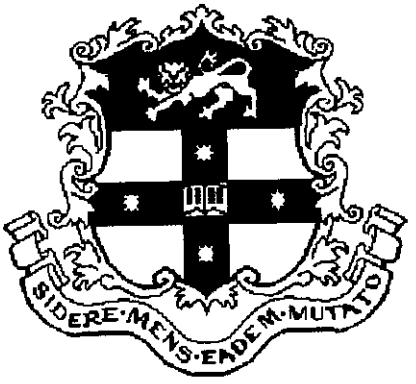
A thesis submitted in fulfillment of the requirements for the degree of

Doctor of Philosophy



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September 2000

To

Ibrahim
Aazim
Aaliya
&
Ayisha

Certificate of originality

The text of this thesis contains no material which has been accepted for the award of any other degree or diploma in any University and to the best of my knowledge, is original and contains no material previously published or written by another person, except where due reference is made.

Ahmed Regina

Acknowledgements

Oars alone can never prevail
To reach the distant coast
The breath of heaven must swell the sea
Or all the toil is lost

(W. Cowper)

I would like to express my deepest appreciation to Dr Peter J. Sharp, University of Sydney, for being my supervisor, and for the guidance and support rendered through out the course of the study. My heartfelt gratitude is due to Dr Matthew K. Morell, CSIRO Plant Industry for being an ideal supervisor, without whose invaluable help this study would not have been materialized. I find no words to thank him for his supervision and inspiration and also for the immense support during periods of mental stress. It was a great honour and pleasure to work with him.

I am also grateful to Dr Sadequr Rahman, CSIRO Plant Industry for his expert guidance on the molecular biology aspects of this project, Dr Ian L. Batey and Ms Barbara Curtin, Grain Quality Research Laboratory, North Ryde for their assistance and valuable suggestions in starch structural analysis, Dr Behjat Hashemi, CSIRO Plant Industry for her assistance in protein analysis, Prof R.A. McIntosh, University of Sydney for his assistance and helpful discussions on aneuploid analysis, Dr Fred Stoddard for providing seed materials and offering help with statistical issues, Dr R. Appels and Dr Z. Li, CSIRO Plant Industry for constructive discussions at various stages of the study.

I am greatly indebted to the staff of the Plant Breeding Institute, Cobbitty, for providing a favourable and healthy environment for the study. Particular thanks are due to Dr H.S. Bariana for his helps in problem solving, Dr Shobha Vengitanagappa for her constant support, encouragement and friendship, Drs C. Wellings, R.F Park, J. Oates and Mr G. Brown for their advice and suggestions, and Mr Peter Abel for the splendid photography.

The stressful period of five years of research has been turned into a tremendous time of pleasant experience and memories by the affectionate company of all my friends. Particular thanks are due to Dr Sheila Kailasapillai for being a true 'friend in need' by all means, Dr Zhao Xiachung for his countless helps in both research and personal issues, Mr Matthew Hayden, Mr Greg Good, Dr Sunita Khatkar, Mr Brant Pawlak, Ms Sue Pfiffer, Dr Mathew Turner and Dr Mohammed Shariflou for their friendships and assistance at various stages of my research. Special thanks are also due to Ms Suvarna Suhas, Ms Manjula Ummer, Ms Gargi Girija, Ms Sujatha Reji, Ms Shabana Hameed and Ms Asmath Hayat for their loving and soothing companionships.

With great pleasure I am dedicating this manuscript to my husband, Mr M. Ibrahim-Kutty, for being exceptionally supportive and understanding, and taking all the pains and efforts in caring the family during my study period, and to my kids, Aazim, Aaliya and little Ayisha who have been patiently waiting for my studies to finish to demand more of my time to themselves. But for their love, affection and concerns I would not have achieved this. I am grateful to my father, Dr M.S. Abdul Kadir who has always been my inspiration in undergoing higher studies. I am also indebted to my brothers Dr A.S M. Sajeev and Mr A. Jewad, and my cousin, Mr Mohammed Haleel for their constant encouragement and motivation.

I gratefully acknowledge the CRC for Plant Science, CSIRO Plant Industry, Goodman Fielder Ltd and Groupe Limagrain for the financial support for the conduct of the study.

Last but not least, I remember my mother, Late Fathima Beevi, who would have been the most joyful person in my achievements.

Abstract

Wheat is the principal, although not exclusive, source of starch in Australia. In order to meet the quality specifications to suit one or other of the enormous end uses of starch, attempts are being made to alter its physicochemical properties mainly through manipulating the ratio of amylose to amylopectin, the two major polymers comprising the starch granule. Wheat lines with low or zero levels of amylose (waxy wheats) were generated through the identification of null lines for the three waxy genes in wheat. Low amylose content starch generated by mutations in one or two of the three waxy genes are preferred for the manufacture of certain types of noodles. On the other hand, high amylose starch is gaining attention as a potential raw material for biodegradable plastics, a preferable alternative for synthetic plastics from petrochemical sources that are often associated with pollution hazards. Complexity arising from the hexaploid nature of wheat and failure in identifying high amylose gene/s in this species have held back, to a certain extent, the efforts in breeding high amylose wheat lines. The current study was undertaken with the objective of investigating the genetic basis underlying the variability in starch in *Triticum* species with a focus to studying the possibilities of increasing the amylose content in wheat.

Since estimation of amylose content is an essential analytical tool required in any amylose breeding experiments, three small scale methods; iodine method, high performance liquid chromatography (HPLC) and concanavalin A (conA) precipitation method, were compared to assess the reliability of each method. The results obtained by HPLC were found to be more consistent over replicates and hence more reliable. The small scale iodine method followed in the study can be a rapid and cheaper method of amylose estimation especially when the amount of starch is limiting as in the case of segregating individual seeds, provided optimum assay conditions are adhered to. Significant positive correlation was observed between the amylose content estimated by HPLC and iodine methods, whereas results of conA did not correlate well with any of the other two methods. Since the three methods were based on three different principles it is not necessary that the methods relate to each other directly.

Previous studies have indicated a narrower range of variability in amylose content in hexaploid wheat compared to a diploid species. A survey conducted among 75 Australian hexaploid wheat cultivars showed a range of 23.5 to 39.8% amylose content (by iodine method). The cultivar Minto was the highest. A similar range (17.6-34.0%) of amylose content was revealed when 40 exotic wheat cultivars were analysed. In both the cases a bimodal distribution of amylose content was observed indicating specific genetic factors influencing low and high amylose contents. Examination of a limited number of tetraploid and diploid species of *Triticum* did not reveal any significant variation in amylose content depending on the number of genomes present. Higher amylose diploid (*T. tauschii*), tetraploid (*T. dicoccoides*) and hexaploid lines were identified which could be used in breeding programmes to widen the genetic variability in amylose content.

The information on the diversity in starch structure and functionality derived by the current study within both hexaploid and diploid wheat starches could be exploited in starch quality breeding experiments. The better gelling ability of *T. tauschii* starches compared to that of hexaploids indicates the scope for selecting suitable donors from among *T. tauschii* accessions for use in breeding improved starch quality suitable for biopolymer manufacture. The distinct starch granule size distribution patterns for hexaploid and *T. tauschii* starches (bimodal and unimodal respectively) is a vital observation made that can be of significance in understanding the genetic basis of starch granule size distribution in wheat.

A complex genetic basis of amylose content was revealed by the identification of several chromosomal regions that had significant effect on amylose content in a survey of CS ditelosomic lines. Absence of the short arm of chromosome 5D resulted in an amylose content as high as 40% (with the euploid CS having 32% amylose content) concurrent with certain changes in amylopectin chain length and starch granule size distributions, and paste viscosity. The information will be of interest in future studies to assign the cause for the considerable increase in amylose content in the line dt5DL.

Analysis of starch branching enzymes revealed variability in the expression of both SBE I and SBE II in hexaploid and diploid (*T. tauschii*, D genome donor) wheats.

Polymorphisms were detected in the genes encoding these enzymes. SBE I analysis also supported the finding that not all *T. tauschii* accessions were involved in the evolution of hexaploid wheat. Chromosome 2 deletion stocks of CS were used to investigate the influence of SBE IIa gene on amylose content. The study did not provide evidence of any significant influence, which is probably due to the buffering capacity of multiple alleles present in hexaploid wheat.

The isolation and partial characterization of SBE IIb (*wSBE II D2*) gene for the first time in wheat accomplished through the current research will have a far-reaching impact on the pursuit of high amylose genotypes in wheat. The fact that the *amylose extender* (*ae*) gene which controls the high amylose phenotype in maize encodes SBE IIb supports the above statement. A 2.26kb fragment of *wSBE II D2* gene was characterized which contained the exons 2,3,4 and 5 in full and exons 1 and 6 in part, and introns 1 to 5 in full. The fragment had an overall similarity of 91.6% over the exons with barley SBE IIb gene and above 80% similarity with maize *ae* gene over the exons 4, 5 and 6. The deduced N terminal amino acids of the wheat SBE IIb pre protein revealed 89.8% similarity with that of barley, 73.5% with that of maize and 76.0% with SBE III of rice.

The current study has also provided some insight into the inheritance of amylose content in wheat. While the *T. tauschii* cross (AUS24242/AUS24230) examined revealed monogenic inheritance of amylose content with partial dominance and additive gene action, the hexaploid cross (Kiata/Lark) showed the possibility of digenic inheritance with partial dominance and additive effect. The breadth of variation observed in the segregating population of AUS24242/AUS24230 indicated the influence of minor and modifying genes affecting amylose content. A QTL (E7.8) was located at the SBE I locus in the Kiata/Lark segregating population which accounted for 8.2% of the variation in amylose content.

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List of abbreviations

ae	Amylose extendet
AGPase	ADP-glucose pyrophosphorylase
AGU	Anhydro-glucose units
AI	Amylose-iodine
API	Amylopectin-iodine
APTS	8-amino-1,3,6-pyrenesulfonic acid
ANOVA	Analysis of variance
ATMI	Australian Triticeae Mapping Initiative
bp	Base pair
CD	Critical difference
CE	Capillary electrophoresis
CL	Chain length
CS	Chinese Spring
2,4-D	2,4-dichlorophenoxyacetic acid
DBE	Debranching enzyme
df	Degree of freedom
DMSO	Dimethyl sulfoxide
DP	Degree of polymerization
DSC	Differential Scanning Calorimetry
dt	Ditelosomic
EDTA	Ethylenediaminetetra-acetic acid
GBSS	Granule-bound starch synthase
HPLC	High Performance Liquid Chromatography
kb	Kilobase pair
kD	Kilodalton
MW	Molecular weight
NITS	Near-Infrared Transmittance Spectroscopy
NMR	Nuclear Magnetic Resonance
NT	Nullisomic-tetrasomic
PAGE	Polyacrylamide gel electrophoresis

PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RS	Resistant starch
RVA	Rapid Visco Analyser
SBE	Starch branching enzyme
SDS	Sodium dodecyl sulphate
SS	Starch synthase
SSP	Starch swelling power.
UHG	Utrahydrogel
Wx	Waxy

CHAPTER 1

Review of Literature

1.1 Utilization of starch

1.1.1 Food industry

Apart from being an energy source in food in its native state derived from grains, fruits, vegetables, roots and tubers, starches perform two basic roles in food industry, as a nutritive stabilizer providing viscosity, texture, mouth feel and consistency to food products and as a processing aid to facilitate manufacturing (Greenwood, 1976). Serving either or both of these purposes, food starches are used in canning, dairy, confectionery, baking, packaged mix, brewing, frozen food and pharmaceutical industries (Osman, 1967, Moore et al., 1984). Depending on the structural composition of the starch used in manufacture, snacks made out of it vary in crunchiness, strength, crispiness and puffiness (Wang, 1997). Pregelatinized starch or cold water swelling starch are widely used in package mixes. Modifications mainly through oxidation, esterification, etherification or hydrolysis lead to the production of starch derivatives which are used in food additives as thickeners and emulsifiers (Rutenberg and Solarek, 1984). Resistant starch, not digested in small intestine, is now realized as a possible source of dietary fibre and is finding its way in many food products (Mauro, 1996). *Wonder White*™ bread made by Quality Bakers Australia Ltd is a typical resistant starch containing product (Brown et al., 1995).

1.1.2 Non-food industry

The ability of starch to provide thickening, gelling, binding, adhesive and film-forming functionality makes it suitable to be used as a raw material in a wide range of industries. Modified starches like hypochlorite-oxidised starches, starch esters, hydroxyalkyl

starches and cationic starches are used in textile and paper industries as binding and sizing agents (Rutenberg and Solarek, 1984). Numerous applications of dextrinised starch have been summarized in Kennedy and Fischer, (1984). Starch has great potential as a raw material for the chemical industry. Glucose, the most important of the starch hydrolysis products, can be further converted to other products such as ethanol, hydrogen gas and antibiotics by fermentation, fructose by enzymic isomerization and sorbitol by chemical dehydrogenation (Kennedy et al., 1988). Cross linked high amylose starches are under development as excipients for the formation of controlled-release solid dosage forms for the oral delivery of drugs (Lenaerts et al., 1998).

Exploring non food applications of cereal products to compete with petrochemical polymers to provide more environmentally benign alternatives started as early as the 1960s and 1970s and the most challenging and remarkable innovation was biodegradable plastics based on natural products like starch. In response to the need for a biodegradable agricultural mulch to obviate the need for removal and disposal of the film at the end of growing season, Otey et al., (1974) developed starch-poly (vinyl alcohol) films and laminated them with poly (vinyl chloride) to reduce water sensitivity (cited in Doane, 1994). The high manufacturing costs of these type films led to the development of films made from polyethylene (Otey et al.,1980). Starch poly (vinyl alcohol) blend materials marketed under the name Mater-BI are suitable for moulding, foaming and making films depending on the copolymer used (Nawrath et al., 1995, Bastioli, 1998). Novon is another marketed product using destructurized starch and other degradable additives such as food gums or proteins. Modified starch resins are water resistant materials which are completely biodegradable and compostable (Bloembergen et al., 1994). The use of kraft lignin as filler for starch films has been reported recently which reduced the water affinity and film resistance to elongation (Baumberger et al., 1998).

In this literature review aspects of starch structure and functionality relevant to the aims of the topic of this thesis are reviewed.

1.2 Starch granule structure

Starch exists as semicrystalline granules composed of two glucose polymers: amylose and amylopectin. The term 'starch' is often used referring to a wide range of amylose/amylopectin ratios, differing in their relative proportion and in their branching degree. The structural characterization of these polysaccharides has been made possible through the use of various amylolytic enzymes such as amylases and debranching enzymes (Kainuma, 1988, Ring et al., 1993). Information on the specificity and action mechanism of the amylolytic enzymes together with the advancements in chromatographic and electrophoretic techniques has accelerated the structural analysis of the major starch polysaccharides.

1.2.1 Fine structure of amylose

Amylose was thought to be a linear long chain of (1→4) linked α-D glucose residues until the 1950s. Investigations using debranching enzymes indicated the lightly branched structure of amylose with some (1→6)-α-D-glucosidic interchain linkages (Kjolberg and Manners, 1963, Banks and Greenwood, 1966). Comparing the degree of polymerization values determined based on the number of non reducing end residues per reducing end residues, Hizukuri et al., (1981) reconfirmed the multibranched structure of amylose, although the overall degree of branching was extremely low. More recently Cura et al., (1995) confirmed through simultaneous enzymatic and chemical methods that amylose is not strictly linear, but contains a small proportion of α1,6 glycosidic linkages.

The moderately branched amylose fraction of sweet potato has tetramodel unit chains with average DP values of 40, 30, 21 and 9 (Madhusudhan et al., 1996). Branching of amylose in corn was observed to be inversely correlated with molecular weight (Mua and Jackson, 1997a). Cheetham and Tao (1998) observed a progressive decrease in the molecular sizes of amyloses with increase in amylose content in maize starches.

The number of branches in amylose varies depending on the botanical origin of the starches. Murugesan et al., (1993) observed more branches in the amylose from potato and sweet potato than in those from maize and wheat. The most frequent branches were short and probably clustered around the reducing terminal of the molecule. The structures of branched and linear molecules of isolated amylose from rice were examined in detail by Takeda et al., (1993b) and revealed a ratio of 0.22:0.78 by mole and 0.32:0.68 by weight of branched to linear molecule. Oat starch was reported to have lower DP values than corn and rice starches (Wang and White, 1994c).

1.2.2 Fine structure of amylopectin

Amylopectin contains both (1-4) and (1-6)-α-D-glucosidic linkages to form a highly branched structure, with three different types of chains: 1) A-chains, short glucan chains unsubstituted except at the reducing end; 2) B-chains, substituted at one or more C_6-OH group of A-chain or other B-chains and also substituted at the reducing end, and 3) C-chains which are substituted at one or more C_6-OH groups, but are unsubstituted at the reducing end (Kainuma, 1988). A:B chain ratio, also referred to as degree of multiple branching, lies within the range of 1:1 to 1.5:1 in an amylopectin molecule (Manners, 1985). Several models have been proposed for amylopectin structure (Whelan (1971), French (1973), Robin et al., (1974)). The overall concept of a cluster model was further refined by Manners and Matheson (1981). This model is compatible with the various enzymic degradation studies which show that the branch points are arranged in tiers or clusters and are not distributed randomly throughout the macromolecule. There is a general agreement that the chains within the granules are radially arranged with their non reducing ends pointing towards the surface and are packed into alternating crystalline and amorphous lamellae with a periodicity of 9nm (Jenkins et al., 1993). Within the clusters, chains associate to form double helices that pack together in ordered arrays to give the crystalline lamellae. The amorphous lamellae contain the branch points (Figure 1.1).

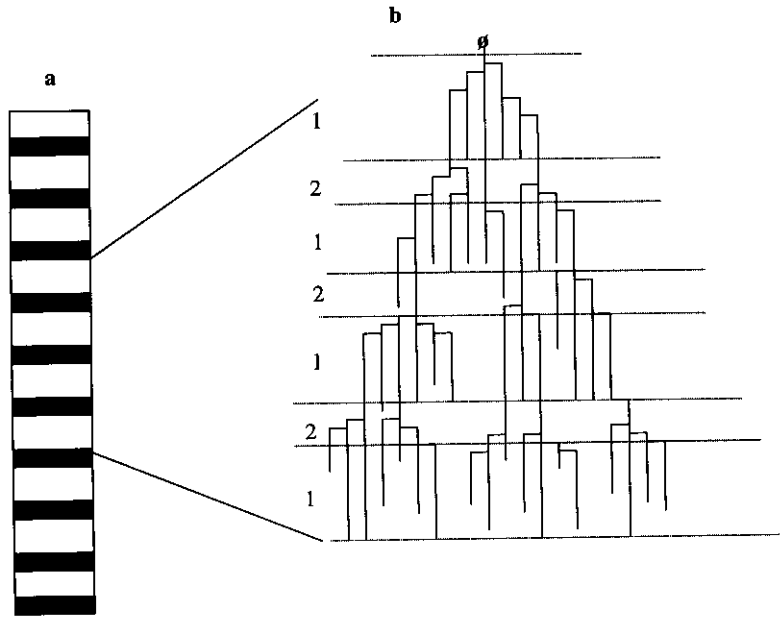


Figure 1.1 Schematic view of a starch granule showing the molecular organization of amylopectin. a) Each black and white section represents an amorphous and crystalline lamella, respectively. b) Succession of seven lamellae in relation to primary structure of a portion of an amylopectin molecule. Each line represents an α-1,4 linked glucan chain (adopted from Ball et al., 1998). ϕ represents the reducing end.

The average chain length (CL) of a branched polysaccharide is defined as the number of glucose residues per non reducing end group and the degree of polymerization (DP) as the number of glucose residues per reducing end group. A summary of structural features of amylopectin in comparison with amylose molecule is given in Table 1.1.

Structural analysis of α-dextrin from potato amylopectin led to the suggestion that amylopectin is built up of structurally different domains, the size of the units of clusters ranging from DP 30-70 and the ratio of A/B chains varying with the size of dextrin (Zhu and Bertoft, 1996). A polymodal behaviour of amylopectin is suggested by Erlander, (1998). According to Blennow et al., (1998), neutral chains of naturally moderately phosphorylated starches showed polymodal distribution with main peaks at DP 14-15 and 50, whereas naturally highly phosphorylated starch showed an increasing proportion

Table 1.1 Comparative structure of amylose and amylopecin molecules (Ball et al., 1998)

Characteristic	Amylose	Amylopectin
Weight percentage in the granule	15-35%	65-85%
Percentage of α-1,6 branches	<1%	4-6%
Molecular mass (Da)	10^4-10^6	10^7-10^8
Degree of polymerization	10^2-10^3	10^3-10^4
Chain-lengths	3-1000	3-50

of chains with mean DP 19. A periodic distribution of the chain length of amylopectin, fractionated to DP 6-12, DP 13-24, DP 25-36, and DP>37, was revealed by high-performance anion-exchange chromatography (Hanashiro et al., 1996. A positive correlation between the amylose content and the average chain length, long chain length, weight ratio and mole ratio of long and short chain length of amylopectin was observed by Cheetham and Tao, (1997). The branching pattern also varies with the molecular weight of amylopectin, with the higher molecular weight amylopectin fraction having longer branched chains than those of its lower molecular weight counterparts (Mua and Jackson, 1997a).

Structural features of amylopectin vary with its botanical origin (Lu et al., 1997, Belloperez et al., 1996, Hoover and Sennanayaka, 1996). The amylopectin of *indica* rice has a lower molecular weight, average DP and average chain number than *japonica* and waxy varieties. Sweet potato amylopectin exhibits a trimodal distribution of unit chains with DP values of 52, 37 and 20 (Madhusudhan et al., 1996). No tissue specific changes in chain length distribution was observed at various stages of development (Noda et al., 1995).

1.2.3 Starch granule size distribution

Amylose, amylopectin and intermediate fractions are tightly packed together to form insoluble granules synthesized within the plant cell. Starch granules vary widely in size, distribution and shape between species. Maize and rice starches exhibit a unimodal granule size distribution ranging from 10-50μm for maize and 1-10μm for rice. Starches of wheat, barley and rye are characterized by a bimodal distribution composed of an A granule population of 10-30μm "diameter" which are lenticular in shape, and a B granule population of generally spherical granules with a diameter less than 10μm (Oliveira et al., 1994, Batey et al., 1996, also reviewed in Morell et al., 1995). In wheat, the larger granules contain 70-80% of the starch by weight and the smaller granules, although more numerous, contain only 20-30% of the total starch in the grain. Granule size distribution varies depending on the genotype within a species as evidenced by high amylose barley containing more small B granules of 4.3-5.7μm diameter (29-43 wt %) than waxy (about 14 wt %) or normal (4.6-15 wt %) (Oscarsson et al., 1997).

Endosperm starch granule development is well known and thoroughly reviewed by Lineback and Rasper, (1988), and Morrison, (1993). The A-type granules in wheat are initiated rapidly during the initial stages of endosperm development up to about 8 days after anthesis following this peak the number of granules per cell drops rapidly and then levels off (Briarty et al., 1976). The B-type granules appear at about 22 days after anthesis and their synthesis continues at a similar rate to that of the larger granules.

The structural composition of the granules and their influence on starch properties may vary depending on their size and location. From studies of A granules in developing wheat and barley, it is suggested that these granules have a core of starch with a high content of amylopectin and a low content of lipid-complexed and lipid-free amyloses. Conversely, the outer layers have much higher content of amylose of both types, but lower content of amylopectin (Morrison and Gadan, 1987, McDonald et al., 1991). Amylose content of smaller granules was less than that of the larger granules at all stages of development (Duffus and Murdoch, 1979). Amylose content of smaller

granules expressed as a percentage of the total starch increased with increasing granule size.

1.3 Starch granule organization

Solid-state NMR studies revealed three distinct components making up the highly ordered and compactly packaged wheat starch granules: 1) highly crystalline regions framed from double-helical starch chains 2) solid-like regions formed from lipid inclusion complexes of starch and 3) completely amorphous regions associated with the branching regions of the amylopectin component of the starch and possibly the lipid-free amylose (Morrison and Tester, 1994). The major crystalline amylopectin fraction is readily oriented in the granule with the characteristic ring structures being formed by alternating clusters of helices formed by external amylopectin chains interspersed with amorphous amylopectin zones. The reversible swelling with no loss of crystalline order that occurs when granules are hydrated at low temperature is due to the swelling of lipid-free amylose found in the amorphous zone. Starch granules provide an insoluble, but readily biodegradable storage system for plants. This balance of mechanical stability with degradability arises from the chemical structure of amylose and amylopectin and from the arrangement of crystalline and amorphous zones within the granules (Calvert, 1997). The various aspects of granule organization especially with regard to the crystalline conformation of α-glucan chains are reviewed in Ring et al., (1993).

1.4 Starch inclusion complexes

1.4.1 Amylose-lipid complex

Recent research reports have shown that lipids in starch are quite important biochemically and technologically. Amylose exists in two different forms: lipid-complexed amylose (L.AM) and lipid-free amylose (F.AM), both of which are amorphous and have different properties (Morrison, 1988). Lipids are suggested to form inclusion complexes with amylose to protect it from branching or breakdown, but do not

prevent its elongation (Morrison et al., 1993b). Lipids in cereal starches are unusual in that they are all monoacyl types, free fatty acids and lysophospholipid, that are able to form inclusion complexes with amylose. In wheat, barley, rye and triticale, they are almost exclusively lysophospholipids while in other starches both free fatty acids and lysophospholipids are present (Morrison, 1993). Starch-lipids interfere in the iodometric determination of amylose because polyiodide can not readily displace lipid complexed with amylose.

High amylose starches are reported to have more lipids than normal starches, while waxy starches have very low or almost none (Morrison et al., 1984, Morrison, 1988, Yasui et al., 1996). Wang and White, 1994b suggested that the chain length of amylose tended to decrease with increased amylose and starch-lipid contents. The influence of starch-lipid content on functional properties of starch is reviewed in Section 1.5.

1.4.2 Starch-iodine complex

The formation of colour by the interaction with iodine is one of the most useful and characteristic reactions of starch (Bailey and Whelan, 1961). The ability of iodine to detect small amounts of starch and to reveal changes in its DP caused by enzymatic and chemical treatments assisted to a great extent in early starch structural analyses (Bates et al., 1943). Amylose binds approximately 20% of its weight of iodine at 20°C giving a blue colour, whereas under the same conditions, amylopectin binds very little iodine, generally less than 0.2%, giving a reddish colour (Banks and Greenwood, 1975).

Minick et al., 1991 reported that starch-iodine complex formation did not involve negatively charged iodine species like I_3, I_5 or I_7, but the linear and non linear polyiodide units, I_6, with inter iodine bond distance of $3.0A^0$ in the amylose helix explains the starch-iodine spectrum. About 17 anhydro-glucose units (AGUs) are bound to six iodine atoms in an AI complex. Characterizing the amylopectin-iodine (API) complex Davis and Khan, (1994) suggested that a nearly linear I_4 unit stabilized within the cavity of a small "amylose like" helix is responsible for the characteristic API spectrum, having 11

AGUs and four iodine atoms in the chromophore. Their study showed that A chains with DP values of less than 17 and more than 10 are involved in the API complex formation. The experimental UV-visible spectrum of the glycogen-iodine complex shows certain features remarkedly similar to that of API complex (Kumari et al., 1996).

Since the AI blue complex gives a broad absorbance band ranging from 580 to 629nm any of these wavelengths can be used for absorbance measurements at different temperatures and hence to characterize the complex (Minick et al., 1991). At temperatures over 15°C the AI complex gradually breaks down, lowering the absorbance values. The starch –iodine complexation behaviour has been determined as a function of degree of polymerization of amylosic chains. At DP 20, there is only a weak interaction with the wave length of maximum absorption (λmax) of the complex in the region of 500nm. As DP increases to above 200 the λmax and the amount of iodine bound approach plateaux values of 640nm and 20% w/w respectively (Banks and Greenwood, 1975).

1.5 Resistant starch (RS)

Resistant starches, that are not degraded in the small intestine and passed into the large intestine, have been classified into three different categories based on nutritional considerations of factors affecting starch digestibility (Englyst et al., 1992). RS Type I (physically inaccessible starches) constitute starch granules that are physically trapped within the matrix of the food, thereby preventing or delaying access of enzymes. RS Type II (resistant starch granules) are native starch granules that are resistant to the action of enzymes. Starches of raw potato, banana and high amylose maize are examples of RS Type II. RS Type III are formed after gelatinization and cooking when the disorganized amylose and amylopectin molecules undergo realignment and crystallization over a period of time in the process known as retrogradation. Enzyme-resistant starch in processed food is of considerable interest due to its analytical and possible nutritional significance in the dietary fibre concept (Sievert and Pomeranz, 1990). Sambucetti and Zuleta, (1996), observed that when amylose content increased

from 18-30% in rice starches the total dietary fibre values increased significantly due to RS. A fourth type of RS (RS Type IV) has been also identified which is chemically or thermally modified starch with reduced susceptibility to digestion. Formation of glycosidic bonds other than α-1,4 or α-1,6 by heat treatment during caramelization is an example (Lineback, 1998).

1.6 Physico-chemical properties of starch

The effective use of starch in food systems and other industrial applications is greatly affected by its physico-chemical properties. Most of the functional attributes of starch can be related to the temperature-dependent interaction of starch with water in the processes known as gelatinisation, pasting and retrogradation (Dengate, 1984, Atwell et al., 1988). Starch gelatinisation takes place when starch granules are heated in the presence of excess water, resulting in granular swelling and subsequent disruption of the molecular order within the starch granule (Yuan et al., 1993), involving loss of birefringence (quantified by polarized light microscopy), loss of double-helical structure (quantified by NMR), loss of crystallinity (quantified by X-ray diffraction) and an endothermic melting or dissociation event (quantified by DSC) (Fisher and Thompson, 1997). Pasting generally refers to the changes in viscosity just before, during and after the event of gelatinisation (Zeng et al., 1997). When starch pastes cool, the starch polymer-water hydrogen bonds are replaced by polymer-polymer hydrogen bonds, resulting in a gel network. The process that occurs when the molecules composing gelatinized starch begin to reassociate leading to the more ordered structure as the starch-water system cools is called starch retrogradation (Atwell et al., 1988).

The techniques of studying gelatinisation include Hot Stage Microscopy, Differential Scanning Calorimetry (DSC), Pulse-Field-Gradient (PFG)-NMR, and flow consistency and gravimetric methods (Konik, 1995, Gomi et al., 1998). Visco-Amylograph or Rapid Visco Analyzer (RVA) is used for starch pasting property analysis. The uptake of water during gelatinization of starch is measured by starch swelling power test. DSC gelatinizaion parameters are significantly correlated with RVA cooling parameters (holding strength, final viscosity and set back) and swelling power, whereas DSC

amylose-lipid peak temperature was strongly associated with RVA heating parameters (peak viscosity and breakdown) and swelling power. Gelatinisation onset and peak temperatures as measured by DSC and hot stage microscopy are significantly correlated with each other (Konik, 1995, Wootton et al., 1998).

Much research has indicated that starch functionality depends on many factors including the molecular structure, size and weight of the amylose and amylopectin components (Sanders et al., 1990, Tester and Morrison, 1990, Lii et al., 1996). Crystallites within the amylopectin molecule determine the onset of swelling and gelatinisation, with the maximum swelling factor being related to the molecular weight and shape of the whole amylopectin molecule. Amylose has an inverse relationship with swelling power A higher proportion of long chains (DP≥35) in amylopectin contribute to increased starch swelling (Sasaki and Matsuki, 1998).

In the RVA, lower apparent and total amylose contents are associated with higher peak, greater breakdown, lower final viscosity, negative set back and lower total set back (Zeng et al., 1997). Starches with comparatively higher molecular weight amylose fractions produce higher setback and final viscosity pastes and result in firmer gels (Mua and Jackson, 1997b). The higher starch paste viscosity associated with many Australian cultivars is a result of reduced amylose content due to the reduced level of granule bound starch synthase (GBSS) associated with the absence of the waxy protein Wx-B1 normally encoded by a locus on chromosome 4A (Zhao et al., 1998). The amylopectin chain length also has a significant role in gelatinisation events. The *aewx* genotype of maize showed high gelatinisation temperature attributed to longer chains in amylopectin which are considered to form long double helices that would require a higher temperature to dissociate completely than that required for shorter double helices (Yuan et al., 1993). Studies on rice varieties revealed that proportion of short chains with DP 6-9 glucose units seemed to inhibit the rate of retrogradation of amylopectin (Lu et al., 1997).

The lipid content, granule size, mechanical damage and botanical source of the starch together with the growth condition and developmental stage of the endosperm are

reported to have influence on gelatinisation parameters (Moss, 1980, Dengate, 1984, Soulaka and Morrison, 1985, Takashi and Seib, 1988, Gudmundsson and Eliasson, 1989, Tester and Morrison, 1990, Morrison et al., 1993a and b, Gudmundsson, 1994, Wang and White, 1994a, Tester, 1997, Fredriksson et al., 1998). Monoacyl lipids are known to play a major role in the paste and gel behaviour of wheat and corn starches. Low–lipid starches showed a decrease in paste temperature, no pasting peak and reduced consistency and setback. Paste clarity was also affected with a negative correlation with starch-lipid content. High growth temperature facilitates amylopectin crystallization and increased gelatinisation temperature, but delays the onset and depress the extent of swelling of the granules when heated in water. Gelatinisation and retrogradation enthalpy of oat starch were found to be lower compared to normal maize and wheat starches, and the possible explanation was the lower level of crystalline order and greater amounts of starch-lipids in oat starch. Potato starch has the highest retrogradation enthalpy, while cereal starches have the lowest with an intermediate value for pea starch.

Information regarding the gelatinisation parameters of starches has been widely used to predict the eating quality of various types of noodles (Crosbie, 1991, McCormick, 1991, Konik et al., 1992, Bhattacharya and Corke, 1996). Reduced amylose content associated with reduced amounts of waxy protein resulted in high swelling power and peak viscosity leading to improved white salted noodle eating quality (Moss and Miskelly, 1984, Crosbie et al., 1992, Yamamori et al., 1992, Miura and Tanii, 1994, Zeng et al., 1997).

High amylose starch is considered to have superior plastic making properties due to formation of an intermolecular network of amylose by the hydrogen bonds when the starch granules swells and disrupt by hydration in warm water (60-80C) (Nawrath et al., 1995). Amylopectin is effective in increasing the flexibility and elongation of the materials because of its high molecular mass and highly branched structure (Soest and Vliegenthart, 1997)

1.7 Starch structure analysis

1.7.1 Estimation of amylose content

The physico-chemical properties of starch which determine its suitability for different end-uses are greatly affected by the ratio of amylose to amylopectin and hence it is of great value to have a reliable procedure to precisely assess the amylose content of starch. Common methods of amylose estimation, potentiometric (Schoch, 1942) and colorimetric (Morrison and Laignelet, 1983) are based on the iodine binding capacity of amylose. When the three dimensional helicoid chains of amylose pack two by two to form a double helix, a central hydrophobic cavity is formed where molecules such as iodine can be fixed (Martinez and Podoliet, 1996). Since amylopectin is usually non linear, its iodine-binding capacity is very small and this is usually ignored during quantitative determination of amylose. However it has to be recognized that there are three types of anomalous amylopectin which can give rise to misleading results while determining amylose content (Morrison, 1993). Type I has a high molecular weight and long external chains with enhanced iodine-binding capacity, typical of the amylose extender (ae) mutant of maize. Type II has a low molecular weight ($<10^6$) similar to that of amylose and usually has long external chains with enhanced iodine-biniding capacity. Type III has a high molecular weight and normal external chain lengths, but longer internal chains which slightly increase the iodine binding. There are numerous reports of simplified modifications of colorimetric methods to reduce or eliminate interference from iodine binding to amylopectin and to predict the amylose content with higher accuracy (Bates et al., 1943, Haunold and Lindesay, 1960, Juliano, 1971, Morrison and Laignelet, 1983, Knutson, 1986). The method suggested by Morrison and Laignelet, (1983), for determining apparent (measured in presence of lipid) and total (measured on lipid-free starch) amylose contents using urea-dimethylsulphoxide as solvent was demonstrated to be repeatable in a collaborative study invoving eight participants by Martinez and Prodolliet (1996).

The iodine binding methods of amylose estimation are greatly affected by many external and internal factors. The estimated amylose shows decreasing variation with increasing wavelength from 590 to 650nm and decreasing pH (4.5-4.7) (Juliano, 1971). While the accuracy of procedures using a single wavelength in cereals is limited due to interference from amylopectin and lipids, a six wavelength multi-component analysis for the simultaneous estimation of amylose and amylopectin from lipid-rich maize starch was demonstrated to be efficient by Sene et al., (1997). The effects of lipids on measured amylose content is not completely predictable because they vary with the quantity of lipid, fatty acid chain length, degree of unsaturation of fatty acids and the source of amylose (Morrison and Laignelet, 1983). They also observed that the absorbance reading decreased linearly with increasing temperature over a range of 15-45°C. Inaccuracies also arise due to the inappropriate choice of standards for the preparation of calibration curve, as the regression equations vary depending on the source of starch to be estimated (Morrison and Laignelet, 1983).

Near-Infrared Transmittance Spectroscopy (NITS), Differential Scanning Calorimetry (DSC) and High Performance Liquid Chromatography (HPLC) are alternative tools for amylose estimation. NITS can be a rapid and nondestructive technique for predicting amylose content with a strong correlation with the colorimetric methods (Campbell et al., 1997). DSC calculates the amylose content on the basis of the melting enthalpy of amylose-lipid complex formed in the presence of a complexing lipid. This method gives accurate values for samples with high amylose content, but overpredicts in the case of samples with low amylose content (Sievert and Holm, 1993). The separation of iso-amylase digested starch into higher molecular weight chains (from amylose) and lower molecular weight chains (from amylopectin) by size-exclusion HPLC on hydrophobic columns provides a repeatable method of amylose estimation unaffected by lipid content of the starch (Batey and Curtin, 1996). The ability of the lectin concanavalin (conA) to specifically complex amylopectin is utilized in yet another approach (Gibson et al., 1997). The complexed amylopectin is precipitated and removed and the amylose content estimated spectrophotometrically by measurement of the amount of glucose produced following complete de-polymerization.

1.7.2 Amylopectin chain length analysis

Since the properties of starch are also profoundly influenced by the chain length distribution of the linear α-1,4 chains and the frequency and spacing of the α-1,6 branch points, chain length analysis is an indispensable tool in starch structural and functional studies. Traditional methods of chain length distribution analysis such as gel permeation chromatography and HPLC with refractive index or light scattering detection methods provide sub-optimal resolution for fine structure determination (O'Shea and Morell, 1996). More sensitive methods like anion exchange chromatography coupled with pulsed amperometric detection for improved resolution have been reported (Hanashiro et al., 1996 Wong and Jane, 1997, Koch et al., 1998). Chain length analysis of flourescently labelled oligosaccharides derived from isoamylase treatment of amylopectins based on high resolution electrophoresis using a DNA sequencer enabled baseline resolution of individual oligomers up to a DP of 80 glucose units at femptomole levels, and quantification of the oligomeric contribution to the distribution of total amylopectin chains (O'Shea and Morell, 1996). The procedure involves the reductive amination of the reducing end of the debranched oligosaccharides with the charged fluorophore 8-amino-1,3,6-pyrenesulfonic acid (APTS) and the electrophoretic separation and detection of the conjugates in polyacrylamide gels within an DNA sequencer. The use of capillary electrophoresis with laser-induced fluorescence (CE/LIF) detection for high resolution analysis was demonstrated by Evangelista, et al., (1995) and O'Shea et al., (1998).

1.8 Biochemistry of starch biosynthesis

A thorough understanding of the metabolic pathways involved in starch biosynthesis and the biochemistry of the enzymes involved in these pathways can guide any genetic modification of starch composition leading to novel starch types. Over the last couple of decades, knowledge of the individual enzymatic steps involved in starch biosynthesis has increased dramatically.

1.8.1 Pathway preceding starch biosynthesis

Views on the regulation of carbon flow and the steps preceding the starch biosynthesis have been reviewed in detail by many authors (Preiss, 1988, Duffus, 1993, Morell et al., 1995). Evidence shows that sucrose, being the major transport material in higher plants, is the main, though not the sole, source of carbon for starch synthesis. Since the amino acids not incorporated directly into protein can be recycled into pathways of carbohydrate synthesis following deamination, it is likely that carbon from amino acids may also be used in starch synthesis Duffus and Rosie, (1978). Sucrose is first converted into UDP-glucose and fructose by a UDP-dependent sucrose synthase during endosperm development in wheat and barley (Hawker et al., 1991, Duffus and Cochrane, 1992). In maize some of the incoming sucrose may be converted to hexoses by invertase (Doehlert et al., 1988). The UDP-glucose formed from sucrose synthase reaction together with any hexose phosphate derived from fructose is converted to glucose-1-phosphate by UDP-glucose pyrophosphorylase and hexokinase coupled to phosphoglucomutase. Evidence that these events take place in the cytosol comes from the work on wheat amyloplasts isolated from endosperm protoplasts (Entwistle and ap Rees, 1988). The identity of the carbon source entering the developing amyloplast where starch biosynthesis takes place is controversial, with ADP glucose, hexose phosphate and triose phosphate suggested by different researchers. Carbon uptake and utilization studies using both isolated amyloplast and endosperm tissues are consistent with the view that hexose phosphates and not triose phosphates are the predominant carbon compounds translocated into the amyloplast to support starch biosynthesis (Morell et al., 1995). More recently, Randhawa and Singh (1998) in their comparative analysis of developing grains of wheat and sorghum gave evidence that triose phosphates act as precursors of starch in sorghum grains, whereas hexose phosphate and /or adenosine 5'-diphosphate glucose may act as direct precursors of starch in wheat grain.

1.8.2 Starch biosynthetic pathway

Following the uptake of hexose phosphate across the amyloplast membrane, starch is biosynthesized *in situ* by the concerted efforts of a set of enzymes, ADP-glucose pyrophosphorylase, starch synthase, branching and debranching enzymes. Analysis of mutants in maize, pea and rice with abnormal endosperm phenotypes has contributed greatly to the identification of genes encoding different isoform of these enzymes. Studies of*Chlamydomonas reinhardtii*, a unicellular, eukaryotic green algae of the order Volvacales have also helped to ascertain the *in vivo* function of many starch biosynthetic enzymes (Ball et al., 1996).

1.8.2.1 ADP-glucose pyrophosphorylase (AGPase)

AGPase, which catalyses the conversion of glucose 1-phosphate to ADP-glucose utilizing ATP molecules, is one of the most extensively studied and characterized starch biosynthetic enzymes at both biochemical and molecular levels. In higher plants it is a tetramer composed of two large and two small subunits of molecular mass of 55-60kDA and 50-55kDa respectively (Morell et al., 1987, Preiss, 1991), whereas in bacteria and cyanobacteria AGPase is a homotetramer with a sub unit size in the range of 50-55kDa. The small and the large subunits of higher plants have about 50-60% amino acid identity to each other and have about 30-40% identity with prokaryotic AGPases. Because of the relatively high identity between the large and small subunits of the plant enzymes and the prokaryotic subunit, it was postulated that the large and small subunits evolved from the bacterial enzyme via gene duplication and divergence (Smith-White and Preiss, 1992). The amino acid sequences of the small subunits are highly conserved among plant groups (85-95%) and between photosynthetic and non-photosynthetic tissue enzyme forms, while the large subunit sequences are less conserved (50-60% identity). Each subunit contains those residues absolutely required for catalysis and the presence of the companion subunit promotes a much more active conformation (Morell, 1995). The AGPases from maize endosperm and potato tuber, as well as from leaves of all plants examined are allosterically activated by 3-phosphoglyceric acid (3-PGA) and

inhibited by inorganic phosphate (Preiss, 1991). Barley endosperm AGPase, on the other hand, is highly active without 3-PGA and is only slightly affected by the activator and inhibitor (Kleczkowski et al., 1993). Similar results of insensitivity in wheat endosperm are reviewed in Preiss (1991) and Duffus (1992).

cDNAs for AGPase subunits are sequenced from many plants including wheat, maize, barley, rice, potato and *Arabidopsis thaliana* (reviewed in Morell et al., 1995, Wasserman et al., 1995). The *Agp1* genes (encoding the small sub unit) and *Agp2* genes (encoding the large sub unit) are differentially expressed in wheat grain (Ainsworth et al., 1995). *Agp2* genes are not expressed until 10 days post anthesis. Both the genes are arranged as triplicate sets of homoeoloci in wheat, *Agp1* genes being located distal to the centromere on the long arm of group 7 chromosomes and *Agp2* on the long arm of group 1 chromosomes.

1.8.2.2 Starch synthase (SS)

The glucose moiety of ADP-glucose is incorporated in the growing starch polymer in a reaction catalyzed by starch synthases, which catalyze the transfer of the glucosyl group from ADP-glucose to the non reducing end of an α-1,4 glucan (Preiss,1991). Four different classes of SS have been identified in plants, granule bound starch synthase (GBSS), SSI, SSII and SSIII (reviewed in Myers et al., 2000). Four groups of SS identified in wheat are the 60kDa GBSS, the 75kDa SSI which exists as both granule bound and soluble forms (Li et al., 1999b), the 100, 108, 115kDa SSIIs which are present in both the starch granule and soluble fraction at the early stages of wheat endosperm development, but that are exclusively granule bound at mid and late endosperm development (Li et al., 1999a) and a 180kDa SS III identical to SS II of maize (Z. Li, personal communication). Two starch synthases have been characterized from soluble extracts of potato (SSII and SSIII) and pea (SSI and SSII) (Denyer and Smith, 1992, Edwards et al., 1995, Abel et al., 1996). In rice, both cDNA and genomic DNA encoding the SSI gene have been cloned and characterized (Baba et al., 1993, Tanaka et al., 1995). In maize the *dull1* locus has been recently characterized and shown

to encode a starch synthase (SSII) related to the starch III of potato (Gao et al., 1998). Cloning of maize cDNA starch synthases zSS IIa and zSS IIb (Harn et al., 1998) have also been reported.

Since the lack of 60kDa GBSS from starch granule resulted in an amylose free or waxy phenotype, this polypeptide is considered to be essential for amylose synthesis in endosperm. The dosage effect of GBSS allele (waxy allele) is found to have a linear relationship for only GBSS activity and not for amylose content. (Flipse et al., 1996a). It has been postulated that amylose content in rice is related to the post-transcriptional regulation of waxy gene (which encodes GBSS/waxy protein) depending on the ability of the cultivar to excise intron I from the leader sequence of the waxy transcript (Wang et al., 1995). An in depth structural investigation of the mutant and wild-type starch in *Chlamydomonas reinhardtii* showed that waxy strains have lost an important component of amylopectin fraction also suggesting that GBSS controls the biosynthesis not only of amylose but also of amylopectin in all types of photosynthesizing or starch accumulating plastids (Delrue et al., 1992).

Starch synthases other than GBSS are thought to be primarily responsible for the elongation of amylopectin chains. However, it is not clear whether the different isoforms of SS play qualitatively distinct roles in amylopectin synthesis or not (Smith et al., 1997). In potato, reduction of SSIII isoform leads to the synthesis of a structurally modified starch with a significant change in granule morphology and an increased level of covalently linked phosphate (Abel et al., 1996). In pea, starches of *rug5* mutants which are defective in SSII activity are characterized by fewer chains of intermediate length (B2 and B3 chains) and more very short and very long chains than the amylopectin from wild-type embryos (Craig et al., 1998).

The three waxy genes (*Wx*) designated *Wx-A1*, *Wx-B1* and *Wx-D1* have been mapped as a triplicate set of single-copy homoeoloci on chromosome arms 7AS, 4AL and 7DS in wheat (Chao et al., 1989, Nakamura et al., 1993b). Both SS1 and class II SSs are located on the short arms of group 7 chromosomes in wheat (Yamamori and Endo, 1996, Li et al., 1999).

1.8.2.3 Starch branching enzyme (SBE)

SBE, belonging to the amylolytic group of enzymes, catalyzes a transferase reaction in which a donor linear α-1,4-glucan chain (either amylose or a linear region of amylopectin) is cleaved and attached to a recipient chain via α-1,6 linkage (Morell et al., 1997). This reaction creates another non reducing end where further chain elongation can take place. Chromatographic studies have revealed multiple forms of SBEs in many species including spinach, pea, potato, maize (Hawker et al., 1974, Boyer and Preiss, 1978, also reviewed in Preiss, 1991), rice (Nakamura et al., 1992) and wheat (Morell et al., 1997). On the basis of their structural features and sequence differences SBE isoforms from plants may be classified into two distinct enzyme families. The SBE I family includes maize SBE I, rice SBE I, pea SBE II, potato SBE I and wheat SBE I, and the SBE II family includes maize SBE IIa, SBE IIb, barley SBE IIa, SBE IIb, pea SBE I, rice SBE III and wheat SBE II (Boyer and Preiss, 1978, Mizuno et al., 1993, Burton et al., 1995, Fisher et al., 1993, Morell et al., 1997, Sun et al., 1998). Members within a group share around 65-70% amino acid identity whereas between families only 45-48% identity is observed.

Biochemical studies and characterization of mutants suggest that there are significant differences between class I and class II SBEs with respect to their substrate specificities, mode of action and physiological roles (Guan and Preiss, 1993, Morell et al., 1997). In general, the SBE I group of enzymes have a higher affinity for amylose than the SBE II group and preferentially transfer longer chains than SBE II enzymes (Takeda et al., 1993a). SBE II produces a much shorter chain length distribution with a peak at DP 6 than SBE I which produces a reproducibly skewed distribution with a peak at DP 10 (Hashemi et al., 1997). Wheat SBE I was demonstrated to have a 2-5 fold higher affinity to amylose than SBE II. Activation by phosphorylated intermediates and inorganic phosphates were also found to be considerably higher for SBE I compared to SBE II (Morell et al., 1997).

SBEs belonging to distinct enzyme families are differentially expressed during the embryo development. SBE I type enzymes are preferentially expressed towards the later

stage of endosperm development (Burton et al., 1995, Gao et al., 1996, Morell et al., 1997). A constant level of expression of wheat SBE II throughout mid and late endosperm development was observed.

Independent genetic control of different SBE isoforms are reported in many species. SBE clones, both cDNA and genomic DNA, that have been isolated and sequenced in cereal plants are summarized in Table 1.2.

Table 1.2 SBE clones characterized from cereals

Species	SBE isoform	Type of clone	Reference
Maize	SBE I	cDNA	Baba et al., 1991
	SBE IIb	cDNA	Fisher et al., 1993
	SBE IIa	cDNA	Gao et al., 1997
	SBE IIb	genomic	Kim et al., 1998
Wheat	SBE II	cDNA	Nair et al., 1997
	SBE I	cDNA and genomic	Rahman et al., 1997, Rahman et al., 1999
	SBE I	cDNA	Repellin et al., 1997
	SBE II	cDNA and genomic	Rahman et al., in prep
Rice	SBE I	cDNA	Nakamura and Yamanouchi, 1992
	SBE I	genomic	Kawasaki et al., 1993
	RBE3	cDNA	Mizuno et al., 1993
Barley	SBE IIa and SBE IIb	cDNA and genomic	Sun et al., 1998

Mutant analysis in maize, rice, and pea have demonstrated that high amylose mutants in each case are deficient in the SBE activity analogous to maize SBE II (Boyer and Preiss, 1978, Mizuno et al., 1993, Sidebottom et al., 1998). The wrinkled seed character of pea is due to the lack of a 114kDa isoform of SBE gene at the *rugosus* (*r*) locus (Bhattacharya et al., 1990). The gene is interrupted by a 800bp transposon-like insertion causing the failure of enzyme production. Barley SBE IIa and SBE IIb were strongly divergent at the 5' ends, primarily due to the 2064bp long intron 2 in SBE IIb (Sun et al., 1998). The predicted protein sequence from maize SBE IIa differed from its SBE IIb

counterpart in having a 49-amino acid N-terminal extension (Gao et al., 1997). Structural differences between the two isoforms at the amino and carboxy terminals are also reported in rice and barley (Mizuno et al., 1993, Sun et al., 1998).

A complex arrangement of genes at SBE I locus in *T. tauschii*, the D genome donor of wheat is described in Rahman et al., (1997). There are approximately ten copies of SBE I type genes in wheat. Nullisomic-tetrasomic analysis confirmed the location of wheat SBE I gene to homoeologous group 7 chromosomes (Morell et al., 1997). The consensus map of hexaploid wheat has located SBE II on the long arm of chromosome 2 (Gale et al., 1993) which was further confirmed by *in situ* hybridization (Mukai et al., 1998).Barley SBE IIa and SBE IIb are located on chromosomes 2 and 5 respectively (Sun et al., 1998).

1.8.2.4 Starch debranching enzyme (DBE)

The role of DBE in starch biosynthesis is a relatively new area of research that emerged on the basis of studies on maize *sugary* mutants which accumulated phytoglycogen – like polysaccharides in developing endosperm instead of amylopectin due to a lesion in R- (debranching) enzyme (Pan and Nelson, 1984). They suggested that DBE plays an important role in adjusting the chain length of amylopectin molecules.

DBE which hydrolyzes α-1,6-glucosidic linkages of α-polyglucans are classified into two classes, direct and indirect DBEs. Direct DBEs which are present in plant and bacteria can hydrolyse α-1,6 branches while indirect DBEs in animals and yeast can remove α-1,6 linkages by the combined actions of 4-α-glucano transferase (EC 2.4.1.25) and amylo-1,6-glucosidase (EC 3.2.1.33) (Jeanningros et al., 1976). Two classes of enzymes have been distinguished in direct DBEs. One class can debranch pullulan and is designated as pullulanase or R-enzyme (EC 3.2.1.41), which is often described as limit dextrinase since α-limit dextrins are better substrates than amylopectin for these enzymes. The second class designated as isoamylase (EC 3.2.1.68) can easily attack α-1,6 branches of glycogen, but not those of pullulan (Nakamura et al., 1996b).

cDNA and genomic clones encoding the pullulanase type of DBE were isolated and sequenced from rice (Nakamura et al., 1996a, Francisco et al., 1998). The amino acid sequence of rice DBE was found to be substantially similar to that of bacterial pullulanase, while it bears little similarity to that of bacterial isoamylase. Comparison of the barley and rice pullulanase genes indicated that most of the exons are highly conserved. The sugary mutation in maize is due to a lesion in *sugary 1* (*su1*) gene which encodes a DBE (James et al., 1995). However in rice, the sugary mutation is not due to a lesion in the DBE gene localized on chromosome 4, but due to a lesion in a regulatory gene located on chromosome 8 (Nakamura, 1996). The properties of starch DBEs and their possible role in amylopectin biosynthesis was thoroughly reviewed by Nakamura, (1996) and Myers et al., (2000).

1.9 Starch mutants

Mutants with altered starch phenotypes, identified in many crops including maize, barley and rice have been instrumental in studying carbohydrate metabolism in seeds and exploring the enzymes and proteins that function in starch biosynthesis. Starch mutants and their significance in starch biosynthetic studies are reviewed in Preiss, (1988) and Sathish et al., (1995). A brief summary of some of the major starch mutants, their phenotypes and effects on starch biosynthetic enzymes are given in Table 1.3.

In order to bring additional insight into the understanding of biogenesis of starch granules, mutants with altered starch structure have been established and studied in *Chlamydomonas reinhardtii*. The STA7 mutant defective in DBE, STA2 defective in GBSS and STA3 defective in SSS II are examples (Delrue et al., 1992, Mouille et al., 1996, Buleon et al., 1997).

1.10 Starch biosynthetic models

There are several models proposed for possible mechanisms of starch biosynthesis as reviewed in James et al., (1995) and Nakamura, (1996). One of the earlier suggestions

Table 1.3 Description of starch mutant in various plant species

Mutant	Phenotype	Plant species	Casual mutations/Enzyme affected secondarily	Reference
Amylose extender (*ae*)	High levels of amylose and abnormal phenotype	Maize	SBE IIb	Garwood et al., 1976, Boyer and Preiss, 1978, Hedman and Boyer, 1982, Fisher et al., 1993, Kasemsuwan et al., 1995
		Rice	RBE III	Mizuno et al., 1993
High amylose (*amo 1*)	Higher level of amylose	Barley	-	Schondelmaier et al., 1992
Low amylose (*lam*)	Low level of amylose	Pea	GBSS	Denyer et al., 1995
Floury-2 (*flo-2*)	Soft, white endosperm which crumbles easily into powder. High amylose content	Rice	RBE I, RBE III and GBSS (not known to be casual)	Yano et al., 1985, Kawasaki et al., 1996
Shrunken (*shx*)	Reduced starch content, altered granule size distribution	Barley	SS-I (not known to be casual)	Tuomi et al., 1993, Schulman et al., 1994, Schulman et al., 1995
Shrunken (sh-2)	High levels of sucrose and low levels of starch	Maize	Large subunit of AGPase	Bhave et al., 1990
Shrunken (shr)	Varying effects on amylose content	Rice	-	Asaoka et al., 1993
Dull (*du 1*)	Mature kernels with tarnished , glassy and dull appearance. High apparent amylose content	Maize	Mutation at SS II, SBE IIa affected secondarily	Gao et al., 1998
Rugosus (*r*)	Wrinkled seed, lowered starch, enhanced amylose, sucrose and lipid levels.	Pea	SBE I	Bhattacharya et al., 1990, Burton et al., 1995
Rugosus 3 (*rug3*)	No starch	Pea	Phosphoglucomutase	Harrison et al., 1998
Rugosus 4 (*rug4*)	Low starch	Pea	Sucrose synthase	Craig et al., 1999
Rugosus 5 (*rug 5*)	Granules with deeply divided lobes, reduced amylopectin synthesis, altered amylopectin chain length	Pea	SS II	Craig et al., 1998
rb	Decreased starch content and increased levels of sucrose	Pea	AGPase	Hylton and Smith, 1992

(to be continued)

Table 1.3 (continued)

Sugary (su1)	High levels of phytoglycogen, increased sucrose and amylose content	Maize	DBE (both isoamylase and pullulanase types affected, but mutation at isoamylase type)	James et al., 1995, Nakamura, 1996
Sugary (su)	High levels of phytoglycogen, increased sucrose content and decreased amylose content	Rice	DBE (similar to *su 1* of maize)	Nakamura et al., 1996a, 1996b
Brittle I (bt1)	Collapsed/shrunken endosperm	Maize	An amyloplast membrane protein	Cao and Shannon, 1997, Singletary et al., 1997, Shannon et al., 1998
Brittle-2 (bt2)	High levels of sucrose and low levels of starch	Maize	Small subunit of AGPase	Preiss et al., 1990, Sathish et al., 1995

was that phytoglycogen was formed as an intermediate product by the action of SSs and SBEs and subsequently converted to amylopectin by the action of DBEs. The lack of substantial evidence showing the occurrence of phytoglycogen in non-sugary sources is one of the criticisms against this model. An alternative model was put forth by James et al., (1995) in which it was suggested individual starch molecules are acted upon simultaneously as they were formed by DBEs, SBEs and SSs. Thus phytoglycogen would accumulate only when DBE was missing and would not be produced in normal kernels. A discontinuous model involving a highly specific glucan trimming mechanism is explained in Mouille et al., (1996). Their suggestion that amylose is synthesized downstream from amylopectin seems to correlate with the absence of reports describing plant mutants accumulating only amylose. *In vitro* studies of purified *Chlamydomonas reinhardtii* starch granules also suggested that amylose is synthesized by extension and cleavage from amylopectin (Vandewal et al., 1998). Labelling experiments indicated that initially the major GBSS extended glucans available on amylopectin. Amylose synthesis occurred thereafter from cleavage of a pre-existing external amylopectin chain.

A role of GBSS in amylopectin biosynthesis was also suggested previously (Delrue et al., 1992, Denyer et al., 1996).

1.11 Inheritance of amylose content

Various types of gene action were reported for the inheritance of amylose content depending on the species and the individual cross under investigation. Monogenic inheritance by completely/partially dominant or recessive genes for high amylose character is observed in several crosses of rice and wheat (Bollich and Webb, 1973, McKenzie and Rutger, 1983, Watanabe et al., 1998, Mohammadkhani et al., 1999). Involvement of two dominant complementary genes was also found (McKenzie and Rutger, 1983). Most of the above reports have suggested the influence of modifying genes of minor effects on amylose content. A single gene with major effect governing low or intermediate amylose content was revealed in rice by Kumar and Khush, (1987) and (1988).

Since the endosperm genotype determines the amylose content, a comprehensive set of models of biometrical genetics for triploid tissues was proposed by Pooni et al., (1992) for rice. Investigations employing these models revealed a complex control of amylose content. Apart from the effect due to two or more genes showing epistasis, amylose content is also highly influenced by cytoplasmic effects and the interaction between cytoplasmic and nuclear genes (Pooni et al., 1993). Effective selection for low or high amylose content is more feasible in crosses involving low and high amylose rice parents than a narrow cross where the parents do not differ considerably in amylose content (Bollich and Webb, 1973, Kumar and Khush, 1987).

1.12 Genetic engineering for altered starch structure

The production of specifically tailored starch to suit various industrial purposes by post harvest chemical modifications is expensive and time consuming.. There is therefore a growing interest in the production of modified starch in transgenic plants. Four basic technologies needed to alter carbohydrate expression in plants are a) plant

transformation b) organ-specific expression, c) suitable cloned genes and 4) plastid targeting. These four combined yield transgenic plants expressing the desired trait (Shewmaker and Stalker, 1992). The mechanisms of co-suppression (sense) and antisense-mediated down regulation of SBEs have induced alterations in the physico-chemical properties of starch (Flipse et al., 1996b, Chibbar et al., 1998). The inhibition of branching enzyme activity by expression of a SBE I anti-sense RNA in the endosperm resulted in a less crystalline starch with reduced gelatinization temperature in transgenic wheat plants (Baga et al., 1999). Transgenic potato plants expressing an antisense of the SBE A isoform resulted in an increased average chain length of amylopectin leading to an increased apparent amylose content (Jobling et al., 1999). Ectopic expression of bacterial genes in transgenic plants to increase corresponding enzyme activities is also being tried for influencing starch characteristics (Muller-Rober and Kobmann, 1994). The increased degree of branching in starch obtained by the expression of glycogen branching enzyme gene (*glgB*) of *Anacystis nidulans* or *Escherichia coli* in potato is a typical example (Korstee et al., 1998). This starch was characterized by a higher proportion of short chains in the amylopectin fraction together with a lower peak viscosity.

1.13 Objectives of the current project

With wheat as the main source of starch in Australia, there is a challenge to wheat quality breeders, during the last few decades, to provide native starches of precise structure to suit specific end uses with either no or minimal manufacturing modifications. The development of waxy wheat with zero percent amylose is an outcome of such an attempt. The discovery of the potential of high amylose starch to provide a benign alternative to petrochemical sources in plastic manufacturing and as a source of dietary fibre has necessitated the need for the development of high amylose wheat varieties. However there are certain key barriers which delay the achievement of this objective. Firstly, no high amylose genes have so far been identified in wheat unlike other cereal crops such as maize and rice. Secondly, common wheat (*Triticum aestivum* L. 2n = 42) is an allohexaploid species consisting of three different diploid genomes

designated A, B and D, each consisting of seven pairs of chromosomes. Most loci controlling enzymes in wheat are known to be triplicated over the three genomes which can result in the masking effect of a mutant gene located on a particular chromosome by the normal homoeologous genes on other chromosomes. Thirdly, lack of sufficient diversity in amylose content in common wheat (Mohammadkhani et al., 1998) demands the screening of land races and wild relatives and introgress any superior trait identified into common wheat through inter-generic and inter-specific hybridizations. With this background, the current project was undertaken with the following objectives:

1) To investigate the available genetic variation in amylose content in *Triticum* species

2) To study the variability in wheat starch branching enzyme, one of the key enzymes in starch biosynthetic pathway

3) To isolate and characterize novel gene/s involved in starch biosynthesis related to high amylose content

4) To study the mode of inheritance of amylose content and breed high amylose wheat lines

CHAPTER 2

Survey of Amylose Content in Wheat Starches

2. 1. INTRODUCTION

Starch, the major energy reserve of many of the agriculturally important plants is composed of two major polymers, amylose and amylopectin. Amylose is essentially a linear polymer of glucose residues, linked by α-1,4 glucosidic bonds with occasional α-1,6 glucosidic linkages. Amylopectin is highly branched with α-1,6 linkages. When starch is used industrially either for food or non food purpose, its effectiveness frequently depends on the relative proportion of amylose to amylopectin, a major determinant of its functional properties. Genetic manipulation of the ratio of amylose to amylopectin to suit various end uses has occurred, a typical example being the development of reduced amylose wheat types for superior noodle quality.

In the non food industry, plastic substitutes produced from starch are attracting attention, mainly due to their biodegradability, making them more environmentally friendly. When starch granules swell and disrupt by hydration in warm water (60-80°C), the amylose molecules released can align and form a hydrogen bonded network leading to film formation, whereas, the highly branched structure of amylopectin results in higher water solubility and a greatly reduced ability to form films (Nawrath et al., 1995). Hence amylose is considered to have superior properties to amylopectin for making 'plastic replacers', due to its predominantly linear macromolecular structure. As the fractionation of normal starch to obtain higher levels of amylose is prohibitively expensive, the development of varieties with high amylose starch is an economically viable way of naturally producing amylose enriched starch.

With the discovery of high amylose genes in maize (*ae*) and barley (*amo1*), breeding for increased amylose content has been achieved in these crops. Attempts to breed high

amylose wheats have not been successful due to its hexaploid nature. Information on the diversity of amylose content in *Triticum* species is one means of identifying germplasm for a breeding programme.

Measurement of the ratio of amylose to amylopectin plays a major role in analysing starch structure, which in turn is essential in determining its end-use. Various analytical procedures involving spectrophotometry and chromatography have been described for the estimation of amylose content, each with its own merits and limitations. As the boundaries between the amylose and amylopectin population of molecules are arbitrary, no single analytical procedure can give an absolute measurement of amylose content. Breeding for altered amylose content requires a simple, quick and reproducible method of amylose estimation with a minimum amount of starch sample.

The results of a comparative study of three different methods of amylose estimation in Australian wheat cultivars (iodometric analysis, high performance liquid chromatography (HPLC) and concanavalinA (conA) precipitation method) and the screening of wheat cultivars for high amylose content using iodometric method are presented in the following section of this chapter.

2.2 MATERIALS AND METHODS

2.2.1 Seed materials

Seed samples for starch analysis were taken from the germplasm collection of the Plant Breeding Institute, Cobbitty. To compare the methods of amylose estimation, seeds were grown under uniform environmental conditions to nullify the environmental variance and well filled grains at maturity harvested for starch extraction. Some of the *T. dicoccoides* and *T. tauschii* accessions were provided by Dr S. Rahman, CSIRO Plant Industry, Canberra.

2.2.2 Starch extraction

2.2.2.1 Small scale method

Small scale starch extraction was performed using the method of Zhao (1999). A single grain was cut into two halves and the embryo less half was crushed and soaked in 1ml of deionized water at 4°C overnight in a 1.5ml eppendorf tube. The softened endosperm tissues were ground with a small plastic pestle. Crude starch was recovered by passing the suspension through a 65µm nylon mesh. After centrifugation for 3 min at 13, 000g, the sediment was resuspended in 1ml of 32% NaCl by vortexing followed by centrifugation for 3 min at 13,000g. The starch granule pellet was washed twice with 1ml of washing buffer containing 55mM Tris-Cl, pH 6.8, 2.3% (w/v) SDS and 10% (v/v) glycerol, each time vortexing and centrifuging as before. This was followed by washing four times with deionized water: in these steps the spinning speed was reduced to 3000g. The yellow layer of impurities on the top of the starch pellets was carefully removed after each centrifugation by pipetting. Finally the pellets were washed once with acetone and centrifuged for 3 min at 13, 000g and then dried at room temperature.

2.2.2.2 Large scale method

The seeds were ground into flour in a coffee grinder (Brevetto). Starch extraction was done the same way as explained in section 2.2.2.1 except that larger volumes of reagents (15ml) were used for extracting starch from 3-5g flour and the various steps were carried out in 50ml tubes.

2.2.3 Amylose estimation

2.2.3.1 Iodine assay

The colorimetric method of Morrison and Laignelet (1983) was followed with slight

modifications. The isolated starch was dried at 65°C for 1 hour and cooled to room temperature to equalize the moisture content before weighing. Approximately 2mg of starch was weighed accurately (accurate to 0.1mg) into a 2ml screw-capped tube fitted with a rubber washer in the lid. For defatting, 1ml of 85% (v/v) methanol was mixed with starch and the tube was heated in a 65°C water bath for 1 hour with occasional vortexing. After centrifugation at 13, 000g for 5 min, the supernatant was carefully removed and the defatting steps repeated once. The starch was dried at 65°C for 1 hour and dissolved in urea dimethyl sulphoxide (UDMSO) solution (9 volumes of dimethyl sulphoxide and 1 volume of 6M urea) in a ratio of 1ml of UDMSO per 2mg of starch. The mixture was immediately vortexed vigorously and kept in a 95°C water bath for 1 hour with intermittent vortexing for complete dissolution of the starch. An aliquot of the starch-UDMSO solution (50µl) was treated with 20µl of I_2-KI reagent: 2mg iodine, 20mg potassium iodide per ml of water. The solution was made up to 1ml with water. The absorbance of 200µl of this sample taken in a microplate was read with an Emax Precision Microplate Reader (Molecular Devices, USA) at 620nm along with standard samples containing amylose ranging from 0 to 100%, made from potato amylose and corn amylopectin (Sigma). The blue value of the starch was converted to percentage amylose using a regression equation derived from the standard samples

2.2.3.2 High performance liquid chromatography (HPLC)

The method of Batey and Curtin (1996) was followed with modification to allow the use of smaller starch samples. Starch (5mg) was suspended in 100µl of 0.25M sodium hydroxide and heated for 5 min at 100°C with stirring. After cooling, 400µl of 0.05M sodium acetate buffer (pH 4.0), 3.2µl of glacial acetic acid and 2.5µl of isoamylase (200 units/ml, Megazyme) were added with continuous stirring followed by incubation at 37°C for 2 hours. The enzyme was denatured on completion of the reaction by heating the solution at 100°C for 20 min. After centrifuging at 12,000g for 5 min, 400µl of the supernatant was treated with 0.2g of mixed bed ion exchange resin (AG 501-X8D, Bio-Rad Laboratories, Richmond, CA, USA) and filtered through a 0.45µm PVDF filter. HPLC was performed on a Waters System (Waters, Milford, MA, USA) using Waters

Ultrahydrogel Column (UHG)-250. Ammonium acetate (0.05M) was used as the mobile phase with a flow rate of 0.5ml/min. The time between the injections was 40 min.

2.2.3.3 ConA method

The assay was conducted using the Amylose/Amylopectin assay kit of Megazyme Int. Ireland Ltd., according to the manufacturer's instructions. Starch (23-25mg) was dissolved in dimethyl sulfoxide (DMSO) (1ml) by heating and intermittent vortexing. The starch was defatted by reprecipitating with 95% ethanol, centrifuging and discarding the supernatant. When the ethanol had drained off thoroughly, the pellets were redissolved in 1ml of DMSO with heating and stirring. The contents were diluted to 25ml with conA solvent (solution 1). Concentrated Con A solvent was prepared by dissolving 49.2g anhydrous sodium acetate, 175.5g NaCl, 0.5g $CaCl_2.2H_2O$, 0.7g $MgCl_2.6H_2O$ and 0.7g $MnCl_2.4H_2O$ per litre of distilled water (pH 6.4, adjusted with glacial acetic acid). Working concentration of conA solvent was prepared at the time of use by diluting 30ml of concentrated solvent to 100ml with distilled water. An aliquot (1ml) of solution 1 was treated with 0.5ml of conA solution (4mg/ml) for 1 hour. To this, 3ml of 100mM sodium acetate buffer, pH 4.5 was added to 1ml of the centrifuged supernatant and heated for 5 min in a boiling water bath to denature conA. After equilibrating to 40°C, 0.1ml of amyloglucosidase/α-amylase enzyme mixture was added and incubated at 40°C for 30 min. To a 1ml aliquot of the supernatant, 4ml of glucose oxidase peroxidase (GOPOD) reagent was added and again incubated at 40°C for 20 min. All the steps excluding the conA treatment were repeated with another aliquot (0.5ml) of solution 1. The absorbance of these two solutions was read at 490nm against a reagent blank prepared from sodium acetate buffer and GOPOD reagent.

2.2.4 Iodine spectrum

The blue starch–iodine complex was developed following the procedure of Batey and Curtin (1996). Starch (approximately 0.1g accurately weighed) was defatted prior to iodine determination by dissolving it in DMSO (10ml) and reprecipitation with ethanol

(30ml). The mixture was allowed to stand at room temperature for 10 min and the precipitate was collected by centrifugation at 12000g for 10 min The precipitate was redissolved in DMSO (15ml) and heated in a boiling water bath for 1 hour with vigorous vortex-mixing at 15 min intervals. After 60 min, distilled water (15ml) was added with mixing and the mixture was returned to the boiling water bath for 30 min. The solution was quantitatively transferred to a 100ml volumetric flask and made up to volume with distilled water. An aliquot (3ml) was accurately transferred to another 100ml volumetric flask containing 90ml distilled water, iodine solution (1ml, containing 0.01M sodium hydroxide, 0.3% w/v iodine and 3% w/v potassium iodide) was added to diluted starch solution and the solution was made up to volume (100ml) within 60 seconds. After standing for 20 min the absorbance of the complex was read from 490-650nm. Spectra were also determined for pure potato amylose and corn amylopectin.

2.3 RESULTS AND DISCUSSION

2.3.1 Iodine spectrum

The iodine spectrum showed an absorption maxima (λmax) of 580nm for pure potato amylose and a broader peak of 520-540nm for amylopectin (Figure 2.1). For wheat starch, the λmax shifted to 560nm due to the combined effects of amylose and amylopectin. The shifting of absorbance peak of amylose to lower wavelengths in this study compared to previously reported peaks of 590-630nm (Minick et al., 1991, Bhatnagar and Hanna, 1994) may be due to the absorbance being read at room temperature. As the temperature increases beyond 15°C, Minick et al., (1991) reported an increase in the nonlinearity of the polyiodine I_6 units, shifting the spectral lines to lower wavelengths depending on the extent of deviation from linearity. Apart from lowering the wavelength, absorbance value is also reduced at temperatures over 15°C due to the breakdown of amylose-iodine (AI) complex (Fonslick and Khan, 1989).

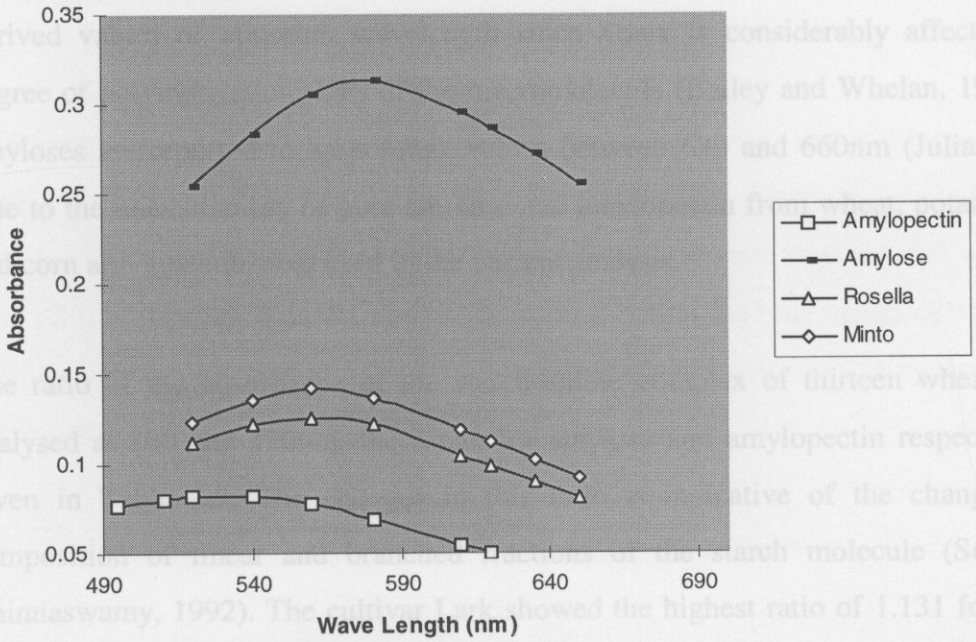


Figure 2.1 Iodine spectra of pure amylose, amylopectin and wheat starches

The present study indicated the optimum wavelengths for amylose analysis to be 580-620nm as shown by the higher difference in absorbance values between the amylose and amylopectin in this range (Table 2.1).

Table 2.1 Difference in absorbance value between amylose and amylopectin at different wavelengths

Wavelength (nm)	Absorbance		Difference in absorbance
	Amylose	Amylopectin	
620	0.287	0.050	0.237
610	0.296	0.054	0.242
580	0.313	0.069	0.244
560	0.306	0.078	0.228
540	0.283	0.082	0.201
520	0.254	0.082	0.172

The source of the starch materials used for the study can have an influence on the derived values of optimum wavelength since λmax is considerably affected by the degree of polymerization (DP) of the macromolecule (Bailey and Whelan, 1961). Rice amyloses are reported to have λmax values between 630 and 660nm (Juliano, 1971). Due to the unavailability of pure amylose and amylopectin from wheat, potato amylose and corn amylopectin were used in the current analysis.

The ratio of the absorbance of the starch-iodine complex of thirteen wheat starches analysed at 580 and 520nm, the λmax for amylose and amylopectin respectively, are given in Table 2.2. The changes in this ratio is indicative of the changes in the composition of linear and branched fractions of the starch molecule (Sokhey and Chinnaswamy, 1992). The cultivar Lark showed the highest ratio of 1.131 followed by Minto (1.107) and Meering (1.100) implying the possibility of a comparatively higher proportion of linear fractions in the starches of these cultivars.

Table 2.2 The ratio of absorbance of starch-iodine complex of wheat starches at 580 and 520 nm, the λmax for amylose and amylopectin respectively

Cultivar	Absorbance ratio
Rosella	1.083 ± 0.007
Minto	1.107 ± 0.008
Gamenya	1.078 ± 0.003
Kulin	1.081 ± 0.025
Lark	1.131 ± 0.018
Meering	1.100 ± 0.006
Gutha	1.078 ± 0.013
Reeves	1.063 ± 0.034
Hartog	1.049 ± 0.033
Kiata	1.048 ± 0.034
Chinese Spring (CS)	1.083 ± 0.004
CS dt5DL	1.090 ± 0.005
CS dt6AS	1.087 ± 0.005

dt = ditelosomic

2.3.2 Iodine method

Calibration curves prepared over a range 0-100% amylose, using mixtures of pure potato amylose and corn amylopectin for intermediate values at intervals of 10% amylose showed high linear correlation between percentage amylose and blue values with $r \geq 0.99$. An example is shown in Figure 2.2, where the blue values of wheat starch samples analysed at the same time were converted into amylose % using the following regression equation.

$$\text{Amylose \%} = -12.74 + 83.3 \times \text{blue value}$$

A calibration curve was constructed each time amylose content analysis was done iodometrically and a regression line fitted, and the regression formula used to convert the blue value to amylose content. The regression equation determined for each individual experiment varied slightly.

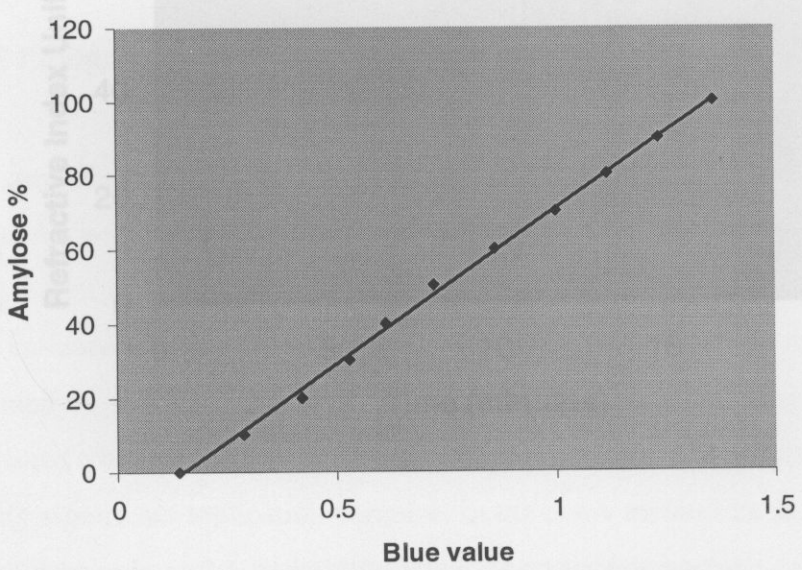


Figure 2.2 Calibration curve for determining amylose content iodometrically derived from potato amylose and corn amylopectin.

As observed in the Figure 2.2, zero % amylose starch gave an absorbance value of 0.141 which is most probably due to the absorbance by the amylopectin fraction of the starch at the wavelength (620nm) used for the measurement.

2.3.3 HPLC

Chromatography of the debranched starch samples on an UHG-250 size exclusion column yielded two peaks (Figure 2.3). The first peak (peak I) to elute contains chains with a high degree of polymerisation (500+) predominantly derived from amylose and the second (peak II) contains chains less than approximately 50 glucose units predominantly derived from amylopectin (Batey and Curtin, 1996). The shoulder observed on the leading edge of the second peak, indicates that this peak does not contain a single distribution of chain lengths (Batey and Curtin, 1996). The percentage area under the first peak is expressed as the amylose content.

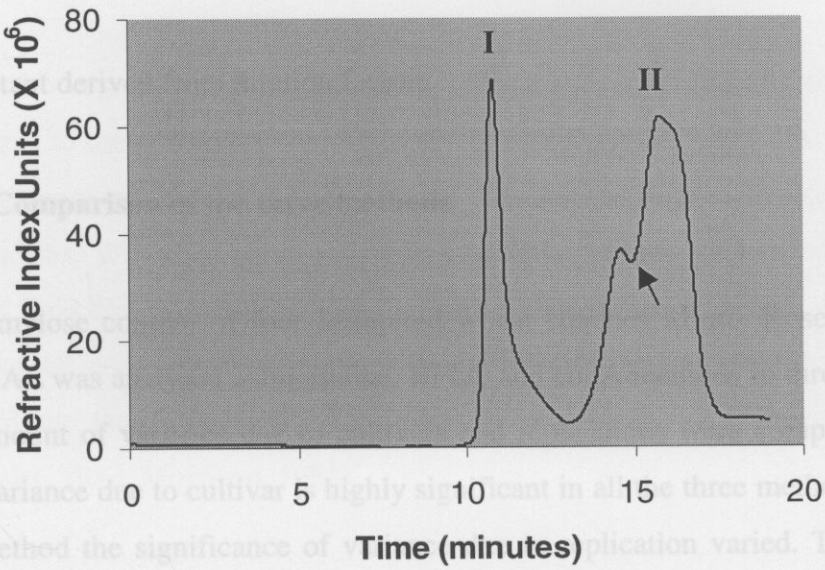


Figure 2.3 Separation of debranched wheat (Minto) starch on an UHG-250 column. Peak I contains chains with >500 DP and peak II contains chains with < 50 DP (Batey and Curtin, 1996). The shoulder observed on the leading edge of peak II is indicated by the arrow.

2.3.4 ConA method

The lectin concanavalin (conA) can specifically complex branched polysaccharides based on ∝-D-mannopyranosyl units at multiple non-reducing end groups with the formation of a precipitate, under defined conditions of pH, temperature and ionic strength (Yuan and Matheson, 1990). The principle involves the removal of the amylopectin components of lipid free starch by precipitation with conA, followed by the enzymatic hydrolysis of the resulting amylose to glucose units. The total starch solution without conA treatment is also subjected to amylolytic hydrolysis to yield glucose. The amylose content is estimated as the ratio of the glucose derived from the conA treated starch solution (after removing the amylopectin fraction) to that derived from the total starch solution which are measured as the absorbance of the respective solutions at 490nm expressed as a percentage.

$$\text{Amylose \%} = \frac{\text{Absorbance of conA supernatant}}{\text{Absorbance of total starch aliquot}} \times 66.8^{*}$$

*Constant derived from dilution factors

2.3.5 Comparison of the three methods

The amylose content of four hexaploid wheat starches Minto, Rosella, CSdt5DL and CSdt6AL was analysed using iodine, HPLC and conA methods in three replications and the amount of variance due to cultivars and replications were compared in Table 2.3. The variance due to cultivar is highly significant in all the three methods. Depending on the method the significance of variance due to replication varied. The HPLC method exhibited a nonsignificant effect due to replication, whereas the iodine method showed a highly significant replication variance. In the conA method the replication variance was significant only at 0.1 probability level. The variance analysis emphasizes the need for replication in the iodine method if reliable data to be obtained by this method.

Table 2.3 Comparison of variance in three different methods of amylose analysis

Method	Cultivar	Replication
Iodine	9.307***	18.390***
ConA	65.425***	1.755*
HPLC	8.799***	0.248

*** significant at 0.01 probability
* significant at 0.1 probability

The mean amylose content of fifteen wheat starches analysed by the three techniques are given in Table 2.4. The results obtained by the HPLC and iodine methods showed a good association between each other with a correlation coefficient of 0.866 (Figure 2.4). ConA method showed a weaker correlation with both the iodine (r=0.626) and the HPLC (r=0.481) methods. Also, the conA method estimated lower values for amylose content compared to iodine or HPLC methods which may be due to the lack of proper standardizations under the existing laboratory conditions.

The highly significant variance due to replication in the iodine method can be mainly attributed to the variable purity of the starch samples and/or inaccuracy in measurements such as weighing and pipetting due to the use of a very small quantity of starch (1-2mg) for analysis. While the assay is highly sensitive it can be influenced by many factors. Use of lipid free starch is essential to get an unbiased estimate of amylose content by this method as the effects of lipids on measured amylose vary with the quantity and type of lipid and the chain length and degree of unsaturation of fatty acids (Morrison and Laignelet, 1983, Morrison, 1988).

Using iodine solution stored at room temperature for the development of the blue iodine complex was found to have variable influence on the measured value, with a considerable underestimation for a high amylose starch compared to a low amylose one (data not shown). Davis et al., (1994) observed that the amylose-iodine complex has 6 iodine atoms lying inside the cavity of amylose helix of about 17 anhydrous glucose

Table 2.4 Amylose content (%) of wheat starches measured by different methods

Cultivar	Iodine[a]	HPLC[b]	ConA[c]
Rosella	30.54 ± 0.80	26.19 ± 0.28	15.47 ± 0.67
Minto	36.68 ± 0.86	30.13± 0.17	18.04 ± 1.67
Lark	34.58 ± 0.34	27.97 ± 0.70	19.41± 1.03
Meering	34.37 ± 0.64	28.25 ± 1.03	19.97 ± 1.87
Gutha	29.92 ± 0.78	25.64 ± 1.03	12.84 ± 1.20
Gamenya	30.75 ± 0.65	26.15 ± 0.40	10.71 ± 0.57
Kulin	33.54 ± 0.67	28.32 ± 0.98	18.08 ± 0.95
Egret	35.16 ± 0.73	33.63 ± 0.68	16.07 ± 1.81
Vasco	36.60 ± 1.25	35.11 ± 0.97	16.51 ± 1.57
Reeves	31.82 ± 0.18	26.85 ± 0.28	14.47 ± 1.12
Hartog	30.75 ± 0.74	24.70 ± 1.26	16.40 ± 0.75
Kiata	33.23 ± 0.98	29.05 ± 0.13	16.42 ± 0.17
CS	31.46 ± 0.40	29.27 ± 0.22	14.28 ± 0.46
CS dt5DL	34.79 ± 0.84	33.57 ± 0.91	23.05 ± 0.80
CS dt6AS	27.15 ± 1.17	21.29 ± 0.60	14.30 ± 1.65

[a] Each sample replicated 5 times and the 4 closer values taken to calculate the mean amylose content
[b] Mean of 3 replicates
[c] Mean of 2 replicates

units (AGU), whereas there are only four iodine atoms involved with every 23 AGUs in an amylopectin-iodine complex. Hence, in higher amylose starch which has the potential to bind more iodine, amylose content could be underestimated due to an evaporation of iodine during storage of the iodine solution at room temperature. To eliminate this anomaly there should be an excess of iodine available for the reaction and a standard curve should be drawn each assay time. Many workers (Minick et al., 1991, Davis et al., 1994) have reported storing stock solutions in the refrigerator and keeping the solution in an ice bath while experimentation. In the present study fresh iodine solution was prepared immediately before the assay in order to avoid this problem. The temperature and wavelength at which the absorbance of the AI complex is read are also critical, as discussed in Section 2.3.1. The multiwavelength component analysis for the estimation

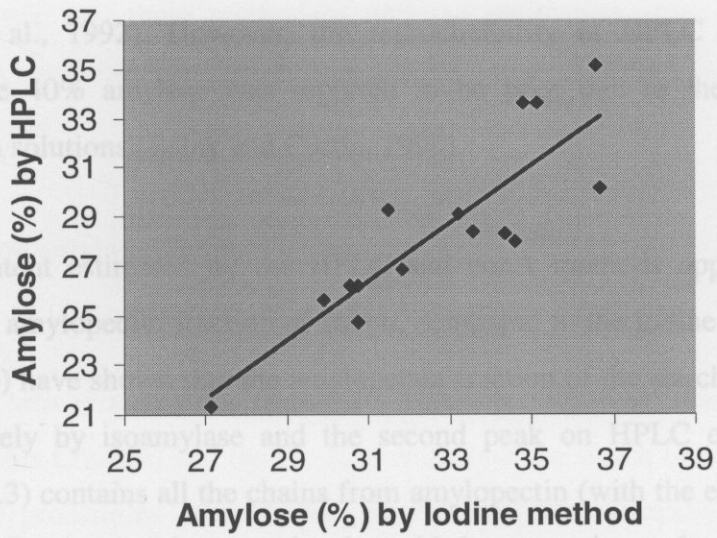


Figure 2.4 Relationship between amylose content estimated by iodine method and HPLC. The data points are means of two replications. The correlation coefficient is 0.866.

of amylose and amylopectin simultaneously, which is reported to be accurate, simple and applicable to routine analysis of genetic variability of starch composition (Sene et al., 1997) is worth trying in future studies.

The lack of significant variance due to replication in the HPLC method indicates its reliability compared to the iodine assay. Batey and Curtin (1996) reported this method to be more reproducible than the blue value method and hence preferable for measuring small differences in starches of similar amylose content. Moreover, the HPLC results are unaffected by the lipid content of the starch which makes this technique even more attractive. While lipid-free starch can be produced by any efficient solvent extraction or bulk precipitation procedures, these are usually time consuming and it is subsequently very difficult to accurately determine the dry-weight of these starches. HPLC of isoamylase debranched starches gives better resolution of short and long chains compared to that of non-debranched starches. While using undebranched starches, there is a chance of amylopectin molecules breaking into fragments of similar size to amylose

molecules, resulting in a difficulty in separation and an overestimation of amylose content (Jane et al., 1992). However, the reproducibility of HPLC of debranched starches of above 40% amylose was reported to be poor due to the instability of debranched starch solutions (Batey and Curtin, 1996).

The amylose content estimated by the HPLC and conA methods appear to be less influenced by the amylopectin fraction of starch, compared to the iodine method. Batey and Curtin, (1996) have shown that the amylopectin fraction of the starch is debranched virtually completely by isoamylase and the second peak on HPLC on a UHG-250 column (Figure 2.3) contains all the chains from amylopectin (with the exception of the central C-chain). ConA precipitates molecules with large numbers of nonreducing end groups, and hence, amylopectin, which has a higher number of nonreducing end groups than amylose, is specifically precipitated. As discussed before, iodine can bind to long linear chained glucose helix resulting in blue inclusion complexes. Hence amylopectin molecules with long linear chains also complex with iodine in a manner similar to that of amylose complex resulting in an overestimation of amylose content influenced by the variation in amylopectin fraction.

The percentage of amylose estimated by conA method in the present study was comparatively low and exhibited a weak correlation with the results from the other two methods. Conversely, Gibson et al. (1997) showed a high correlation of this technique with various colorimetric methods. In my experience, it was difficult to obtain consistent and satisfactory results with the conA method. Further investment of time in perfecting this method would be required to improve the assay procedure, however, this was not justified by the aims of the project.

Since the analytical procedures discussed here are based on different separation principles, each method provides a different window through which we can measure the amylose and hence the results from various methods may not relate to each other in a strong manner. The small scale HPLC method described was found to be reliable and repeatable within the limitations mentioned above. However the requirement of an expensive working system limits its use for routine analysis by many laboratories. When

optimum assay conditions are followed the iodine method used in the current analysis can be an economic and rapid method of amylose estimation especially when the quantity of starch available for the assay is limiting and there are large number of samples to be assayed.

2.3.6 Screening for amylose content

The strategy used was to screen germplasm for amylose content by iodometric method (Section 2.3.2 of this Chapter) and then to further analyse the starch of selected lines by various analytical procedures (see Chapter 3). Seventy five Australian varieties, 45 exotic varieties 12 tetraploid lines and 7 diploid accessions were screened. There were statistically significant differences among the Australian varieties with a critical difference $(CD)_{0.05}$ of 3.5. The mean amylose content along with the granule bound starch synthase (GBSS) status as observed by Zhao et al., 1998, of each line is given in Table 2.5. The values ranged from 23.5% in Tincurrin to 38.9% in Minto. The frequency distribution graph (Figure 2.5) appears to be bimodal, the first peak falling within the range of 23-30% amylose, representing the low amylose group and the second peak within the range of 31-39%, representing an intermediate to high amylose group. Out of the 75 lines analysed 28 fell under the first peak and the rest in the second. The granule bound starch synthase (GBSS) status of 57 cultivars were known (Zhao et al., 1998), out of which 49 were normal (with all three GBSS protein isotypes present), 7 had null phenotypes for the isoform controlled by chromosome 4A (null 4A) and 1 was a biotype heterogeneous for normal and null4A. The mean amylose content for the null 4A lines were lower when compared to that of normal lines (normal mean = 31.6%, null 4A mean = 28.5%, $t = -2.0$, $P < 0.026$). Although the distribution of the two GBSS types is overlapping within the amylose groups, the number of null 4A types observed was higher in the low amylose group than in the intermediate-high amylose group.

Significant differences in the amylose/amylopectin ratio were reported previously among Australian wheat varieties (Moss and Miskelly, 1984). Studies on GBSS mutants

Table 2.5 Amylose content and GBSS status[a] of Australian wheat cultivars, ranked in order of amylose content

Cultivar	Pedigree	Mean Amylose %[b]	GBSS
Tincurrin	Gluclub/3/Chile1B//Insignia/Falcon	23.5	Normal
Olympic	Baldmin/Quadrat	24.1	Normal
Angas	Schomburgk*3//Aroona/Moro	24.4	Null4A
Goroke	TM56*2/AUSEN$-21//3Ag3/4*Condor	24.8	Normal
Rosella	Farro Lunga/Heron//2*Condor/3/Quarrion sib	25.1	Null4A
Vulcan	Condor/Pitic 62//Condor sib	25.3	Normal
Bodallin	Not known	25.3	Null4A
Cunningham	3Ag3/4 Condor//Cook	26.4	Normal
Owlet	M2256//Fleche D'or/3*Kite/3/M2293	26.4	Normal
Oxley	WW80/2*WW15	26.4	Normal
Grebe	Skorospelka/3*Egret	26.6	Normal
Suneca	Ciano/2/Spica/Amber Mutant Sonora 64	26.8	Normal
Gutha	Gamenya//Gabo*Khapstein/3/Falcon*3/Chile	26.9	Biotype
Schomburgk	[(W3589*Oxley)*Warigal**2]*Aroona**2	27.4	Normal
Pinnacle	Pindar selection	27.5	-
Perouse	3Ag#14/4*Condor//Oxley/3/3*Cook	27.6	Normal
Gamenya	Gabo*6/Mentana W1124//Gabo*2/Kenya 117A W1347	28.1	Null4A
Osprey	Condor*2/WW33B	28.7	Normal
Sunbri	Cook*2/VPM/3/3*TG/CNDR//WW80/2* WW15	29.1	Normal
Corella	Huelqen/4/Mayo/Norin 10/2/Yaktana 54/3/Kenya/Lincoyan/5/2*Egret	29.5	Normal
Pelsart	Potam 70/4*Cook	29.5	Normal
Moray	Loros/5*Halberd	29.8	Normal
Lowan	Olympic/Potam 70	30.1	Normal
Ouyen	Takari/TM56//Cocamba	30.2	Normal
Sunfield	Complex	30.5	Normal
Stretton	Tom Poule Barbu Rouge. IRN62-101:Z501 (Aus 18446)/Bodallin	30.7	Normal
Warrigal	LR/N10B//3*ANE/4/MY48/Uruguay 1084//)F/3/4* Dirk 4	30.8	Null4A
Kite	Norin 10 Brevor Seln.14//4*Eureka 2/3/T-A/3*Falcon/4/T-A/4*Fqalcon/5/ etc.	30.8	Normal
Warbler	Kavkaz/Timgalen//3*Oxley	31.5	Normal
Egret	Heron/2*WW15	31.6	Normal
Sundor	CS/AGEL(2n=14)//4*WW80/2*WW15	32.0	-
Longbow	TJB268175/Hobbit	32.1	-
Condor	WW80/2*WW15	32.1	Normal
Combination 3	Ernstein/C112632	32.1	-
Tern	Not known	32.4	-
Hybrid Pulsar	Sunfield sib/R5084	32.4	Normal
Lance	CLLF/Raven	32.5	Null4A
Katyil	Aus 10894//4*Archon/QR	32.7	Normal
Lilimur	IBWSN12-127/ASN3-61	32.7	Normal
Cranbrook	WE/Ciano'S'/Noroeste 66/3/Zambezi	32.9	Normal
Isis	Ghurka/Waigal//Macquaire/3/Macquaire/ Celebration	32.9	-
Quarrion	Condor/TA3PNB3P//WW33G/3/Condor/ WW33B	32.9	Normal

(to be continued)

Table 2.5 (continued)

Cultivar	Pedigree	Mean Amylose %[b]	GBSS
Songlen	LR64A/SN64A//TG	33.1	-
Katunga	RD28*2/Millewa	33.2	Normal
Mokoan	WW15/OLY//KAL/OLY	33.2	Normal
Currawong	KVZ/BVHO//KAL/BB14/CNDR/TA3P NB3P//WW33-G/3/CNDR/WW33-B	33.5	-
Meteor	CNDR*2//YT54/N10B/5/Kite/4/MX77/ 3/8156//HRWREST/PMP	33.5	-
Sunco	SUN9E-27*4/3Ag14//WW15/3/3*Cook	33.6	Normal
Sunkota	LR64A/SN64A//TG/3/IRN67-451	33.9	-
Bass	FDRS/2*CNDR	33.9	-
Avocet	WW119/WW15//EGT	34.1	-
Canna	K117A/2*GB.AUS//MTA/6GB.AUS/3/ PJ62/GB55	34.1	Normal
Neepawa	RL-4031//TH*6/KF/3/TH*2//FN/TH	34.2	-
Kelelac	TM56/3/Summit/Aus11577//WW15	34.3	Normal
Torres	Not known	34.3	-
Skua	3AG14/4*CNDR	34.5	-
Cocamba	Aus10894/4*Condor	34.7	Normal
Hyden	Gamenya/Inia	34.7	Null4A
Kulin	Bodallin//Gamenya/Inia	34.9	Normal
Kendee	Dundee/Kenya C6042	34.9	-
Matong	Kalyanasona/Olympic	35.0	Normal
Sunbird	GLL/CUL//L-1032-4L-OKE-OS-4S-OAP	35.0	-
Vasco	3Ag14/4*Condor//4*Spear	35.3	Normal
Swift	Condor/3Ag14//Romany/4189	35.4	Normal
Shrike	WW15/M12382//Kite/3/WW15/4/ Condor/5/WW336	36.2	Normal
Meering	Condor selection	36.3	Normal
Diaz	Combination 3/3*OXY//3*Cook	36.3	-
Kiata	Loros/5*Condor	36.4	Normal
Sunmist	Miskle selection	36.5	Normal
Kewell	PeanutOil OLYMTL132A/Aus1278// OLY	36.8	Normal
Lark	Canrock 1/ 2 CSP44/Banks	37.0	Normal
Banks	PWTH/Condor sib//2*Condor	37.1	Normal
BD159	Jabiru/2*Millewa/3/DX679RR/4/ Cocamba sib	37.4	Normal
Miskle	Cook//SUN27B/Dougga	37.4	Normal
Minto	Condor/Olympic/Egret	38.9	Normal

- GBSS status not known
a Data taken from Zhao et al., (1998)
b Mean of two replicates

have established that GBSS is required for amylose synthesis and plants that lack activity of GBSS have undetectably low levels of amylose in their storage starches.

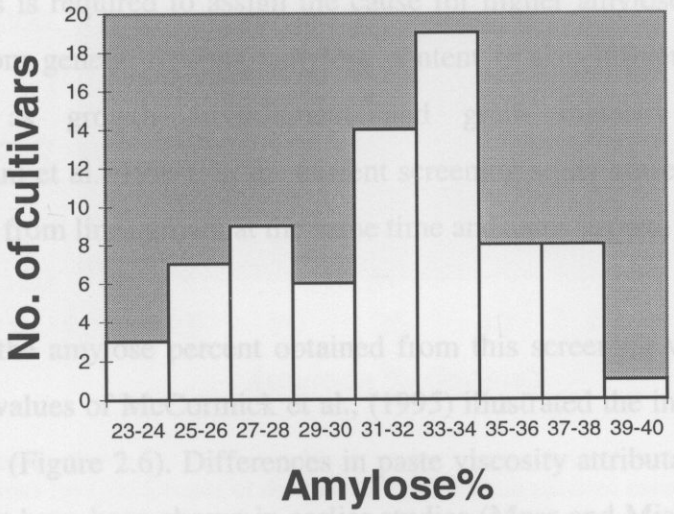


Figure 2.5 Frequency distribution of % amylose content in Australian cultivars.

In wheat the waxy genes (*Wx*) which control the GBSS are organized as a triplicate set of single copy homoeoloci on chromosome arms 7AS, 7DS and 4AL (due to translocation of a portion of the short arm of chromosome 7B to 4A), each locus encoding a GBSS protein (Chao et al., 1989, Liu et al., 1992). However the influence of the 4AL locus on amylose content is greater than the other two loci (Miura et al., 1994). Hence, all the null 4A lines screened here except two had comparatively low values for amylose content. The varieties Lance and Hyden in spite of their null 4A status had higher amylose content.

Starch is biosynthesized by the concerted efforts of a set of enzymes, ADP-glucose pyrophosphorylase (AGPase), starch synthase (SS), starch branching (SBE) and starch debranching (DBE) enzymes. Mutations affecting some of these enzymes, resulting in

higher amylose content, have been revealed in many crops. The high amylose mutants of maize and rice, amylose extender (*ae*) which lack a SBE IIb isoform, the sugary (*su 1*) mutation in maize which affects the DBE activity and dull 1 (*du1*) mutation in maize which affects the SS II and SBE IIa activities are typical examples (Garwood et al., 1976, Mizuno et al., 1993, James et al., 1995, Gao et al., 1998). Consequently, further genetic analysis is required to assign the cause for higher amylose content in null 4A lines. Apart from genetic reasons, amylose content is also influenced by non-genetic factors such as growth environment and grain maturity (Fergason, 1994, Mohammadkhani et al., 1999). In the current screening study close attention was given to collect seeds from lines grown at the same time and same growth conditions.

Correlation of the amylose percent obtained from this screening with the starch paste peak viscosity values of McCormick et al., (1995) illustrated the inverse relationship of these two traits (Figure 2.6). Differences in paste viscosity attributable to their inherent amylose content have been shown in earlier studies (Moss and Miskelly, 1984). In their assessment of starch pasting properties of wheat cultivars important to Victorian wheat breeding, McCormick et al., (1995) suggested that Currawa, a cultivar released in 1912, was the common ancestor of the high peak viscosity varieties including the currently grown cultivar, Rosella which is a low amylose variety as per the current study. McCormick et al., (1995) also suggested that low peak varieties were related to WW15, a semi dwarf parent from CIMMYT that was used extensively in Australian breeding programmes in the past two decades. The majority of the cultivars with above 35% amylose in this study have the variety 'Condor', which is derived from the cross between WW80 and WW15, in their pedigree (Table 2.5). However, some of the low amylose cultivars also have Condor in their pedigree. Null4A genotypes exhibited higher RVA peak viscosity values compared to genotypes with normal GBSS status (Figure 2.6), agreeing with earlier reports (Zhao et al., 1995, Zeng et al., 1997, also see Section 3.3.2 of Chapter 3). The results have shown that GBSS is not the only cause of

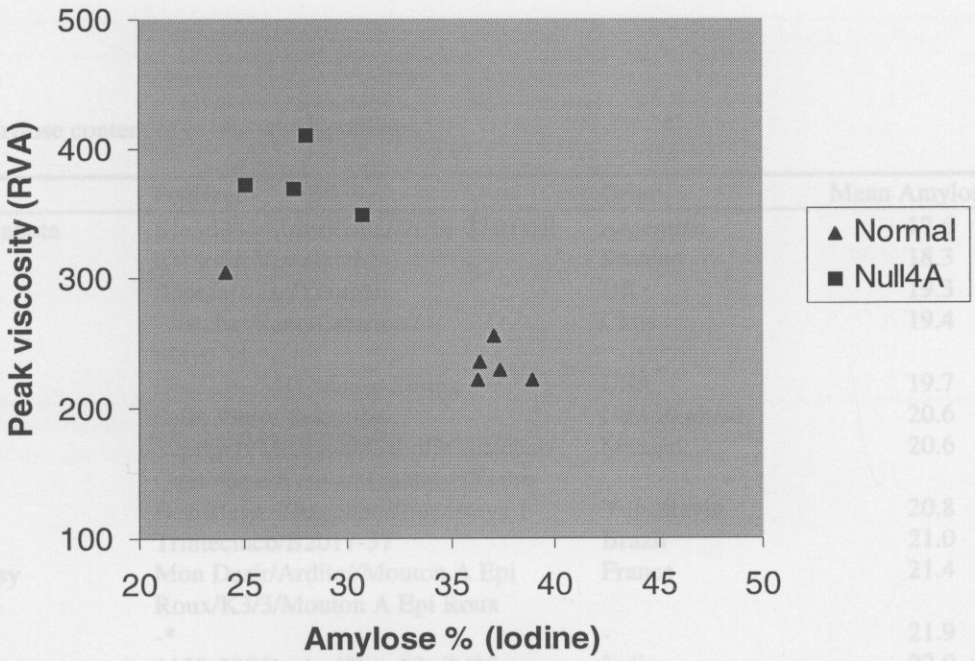


Figure 2.6 Relationship of amylose content and flour peak viscosity of Australian wheat cultivars. Normal and Null4A represents the GBSS status of the cultivars.The data on amylose content and peak viscosity are from different samples, the amylose %from the current study (Table 2.5), and the peak viscosity from McCormick et al., (1995).

variation in amylose content in wheat, but that determining the cause of differences would require complex genetic analysis.

Significant differences ($CD_{0.05}$ = 2.5) were observed in the mean amylose content among the 46 exotic cultivars analysed (Table 2.6). A clear bimodal distribution was noticed with two distinct categories of 18-26% and 27-38% (Figure 2.7). The cultivars tested were almost equally distributed within the two categories. No geographic barrier could be identified between the two groups. Pergamino Gabota, a variety from Argentina recorded the lowest amylose content of 17.6% and Ceyhan, a Turkish variety, showed the highest amylose content of 37.7%. Since the cultivars were selected randomly from the germplasm collection, a wide variation in the genetic background is expected which can be exploited in future breeding programmes.

Table 2.6 Amylose content of exotic wheat cultivars

Cultivar	Pedigree	Origin	Mean Amylose %[a]
Pergamino Gabota	KleinH44/Sinvalocho//2018-37Brazil	Argentina	17.6
Viken SS	LV from Vermland	Sweden	18.3
Warden	Benefactress/Yeoman	UK	19.3
Platifen	Thatcher/SantaCatarina// Mayo 54	Chile	19.4
Transfer	DM/AEUM//Chinese Spring	USA	19.7
Titan	Saint Pierre Selection	New Zealand	20.6
Nainari 60	Supremo/Mentana//Gabo/3/Thatcher/ Queretaro//Kenya/Mentano/5/Gabo	Mexico	20.6
Vojvodanka	Bezostaya 4/Argelato//Bezostaya 1	Yugoslavia	20.8
Veranopolis	Trintecinco/B2017-37	Brazil	21.0
Etiol Dechoisy	Mon Desir/Ardito//Mouton A Epi Roux/K3/3/Mouton A Epi Roux	France	21.4
Sambtalia	-*	-	21.9
Sonalika	1153-388/Andes//Pitic62 sib/3/Lerma Rojo 64	India	22.0
Wren	Yaqui 50-E/8156//Kalyan	Mexico	22.0
VQ 106	-	-	22.2
VQ 115	-	-	22.3
Norin 40	Turkey Red 11/MartinAmber// Konoso 26	Japan	22.9
Manitou	Canthatch/ThatcherX$_7$/Frontana	Canada	23.2
Kalyan	AKP562/GB55	India	23.6
PA150-2K	-	-	24.2
Sarastovskaja	Albidum24/Lutescens 55-11	Russia	26.6
Justin	ND4/NS.3880.227//Conley	USA	28.2
Romany	Colotana 261-51/Yaktana54A	Kenya	29.0
Potam 70	Inia sib/Napo63	Mexico	29.0
Carthage	Napo63/Tobari66 sib//8.56	Tunisia	30.1
Kudu	Kenya131/Kenya184P	Kenya	30.4
Chile 8	Trigo De Nueva-Holanda	-	30.4
Saric 70	Ciano sib//Sonora64/Klein Rendidor/3/8156	Mexico	30.5
Iumello	BP in USA	Mediterranean	31.7
Koolsie	-	-	32.1
Chile 10	-	-	32.4
Khano	LV	India	32.9
Pato	Tezanos Pinto Precoz/Sonora64A//Narino59	Argentina	33.0
Cappetti	Jeanah Rhetifah selection	Italy	33.2
Ciano	Pitic62/Chris sib//Sonora64	Mexico	33.6
Gano	-	-	33.9
Penz	-	-	34.1

(to be continued)

Table 2.6 (continued)

Cultivar	Pedigree	Origin	Mean Amylose %
Comet	Marquis/Hard Federation	USA	34.3
Kador	Champlein/Cappelle//B21	France	34.8
Kamilaroi	Ciano sib/3/Sonora64/Klein Rendidor//Siete Cerros sib	Mexico	34.9
Cajemi 71	-	Mexico	35.3
Karkas	-	-	35.7
Choti Lerma	Lerma Rojo64 sib/Huamantla Rojo	India	36.6
Chenado	-	-	37.5
Kenora	-	-	37.5
Ceyhan	-	Turkey	37.7

a Mean of two replicates

* Unkown details are represented by a dash

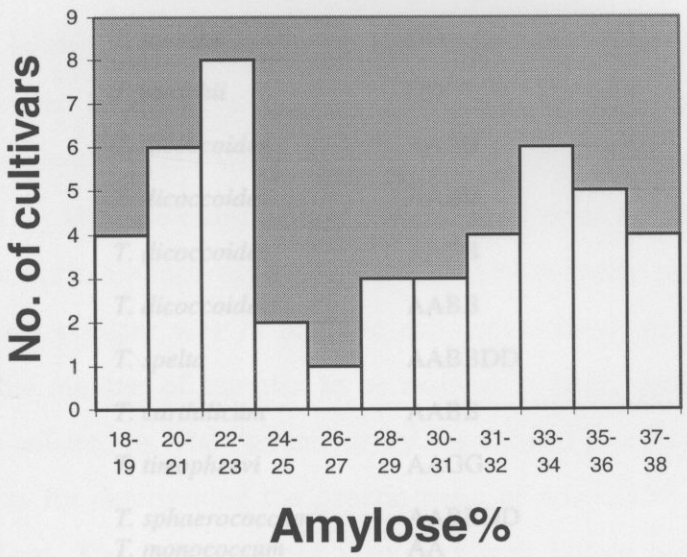


Figure 2.7 Frequency distribution of % amylose in exotic wheat cultivars.

Among the species other than *T. aestivum* analysed, AUS21401 (*T. dicoccoides*), an accession from Israel, recorded the highest amylose content of 35.6% (Table 2.7). The Russian accession AUS24242 (*T. tauschii*) was the highest ranking diploid (34.7%). Variation for apparent amylose content of endosperm starch in *T. durum* and *T. tauschii* has been assessed by Watanabe et al., (1998). In their extensive screening of 665 *T. durum* accessions and 732 *T. tauschii* accessions, they identified 12 lines with 40-45% amylose content.

Table 2.7 Amylose content of alien species of *T. aestivum*

Accession No.	Species	Genome	Mean amylose %
AUS24242	*T. tauschii*	DD	34.7
AUS24092	*T. tauschii*	DD	30.1
AUS23890	*T. tauschii*	DD	32.2
AUS24048	*T. tauschii*	DD	18.2
AUS23986	*T. tauschii*	DD	22.2
AUS24230	*T. tauschii*	DD	22.4
AUS21401	*T. dicoccoides*	AABB	35.6
AUS21499	*T. dicoccoides*	AABB	27.8
AUS21381	*T. dicoccoides*	AABB	17.5
AUS21317	*T. dicoccoide*	AABB	21.6
W297	*T. spelta*	AABBDD	28.1
W995	*T. carthilicum*	AABB	31.2
W1899	*T. timopheevi*	AAGG	21.7
W52	*T. sphaerococcum*	AABBDD	23.1
W2698	*T. monococcum*	AA	28.1
W43	*T. dicoccum*	AABB	27.7
W76	*T. persicum*	-*	14.3
W59/1	*T. dicoccoides*	AABB	17.4
W48/3	*T. turgidum*	AABB	22.3

* genome not known

The significant variants identified within the diploid and tetrapoid wheat lines could be used to alter amylose content in cultivated hexaploid wheat through alien gene transfer techniques. Genes can be transferred directly to bread wheat from the tetraploid and diploid by crossing them with *T. aestivum*, with the hexaploid as the female parent and back crossing with *aestivum* a few times. Development of synthetics by crossing between the tetraploid and the diploid and subsequent chromosome doubling is another possible method.

2.4 CONCLUSION

The effectiveness of a high amylose breeding program, as in any other, is dependent on the type and level of genetic variability present in the available breeding population and the ability of the breeder to select for the most desirable combinations of genetic factors involved (Fergason, 1994). Since it is not possible to visually assess the differences in amylose content, the success is dependent on chemical starch analysis to determine amylose content. As the boundaries between the amylose and amylopectin population of molecules are arbitrary, no one analytical procedure can give an absolute measurement of amylose content. However, most of the methods serve the purpose of a comparative ranking for amylose content which is sufficient in most breeding experiments. The HPLC method for amylose estimation was found to be more reliable due to the efficacy of achieving uniform replicate estimates. The small scale iodine method followed in this study provides a cheaper way of amylose estimation when the quantity of starch is limiting and the number of samples to be assayed is large, provided optimum assay conditions are adhered to. The current survey has defined excellent material to conduct genetic analysis for determining the genetic basis of non-GBSS variation in amylose content in wheat. The cultivars/lines identified with higher amylose content in the present study could be utilized in breeding programmes aimed at developing high amylose wheat varieties. Cyclic breeding for accumulating favourable alleles is a suggested approach.

CHAPTER 3

Characterization of Wheat Starch: Structural and Functional Attributes

3.1 INTRODUCTION

The utility of starch for specific purposes is dependent mainly on its structural and functional properties. Starches isolated from different sources are known to have different structural and functional properties (Lii and Lineback, 1977, reviewed by Whistler et al., 1984). For example, distinct structural differences in amylopectin molecules of starch were revealed among various cereal species (Lii and Lineback, 1977). Wheat starch has lower thickening power and lower gelatinization temperature than corn starch (Knight and Olson, 1984). Potato starch excels in film forming and binding characteristics (Mitch, 1984). Mutants with varying starch characteristics are reported within a species. *Waxy* mutants with zero percentage amylose, *amylose extender* with higher amounts of amylose and *sugary* mutants with accumulated phytoglycogen are typical examples (reviewed in Sathish et al., 1995 and Nakamura, 1996). While normal corn starch produces an opaque and short paste which sets to a strong gel, waxy starches produce clear and long pastes which have lower tendency to form gels (Jane and Chen, 1992). Accumulation of highly branched phytoglycogen in *sugary* mutants is accompanied by lower gelatinization temperature in the starch (Nakamura et al., 1998)

The thickening and gelling features of starch are important for various food purposes. High starch/flour peak viscosity and swelling power are negatively associated with alkaline noodle firmness, producing softer and less desirable noodles (Moss, 1982, Miskelly and Moss, 1985, Konik et al., 1994). On the other hand, these characteristics are sought after in Japanese white salted noodles where soft and elastic eating qualities are important (Crosbie, 1991, Crosbie et al., 1992, Konik et al., 1992, Konik et al.,

1993). Starches with high amylose content are suitable for development of plastics as a biodegradable alternative to many of those derived from petroleum (Christie et al., 1997). The formation of a network of amylose in high amylose material due to strong physical crosslinking, mainly by hydrogen bonding and crystallization of linear amylose chains, leads to a product with high strength and stiffness (Soest and Vliegenthart, 1997).

Starch granules provide a water insoluble but readily biodegradable storage system for plants. This balance of mechanical stability with degradability arises from the chemical structures of the amylose and amylopectin, and from the arrangement of amorphous and crystalline zones within the granules (Calvert, 1997). Apart from the ratio of amylose to amylopectin, variation in the fine structure of these components including chain length distribution profile, number of chains, chain length and degree of polymerization (DP) can result in different physicochemical properties. Minor components such as integral proteins, lipids and phosphorous also contribute to starch functionality (Morrison, 1993, Jane et al., 1996).

Starch functionality can be related largely to the temperature dependent interactions of starch with water in such processes as swelling, gelatinization, pasting and retrogradation (Dengate, 1984, Atwell et al., 1988, Zeng et al., 1997). Various analytical techniques and instruments have been designed for efficient starch structural and functional analyses. Some of these have been employed for a comparative study of hexaploid and diploid wheat starches and the results are presented in this chapter.

3.2 MATERIALS AND METHODS

3.2.1 Seed materials

Triticum aestivum seeds were taken from the germplasm collection of the Plant Breeding Institute, Cobbitty. Seeds of *T. tauschii* were kindly supplied by Dr S.

Rahman, CSIRO Plant Industry, Canberra. Plants were grown in pots in a bird proof cage under uniform growth conditions and well filled grains harvested at full maturity for starch extraction.

3.2.2 Starch extraction

Large scale starch extraction for structural and functional analyses was done following the method described in section 2.2.2.2.

3.2.3 Amylopectin chain length distribution analysis

Fluorophore assisted carbohydrate electrophoresis (O'Shea and Morell, 1996, O'Shea et al., 1998) was carried out to study the amylopectin chain length distribution. Purified starch (20mg) was suspended in 800µl of 0.125M sodium hydroxide and boiled for 5 min in a water bath. The suspension was neutralized with 12.8µl of glacial acetic acid, made up to 2ml with 50mM sodium acetate buffer, pH 4.0 and digested with 10µl of isoamylase (200U/ml, Deltagen, Boronia, Vic., Australia). After incubation at 37°C for 2 hours, the sample was boiled for 20 min to inactivate the isoamylase. The mixture was centrifuged at 14,000g (2 min, room temperature), and the supernatant transferred to a tube containing approximately 200mg of ion-exchange resin (AG501-X8(D), Bio-Rad, Hercules, CA, USA) and mixed on a rotor for 30 min to remove buffer salts. After settling the resin, the supernatant was transferred to a new tube and diluted to 4ml with deionized water. Aliquots (50µl) of the mixture were dried under vacuum, mixed with 5µl each of 0.2M APTS (1-aminopyrene-3,6,8-trisulfonic acid, Lambda Fluoreszenztechnologie, Graz, Austria) and 1M sodium cyanoborohydride (Sigma, St. Louis, MO, USA) and then incubated for a minimum of 16 hours at 37°C. The mixture was made up to 25µl with 3M aqueous urea and applied to a P/ACE 5510 capillary electrophoresis system with argon-laser LIF detection. The raw data from the analysis was normalized by dividing the fluorescence detected for each oligosaccharide peak by the total fluorescence detected for all of the oligosaccharide peaks with a degree of

polymerization between 6 and 60. The analysis was performed at CSIRO Plant Industry, Canberra.

For electrophoresis using a DNA sequencer, sample volumes of 1-5µl were loaded into the wells of 10% uniform polyacrylamide gel (37.5:1 ratio of acrylamide to N,N'-methylenebisacrylamide as crosslinker) containing 8.3M urea and were electrophoresed using a buffer containing 0.089M Tris base, 0.089M boric acid and 0.002M EDTA for a period of 15 hours at 40°C at a constant current of 30mA using an Applied Biosystems (Perkin-Elmer Corporation, Applied Biosystems Division, Foster City, CA, USA) 373A DNA sequencer. The fluorescence data was analysed using 672 Genescan™ software.

3.2.4 Starch paste viscosity analysis

Starch paste viscosity was determined with a Rapid Visco Analyser (RVA, Newport Scientific, Sydney) at CSIRO, Grain Quality Research Laboratory, North Ryde. Starch sample (3g) was accurately weighed and added to 25g of water in a can by gentle rotation. Starch and water were mixed by hand over a period of 10 sec and the can was fixed to the apparatus. The temperature profile for the RVA comprised the following stages: hold at 60°C for 2 min, heat to 95°C over 6 min, hold at 95°C for 4 min, cool to 50°C over 4 min, and hold at 50°C for 4 min. The software Thermocline for Windows was used for collection and analysis of data.

3.2.5 Starch granule size distribution analysis

Starch granule size distributions were measured with a Malvern Laser Diffraction Particle Size Analyzer, following the method of Batey et al., 1996. Approximately 10mg of starch was dispersed in a 15ml stirring cell and subjected to analysis. Results were obtained as the proportion of starch (by volume) with granule diameters divided into 16 classes, which corresponded to <1.9, 1.9-2.4, 2.4-3.0, 3.0-3.8, 3.8-4.8, 4.8-6.2, 6.2-7.9, 7.9-10.1, 10.1-13.0, 13.0-16.7, 16.7-21.5, 21.5-28.1, 28.1-37.6, 37.6-53.5, 53.5-87.2 and 87.2-188µm. The B granule content was taken as the proportion of granules with a

diameter of less than 10.1μm and the A granule content as the proportion of granules with above 10.1μm. Each sample was measured in duplicate.

3.2.6 Measurement of starch swelling power (SSP)

The method of Crosbie (1991) with slight modifications to suit small scale analysis was followed for SSP measurement. A starch sample of 30mg was weighed into a 2ml screw-capped tube and dehydrated by incubation in a 130°C oven for 1 hour. The tube was capped and reweighed after cooling to room temperature. After adding 1.8ml of 2% calcium chloride, the contents were mixed immediately by vortexing. The tube was then placed in a boiling water bath and constantly inverted for the first 5 min, twice at regular intervals of 30 sec for the next 5 min followed by 1 min interval for 5 min, then every 2.5 min for 10 min, and finally every 5 min for a total time of 35 min. After cooling to room temperature under tap water, the tube was centrifuged at 12,000g for 8 min. The supernatant was then carefully removed by suction. Swelling power was calculated as the weight of sedimented gel, divided by the original weight of the initial dehydrated starch.

3.2.7 Statistical analyses

Analysis of variance and correlation analysis were conducted using Microsoft Excel, 1997 and Minitab for Windows version 11.21.

3.3 RESULTS AND DISCUSSION

3.3.1 Amylopectin chain length study

The chain length distribution of amylopectin of five hexaploid and four diploid starches were analysed by flourophore assisted slab gel electrophoresis using a DNA sequencer

and by capillary gel electrophoresis. Both the methods involve the separation of the oligosaccharides derived from isoamylase debranched amylopectin which was fluorescently labelled with 8-amino-1,3,6-pyrenetrisulfonic acid (APTS) via reductive amination, thereby introducing a single label per oligomer and subsequent laser induced fluorescence detection. A baseline resolution of individual oligomers to chain lengths beyond a DP of 50 was obtained by both the methods as demonstrated initially by O'Shea and Morell., 1996, O'Shea et al., 1998).

A direct comparison of the composition of the proportional quantities of each oligomer present in the distribution of the starches of the varieties Kiata, Lark, Meering and CS was made with that of the variety Minto by subtracting the results of those varieties from that of Minto (Figure 3.1). A negative value in the DP 5-18 indicated the occurrence of a lower proportion of mass in those chain lengths in Minto compared to other lines. Among the lines compared, CS showed the highest proportion of shorter chain lengths compared to Minto. A slight increase in the proportion of mass in the longer chains (DP 20-50) is observed in Minto with respect to other lines.

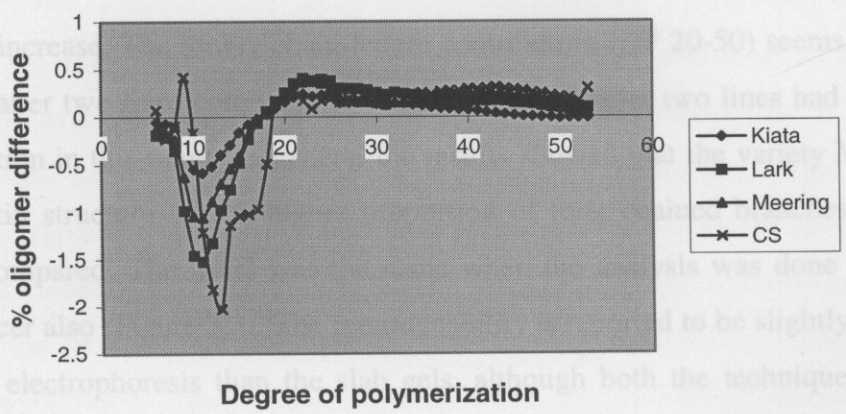


Figure 3.1 Chain length profile comparison of hexaploid wheat starches with respect to that of the variety Minto as obtained by capillary electrophoresis. The percentage of the total mass present in each individual oligosaccharide of starches from Kiata, Lark, Meering and CS was subtracted from the corresponding value for Minto starch.

An almost similar result was obtained when the diploid lines were compared with Minto (Figure 3.2). AUS24242 and AUS24092 exhibited a considerably higher proportion of shorter chain lengths whereas the other two lines, AUS23986 and AUS24230 showed

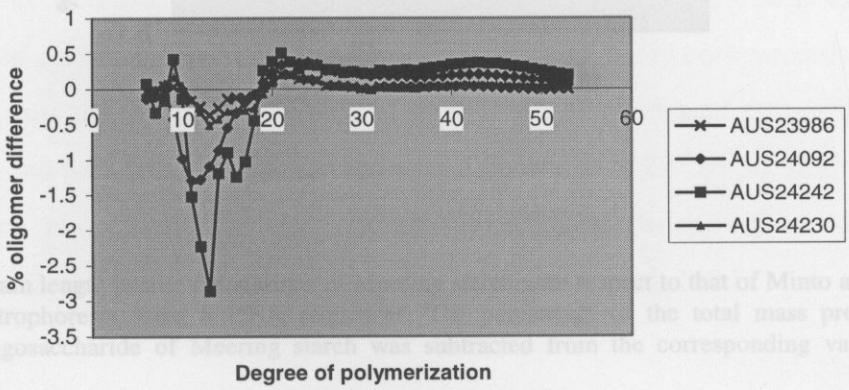


Figure 3.2 Chain length profile comparison of *T. tauschii* starches with respect to that of the variety Minto as obtained by capillary electrophoresis. The percentage of the total mass present in each individual oligosaccharide of *T. tauschii* starches was subtracted from the corresponding value for Minto starch.

only a slight increase. The longer chain length contribution (DP 20-50) seems to be the same in the latter two lines compared to Minto, but the former two lines had a slightly lower proportion in this range. In general the results showed that the variety Minto had an amylopectin structure with a higher proportion of long chained branches than the other lines compared. The trend was the same when the analysis was done using the DNA sequencer also (Figure 3.3). The reproducability is reported to be slightly better on capillary gel electrophoresis than the slab gels, although both the techniques provide substantially high resolution and allow a comparison of chain length distribution by subtraction (O'Shea et al., 1997).

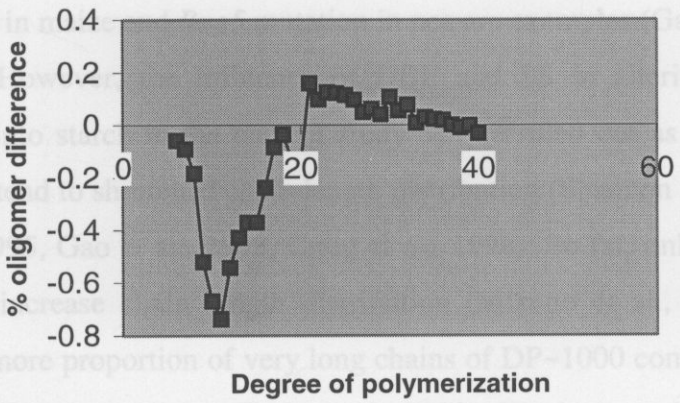


Figure 3.3 Chain length profile comparison of Meering starch with respect to that of Minto as obtained by slab gel electrophoresis using a DNA sequencer. The percentage of the total mass present in each individual oligosaccharide of Meering starch was subtracted from the corresponding value of Minto starch.

The amylopectin branch network appears to be the result of the action of different isoforms of starch branching enzyme (SBE) and hence the variation observed in the chain length distribution may be attributed to the differential activity of any of these isoforms. The SBE cleaves linear α-1,4 glucan and transfers the severed chain to a second chain by α-1,6 linkage and the rate and specificity of this reaction influences both amylose/amylopectin ratios and the fine structure of amylopectin. It has been demonstrated that the SBE 1 isoform of maize preferentially transfers longer chains of α-1,4 glucan compared to SBE IIa and SBE IIb (Takeda et al., 1993a). Studies on *STA7* mutants of *Chlamydomonas reinhardtii* which is defective of debranching enzyme (DBE) led to the proposal that amylopectin clusters are synthesized by a discontinuous mechanism involving a highly specific glucan trimming mechanism (Mouille et al., 1996) and hence it is reasonable to assume that variation in the DBE activity also can cause alterations in the amylopectin structure. More recently Vandewal et al., (1998) hypothesized that amylose is synthesized by cleavage and extension from amylopectin. They suggested the possibility of some hydrolytic enzymes mediating the cleavage or the GBSS 1 holding the dual activities of synthase and hydrolase. Mutations affecting

soluble starch synthases (SS) also influence amylopectin chain length distributions. *Dull1* mutation in maize and *Rug5* mutation in pea are examples (Gao et al., 1998, Craig et al., 1998). However, the influence of DBE and SS in altering the amylopectin structure of Minto starch in the current study can be ruled out as mutations affecting these enzymes tend to shortened chain length distribution (Shannon and Garwood, 1984, James et al., 1995, Gao et al., 1998, Craig et al., 1998). So far, only mutations in SBE are known to increase chain length distribution (Mizuno et al., 1993) although the occurrence of more proportion of very long chains of DP~1000 compared to normal are reported in rug5 mutants of pea (Craig et al., 1998). The increased proportion of chain length recorded for Minto in this study is within the range of DP 20-50. Multiple mutant combinations also affect the chain length distribution depending on the type of mutations involved (Ikawa et al., 1981, Shannon and Garwood, 1984)

Minto had the highest amylose content in the varietal screening tests (Chapter 2). Increase in the chain length of amylopectin with increased amylose content is reported in several studies (Salomonsson and Sundberg, 1994, Kasemsuwan et al., 1995). Wang and White, (1994b) observed differences in chain length and chain length distribution of amylopectin with the short chain length (A and short B chains) increasing with increased amylose and starch-lipid contents in oat starches. A higher proportion of longer chains seemed to be associated with amylose content as observed by the larger proportion of longer chains in Minto starch. However, the analysis of diploid starch did not agree with this. The two high amylose lines AUS24242 and AUS24092 showed a higher proportion of shorter chains compared to the low amylose lines, AUS23986 and AUS24230, with no noticeable difference in the long chain fraction, indicating that the proportion of longer chains need not necessarily increase with increasing amylose content. It is possible that the higher amylose in these lines is not connected with SBE, but with other enzymes as in the case of *dull1* mutants which affects the SS II activity. In *dull1* mutants the starch exhibits a higher amylose content together with higher degree of amylopectin branching (Gao et al., 1998).

As amylopectin is the major component of most starches, variation in its fine structure including the DP and chain length can lead to differences in the functional properties of

the starch. The chain length distribution profile of the amylopectin influences the intrinsic viscosity and retrogradation of starches by forming a mixture of crystallites of different sizes, with longer chain lengths forming longer double helices (Yuan et al., 1993, Lu et al., 1997). The shorter average chain lengths in cereals result in slower retrogradation compared to pea and potato starch. Gel strength analysis of reconstituted starches proved that long branch chain amylopectin had a strong tendency to gel with greater gel strength (Jane and Chen, 1992). Hence, the starch of Minto, having a higher number of longer chains in the amylopectin fraction and high amylose content, appears to be interesting and its end uses should be further examined.

3.3.2 Pasting property analysis.

The starch pasting viscosity of different wheat starches was studied using the Rapid Visco Analyser. A typical pasting cycle curve of a wheat starch derived from the RVA is shown in Figure 3.4. The pasting profile exhibits a maximum viscosity followed by a minimum called the trough and the difference between these two values represents the breakdown. During cooling the viscosity increases again to give the final viscosity which then remains more or less constant. The setback and total setback correspond to the relatedness of the final viscosity with peak viscosity (setback) and minimum viscosity (total setback). The peak viscosity is related to the situation where the starch granules show an optimum balance between swelling and rigidity (reviewed by Thiewes and Steeneken, 1997). Breakdown is a measure of the degree of disintegration of the granules with stirring. The aggregation of the amylose fraction by the formation of bonds between and within them leading to reinforcement of swollen granules and fragments results in setback which often represents the gelling ability or retrogradation tendency of the starch (reviewed by Dengate, 1984).

The RVA parameters derived from the curve varied considerably depending on the genotype analysed (Table 3.1). The programme temperature at which the onset of gelatinization (pasting temperature) occurred ranged from 65.9°C (CS) to 78°C (Minto) and the time taken to reach the peak viscosity varied from 7.13min (CS) to 8.33min (AUS24092). Minto starch exhibited the lowest peak (265.42) and minimum (76.33)

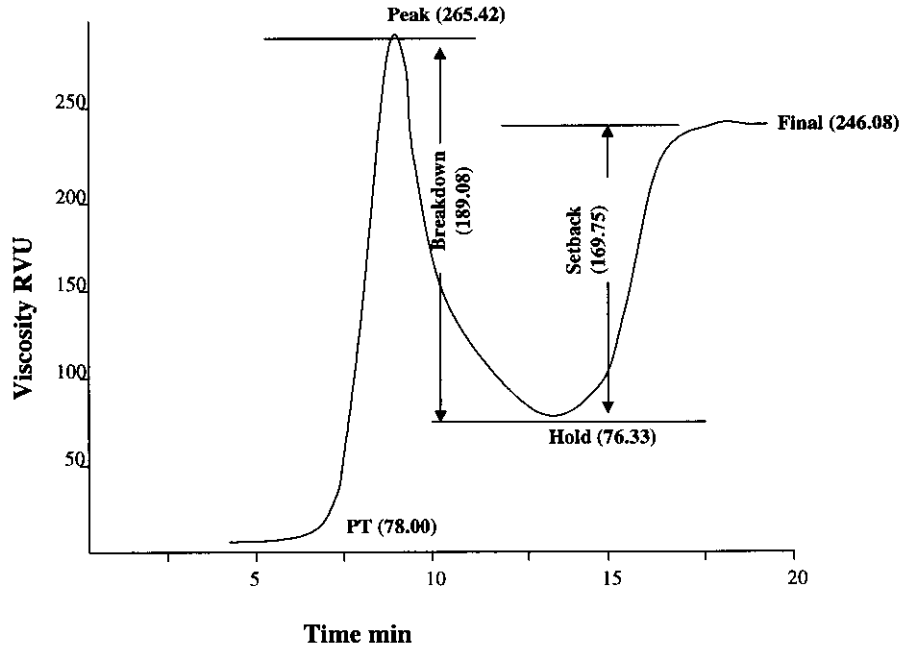


Figure 3.4 Pasting curve obtained using RVA for Minto starch. PT: pasting temperature, Peak: peak viscosity, Hold: minimum viscosity, Final: final viscosity.

viscosities. The highest values for these two parameters were recorded by CS (385.42) and AUS24230 (103.67) respectively. The highest peak and the lowest minimum viscosity values for CS resulted in the highest breakdown in this variety. While the diploid line AUS 23986 showed the highest final viscosity (284.5) and setback (204.08) the lowest values for these two parameters were observed in the hexaploid cultivars Lark and Kiata respectively.

Correlation studies among the hexaploid starches revealed significant positive association between the peak viscosity and the breakdown, final viscosity and setback, and peak time and pasting temperature (Table 3.2). Both peak viscosity and breakdown were negatively correlated with peak time and pasting temperature. The pasting parameters behaved differently regarding their relationship with amylose content. While the peak viscosity and breakdown displayed a significant negative association with amylose content, the peak time and pasting temperature showed a strong positive

Table 3.1 Pasting parameters derived from Rapid Visco Analyser of wheat cultivars/lines

Cultivar/Line	Peak viscosity	Trough	Breakdown	Final viscosity	Setback	Peak time	Pasting temperature
Meering	269.83	86.67	183.17	254.17	167.50	8.00	74.45
Minto	265.42	76.33	189.08	246.08	169.75	8.13	78.00
Kiata	299.67	84.92	214.75	234.67	149.75	7.87	74.40
Lark	298.00	80.42	217.58	230.50	150.08	7.87	73.50
CS	385.42	79.00	306.42	233.25	154.25	7.13	65.90
AUS23986	345.42	80.42	265.00	284.50	204.08	7.60	72.25
AUS24092	296.83	95.75	201.08	274.08	178.33	8.33	75.00
AUS24230	354.50	103.67	250.83	283.17	179.50	7.73	67.90
AUS24242	370.25	100.75	269.50	275.67	174.92	7.67	69.40
Std. Error	4.10	0.29	4.10	2.63	2.34	0.23	0.08

Table 3.2 Correlation between RVA parameters and amylose content in hexaploid wheat starch

	Peak 1	Trough 1	Breakdown	Final Viscosity	Setback	Peak time	Pasting temperature
Trough 1	0.2297						
Breakdown	0.9965**	-0.3104					
Final viscosity	-0.6138	0.2969	-0.6250				
Setback	-0.5357	-0.1330	-0.5118	0.9069*			
Peak time	-0.9935**	0.1757	-0.9855**	0.5329	0.4756		
Pasting temperature	-0.9628**	0.0485	-0.9446*	0.4871	0.4842	0.9832**	
Amylose %[+]	-0.9190*	-0.1188	-0.8875*	0.3648	0.4311	0.9514*	0.9763**

* Significant at 5% level
** Significant at 1% level
[+] Based on iodine method

correlation. Although the correlation of the final viscosity with amylose content was positive, this was not statistically significant at the 5% level. Diploid starches showed significant positive correlation only between peak viscosity and breakdown (r= 0.946, p=0.054), and peak time and breakdown (r= 0.983, p=0.017).

Genetic differences as significant source of variation for starch peak viscosity is substantiated by many researchers (Loney and Meredith, 1974, Batey and Curtin, 1997). A considerable variability in pasting properties among Australian wheat cultivars was demonstrated in many preceding studies also (reviewed in Zeng et al., 1997). The interrelationship of the pasting parameters of hexaploid wheat starches derived in the current study was supported by the findings of Zeng et al., (1997), although some of their significant associations were found to be insignificant in this study. Their results revealed a high negative correlation of peak viscosity with minimum and final viscosities, but this relationship was not strong in the current analysis even though the trend was the same. Significant correlation with r >0.50 ($p<0.001$) between all the pasting parameters were reported by Konik et al., (1992). The average setback and final viscosity were higher for the *T. tauschii* lines (184.20 and 279.35) compared to the hexaploids (158.27 and 239.79). Generally, high setback values are associated with greater starch paste retrogradation (Wang and White, 1994a). However, Hoover and Vasanthan (1992) remarked that setback values reflect the extent of water immobilization around the charged centres of starch components, rather than starch paste retrogradation. The association of water molecules with these charged centres would decrease the effective water concentration in the continuous phase, resulting in a rise in viscosity during cooling cycle. The lower peak viscosity and higher pasting temperature of oat starch compared to that of corn was attributed to the large quantities of starch lipids present in oat starches, since defatting decreased pasting temperature due to increased granular strength (Wang and White, 1994a and 1994b). The lipid content of the lines in this study was not analysed and hence the above relationship can not be commented on based on the current results.

Paste viscosity can be influenced greatly by the presence of α-amylase activity (Batey et al., 1993, Mares and Mrva, 1993, Ross et al., 1997). In the absence of sprouting some wheat cultivars produce α-amylase during the later stages of grain ripening (late

maturity α-amylase, LMAA) sufficient to reduce the amylograph peak viscosity to unacceptable low levels (Mares and Mrva, 1993). The hexaploid cultivars used in the current study were of low LMAA types (D. Mares, personal communication, Mrva, 1993). Information on the LMAA status of *T. tauschii* lines was not available and hence an effect on the viscosity due to LMAA cannot be ruled out. Elimination of α-amylase activity by using inhibitors such as silver nitrate (Batey et al., 1993) should be looked at in any future work.

As amylose is a major determinant of starch structure, its association with the starch functional properties has been a subject of extensive study. Several reports have established the high negative correlation of amylose content and peak viscosity (Moss, 1980, Moss and Miskelly, 1984, Zeng et al., 1997, see also section 2.3.6 of Chapter 2). Cultivars with even small differences in amylose content were found to vary appreciably in starch paste peak viscosity and eating quality (Crosbie, 1991). Minimum and final viscosities did not show any significant correlation with amylose content in this study. Zeng et al., (1997) observed that amylose content correlated with these two parameters only at 16 min hold of 93°C and not at 4 min hold. The low amylose lines AUS23986 and AUS24230 tended to reach peak viscosity at a lower temperature (and in less time) and also had greater breakdown which was in agreement with the observation of Oda et al., (1980). However, the line AUS24242 was unique in its pasting behaviour in relation to its amylose content.

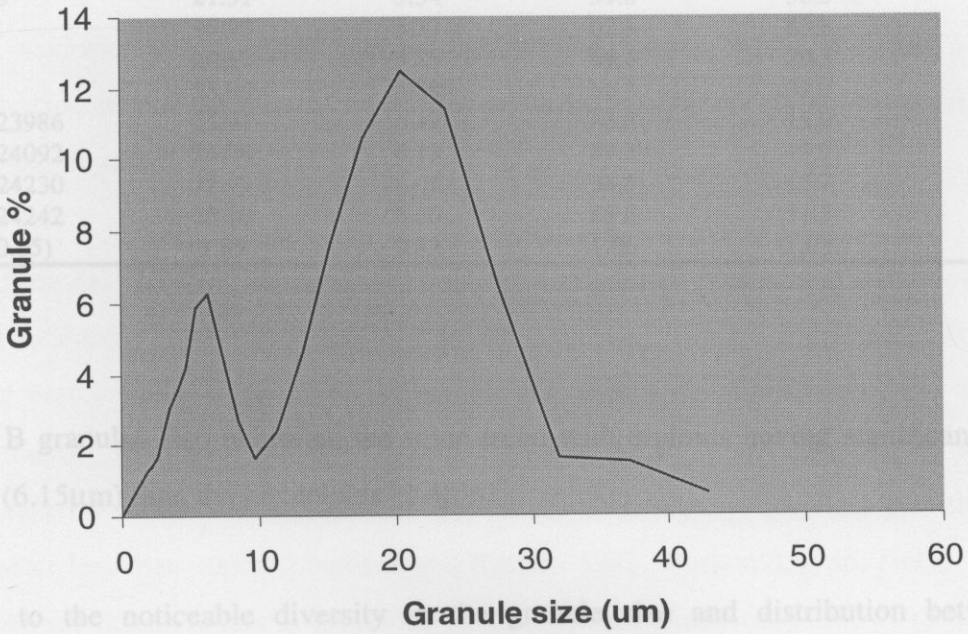
The higher starch paste viscosity associated with many Australian cultivars was reported to be associated with a reduced amylose content due to the lesser level of GBSS related to the Null4A genotypes (Zhao et al., 1995, Zeng et al., 1997). All the hexaploid cultivars analysed here are normal for GBSS alleles (section 2.3.6). The *T. tauschii* lines, bearing only the D genome, showed considerable variability in peak viscosity (296.83-370.25). There may be functional variability among the GBSS alleles. Genetic factors other than GBSS associated with pasting properties are also a possibility. In a study on fractionated amylose and amylopectin, it was seen that the amylose fraction did not give any peak viscosity, peak time, peak temperature or shear thinning, unlike amylopectin fraction (Mua and Jackson, 1997b), which suggests that the amylopectin is more important in determining the peak viscosity than amylose. The same study showed

that amylose fraction has a greater role in the retrogradation of starch as shown by its higher setback values and that the low molecular weight amylopectin fractions with high branching ratios, short branch chains and crystallinity gave high peak temperatures and low peak viscosity. It is not clear whether the variation observed in the chain length distribution analysis (Section 3.3.1) is large enough to be reflected in the functionality differences among the various starches. Synergistic effects on paste viscosities due to interactions of amylose and amylopectin are also reported in which long branch chain amylopectin and the intermediate molecular size amylose produce the greatest synergistic effect on viscosity (Jane and Chen, 1992). In general, starch functionality depends to a great extent on the molecular structure of both the amylose and amylopectin components.

3.3.3 Granule size distribution

A clear bimodal distribution pattern was observed for the hexaploid wheat cultivars analysed with small granules (B) having a diameter ranging from 2-10μm and large granules (A) ranging from 11-50μm diameter (Figure 3.5A). A more or less unimodal type of distribution was observed for all the diploid lines analysed (Figure 3.5B). (However, the terms A and B are used here also to describe the granules of size above and below 10μm respectively). There was statistically significant difference in the proportion by volume of each granule type among the different lines analysed (Table 3.3). Considerable variation was observed between hexaploids and diploids. Among the hexaploids, the proportion of A granules ranged from 59.8% (Minto) to 69.9% (Lark). The *T. tauschii* lines had a greater proportion of A granules compared to hexaploids ranging from 83.6% (AUS23986) to 89.5% (AUS24092) and a lesser fraction of B granules within the range of 10.7% (AUS24092) to 15.9% (AUS23986). The average granule diameter also varied significantly among the lines with distinct difference observed between the hexaploids and the diploids (Table 3.3). While the mean A granule diameter was 22.0μm for the hexaploids, it was 26.2μm for the *T. tauschii* lines.

A)



B)

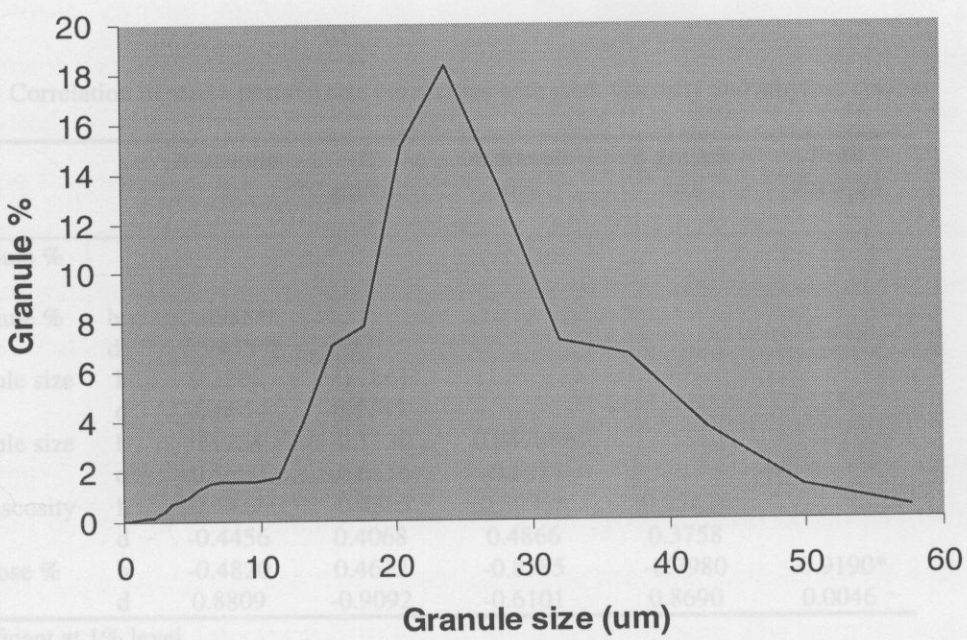


Figure 3.5 Particle size distribution pattern of wheat starches: A) Minto B) AUS24242.

Table 3.3 Starch granule size, distribution and swelling power among wheat cultivars/lines

Cultivar/Lines	Granule Size (μm)		Granule Distribution (%)		Swelling power (g/g)
	A granule	B granule	A granule	B granule	
Meering	21.22	5.16	64.8	34.1	20.4
Minto	21.31	5.34	59.8	38.5	20.92
Kiata	20.90	5.37	62.6	35.8	20.58
Lark	20.57	5.25	69.4	29.3	20.9
CS	25.96	5.89	66.5	32.3	20.19
AUS23986	25.91	6.12	83.8	15.9	20.22
AUS24092	25.08	6.13	89.3	10.7	19.65
AUS24230	27.93	6.05	84.5	15.7	20.34
AUS24242	25.70	6.30	88.5	11.5	18.57
CD (0.05)	1.85	0.12	1.06	1.08	1.56

The B granules also exhibited the same trend with diploids having significantly larger size (6.15μm) than the hexaploids (5.4μm).

Due to the noticeable diversity in the granule size and distribution between the hexaploids and the diploids, the correlation analysis was done separately for the two groups (Table 3. 4). The starch granule dimension showed significant positive correlation with peak viscosity and a negative association with amylose content among

Table 3.4 Correlation of starch particle size parameters with peak viscosity and amylose content

		A granule %	B granule %	A granule size	B granule size	Peak viscosity
A granule %						
B granule %	h	-0.9988**				
	d	-0.9955**				
A granule size	h	0.1860	-0.1661			
	d	-0.6654	0.7145			
B granule size	h	0.1254	-0.1120	0.9478**		
	d	0.5500	-0.6156	-0.5517		
Peak viscosity	h	0.4437	-0.4385	0.8996*	0.9342*	
	d	-0.4456	0.4068	0.4866	0.3758	
Amylose %	h	-0.4820	0.4622	-0.8585	-0.7980	-0.9190*
	d	0.8809	-0.9092	-0.6101	0.8690	0.0046

** Significant at 1% level
* Significant at 5% level
h hexaploid wheat
d diploid wheat

the diploids. The proportion of A granules had a positive relationship and that of the B granules a negative correlation with amylose content in the diploids. However this relationship was reversed and weak in the case of hexaploids. Though not strong, the peak viscosity had a positive association with A granule proportion and a negative association with B granule ratio among the hexaploids. Again, the diploids exhibited an opposite trend.

The bimodal distribution based on the size of the starch granules in bread wheat have been established by earlier researchers. Wheat starch is one of three starches(the others being barley and rye starch) in which the granules are considered to have a bimodal distribution, composed of an A granule population 10-30μm in diameter which are lenticular in shape and a B granule population of generally spherical granules with a diameter less than 10μm (Lineback and Rasper, 1988, Stark and Lynn, 1992). A similar pattern for durum wheat is described in Soulaka and Morrison, (1985). About 70-80% of starch mass in wheat was reported to be constituted by A granules (reviewed in Morell et al., 1995). A wider range observed in the current study (59.8%-89.5% by volume) is probably due to the greater genetic diversity of the materials used for the study. While genetic differences do affect the granular proportion, the growth environment has also a crucial role in the variation observed (Batey et al., 1996). The contribution of A type and B type granules to total starch weight at maturity varies according to grain size and degree of grain filling, which are influenced by differences in rates and condition of grain growth (Duffus and Murdoch, 1979). The environmental errors are minimised here by using samples grown in the same location and season, and the grains collected at full maturity.

The relative proportion of A and B granules at maturity is determined through a sequential pattern of development during the grain growth (Morrison, 1993, Morell et al., 1995). Generally there are two phases of starch accumulation in wheat which may each be broken down into several stages of grain development. The number and size of large A granules increases during phase 1. In phase 2, no further A granules are initiated, but existing A granules increase greatly in size and at the same time many

small B granules are initiated and they increase in both number and size. However, the size of B granules is limited to 10μm as described elsewhere (Bechtel et al., 1990, Morell et al., 1995). Whether these explanations of development of A and B granules can be applied to the D genome starch seems dubious as there is no clear cut demarcation between the two granule types as revealed by the *T. tauschii* starch analysis. These starches had a 50% lower proportion of smaller granules compared to hexaploid starches. Hence, it is a possibility that the starch granule development in these diploids takes place in a manner similar to that of other diploid plants such as maize and rice, where the average granule size increases with increasing age of the storage tissue. Conversely, in wheat, rye and barley where a second population of small granules are formed late in the development, the average granule size initially increases and as the small granules are formed, the average size decreases towards maturity (reviewed by Shannon and Garwood, 1984). On the basis of this explanation, the higher average granule size for the *T. tauschii* starches compared to that of the hexaploids could be substantiated.

Although it is well understood that A granules have higher amylose content than B granules (Soulaka and Morrison, 1985, Duffus and Murdoch, 1979), a negative correlation between granule size and amylose content is reported in oats (Wang and White, 1994b) and barley (Oscarsson et al., 1997), which is in parallel with the findings of this study. High amylose barley was found to have smaller A granules than the waxy types. An association between high amylose content and granule size was also revealed in their work. Conflicting results were reported on the relationship between starch granule size and pasting parameters (reviewed by Dengate, 1984, Soulaka and Morrison, 1985, Vasanthan and Bhatty, 1996). In their analysis of barley starches, Vasanthan and Bhatty, (1996) concluded that the differences in physico-chemical properties were greater among the genotypes than between small and large granule starches from the same genotype. The relationship of size fractionated granules to physico-chemical properties need not be consistent when starch as such is taken due to the synergistic effects of the various structural components. The discrepancy observed in the behaviour of diploid and hexaploid starches could also be attributed to the variable genic interactions in these genotypes.

The observation that *T. tauschii* (D genome) starch has an altered pattern of granule size distribution compared to bread wheat (ABD genome) and durum wheat (AB genome) throws some light on the genetics of granule size distribution. It is a possibility that the controlling factors for the formation of secondary particles of smaller size in the later stage of granule development are located in A and/ or B genome, the presence of which generates a bimodal distribution pattern. Indications of certain loci having an effect on B granule population were given in Batey et al., (1996), in which chromosomes 3A, 3B, 1A and 1B were found to influence the number of smaller granules. Evidence of a factor in the short arm of chromosome of 5D was found in this work which is discussed in Section 4.3.4.3 of Chapter 4. Initiation and development of starch granules being a complex process involving many steps, it is feasible that different loci are entailed in controlling the various key steps.

3.3.4 Starch swelling power (SSP)

Although statistical analysis revealed significant differences in the SSP among the 10 wheat cultivars/lines analysed, all the hexaploid cultivars did not vary regarding this trait (Table 3.3). However, among the *T. tauschii* lines, the higher amylose starches AUS24092 and AUS24242 had a lower SSP than the lower amylose lines AUS23986 and AUS24230. Differences in swelling power between the different starches are attributed to differences in amylose and lipid contents and in granular organisations (Dengate, 1984, Tester and Morrison, 1990, Wang and White, 1994b, Vasanthan and Bhatty, 1996, Yasui et al., 1996, Zeng et al., 1997). Smaller starch granules hydrate and swell more efficiently than the larger granules due to their higher ratio of surface area to unit weight of starch (Vasanthan and Bhatty, 1996). The higher proportion of B granules (Section 3.3.3), hence, may be a cause for the higher swelling in AUS24230 and AUS23986. Variation in flour swelling volume accounted by some starch properties other than the amylose content *per se* resulting from the null 4A GBSS condition was suggested by Zhao et al., (1995). Change in amylose distribution within the starch granule rather than the gross amylose percent may also lead to alteration in swelling power.

The differences in swelling power among the diploids is reflected in the RVA parameters also. High swelling starch granules occupy a large volume fraction in a paste and pack very close to each other (Vasanthan and Bhatty, 1996). They would thus impart higher intergranular friction during movement and hence low pasting temperature and high peak viscosity. Furthermore, the susceptibility of starch granules to shear deformation (breakdown) has been shown to increase with increase in degree of swelling due to granule softening (Vasanthan and Bhatty, 1996). However, it is to be noted that AUS24242 was unique in its pasting properties in relation to amylose content.

Wang and White, 1994b, pointed to discrepancies in starch swelling power accounted for by the differences in the methodologies. It is doubtful whether the hexaploid cultivars analysed in this study genuinely do not vary in their starch swelling capacity or the small scale analytical method has failed to pick up any variation among the cultivars. When Crosbie, (1991) analysed starches of cultivars significant to wheat production in Western Australia over a range of Barbender peak viscosity of 440-1115 amylograph units, the starch swelling power varied from 17.0-21.9. They observed a high correlation between SSP and peak viscosity and suggested SSP as a useful alternative to starch paste peak viscosity in characterising the quality of starch isolated from flour, offering advantages in terms of time and sample size.

3.4 CONCLUSION

This study has revealed diversity in the structure and functionality among hexaploid and diploid (D genome) wheat starches. Minto starch was found to have an amylopectin component with a slightly higher proportion of chains with >20 DP and a lower proportion of shorter chains than any of the lines compared. The most obvious structural difference between the hexaploid and diploids was in the pattern of starch granule distribution, where the diploids exhibited a unimodal distribution as against the bimodal distribution in the hexaploids. In general, the diploid starches were found to have better

gelling ability (as indicated by higher setback) than the hexaploids, indicating a scope for selecting suitable donors from among the *T. tauschii* lines for breeding for improved starch quality suitable for biopolymer manufacturing. Although some general assumptions regarding the relationship between various structural and functional attributes could be made, a larger study is required to further examine relationships between starch structure and function. Genetic analysis of segregating populations would be very useful to pin down the chromosomal regions controlling key starch traits in wheat. In such a study, the ability to measure phenotypes accurately would be crucial.

CHAPTER 4

Aneuploid Analysis for Amylose Content

4.1 INTRODUCTION

Aneuploidy, an increase or decrease in chromosome number not involving an entire genome can be exploited in crop plants to locate genes on chromosomes, to map gene-to-centromere distances, to transfer chromosomes from one cultivar or species to another and to identify chromosome homologies. Telosomics involve a change in a single chromosome arm whereas nullisomic-tetrasomics involve deletion of one chromosome pair with a compensating doubling of a homoeologous chromosome. Deletion stocks are powerful aneuploid stocks with various sized terminal deletions in individual chromosome arms useful for sub-arm localization of genes (Endo and Gill, 1996).

The development of wheat aneuploid lines by Sears (1954) has made possible an unprecedented approach to studying the genetic structure of complex characters in hexaploid wheat (Miura and Tanii, 1993). Because of the large number of chromosomes and the paucity of known gene locations, aneuploid analysis was suggested to be the most direct approach to identification of gene:chromosome relationships in wheat (McIntosh, 1987). Genes controlling the starch quality have been located using this approach by many workers (Chao et al., 1989, Yamamori and Endo, 1996, Morell et al., 1997). While utilisation of aneuploid lines to study the effects of specific genes on particular phenotypes is commonplace, deletion stocks provide particularly effective tools as the effects observed are not confounded by the presence of additional chromosomes as in nullisomic-tetrasomic lines. The current study aimed at utilizing ditelosomic lines of the wheat cultivar Chinese Spring (CS) to better understand of the influence of specific chromosomes on amylose content. The lines identified to have

significant alteration in amylose content from the control were further analysed for any starch structural, molecular or biochemical changes.

The consensus map of hexaploid wheat has located a starch branching enzyme II (SBE II) gene on the homoeologous group 2 chromosomes (Gale et al., 1993). A CS deletion stock with various sized terminal deletions of group 2 chromosome set was also utilized for the sub arm localization of SBE II gene through molecular and biochemical approaches. The influence of the gene on amylose content and amylopectin chain length distribution was further investigated by analyzing the deletion lines with missing allele of the gene.

4.2 MATERIALS AND METHODS

4.2.1 Aneuploid lines

Chinese Spring (CS) ditelosomic lines grown in three different years were kindly provided by Prof R.A. McIntosh, University of Sydney. The CS chromosome 2 deletion stocks were supplied by Dr. B.S. Gill, Kansas State University. The deletion stocks were grown under quarantine to collect leaf samples for DNA extraction and to harvest seeds for protein and amylose analysis.

4.2.2 Starch analysis

Starch extraction was as described in Section 2.2.2. Amylose estimation was done by iodine method, HPLC or conA method according to the protocols explained in Section 2.2.3. Starch structural analysis included amylopectin chain length study by capillary electrophoresis followed by laser induced detection (Section 3.2.3) and particle size distribution using the Malvern Particle Sizer (Section 3.2.5). Starch paste viscosity and starch swelling power were analysed as described in Sections 3.2.4 and 3.2.6

respectively. Resistant starch analysis was done at Grain Quality Research Laboratory, North Ryde, following the Amyloglucosidase/α-Amylase method of Megazyme

4.2.3 Isolation of total genomic DNA

Healthy leaf samples were collected from 10-20d old glasshouse-grown plants or from 6-8d old leaves of field-grown plants. Leaf materials were used fresh or stored at -80°C until required.

Approximately 2-3g of fresh or frozen leaves were ground into fine powder in liquid nitrogen using a mortar and pestle. The ground tissues were transferred to 15ml preheated DNA extraction buffer (100mM Tris-HCl pH 8.0, 50mM EDTA pH 8.0, 500mM sodium chloride, 2.5% SDS) and incubated at 65°C for 20-40 min. Then 4.5ml 5M potassium acetate was added and incubated at 4°C for 20 min and centrifuged at 6,000g. The supernatant was filtered through gauze and added to 25ml isopropanol to precipitate the DNA, which was collected using a glass hook. DNA was dissolved in 1 x TE buffer (10mM Tris pH 8.0, 1mM EDTA pH 8.0). RNaseA was added to a final concentration of 100µg/ml and incubated for 30 min at 37°C. An equal volume of phenol/chloroform was added and centrifuged to remove protein contaminants. Phenol/chloroform extraction was repeated 2-3 times to produce a clear supernatant and the DNA was precipitated with 1/10 volume of 3M sodium acetate and 2 volumes of ethanol. The precipitated DNA was collected again using glass hook, washed in 70% ethanol; air-dried 3-5 min and dissolved in 500µl deionized water.

DNA concentration was determined by running 1µl on a 0.8% agarose gel alongside a series of standards made from λDNA. Agarose mini-gels were cast from 50ml of 0.8% melted agarose solution containing 1 x TAE buffer (0.04M Tris-acetate, 1.0mM EDTA, pH 8.0). DNA samples were mixed with 6 x gel loading buffer containing 0.25% bromophenol blue, 0.25% xylene cyanol FF and 15% Ficoll 400 and electrophoresed at 70-80V in 1 x TAE buffer. The gel was stained in a 0.5mg/l solution of ethidium bromide for 10 min, destained by rinsing in water and photographed using UV light of

wavelength 302nm.

4.2.4 DNA Southern hybridization

4.2.4.1 DNA Southern blotting

The restriction enzymes *Eco*R1, *Dra*1 or *BamH*1 were used to digest genomic DNA. Approximately 15-20μg of DNA was digested with 30 units of enzyme at 37°C overnight. The digested DNA was run on a 0.8% agarose gel in 1 x TBE (0.045M Tris-borate, 1.0mM EDTA, pH8.0) at 40V for 16h until the bromophenol blue dye had migrated approximately 13cm. The DNA was transferred to Hybond N^+ nylon membrane (Amersham) under alkaline conditions. The gel was soaked in 0.25M hydrochloric acid to depurinate the DNA fragments, followed by rinsing briefly with deionized water. Two washes with 0.4M sodium hydroxide for 15-20 min were done for denaturation. DNA was transferred to membrane using 0.4M sodium hydroxide as the transfer solution. After transfer the membrane was rinsed in 2 x SSC (1.75% NaCl, 0.88% sodium citrate, pH 7.0) briefly, and then blotted dry between 2 sheets of 3MM filter paper at room temperature.

4.2.4.2 Preparation of RFLP probes

Probes of various starch biosynthetic enzymes were obtained from Dr. T. Musket, University of Missouri, Columbia and Dr. S. Rahman, CSIRO Plant Industry, Canberra. The probe psr628 was selected from the Australian Triticeae Mapping Initiative (ATMI) collection.

The ATMI probe was maintained in *E. coli* strains as LB (Luria Broth: 10g/l Bacto-tryptone, 5g/l Bacto-yeast extract, 5g/l NaCl, pH 7.0) glycerol stock at -80°C. Cells were scraped from frozen glycerol stocks and transferred into LB plates containing ampicillin (100μg/ml) and incubated at 37°C overnight. A single bacterial colony was transferred

into 2ml of LB medium containing ampicillin (100µg/ml) at 37°C overnight while shaking vigorously. Cells were centrifuged and re-suspended in 100µl lysis buffer (25mM Tris-HCl pH 8.0, 10mM EDTA, 50mM sucrose) and left on ice for 5 min. The cells were lysed with 200µl of 0.2M NaOH and 1% SDS. The contents of the tube were gently mixed and again left on ice for 5 min. Then 150µl of 5M potassium acetate at 4°C was added and mixed by inverting and incubated again on ice for 5 min. Following centrifugation at 13,000g for 10 min at room temperature, plasmid DNA from the supernatant was purified by phenol/chloroform extraction and ethanol precipitated.

The plasmid inserts were obtained by PCR amplification of inserts using the primers M13 forward and reverse for pUC vectors. PCR reaction was conducted in 20µl total volume of 1 x PCR buffer (Advanced Biotechnologies), 2.5mM $MgCl_2$, 1µl of dNTP mixture containing 1.25mM of each dNTP, 5pmol of each primer, 20-50ng miniprep DNA and 1 unit of *Taq* DNA polymerase (Advanced Biotechnologies). The temperature profile consisted of 35 cycles of 30 sec at 94°C, 30 sec at 50°C and 90 sec at 72°C with a final extension time at 72°C at the end of the cycling period. The probe DNA was checked for the correct size and concentration on a 0.8% agarose gel compared with the molecular weight standard Spp1/*Eco*R1 (Bresatec).

4.2.4.3 ^{32}P-labelling of DNA probes

Radioactively labeled probes were synthesised by random priming, essentially as described by Feinberg and Vogelstein (1983). Labeling was done using the Gigaprime labeling kit from Bresatac. Approximately 50-100ng of PCR-isolated insert was denatured by incubation at 95°C for 5 min and quickly chilled on ice. The labeling mixture contained nucleotide/labeling buffer cocktail (6µl), decanucleotides (6µl), Klenow fragment (1µl), 32P-dCTP (3µl) and 50-100ng denatured probe DNA made up to 24µl with deionized water. The reaction was incubated at 37°C for 2h.

4.2.4.4 Hybridization and autoradiography

Membranes were pre-hybridised for 3-4h at 65°C in 20ml buffer (0.6M sodium chloride, 20mM PIPES pH 6.8, 5mM EDTA, 0.2% BSA, 0.2% ficoll, 0.2% PVP, 1% SDS, 0.5% tetrasodium pyrophosphate and 500µg/ml denatured salmon sperm DNA) in glass tubes in a Hybaid oven. Probe and salmon sperm DNA were denatured by placing in a boiling water bath for 10 min followed by cooling on ice. The pre-hybridisation buffer was replaced by the same buffer containing the denatured probe DNA labelled with ^{32}P-dCTP and hybridised overnight at 65°C. The hybridised membrane was rinsed with 2 x SSC, 0.1% SDS, followed by two further washes with the same solution at 65°C for 15 min each and twice with 0.5 x SSC, 0.1% SDS at 65°C for 15 min each. Autoradiography was performed for 3-10d using Kodak BioMax MS film with one intensifying screen at –80°C.

4.2.5 PCR amplification using SBE II primers

Two sets of primers, Sr913F (5' ATCACTTACCGAGAATGGG 3') and E6R (5' CTGCATTTGGATTCCAATTG 3'), and da5.seq (5' GGCTTGGATACAATGC AGTGC 3') and E11R (5' CTGGAGTTCCAAAACGGCTAC 3') which are specific for the starch branching enzyme-2 (SBE II) were kindly provided by Dr. S. Rahman and Ms. L. Preston of CSIRO Plant Industry, Canberra. PCR was conducted in 10µl volume containing 50ng template DNA, 1µl of PE 10 x reaction buffer containing 1.5mM $MgCl_2$, 1µl of dNTP mixture containing 2mM of each dNTP, 0.04µl dUTP, 1µl of each of the primers and 0.3µl Amplitaq Gold. The PCR programme included an initial denaturation step of 95°C for 5 min, followed by 5 cycles of 94°C for 1 min, 65°C for 1 min and 72°C for 1min 20 sec. The third step involved 5 cycles of 94°C, 60°C and 72°C, each for 1 min 20 sec. The next 5 cycles had the same denaturing and extension temperatures, but the annealing temperature was reduced to 55°C, which was further reduced to 50°C in the next 35 cycles with the denaturing and the extension temperatures remaining the same. There was a final extension at 72°C for 5 min with a holding at 25°C. The products were run on a 1.8% Metaphor agarose gel.

4.2.6 Analysis of starch branching enzyme isoforms

All the protocols used were developed at the CSIRO Plant Industry, Canberra.

4.2.6.1 Tissue extraction and protein estimation

Ears were harvested 25d after anthesis and snap-frozen in liquid nitrogen and stored at –80°C until use. Tissue extraction was done at 4°C. Endosperm dissected away from all maternal tissues (0.2g) was homogenized in 600µl of 50mM KPi buffer (42mM K_2HPO_4 and 8mM KH_2PO_4), pH 7.5, containing 5mM EDTA, 20% glycerol, 5mM DTT and 1mM pefabloc. The ground samples were centrifuged for 10 min at 13,000g and the supernatant aliquoted and frozen at –80°C until use. For total protein estimation, a BSA standard curve was set up using 0, 20, 40, 60, 80 and 100µl aliquots of 0.25mg/ml BSA standard. The samples (3µl) were made up to 100µl with distilled water and 1ml of Coommassie Plus Protein reagent was added. After incubation for 5 min, the absorbance was read at 595nm, using the zero BSA sample from the standard curve as the blank.

4.2.6.2 Gel electrophoresis of SBEs

Nondenaturing PAGE was carried out using 10% acrylamide gels to analyse SBE I. The gel was cast on 16 x 18cm^2 gel plates with 30ml volume of separating gel and 10ml volume of stacking gel. The separating gel composition was 0.33M Tris-HCl pH 8.8, 0.05% CHAPS, 10% Glycerol, 10% acrylamide 37.5:1, 0.07% TEMED and 0.05% APS. The stacking gel was composed of 0.13M Tris-HCl pH 6.8, 0.05% CHAPS, 6% acrylamide 37.5:1, 0.1% TEMED and 0.06% APS. Sample volumes required to get 20µg of protein were mixed with 1µl loading buffer (80% glycerol, 1% BPB) per 10µl of the sample. The gel was run for 16h at 138V and 16°C constant temperature in a running buffer containing 190mM glycine, 25mM Tris pH 8.3 and 2mM thioglycolate.

SBE II was analysed on a 6% nondenaturing polyacrylamide gel using 25μg of protein. The separating gel contained 0.15% β-limit dextrin, 0.2mM α-cyclodextrin, 0.06% APS, 0.1% TEMED while the concentration of Tris-HCl, CHAPS and glycerol remaining the same as in the case of SBE I. The stacking gel composition was the same as that of SBE I except that 5% acrylamide 37.5:1 was used instead of 6%.

4.2.6.3 Immunoblotting of SBE gels

Primary antibodies, anti-wheat BE I and anti-wheat BE II, specific for wheat SBE I and SBE II respectively were kindly provided by Dr. Matthew Morell, CSIRO Plant Industry, Canberra. Gels were soaked in running buffer containing 0.25% SDS for 20 min prior to transfer. Western blotting was done using Novablot apparatus according to the suppliers protocol. Transfer buffer was composed of 48mM Tris-HCl, 39mM glycine, 0.0375% SDS and 20% methanol. A constant current of 0.23A was maintained throughout the transfer. After transfer, the membranes were soaked in washing buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 0.1% Triton X-100) containing 5% skim milk for at least 1h for blocking non specific binding sites, followed by washing five times with washing buffer (2 x 1 min., 1 x 5 min., 1 x 10 min., and 1 x 5 min.). Then the membranes were incubated with primary antibody (diluted 1:5000 in washing buffer containing 2% BSA) for 45 min. on a platform rocker, which was followed by washing seven times with washing buffer (2 x 1 min, 2 x 5 min, 1 x 10 min, 1 x 15 min, 1 x 5 min). Incubation with secondary antibody (GAR-HRP), diluted 1:3000 in washing buffer containing 2% BSA, was done for 30 min, followed by seven washings as before with washing buffer and two washings with buffer without Triton X-100. The membranes were drained and immunoreactive bands were revealed using an Amersham ECL detection system, according to manufacturer's instructions.

4.3 RESULTS AND DISCUSSION

4.3.1 Analysis of CS ditelosomic lines

4.3.1.1 Amylose content of CS ditelosomic lines

Twenty nine ditelosomic (dt) lines of the variety Chinese Spring (CS) were analysed iodometrically for amylose content. Statistical evaluation by analysis of variance showed that there were significant differences in the amylose content between the lines with a critical difference (CD) at 5% confidence level of 4.1, the values ranging from 43.5% in the line dt5DL to 19.9% in dt6AS. The mean deviation in each ditelosomic line from the euploid CS was considered as an estimate of the individual effects of specific chromosomal arms on amylose content and is depicted in Figure 4.1. The results showed significant variation in amylose content due to missing specific chromosomal arms. The amylose content was significantly increased compared to the euploid CS in the lines dt1BL, dt5DL, dt6DS and dt7DS, whereas, dt2BL, dt3BL, dt3DL, dt7BS and dt6AS had significant reductions in amylose content. Among these, the largest effects were observed in the lines dt5DL and dt6AS, which exhibited the most extreme phenotypes in the positive and negative directions respectively.

The effects due to the waxy genes encoding the granule bound starch synthase (GBSS) located on chromosomes 7AS, 4AL and 7DS in wheat (Nakamura et al., 1993b) have been analysed thoroughly in monosomic and nullisomic-tetrasomic lines of CS by many workers. In spite of the missing GBSS allele in the line dt7AL, the current study did not reveal any significant change in the amylose content in this line compared to the control, which was in agreement with the findings of Miura et al., (1994) in monosomic lines. They observed a reduction in the amounts of proteins coded by the genes on chromosome arms 7AS and 7DS, but with no effect on amylose content. The major effect of waxy locus on chromosome 4AL could not be studied here due to the unavailability of the line dt4AS missing the long arm of chromosome 4A.

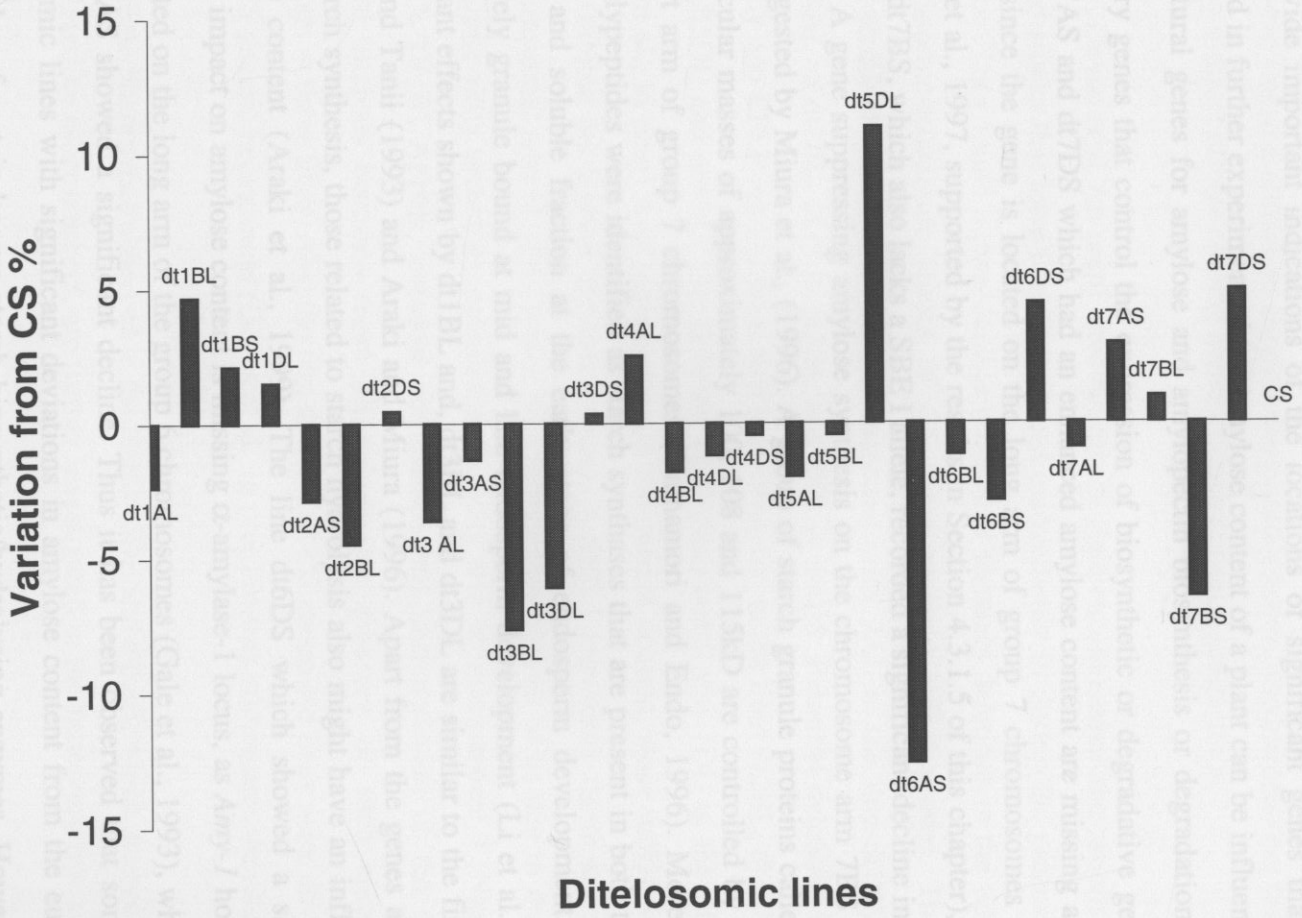


Figure 4.1 Variation in amylose content in CS ditelosomic lines expressed as change from CS.

Since ditelosomic lines are missing a whole arm of particular chromosomes, they are lacking many genes. Hence it is difficult to attribute an altered phenotype to the lack of any particular gene (unless supported by further examination). However this analysis can provide important indications of the locations of significant genes that can be examined in further experimentation. Amylose content of a plant can be influenced both by structural genes for amylose and amylopectin biosynthesis or degradation, and by regulatory genes that control the expression of biosynthetic or degradative genes. The lines dt7AS and dt7DS which had an enhanced amylose content are missing a locus of SBE I, since the gene is located on the long arm of group 7 chromosomes in wheat (Morell et al., 1997, supported by the results in Section 4.3.1.5 of this chapter), whereas the line dt7BS, which also lacks a SBE I allele, recorded a significant decline in amylose content. A gene suppressing amylose synthesis on the chromosome arm 7BS in wheat was suggested by Miura et al., (1996). A group of starch granule proteins called SGP-1 of molecular masses of approximately 100, 108 and 115kD are controlled by genes on the short arm of group 7 chromosomes (Yamamori and Endo, 1996). More recently these polypeptides were identified as starch synthases that are present in both the starch granule and soluble fraction at the early stage of endosperm development, but are exclusively granule bound at mid and late endosperm development (Li et al., 1999a). Significant effects shown by dt1BL and, dt3BL and dt3DL are similar to the findings of Miura and Tanii (1993) and Araki and Miura (1996). Apart from the genes associated with starch synthesis, those related to starch hydrolysis also might have an influence on amylose content (Araki et al., 1999). The line dt6DS which showed a significant positive impact on amylose content is missing α-amylase-1 locus, as *Amy-1* homoeoloci are located on the long arm of the group 6 chromosomes (Gale et al., 1993), whereas the line dt6AS showed a significant decline. Thus it has been observed that some of the ditelosomic lines with significant deviations in amylose content from the euploid CS lack alleles of certain known starch biosynthetic/hydrolysing enzymes. However, from the current analysis it is not possible to elucidate the extent to which these missing alleles have influenced the change in amylose content.

Studies on gene-enzyme relationships have demonstrated that mutant alleles affecting the starch pathway not only have site-specific effects on metabolism but also influence

non target enzymes (Singletary et al., 1997). Their observations on *amylose extender*-1, *brittle*-2, *shrunken*-1 and *sugary*-1 mutants indicate that all these mutants except *sugary*-1 displayed large increases (approximately 2- to 5-fold) in the activity of various enzymes unrelated to the structural gene. Hence it is possible that apart from any direct effect due to missing allele/s of structural/regulatory genes, amylose content may also be altered by indirect effects coming about through epistatic interactions.

Recently, Devos et al. (in prep) have brought to light chromosomal aberrations in wheat aneuploid stocks that have been introduced inadvertently in during the process of their generation. They found a considerable number of terminal deletions in the ditelosomic and nullisomic-tetrasomic stocks of CS using mapped RFLP probes. Their work revealed terminal deletions in the chromosomal arms 7AL in the line dt1BS, 3BS in dt2AS, 4AL in dt2BL, 2DS in dt5BL and 2BS in dt6BS. Potential deletions identified using only one RFLP marker have also been observed in the lines dt2BL, dt3BS, dt4AS, dt4AL, dt5DL and dt6AL. The deletion in chromosomal arm 1DL in the line dt5DL (Devos et al., in prep) is of significance in the current context, since dt5DL is the line which exhibited the highest amylose content. A SBE 1 regulatory factor is detected in the chromosomal arm of 1DL, which was further investigated to be due to the missing SBE 1 allele via a secondary deletion in the long arm of chromosome 7 (Nagamine et al., 1997, Dr R. Appels, personal communication). Interpretation of the results of aneuploid analysis becomes more difficult in the presence of such chromosomal aberrations in these lines. Also the fact that not all CS aneuploid lines were developed in a pure CS genetic background (Devos et al., in prep) further compicates the situation.

The extreme phenotypes observed in the lines dt5DL and dt6AS, which are not observed and analysed by other workers, are further investigated in the following few sections of this chapter.

4.3.1.2 Multi-year trial with lines dt5DL and dt6AS

In order to verify whether the effects of chromosomes 5D and 6A on amylose content were stable over different growing conditions, the blue value of the lines dt5DL, dt6AS

and euploid CS grown in three different years – 1994, 1995 and 1997 were analysed. The analysis of variance showed a significant year × line interaction effect and hence the variation in amylose content among the lines was tested against year × line interaction as error. The trial confirmed the effects of chromosomes 5D and 6A in altering the amylose content. Regardless of the growth year the line dt5DL recorded a significantly higher amylose content, whereas the line 6AS showed a significantly lower value compared to the control (Table 4.1) even though the extent of response varied from year to year. These results indicate the existence of a genetic basis for the phenotypic effects of these two chromosomes, which is modulated by environmental effects.

Table 4.1 Amylose content of ditelosomic lines of CS grown in three years

Line	Mean amylose content (%)		
	1994	1995	1997
Dt5DL	34.9*	43.5**	35.2*
Dt6AS	28.3*	21.2**	26.1**
CS	31.1	32.5	31.6

* Significantly different from CS at 5% level
** Significantly different from CS at 1% level

4.3.1.3 Confirmation of amylose content with HPLC and conA methods

Since the iodine method gives an apparent amylose content that can be influenced by iodine binding by amylopectin, the trait value of these ditelosomic lines were also estimated by the alternate approaches of HPLC and conA methods (Table 4.2). The estimated amylose content was significantly higher in the line dt5DL compared to CS by both HPLC and conA methods. The line dt6AS had a significantly lower value than the control in the case of HPLC, whereas no significant decrease was observed with the conA method. The results obtained by both these methods appeared to be less influenced by the amylopectin fraction of the starch, as the amylose and the amylopectin

components are separated before the estimation of amylose (Batey and Curtin, 1996 and Gibson et al., 1997). These results confirm that the 5DL effect on amylose content was stable over different growing seasons and is caused by an increase in amylose content, not an artefact of the iodine blue value measurement procedure.

Table 4.2 Amylose content of ditelosomic lines measured by HPLC and conA methods

Line	Mean amylose content (%)	
	HPLC	ConA
Dt5DL	33.6*	23.1
Dt6AS	21.3*	14.3
CS	29.3	14.3

* Significantly different from CS at 5% level

Seed production in the line dt6AS was poor, yielding very low amount of starch. Hence only line dt5DL was carried forward for further starch structural analysis.

4.3.1.4 Further characterisation of dt5DL starch

4.3.1.4.1 Amylopectin chain length analysis

The molecular structure of the amylopectin of the line dt5DL was studied by analysing the chain length distribution in comparison with that of the euploid CS by fluorophore assisted carbohydrate electrophoresis followed by laser induced detection (O'Shea, et al., 1998). The distributions were normalized by summing the fluorescence over the distribution and setting the total value to 100. The percentage contribution for each oligomer of the dt5DL was subtracted from the percentage contribution for the corresponding oligomer of CS (Figure 4.2). The results showed that dt5DL starch had a lower proportion of mass in shorter chains (below DP=20), and had a higher mass

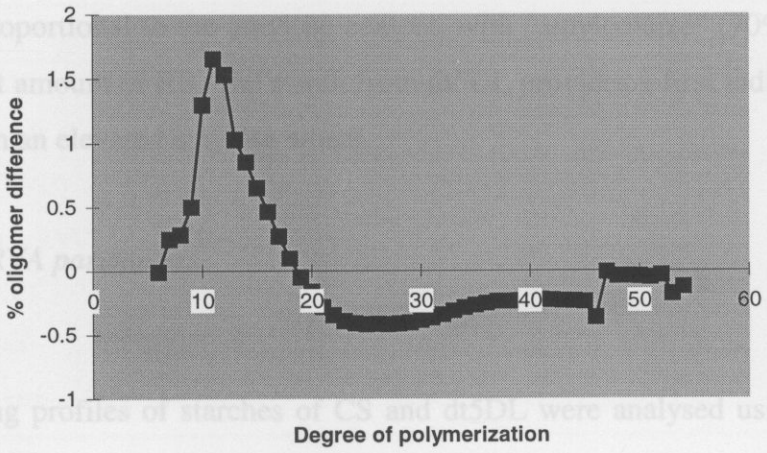


Figure 4.2 Amylopectin chain length comparison of dt5DL starch with respect to euploid CS. The percentage contribution for each oligomer of the dt5DL was subtracted from the percentage contribution for the corresponding oligomer of CS.

contribution from chains of a length greater than 20 glucose units compared to the control CS starch. The higher blue value observed in dt5DL starch might also reflect the long chained amylopectin fraction, apart from the actual amylose content. Altered amylopectin structure in conjunction with higher amylose content is a feature of amylose extender (*ae*) genotypes where the absence of a branching enzyme isoform results in increased amylose content, amylopectin with longer branches and an intermediate material with a lower molecular weight than that of amylose (reviewed in Mizuno et al., 1993, see also Section 3.3.1 of Chapter 3).

4.3.1.4.2 Resistant starch analysis

In the resistant starch (RS) analysis done at CSIRO, N. Ryde, the line dt5DL yielded some RS (2% compared to 0.9% and 0.3% for the controls, Sunelg and Janz respectively). Unfortunately, the data on euploid CS was not available. The existence of

a relationship between the amylose content and the yield of RS had been reported previously (Berry, 1986, Berry et al., 1988, Wyatt and Horn, 1988). Among the various type of starch studied, Sievert and Pomeranz (1989) observed the yield of RS to be directly proportional to the amylose content, with "amylomaize" (70% amylose) giving the highest amount of RS. The starch from dt5DL provides a first indication of this type of starch in an elevated amylose wheat.

4.3.1.4.3 RVA parameters

The pasting profiles of starches of CS and dt5DL were analysed using a Rapid Visco Analyser (RVA). The results obtained were assumed to be free of the influence of α-amylase mainly due to three reasons: 1) CS is reported to be a variety with low levels of late maturity α-amylase (Mrva, 1994), 2) No α-amylase gene has so far been reported in wheat chromosome 5D (Gill and Raupp, 1996) and hence the chances of any alteration in α-amylase level in dt5DL line are less, and 3) Plants of both the lines were grown under the same environmental conditions and the harvested seeds were free of rain damage. It is observed that the starches vary in their inherent paste viscosity, the parameters recorded are given in Table 4.3. Lower peak viscosity and breakdown were evident for dt5DL starch compared to CS, whereas all the other parameters were higher for dt5DL. Previous studies showed that variation in amylose content of starch accounted for approximately half the variation in peak viscosity (Moss, 1980, Miskelly, 1984). Each 1% reduction in apparent or total amylose content correspond to an increase in peak viscosity of about 22 and 25 RVU, respectively as observed by Zeng et al., (1997).

The minimum viscosity (trough), which is the lowest apparent viscosity obtained upon continuous mixing (shear thinning) of the gelatinized system and the final viscosity which represents the increase in viscosity associated with gelation and retrogradation during cooling are found to be positively associated with amylose content in their study,

Table 4.3 RVA pasting properties of dt5DL and CS starches

RVA Parameters	Dt5DL	CS
Peak 1 (RVU)	323.75	385.42
Trough 1 (RVU)	86.25	79.00
Breakdown (RVU)	237.50	306.42
Final viscosity (RVU)	286.50	233.25
Set back (RVU)	200.25	154.25
Peak time (min)	7.87	7.13
Pasting temperature (°C)	72.45	65.90

whereas the breakdown, the difference between the peak and minimum viscosities showed a significant negative correlation. Gelation and retrogradation have been attributed to the increased thermodynamic stabilization of a starch gel through increased polymer – polymer hydrogen bonding, especially involving amylose. Consequently, those starches with reduced amylose content tend to exhibit reduced final viscosities upon cooling. The lower peak viscosity, lower breakdown, higher trough and final viscosity and greater set back observed in dt5DL starch compared to CS reflect its higher inherent amylose content revealed by various chemical analyses.

Variation in starch functionality indicates structural changes in amylopectin also (Jane and Chen, 1992, Mua and Jackson, 1997b). A decrease in amylopectin molecular weight can cause an increased set back and final viscosity as observed in dt5DL starch.

4.3.1.4.4 Granule size distribution

The size distribution analysis of A (>10μm) and B (<10μm) granules measured in a Malvern Particle Sizer revealed significant variation among the dt5DL and CS starches. Dt5DL has a 6% higher proportion by volume of B granule (38.6%) compared to CS

(32.3%) (t=7.8, P=0.008). Previous reports have shown that growth environment and the methods of extraction of starch granules affect B granule proportions (Duffus and Murdoch, 1979, Batey et al., 1996) and hence the current analysis was done using samples grown at the same location and extracted by the same procedure

The occurrence of a higher proportion of small granules (by weight) in high amylose barley has been demonstrated (Oscarsson et al., 1997), even though other studies have provided evidence that there is more amylose in A granules than in B (Soulaka and Morrison,1985). Whether the variation in the granular proportions in dt5DL starch is related to its higher amylose content or not needs to be investigated. In an analysis of the nullisomic-tetrasomic lines of CS, Batey et al., (1996) found controlling factors in homoeologous chromosome groups 3 and 1. No significant effect was noticed in chromosome 5D, in contrast to the present study, which is probably due to the complementation by the additional 5A chromosome in the N5DT5A line used for their analysis.

4.3.1.4.5 Starch swelling volume (SSV)

No significant difference was observed in SSV between the CS and dt5DL starches. Swelling is primarily a property of amylopectin, although amylose and lipids in the granule strongly inhibit swelling (Tester and Morrison, 1990). Within the amylose fraction, 80% of the lipid free amylose is assumed to swell with amylopectin, whereas the lipid complexed amylose inhibit swelling (Morrison et al., 1993b). An interplay of internal lipid content and granule size is found to be responsible for net swelling of barley starch by Vasanthan and Bhatty, (1996). These results indicate that analysing the lipid content of the starches, apart from other structural studies will be useful in substantiating the swelling behaviour of starch. The association between greater swelling with reduced amylose content and higher pasting is well documented (Crosbie, 1991). However, Konik et al., (1994) found no significant correlation between swelling power and RVA peak paste viscosity.

4.3.1.5 Biochemical analysis of dt5DL and dt6AS lines

Non denaturing PAGE analysis using developing endosperms from the ditelosomic lines of CS was performed, and revealed immunoreactive proteins following a reaction with primary antibodies raised against synthetic polypeptide designed from an amino acid sequence at N-terminus of wheat SBE I. Four SBE I polypeptides – A, D-i, D-ii and B (designated as per Morell et al., 1997) with variable intensities were revealed in all 16 lines analysed except dt7DS in which D-i and D-ii bands were missing (Figure 4.3). This is due to the absence of the gene encoding SBE I D-i and D-ii polypeptides in the line 7DS which lacks the long arm of chromosome 7D. This result confirms the location of gene controlling the expression of the SBE I isoforms D-i and D-ii in the long arm of chromosome 7D. Evidence of the assignment of wheat SBE I polypeptides to chromosome 7 of wheat has been provided by Morell et al., (1997) by nullisomic – tetrasomic analysis. Differential expression of the SBE I genes residing on different wheat genomes is also being suggested which provides basis for the variable intensities of the bands (Baga et al., 1999). Since lines 5DL and 6AS, which are of interest in the current study, did not show any polymorphism, within the limits of the assay conditions and the antibodies used to pick up the polypeptides, we can say that the short arms of chromosome 5D and long arm of 6A do not carry any gene controlling the SBE 1 class of enzymes.

4.3.1.6 Molecular analysis of dt5DL and dt6AS lines

The chromosomal arm deletion of dt5DL was confirmed by Southern blotting using the plasmid probe psr628 specific for the short arms of group 5 chromosomes in wheat (Figure 4.4). Attempts were made using RFLP analysis to assign the cause of the altered starch structure in the line dt5DL to any of the known enzymes involved in the starch biosynthetic pathway. Three probes, E 7.8 specific for SBE I gene of wheat, sbe 9 specific for SBE IIa of wheat, and 5C04 B10 specific for soluble starch synthase (SSIIb) of maize were used for detecting polymorphism. None of the probes picked up any

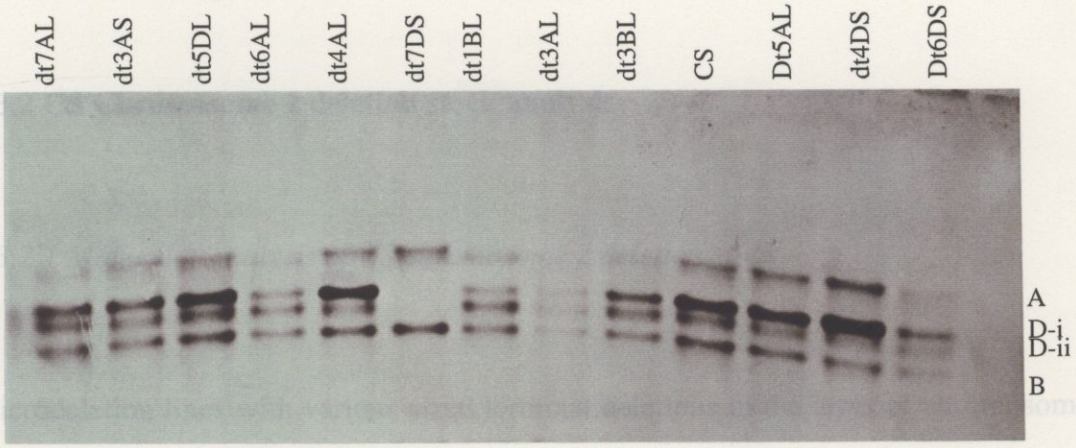


Figure 4.3 Nondenaturing PAGE immunoblot of wheat endosperm from ditelosomic lines of CS using a primary antibody raised against synthetic peptide designed from an amino acid sequence at N-terminus of wheat SBEI.

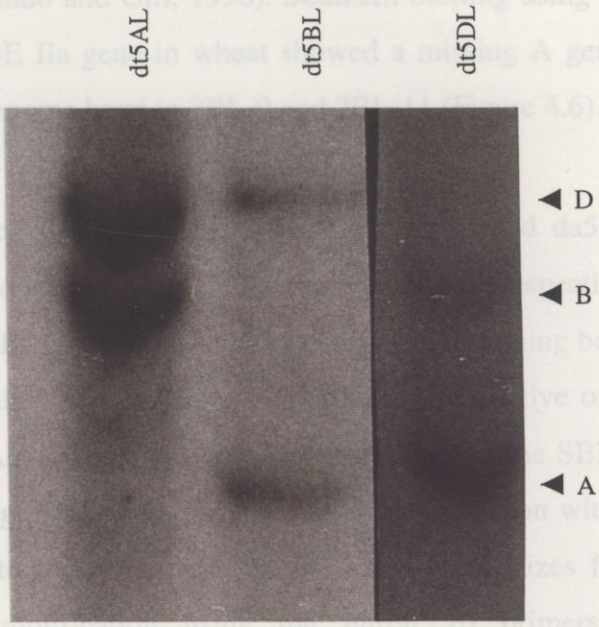


Figure 4.4 Autoradiograph showing the hybridization of RFLP probe psr628 to *Dra*1 digested genomic DNA from chromosome 5 ditelosomic lines of CS.

polymorphism between dt5DL and the euploid CS (results not shown), indicating the need for more analysis using a wide range of probes.

4.3.2 CS Chromosome 2 deletion stock analysis

4.3.2.1 Molecular analysis of CS chromosome 2 deletion stock

Microdeletion lines with various sized terminal deletions in the arms of chromosome 2 were used to confirm the location of starch branching enzyme II gene and to analyse its effect on the amylose content. Figure 4.5 shows the C – banding diagram of chromosome 2 of CS showing the breakpoints of the deletions. The deletions were designated by using the chromosome arm hyphenated with consecutive numbers, for example, 2AL-1 (Endo and Gill, 1996). Southern blotting using the RFLP probe sbe 9 specific for the SBE IIa gene in wheat showed a missing A genome band in the line 2AL-2 and the B genome band in 2BL-9 and 2BL-11 (Figure 4.6).

PCR was conducted using primers Sr913F and E6R, and da5seq and E11R, which amplify intron 5 and intron 10 regions of the SBE II gene respectively. With the intron 5 primers, the lines 2BL-9, 2BL-11 and 2BL-2 showed a missing band (B) whereas all the other lines tested showed similar banding pattern irrespective of the deletions (Figure 4.7). In the line 2AL-2, known to be lacking an allele of the SBE II gene by the RFLP analysis, no missing bands were observed after amplification with the intron 5 primers. This may be due to the production of uniform product sizes from the A and the D genomes. PCR amplification using the intron 10 primers did not show any polymorphism among the lines in both agarose and PAGE gels.

4.3.2.2 Biochemical analysis of CS chromosome 2 deletion stock

Immunological analysis of SBE II protein isoforms from the chromosome 2 microdeletion lines using anti-wheat BE II antibody raised against a synthetic peptide

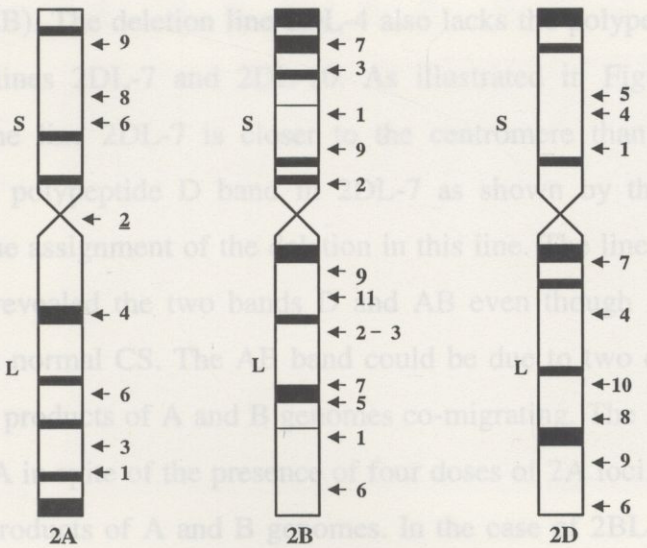


Figure 4.5 C-banding pattern of CS chromosome 2 deletion stock showing the break points. 'S' and 'L' denote the short and long arms of the chromosome respectively. Underlined number at the centromeric position indicates the deletion that lost the entire long arm. The diagram is based on Endo and Gill (1996).

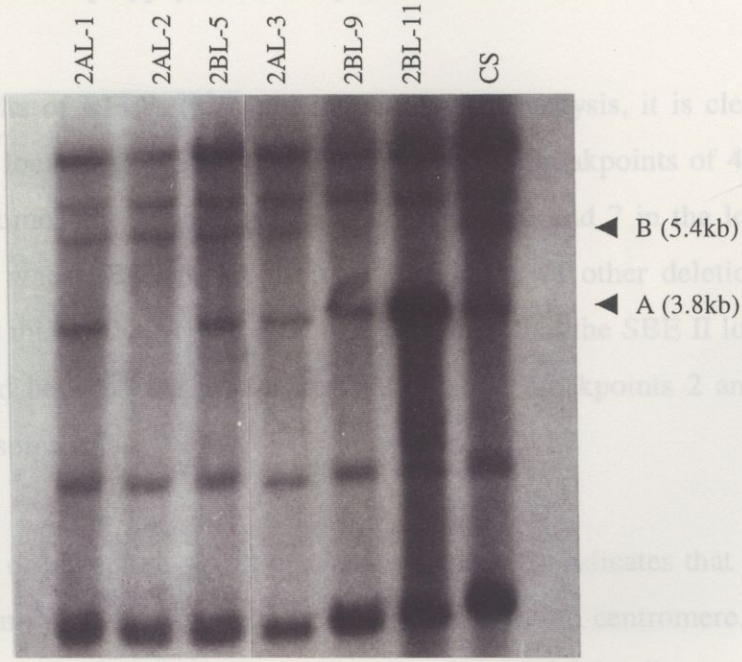


Figure 4.6 Autoradiograph showing polymorphism obtained using RFLP probe sbe9 hybridised to *Eco*R1 digested genomic DNA from CS group 2 chromosome deletion lines.

with sequence similarity to the N-terminal sequence of wheat SBE IIa resolved two distinct bands designated as D and AB (Figure 4.8). The band D represents the SBE II isoform encoded by the D genome as evidenced by its absence from the nulli 2D tetra 2B line (N2DT2B). The deletion line 2DL-4 also lacks the polypeptide D whereas it is present in the lines 2DL-7 and 2DL-10. As illustrated in Figure 4.5, the deletion breakpoint in the line 2DL-7 is closer to the centromere than that in 2DL-4. The presence of the polypeptide D band in 2DL-7 as shown by the immunoblot raises concern about the assignment of the deletion in this line. The line lacking chromosome 2B (N2BT2A) revealed the two bands D and AB even though AB band is of lower intensity than in normal CS. The AB band could be due to two different polypeptides representing the products of A and B genomes co-migrating. The lower intensity of AB band in N2BT2A in spite of the presence of four doses of 2A loci indicates the variable amount of the products of A and B genomes. In the case of 2BL-2 and 2BL-9 lacking one of the SBE II alleles (shown by PCR and RFLP) the AB band was weaker as against 2BL-5, which has stronger AB band indicating the presence of both the alleles. The deletion line 2AL-2 also exhibited a weaker AB polypeptide band which is interpreted to be due to the absence of polypeptide A compared to 2AL-3 line.

Combining the results of RFLP, PCR and immunological analysis, it is clear that the SBE II gene can be located to a point between the deletion breakpoints of 4 and 10 in the long arm of chromosome 2D and between breakpoints 2 and 7 in the long arm of chromosome 2B in wheat. Because of the non availability of other deletion lines of chromosome 2A for this study it could only be established that the SBE II locus on the A genome is located between the broadly spaced deletion breakpoints 2 and 3 of the long arm of chromosome 2A.

The consensus map of hexaploid wheat of Gale et al., (1993) indicates that the SBE II gene on the long arm of chromosome 2 is close (1.3cM) to the centromere. However, discrepancies between physical and genetic maps were observed for distances of loci in relation to the centromere (Lukaszewski and Curtis, 1991). Loci that genetically map close to the centromere are physically localized to more distal chromosome regions due

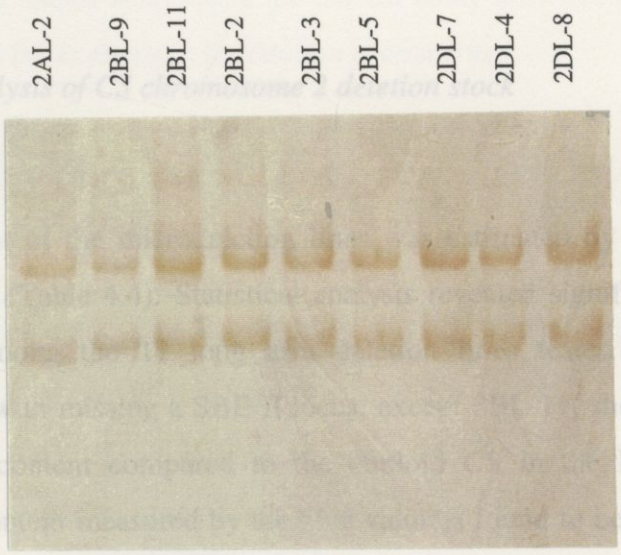


Figure 4.7 Nondenaturing PAGE gel showing the PCR product of CS group 2 chromosome deletion lines using primers specific for intron 5 region of the SBE II gene.

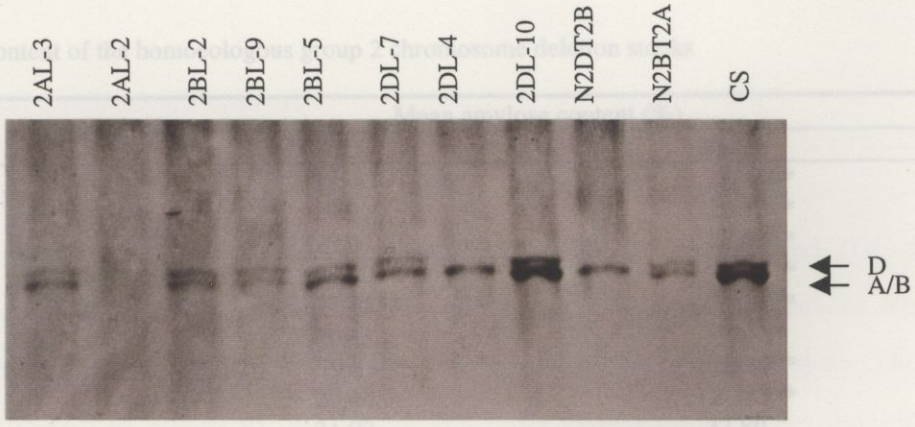


Figure 4.8 Nondenaturing PAGE immunoblot of wheat endosperm from CS group 2 chromosome deletion lines using anti-wheat SBE II antibody. N2DT2A (nulli 2D tetra 2A), N2BT2A (nulli 2B T 2A) and CS were used as controls.

to uneven distribution of recombination. It will be important to map SBE II on high density maps, such as the Synthetic W7984 x Opata85 map (Van Deynze, 1994)

4.3.2.3 Amylose analysis of CS chromosome 2 deletion stock

The amylose content of the microdeletion lines was estimated by the HPLC and the iodometric methods (Table 4.4). Statistical analysis revealed significant differences in amylose content among the 17 long arm deletion lines tested by both methods. However, the lines with missing a SBE II locus, except 2BL-11, showed no significant effect on amylose content compared to the euploid CS in the HPLC whereas the apparent amylose content measured by the blue value is found to be significantly lower in all the deletion lines irrespective of the presence or absence of the SBE II allele. Wheat being a hexaploid crop, the depletion of one protein isoform due to a missing locus can be compensated by the isoforms encoded by the other two alleles. Analysis of GBSS proteins in wheat has revealed that a reduction in either of the Wx-A1 protein or the Wx-D1 protein can be compensated by the more abundant Wx-B1 protein (Miura et al., 1994). Only a decrease in the dosage of the Wx-B1 gene gave a sufficient reduction in Wx-B1 protein to reduce the level of amylose synthesis. While the absence of Wx-B1 gene led to a reduction of amylose content by more than 3%, the absence of Wx-A1 and

Table 4.4 Amylose content of the homoeologous group 2 chromosome deletion stocks

Line	Mean amylose content (%)	
	HPLC	Iodine
2AL-1	22.36**	27.46**
2AL-2[a]	20.92	29.13**
2BL-9[a]	20.47	27.04**
2BL-10	22.10**	25.38**
2BL-11[a]	17.45**	26.38**
2DL-4[a]	20.43	30.32*
2DL-6	19.33**	28.30**
2DL-7[a]	20.40	27.83**
CS	21.03	32.89
CD (0.05)	0.73	1.68
CD (0.01)	1.00	2.69

[a]Lines missing an allele of SBE II
CD: Critical difference
* significantly different from CS at P=0.05
** significantly different from CS at P=0.01

Wx-D1 decreased the amylose content less than 2% (Miura and Sugawara, 1996). However, the SBE II alleles analysed in the current study does not seem to vary among the three genomes as far as amylose production is concerned.

Sidebottom et al., 1998 has reported an increased amylose and reduced high molecular weight amylopectin contents in a low amylopectin maize variety starch due to the absence of a SBE II isoform. Although only one type of SBE II has been reported so far in wheat (Nair et al., 1997), evidence for other forms has been obtained during the course of this study (Chapter 5) as in the case of maize and barley where two types of SBE II, SBE IIa and SBE IIb, are known (Gao et al., 1997, Sun et al., 1998). In several *amylose extender* (*ae*) alleles analysed in maize, normal levels of SBE I and SBE IIa activity were found with the absence of SBE IIb activity, which led to the assumption that *ae* is the structural gene for SBE IIb. It was clear from the phenotype of *ae* mutant kernels that the loss of SBE IIb function could not be compensated for by SBE IIa or SBE I. Independent genetic control, differential expression and catalytic properties of the two types of SBE II have also been established (Takeda et al., 1993a, Gao et al., 1997, Sun et al., 1998). Very recently the SBE II gene located on the homoeologous group 2 chromosomes of wheat was identified to be a SBE IIa type gene. (Rahman et al., in prep). Hence it can be concluded that the current study was focussing on a SBE IIa gene.

4.3.2.4 Amylopectin chain length analysis

The chain length profiles of the microdeletion lines 2AL-2, 2BL-9, 2BL-11 and 2DL-4 were compared with that of CS by subtracting the percentage of total mass present per oligomer in CS from that present in each of the microdeletion lines (Figure 4.9). The amylopectin of 2BL-9 was found to be distinct from the others with a considerable reduction in the proportion of mass in shorter chains of around DP 7-12 and a significant increase in the proportion of mass in longer chains (DP 12-30) compared to

CS. The other three deletion lines had comparatively more contribution from shorter chains, while the longer chain contribution remains the same with respect to CS.

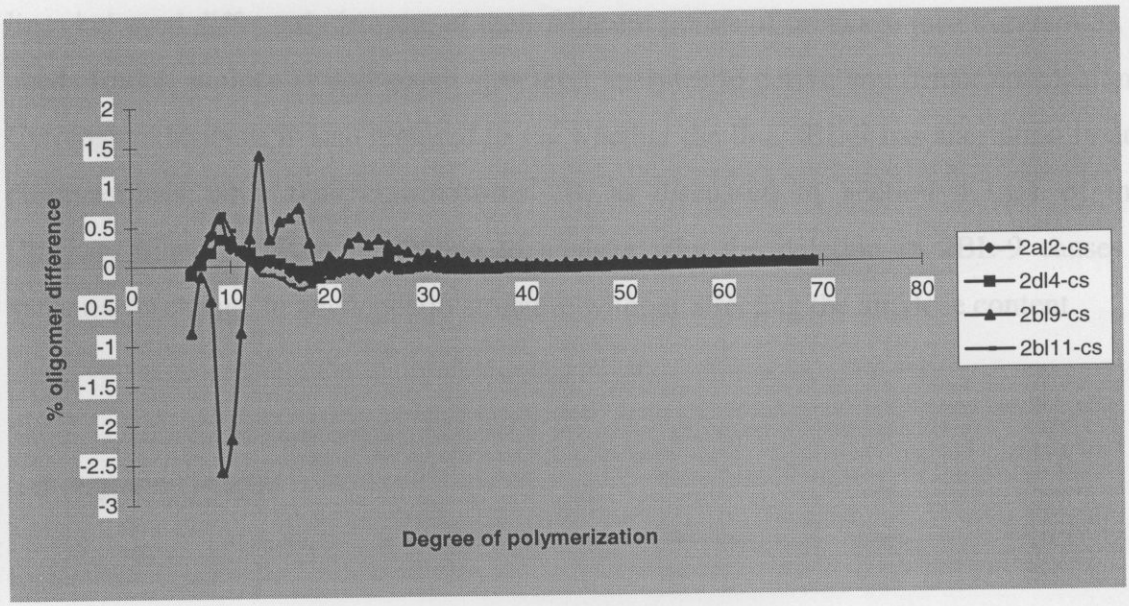


Figure 4.9 Amylopectin chain length profile comparison of chromosome 2 deletion stock starches with respect to that of CS. 2al2-cs indicates that the percentage contribution for each oligomer of CS subtracted from the percentage contribution for the corresponding oligomer of 2AL-2. Similarly 2dl-4-cs represents 2DL-4 compared with CS, 2bl-9-cs represents 2BL-9 compared with CS, and 2bl11-cs represents 2BL-11 compared with CS.

The result clearly revealed an altered amylopectin structure in 2BL-9. The effect of *ae* genotypes which lack the SBE IIb isoform on amylopectin to produce abnormal types with longer branches has been well documented (Kasemsuwan et al., 1995, Mizuno et al., 1993, O'Shea and Morell, 1996, Hashemi et al., 1997). Distinct differences in chain length distribution of products produced by the action of wheat SBE I and SBE II on synthetic amylose are demonstrated by Hashemi et al., (1997) and their results suggested that SBE II is required to attain the chain length distribution found in normal starch fine structure and cannot be complemented by the presence of SBE I.

Although all the deletion lines used for the current chain length study lack a SBE IIa isoform, only 2BL-9 exhibited a significantly altered amylopectin structure, which leads to the assumption that there is a second influencing factor missing from 2BL-9 which is present in the other deletion lines. This could be assigned to a region in between the deletion break points of 2BL-9 and 2BL-11 due to the fact that the starches of these two lines behaved differently in spite of their adjacent points of breakage (see Figure 4.5). It needs further molecular and starch structural analyses to derive any firmer conclusions. Cytological analysis is also required to see whether the line 2BL-9 has aberration in any chromosomes other than chromosome 2B, as discussed in Section 4.3.1.1 of this Chapter. It will also be interesting to analyze why the deletion in 2BL-9 causes a measurable change in amylopectin structure without affecting the amylose content.

4.4 CONCLUSION

Analysis of CS ditelosomic lines revealed significant alterations in amylose content compared to euploid CS due to deletions of specific chromosome arms. The lines dt5DL exhibited the highest amylose content and the line dt6AS the lowest. The increase in amylose content was accompanied by changes in amylopectin chain length and starch granule distributions, and paste viscosity in dt5DL starch. The CS homoeologous group 2 chromosome deletion stock analysis did not reveal any significant effect on amylose content due to a missing allele of SBE IIa. Aneuploid lines lack either parts of chromosomes or complete arms of chromosomes or whole chromosomes. In all of these cases there is the possibility of missing many genes which make the interpretation of the results difficult. The hexaploid nature of wheat adds to the complexity since the phenotypic effects due to missing allele may be compensated by presence of other alleles.

CHAPTER 5

Starch Branching Enzyme Analyses

5.1 INTRODUCTION

Starch biosynthesis is a complex process that has attracted increasing interest over the last few years. Amylose and amylopectin, the two major polymers of starch and their individual structures are determined by the concerted action of at least four known classes of enzymes, ADPglucose pyrophosphorylase (EC 2.7.7.27), starch synthase (EC 2.4.1.21), branching enzyme (EC 2.4.1.18) and debranching enzyme (EC 3.2.1.41). Although various models have been proposed for the possible mechanisms of starch biosynthesis (reviewed in James et al., 1995 and Nakamura et al., 1996b), the fine details of amylose and amylopectin biosynthesis are still unclear. More recent proposals that amylopectin is synthesized by processing a more highly branched intermediate and amylose is synthesised downstream from amylopectin (Mouille et al., 1996, Vandewal et al., 1998) will be rigorously tested over time.

The final branching structure of the starch polymers appears to be determined, at least in part, by a balance between the activities of debranching and branching enzymes (Kim et al., 1998). While debranching enzyme hydrolyses the α-1,6 glycosidic linkages, starch branching enzymes (SBE) catalyze a transferase reaction in which a donor linear α-1,4-glucan chain (either amylose or linear regions of amylopectin) is cleaved and transferred to a recipient chain via the synthesis of an α-1,6 linkage (Morell et al., 1997). Multiple isoforms of SBE are reported in plants, and these are generally classified into two distinct enzyme families SBE I and SBE II, based on structural features and sequence differences (Burton et al., 1995). Evidence from biochemical studies and the characterization of mutants suggested that there are significant differences between SBE I and II with respect to substrate specificities, modes of action and physiological roles. For example, the SBE I group of enzymes, in general, have a higher affinity for amylose

than SBE II groups and preferentially transfer longer chains than SBE II enzymes (Takeda et al., 1993a).

High amylose mutants identified in maize, rice and pea were found to be deficient in the SBE activity analogous to maize SBE II (Boyer and Preiss, 1978, Morell et al., 1995, Mizuno et al., 1993, Sidebottom et al., 1998). Two different forms of SBE II that differ slightly in sequence and catalytic properties have been identified in maize and barley (Boyer and Preiss, 1978, Kim et al., 1998, Sun et al., 1998). It has been demonstrated that maize kernels homozygous for the recessive amylose extender (*ae*) allele lack the SBE IIb activity resulting in a high amylose phenotype (Hedman and Boyer, 1982).

In wheat, multiple copies of SBE I type genes have been reported and assigned to group 7 chromosomes (Morell et al., 1997, Rahman et al., 1997, Rahman et al., 1999). Only one form of SBE II has so far been reported in wheat (Morell et al., 1997, Nair et al., 1997). This chapter deals with the studies undertaken to detect polymorphisms in SBEs in *Triticum* species and isolation of variants of SBE II from *Triticum tauschii*, the D-genome donor to wheat.

5.2 MATERIALS AND METHODS

5.2.1 Plant materials

Triticum tauschii lines used for the analyses are detailed in Table 5.1. Seeds of these lines were kindly provided by Dr Fred Stoddard, University of Sydney. Hexaploid and tetraploid wheat cultivars/lines were taken from the germplasm collection of Plant Breeding Institute, Cobbitty. Plants were grown in pots under uniform growth conditions and ears snap frozen 25d after anthesis.

Table 5.1 Details of *T. tauschii* lines used for Western blot analyses of starch branching enzymes

Accession No.	Origin	Sub species
AUS24104	Afghanistan	*typica*
AUS24115	Afghanistan	*typica*
AUS24121	Afghanistan	*typica*
AUS24241	Russia	*typica*
AUS24190	Iran	*typica*
AUS24035	"unknown"	"unknown"
AUS24122	Iran	*typica*
AUS24192	Iran	*typica*
AUS24238	Russia	*typica*
AUS24050	Russia	*typica*
AUS24034	Japan	"unknown"
AUS24207	Afghanistan	*typica*
AUS24043	"unknown"	"unknown"
AUS24053	"unknown"	*anathera*
AUS24171	Iran	*typica*
AUS24210	Afghanistan	*typca*
AUS24090	Afghanistan	*typica*
AUS24242	Russia	*typica*
AUS24092	Afghanistan	*typica*
AUS23986	Iran	"unknown"
AUS24230	Russia	*Typica*
AUS23964	Turkey	"unknown"
AUS23995	Russia	"unknown"
AUS24089	Afghanistan	*typica*
AUS24095	Afghanistan	*anathera*
AUS23992	Japan	"unknown"

5.2.2 Starch branching enzyme analyses

The protocols described in Section 4.2.6 were followed for protein extraction, electrophoresis, and immunoblotting of starch branching enzymes I and II.

5.2.2.1 Two-dimensional gel electrophoresis of SBE II

First dimensional gel was cast on 9 x 8cm^2 gel plates with a 10ml volume of 6% acrylamide gel and 5ml volume of stacking gel. The separating gel composition was 1M Tris-HCl pH 8.8, 0.05% CHAPS, 10% glycerol, 6% acrylamide 37.5:1, 0.07% TEMED, and 0.05% APS. The stacking gel was composed of 1M Tris-HCl pH 6.8, 0.05% CHAPS, 6% acrylamide 37.5:1, 0.07% TEMED and 0.05% APS. Samples were prepared as in Section 4.2.6.1. The required volume of sample to load 35µg of protein was mixed with 1/10th volume of loading buffer (80% glycerol, 1% BPB. The gel was run for 2-3 hours at 138V and 4°C constant temperature in the running buffer described in Section 4.2.6.2.

The second dimension gel was cast the same way as described in Section 4.2.6.2 except that the separating gel contained 6% acrylamide 37.5:1 and 0.08% amylopectin. The stacking gel was poured to 1cm below the top edge of the plates and the top portion was filled with running buffer (section 4.2.6.2). No wells were created in the stacking gel. After the first gel was run the plates were carefully separated and the bottom 1.5cm portion from the gel was discarded. The next 4.3cm strip from each lane of the gel was removed and inserted horizontally into the running buffer on top of the stacking gel of the second dimension gel. Three strips were placed on one gel. Following running overnight at 138V and 6°C in running buffer as above, the gel was stained.

The gel was fixed in 50% methanol, 12% acetic acid and 0.04% formaldehyde for 1h. Following fixing, the gel was washed thrice in 50% ethanol, 20 min each time. The gel was pretreated with 0.02% sodium thiosulphate for 1 min followed by rinsing with distilled water thrice, 20 min each time. Staining of gel was done for 20 min in 0.2% silver nitrate and 0.03% formaldehyde followed by rinsing twice with distilled water. The gel was developed in 6% sodium carbonate solution containing 0.05% formaldehyde and 0.0004% sodium thiosulphate until the bands were sufficiently visible. Following rinsing with distilled water for 2 min twice, the development was

stopped with stopper solution (50% methanol and 12% acetic acid). Finally the gel was washed with 30% methanol for 1 hour.

5.2.3 Screening of *Triticum tauschii* genomic library

A genomic DNA library constructed in λGEM 12 (Promega) system with *Sau*3A partially digested genomic DNA from *T. tauschii* var. *strangulata* (CPI accession No.110799) was prepared by Dr S Rahman, CSIRO Plant Industry and the screening was conducted at CSIRO Plant Industry, Canberra.

5.2.3.1 Plating of genomic DNA library

Ten petri dishes containing LB agar medium were plated with 80µl amplified genomic library, 150µl PMC 103 strain of *E. coli* cells, 4ml of LB liquid medium and 4ml of melted and cooled (about 50°C) top agar per plate under a laminar flow hood. The plates were incubated at 37°C overnight followed by chilling at 4°C for at least one hour. It was estimated that each plate contained 5 x 10^4 plaques.

Hybond N+ membrane filters were placed on top of the plaques in each plate. The filters were then marked in three asymmetric locations by stabbing through it and into the agar beneath with a 10-gauge needle. When the filters were thoroughly wetted (1-2 min), they were peeled from the plates, numbered and placed numbered side up, on top of 2 layers of 3MM filter paper. When all the plates were processed, the membranes were washed in 1 x denaturing buffer (1.5M NaCl, 0.5M NaOH) for 2 min, neutralized in two changes of neutralizing buffer (1.5M NaCl, 0.5M Tris-HCl, pH 7.0, 0.01M EDTA) for one min each time, washed in 5x SSC for 2 min and finally blotted dry between two layers of 3MM filter paper. The filters were then fixed by baking for 2h.

5.2.3.2 Screening by DNA hybridization

The filters were soaked briefly in 2x SSC and then transferred, along with a 3MM filter paper cut to the size of the membranes on top of them, to a hybridization container containing enough prehybridization buffer to just cover the membranes and incubated at 42°C for at least 4 hours with gentle shaking. The prehybridization solution contains 50mM Tris-HCl pH 7.5, 1M NaCl, 25% formamide, 10x Denhardt's solution, 1% SDS and 0.1mg/ml herring sperm DNA. Starch branching enzyme probe DNA supplied by Dr. S. Rahman was labelled with ^{32}P by an Amersham (USA) kit. Probe DNA (20ng) was combined with 6.0µl of random oligonucleotides (provided by the manufacturer). This mixture was incubated at 95°C for 5 min to denature the DNA and quickly chilled on ice. Then 10µl probe labeling buffer (0.5M HEPES, 0.125M Tris-HCl, 12.5mM DTT, 12.0mM $MgCl_2$, 1.0mg/ml BSA), 2.5µl dNTP mixture (0.2mM dATP, 0.2mM dTTP, 0.2mM dGTP), 1.0 U Klenow fragment, 3.0µl ^{32}P-dCTP (10 Ci/l), and enough milliQ water were added to make the total volume 25µl. Probe labeling reactions were incubated at 37°C for 1h. To remove the unincorporated dNTPs, the probe was purified by passing through a Sephadex® G-50 NICK®Column (Pharmacia) according to manufacturer's instructions.

Following prehybridization the labeled probe was denatured in a 95°C water bath for 5 min and chilled rapidly on ice. This was added to the filters along with prehybridization buffer containing 10% dextran sulphate and 1% sodium pyrophosphate, and the hybridization was allowed to occur at 42°C for 16 hours. On completion of hybridization, the hybridization solution was removed and the filters were immediately washed in prewarmed 2 x SSC, 0.1% SDS briefly at 65°C. This was followed by two consecutive washes in 0.2 x SSC, 0.1% SDS for 15 min each. After washing, the excess liquid was removed by blotting on Whatman 3MM filter paper. The filters were then placed between two sheets of plastic wrap in a cassette and exposed to X-ray film (Kodak) overnight at -80°C with an intensifying screen.

Following the development of the film, the film and filters were lined up using mark numbers and the asymmetrically located needle holes. The positive plaques were identified by locating the origin of signals in the stock plates. Using an inverted pasteur pipette, the portion of the stock plate where the putative clone lined up with the film spot was cut and transferred to 1ml of SM buffer (5.8g/l NaCl, 2.0g/l $MgSO_4.7H_2O$, 50.0mM Tris-HCl pH 7.5, 0.01% gelatine) and 20µl of chloroform and mixed well by vortexing. Plating and hybridization were repeated using appropriate dilution of the phage suspension following the same procedure as that of the first plating until 50-100 individual plaques were obtained per plate and individual plaques could be clearly picked.

5.2.3.3 Purification of λ phage DNA

A single colony of PMC 103 was grown in 10ml LB + 10mM $MgSO_4$ and 0.2% maltose overnight at 37°C. The culture (50µl) was added to 2.5ml of top agar at 48°C, mixed well and laid on top of the bottom agar in a 90mm petri dish. A few (1-6) drops of the purified λ phage were added onto the solidified top agar and the plate was incubated overnight at 37°C. One 5mm diameter plaque or five 1mm plaques were transferred to 1ml of SM buffer. The overnight culture (50µl) of PMC 1103 was mixed with 10, 50 or 250µl of eluted phage particles and incubated at 37°C for 30 min. Each bacterium phage mixture was transferred to a 250ml flask containing 20ml LB medium and 0.2% maltose and incubated at 37°C with shaking. Then 4 drops of chloroform were added to the flask which contained lysed cultures. The culture was poured into a Corex tube and spun at 10,000g at 5°C for 10 min. The supernatant was transferred into a fresh tube and incubated at 37°C for 1h after adding 20µl DNase (10mg/ml) and 20µl RNase (10mg/ml). This was followed by addition of 1/4 volume of 25% PEG_{8000} and incubation on ice for at least one hour prior to centrifugation at 10,000 rpm at 5°C for 10 min. The pellet was resuspended in 500µl SM buffer. On addition of 3µl of DNase (10mg/ml) and 3µl RNase (10mg/ml), the phage DNA was incubated at 37°C for 30 min, followed by the addition of 0.5% SDS and 5µl proteinase K (10mg/ml). The tubes were incubated at 37°C for a further 30 min. After adding 500µl phenol and 200µl

chloroform and mixing, centrifugation was carried out for 5 min. The upper phase was transferred to a fresh tube and phenol-chloroform extraction was repeated. Following a final extraction with 500µl chloroform the phage DNA was precipitated with 2 volumes of ethanol and $1/10^{th}$ volume of sodium acetate. The pellets were washed with 500µl of 70% ethanol, dried briefly in a Speedvac and resuspended in 20µl TE.

5.2.3.4 Transformation of E. coli with plasmids

The genomic clones were digested with *Bam*H1 and *Eco*R1 and the fragments were ligated into linearized and dephosphorylated pUC19 vector according to Sambrook et al., (1989). Transformation of competent cells (JM 101) with plasmid was performed by either heat shock at 42°C for 50 seconds or by high voltage electroporation according to recommendations supplied with a Gene-Pulser (BioRad). Competent cells (50µl) were combined with 5µl deionized water containing 60ng DNA from a ligation reaction. This mixture was transferred to pre-chilled disposable Gene-Pulser®/E. coli Pulser™cuvette, 0.2cm (BioRad), and after electroporation, the cells were mixed with 600µl LB broth without antibiotic, transferred into a new eppendorf tube and incubated at 37°C for half a hour. Aliquots of 100µl of the culture were then placed onto a plate of solid LB media supplemented with ampicillin (100µg/ml), 40µl of a stock solution of X-gal (20mg/ml in dimethylformamide) and 4µl of a solution of isopropylthio-β-D-galactoside (IPTG) (200mg/ml) and grown at 37°C overnight. Positive white colonies were selected for recombinant plasmid extraction.

5.2.3.5 Purification of plasmid DNA for DNA sequence determination

A single colony was transferred to 2ml of Terrific Broth (TB) medium containing ampicillin (100µg/ml) and incubated overnight at 37°C. One litre of TB medium was made by mixing 100ml of a sterile solution of 0.17M KH_2PO_4, 0.72M K_2HPO_4 and 900ml of sterile, cool solution containing 12g Bacto-tryptone, 24g Bacto-yeast extract and 4ml glycerol. Aliquots (1.5ml) of the culture was pelleted by centrifugation for 1

min in a microcentrifuge. The supernatant was removed and the bacterial pellet was resuspended in 200µl of GET buffer (50mM glucose, 10mM Na_2EDTA, 25mM Tris-HCl, pH 8.0) by repeated pipetting and 300µl of freshly prepared lysis buffer (0.2N NaOH and 0.1% SDS) was added and mixed by inversion and then incubated on ice for 5 min. This solution was neutralized by adding 300µl of 3.0M potassium acetate (pH 4.8) and mixed by inverting the tube, and incubated on ice for 5 min. The cellular debris were removed by centrifugation for 10 min at room temperature, and the supernatant was transferred to a clean tube. RNaseA (Sigma, USA) was added to a final concentration of 20µg/ml and incubated at 37°C for 20 min. The supernatant was extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The plasmid DNA was precipitated by adding an equal volume of 100% isopropanol and pelleted by centrifugation for 10 min at room temperature. The DNA pellet was washed with 700µl of 70% ethanol and then dried in a Speedvac. The pellet was dissolved in 32µl of dH_2O and then precipitated by adding 8.0µl of 4M NaCl and 40µl of autoclaved 13% PEG_{8000}. After thorough mixing, the sample was incubated on ice for 20 min, and then the plasmid DNA was pelleted by centrifugation for 15 min at 4°C. The supernatant was carefully removed and the pellet was rinsed with 700µl of 70% ethanol. The DNA pellet was dried in a Speedvac and resuspended in 20µl of dH_2O.

5.2.4 Sequencing and sequence analysis

Sequencing was performed on an ABI sequencer at the CSIRO Division of Plant Industry, Canberra or the Australian Genome Research Facility, University of Queensland. Sequence analysis was carried out using Genetics Computer Group (GCG) package within ANGIS (Australian National Genomic Information System). Genebank/EMBL databases were searched for homologous sequences.

5.2.5 Genomic DNA analysis

Southern blot of SBE I and II genes referred to in this Chapter are explained elsewhere

(Sections 4.2.4). PCR amplification of SBE II gene using the primers sr913F and E6R is described in Section 4.2.5. The SBE I primers sr406F (5' TGGCGCCGGAGACGC AACGG 3') and sr412R (5' TGACGAAGGCTGAAAAGTGGC 3') and the SBE IIa primers, DA12 (5' AGTGACTCTGGTCGTTTAGG 3'), DA13 (5' CCCTTACAGAG TAAGAACCAG 3'), sr854.1180F (5' CTGGCTGACTCAATCACTACG 3'), sr866F (5' TATCTTCAGGTATCTACAGC 3'), sr2370, sr2700, sr861F (5' CAGACCTTGTC ACCATATATGC 3') and sr862R (5' TGGCAACACAATTTG ATGCC 3') were kindly provided by Dr. S. Rahman, CSIRO Plant Industry, Canberra. A 20µl PCR reaction mixture contained 80µM dNTP, 1 x PCR buffer (Advanced Biotechnologies), 2mM $MgCl_2$, 5pmol of each of the primers, 50ng template DNA and 1 unit of *Taq* polymerase. PCR was conducted with an initial denaturation step of 94°C for 2 min followed by 36 cycles of 94°C for 1 min, 55°C for 30 sec and 72°C for 30 sec, one cycle of 72°C for 5 min, and finally holding at 25°C.

5.3 RESULTS AND DISCUSSION

5.3.1 Detection of polymorphisms in SBEs

5.3.1.1 Analysis of SBE I

Nondenaturing PAGE immunoblot analysis using a primary antibody raised against a synthetic peptide designed from the amino acid sequence at N-terminus of wheat SBE I was performed on a total of 40 lines of various species of *Triticum*. In general, the analysis of hexaploid wheat cultivars revealed the presence of four SBE I polypeptides (Figure 5.1). A previous report has shown that in hexaploid wheat CS the top band corresponds to polypeptide A encoded by chromosome 7A, the middle two bands, polypeptides D-i and D-ii, by the chromosome 7D and the bottom band, polypeptide B,

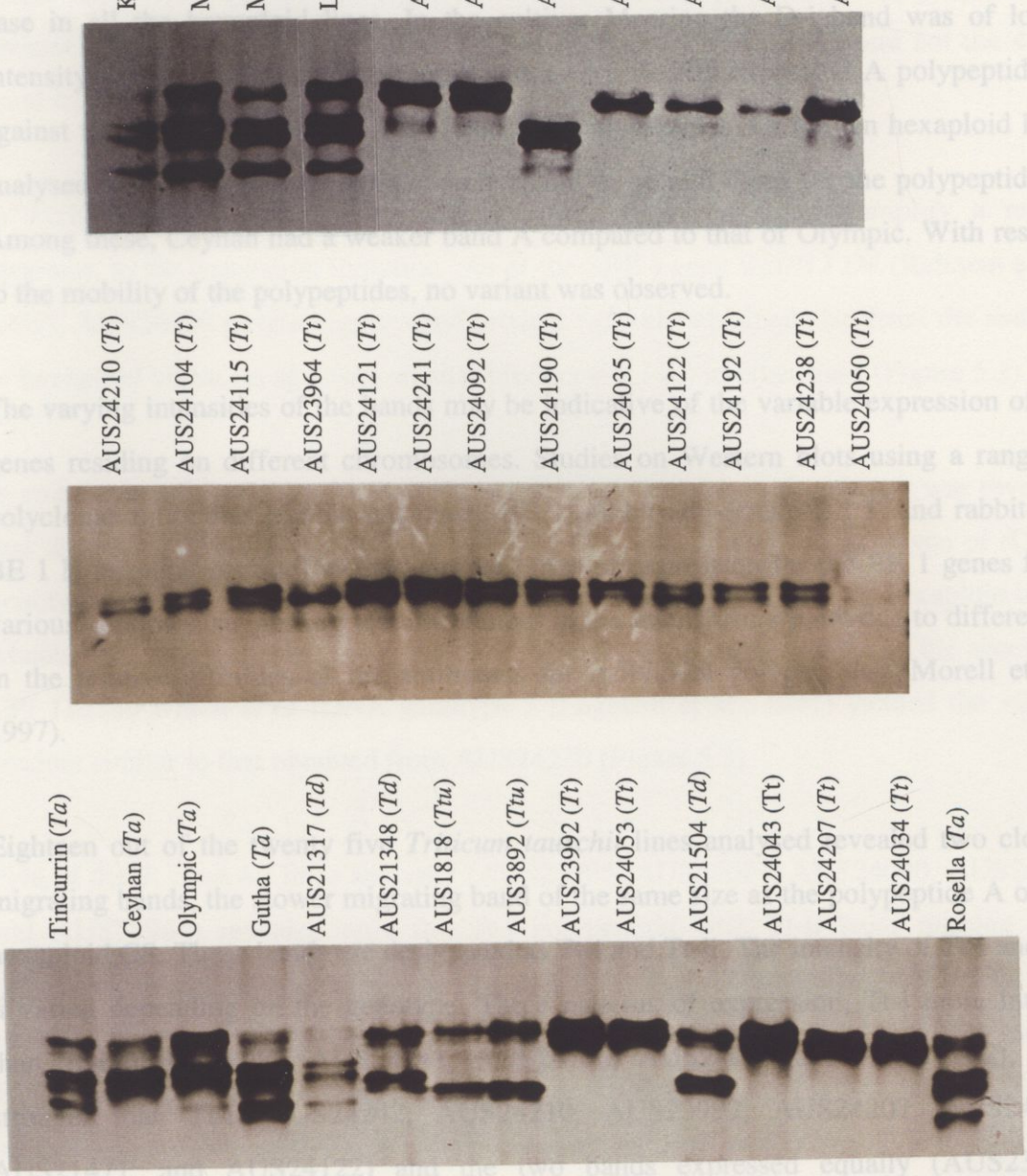


Figure 5.1 Nondenaturing PAGE immunoblot of wheat endosperm samples using a primary antibody raised against synthetic peptide designed from an amino acid sequence at N-terminus of wheat SBE 1. *Ta*: *Triticum aestivum* (hexaploid), *Tt*: *Triticum tauschii* (diploid), *Td*: *Trticum dicoccoides* (tetraploid) and *Ttu*: *Triticum turgidum* (tetraploid) The bands are designated from top to bottom as A, D-i, D-ii and B (as in Figure 4.3) in all hexaploid lines, Tt-i and Tt-ii in all the diploid lines except AUS24230, D-i and D-ii in AUS24230, and A and B in all the tetraploid lines.

by 7B (Morell et al., 1997). The location of gene/s for D-i and D-ii polypeptides on the long arm of chromosome 7D is confirmed by the ditelosomic analysis reported in Chapter 4 (Section 4.3.5, Figure 4.3). The relative intensities of the bands varied among the genotypes. Although an expression level in the order of SBE-1_{Di} >SBE-1_B >SBE-1_A >SBE-1_{Dii} was observed in CS (Figure 4.3) supporting the earlier report, this was not the case in all the hexaploid lines. In the cultivar Meering the D-i band was of lower intensity than D-ii. The cultivar Gutha had a very weakly expressed A polypeptide as against the strongly expressed B, D-i and D-ii polypeptides. Of the ten hexaploid lines analysed two, Ceyhan and Olympic were found to be null types for the polypeptide B. Among these, Ceyhan had a weaker band A compared to that of Olympic. With respect to the mobility of the polypeptides, no variant was observed.

The varying intensities of the bands may be indicative of the variable expression of the genes residing on different chromosomes. Studies on Western blots using a range of polyclonal antibodies (rabbit anti-maize BE 1, rabbit anti-potato BE 1, and rabbit and BE 1 N-terminal peptide) yielded similar ratios of expression for the BE 1 genes from various genomes, suggesting that differences in the intensities are not due to differences in the relative affinities of the antibodies for individual polypeptides (Morell et al., 1997).

Eighteen out of the twenty five *Triticum tauschii* lines analysed revealed two closely migrating bands, the slower migrating band of the same size as the polypeptide A of the hexaploid CS. These bands are designated as Tt-i and Tt-ii. The intensity of Tt-i and Tt-ii varied depending on the genotype. Three patterns of expression, Tt-i more intense than Tt-ii (AUS24115, AUS24241, AUS24190, AUS24035 and AUS24192), Tt-ii stronger than Tt-i (AUS24242, AUS24210, AUS23992, AUS24207, AUS24034, AUS21471, and AUS24122) and the two bands expressed equally (AUS23986, AUS24043, AUS24104, AUS24121, AUS24092 and AUS24238) were noticed. AUS24230 is the only diploid line with bands of similar mobility to the D genome encoded D-i and D-ii bands of the hexaploid CS. It is not clear whether D-i and D-ii are products of separate genes or whether one is derived from the other through post transcriptional or post translational modification (Morell et al., 1997). Similarly the

relationship of Tt-i and Tt-ii polypeptides is also not clear. Southern blot analysis using the region encoding exons 3, 4 and 5 of the SBE I gene *wSBE I-D2* (E 7.8) as probe (Rahman et al., 1997, 1999) revealed two types of banding patterns among the *T. tauschii* lines on *Bam*H1 digest (Figure 5.2). Pattern 1 with a major 4.8kb band is exhibited by the lines with the Tt-i and Tt-ii polypeptides whereas pattern 2, with a major 1.2kb band, was observed only in AUS24230 where D-i and D-ii polypeptides are present. Hexaploid wheat carries the 1.2kb band seen in AUS24230 and not the 4.8kb band from the other lines.

In PCR amplification using primers sr412R and sr406F which amplify a region upstream to the translation initiation site of the SBE I gene *wSBE I D4* (Rahman et al., 1999), AUS24230 gave a bigger sized product (~2.4kb) obtained also from the analysis of hexaploid wheat, as against a smaller product (~2.1kb) in other lines (Figure 5.3).

In an analysis of the *Nor-D3* locus of *T. tauschii* by Lagudah et al., 1991, it was revealed that out of the 20 different genotypes of rDNA identified, only one genotype of rDNA, genotype 7, carries the allele similar to the *Nor-D3a* allele present in all hexaploid bread wheats. PCR amplification using the SBE I primers sr412R and sr406F of the accession CPI 110799 which is of rDNA genotype 7 (Lagudah et al., 1991) yielded the ~2.4kb product similar to that obtained from AUS24230 (Figure 5.3).

Five of the *T. tauschii* lines analysed (AUS23995, AUS24089, AUS24090, AUS24095 and AUS23964) revealed only the Tt-i polypeptide with Tt-ii band missing. The expression of the Tt-i band was stronger in all these lines except AUS24090. It is possible that the weaker intensity of the band in AUS24090 is due to the use of immature endosperm for analysis. It has been demonstrated in wheat that the SBE I proteins (both WBE-1AD and WBE-1B in hexaploids) are not detectable until 18 d after anthesis and increase in relative expression late in endosperm development (Morell et al., 1997). A similar report comes from pea where the maximum expression of SBE II (analogous to the maize SBE I type protein) occurs later in the embryo development than the SBE I protein (Burton et al., 1995). No lines missing the Tt-i polypeptide were

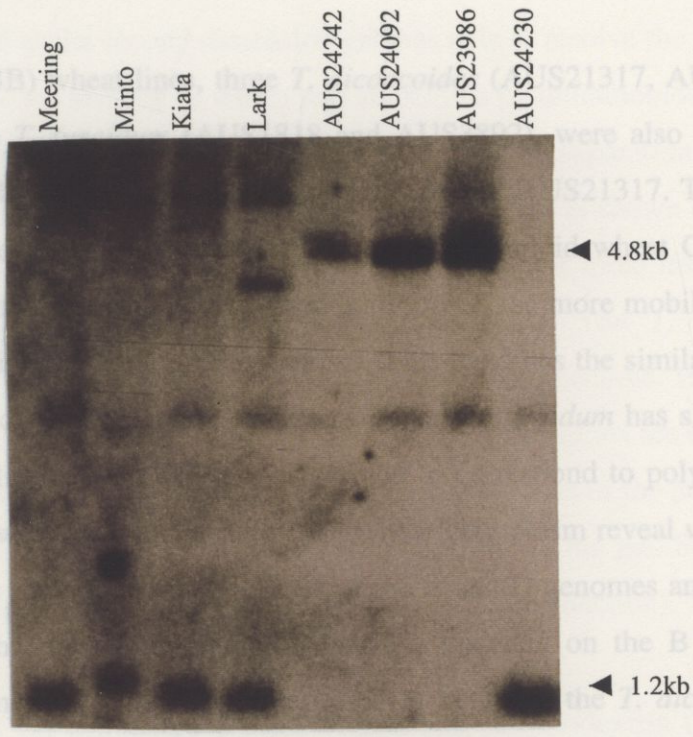


Figure 5.2 Autoradiograph showing polymorphism among *Triticum* species detected by the probe E7.8 specific for SBE I of wheat.

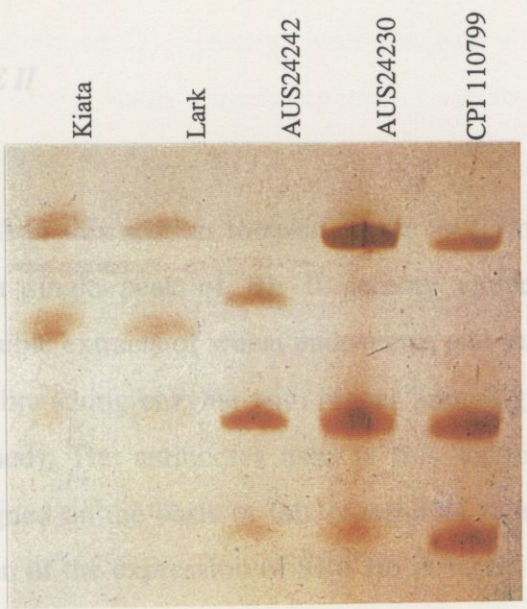


Figure 5.3 Comparison of banding patterns among *Triticum* species amplified with primers sr412R and sr406F specific for wheat SBE I gene as detected on a denaturing PAGE gel.

observed. Variation in the SBE I polypeptide among the *T. tauschii* lines was not related to geographic origin or sub species classification (see Table 5.1).

Five tetraploid (AABB) wheat lines, three *T. dicoccoides* (AUS21317, AUS21348 and AUS21504) and two *T. turgidum* (AUS1818 and AUS3892), were also studied. Two immunoreactive bands were revealed in all the lines except AUS21317. The first band (band A) corresponded to the polypeptide A from the hexaploid wheat CS and hence may be encoded by the A genome. If this logic is followed, the more mobile band (band B) in *T. dicoccoides* is encoded by the B genome. This band has the similar mobility to the D-ii polypeptide of CS. The corresponding band in *T. turgidum* has slightly higher electrophoretic mobility. None of the bands seemed to correspond to polypeptide B of hexaploid wheat. However, extensive studies of wheat germplasm reveal wide diversity in the electrophoretic mobility of the products of the B and D genomes and therefore it is highly probable that the faster migrating band is encoded on the B genome (M. Morell, personal communication). It is interesting to note that the *T. dicoccoides* line AUS21317 showed a banding pattern very similar to hexaploid wheat. A misclassification is unlikely in the case of AUS21317, since phenotypically the line resembled a typical *T. dicoccoides* line.

5.3.1.2 Analysis of SBE II

The analysis of SBE II in this section focuses on the isoform SBE IIa. Morell et al., (1997) showed that a single peak of BE II activity eluted from anion exchange chromatography of soluble extracts of wheat endosperm and that this peak yielded a N-terminal sequence for branching enzyme with higher homology to maize SBE IIa than IIb (Morell, unpublished). The antibodies used in this section were raised against a synthetic peptide designed on the basis of this N-terminal sequence (see Materials and Methods). The question of the expression of SBE IIb in wheat endosperm has not been directly addressed in this work although the available information suggests it is either not present in the soluble fraction or only very weakly expressed relative to SBE IIa (M. Morell, personal communication).

The nondenaturing gel protocol followed for the separation of SBE I proteins did not resolve the SBE IIa proteins encoded by the different genomes. A two dimensional gel using a standard non-denaturing gel in the first dimension and an amylopectin containing affinity gel as the second dimension gel was able to resolve the proteins from the different genomes, the identity of which was confirmed by immunoblotting followed by detection using the antibody mentioned above. Typical separation of SBE IIa polypeptides in hexaploid and diploid (*Triticum tauschii*) wheat is shown in Figure 5.4. As expected for a hexaploid wheat, the cultivar Minto revealed three immunoreactive signals, probably encoded by the three different genomes. The *T. tauschii* line AUS24092 showed only one protein that is encoded by the D genome. However, only two bands are resolved in the hexaploid cultivar CS. The possibilities are either that CS is carrying a null allele for one of the SBE IIa polypeptides or two of the polypeptides in CS are of the same size and affinity, and hence comigrating in the gel. Further studies were supportive of the second suggestion (discussed later in this Section).

A 6% nondenaturing gel containing β-limit dextrin and α-cyclodextrin (see Materials and Methods) yielded a high resolution of SBE IIa polypeptides in a single dimension gel. This protocol was used to screen a total of 36 wheat cultivars/lines which included diploids, tetraploids and hexaploids (Figure 5.5). All 24 *T. tauschii* lines screened revealed a single band (band D) agreeing with the two dimensional gel analysis. Although the intensity of the bands varied, suggesting variability in expression of the encoding genes depending on the genotypes, the diversity is less than that in SBE I. Neither null types for SBE IIa nor any variants with respect to mobility could be identified among the *T. tauschii* lines.

The *T dicoccoides* and the *T. turgidum* lines analysed revealed two immunoreactive proteins (bands A and B) as expected for a tetraploid species, with the exception of AUS21317. The mobility of both the polypeptides in *T. turgidum* lines is slightly higher than those in the *T. dicoccoides* lines. Although the bands were designated as A and B it is not certain which is the genome encoding each of them. The banding pattern of the *T. dicoccoides* line AUS21317 resembled that of a hexaploid wheat. A similar observation regarding this line was also made for SBE I.

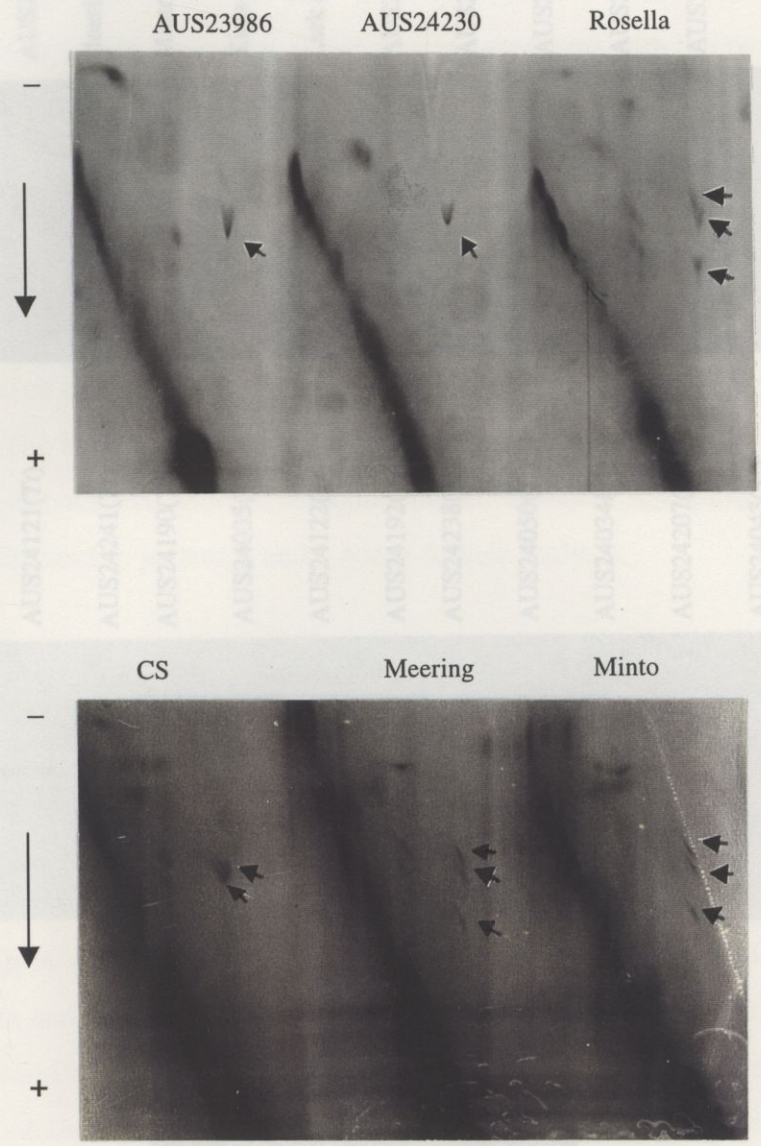


Figure 5.4 Two dimensional PAGE gel showing the separation of SBE II polypeptides in *Triticum* species. The orientation of electrophoretic separation in the second dimension gel is indicated by the arrows.

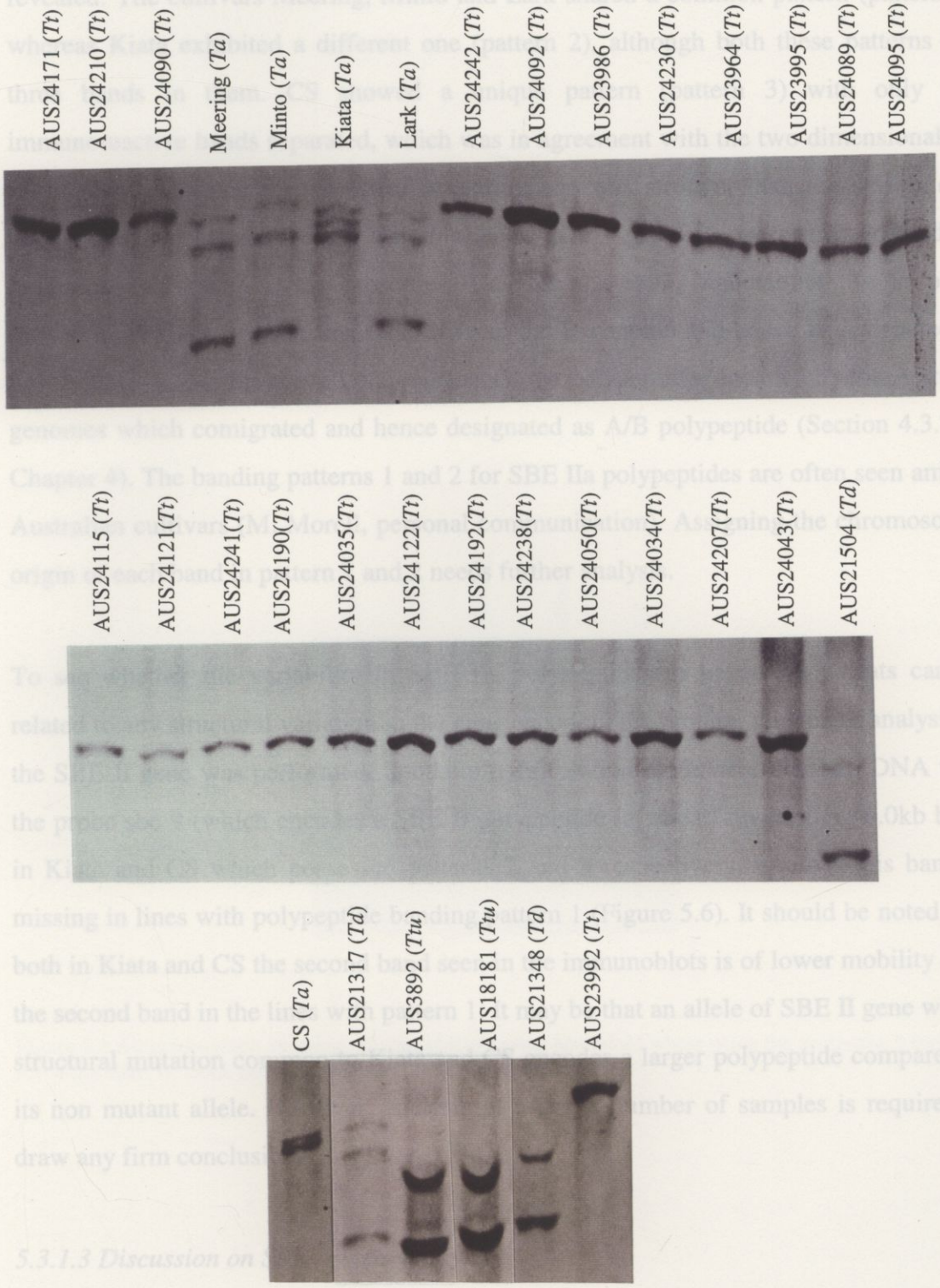


Figure 5.5 Nondenaturing PAGE immunoblot of wheat endosperm using a primary antibody raised against a synthetic peptide designed from the amino acid sequence at the N-terminus of wheat SBE IIa. *Ta*: *Triticum aestivum* (hexaploid), *Tt*: *Triticum tauschii* (diploid), *Td*: *Triticum dicoccoides* (tetraploid) and *Ttu*: *Triticum turgidum* (tetraploid).

Among the five hexaploid cultivars analysed three different banding patterns were revealed. The cultivars Meering, Minto and Lark shared a common pattern (pattern 1), whereas Kiata exhibited a different one (pattern 2), although both these patterns had three bands in them. CS showed a unique pattern (pattern 3) with only two immunoreactive bands separated, which was in agreement with the two dimensional gel result. Of the two bands the faster migrating one was stronger than the slower one. Analysis of the chromosome 2 deletion stock of CS (SBE IIa isoform is located on homoeologous group 2 chromosomes, Gale et al., 1993, Rahman et al., in prep) provided evidence that the first band is from the D genome and hence is designated as polypeptide D, and the second one represents two polypeptides encoded by the A and B genomes which comigrated and hence designated as A/B polypeptide (Section 4.3.7 of Chapter 4). The banding patterns 1 and 2 for SBE IIa polypeptides are often seen among Australian cultivars (M. Morell, personal communication). Assigning the chromosomal origin of each band in pattern 1 and 2 needs further analysis.

To see whether the variability in SBE IIa polypeptides in hexaploid wheats can be related to any structural variation in the gene encoding the protein, molecular analysis of the SBE II gene was performed. Southern hybridisation of *Bam*H1 digested DNA with the probe sbe 9 (which encodes a SBE II polypeptide in wheat) revealed a ~6.0kb band in Kiata and CS which possessed patterns 2 and 3 respectively, whereas this band is missing in lines with polypeptide banding pattern 1 (Figure 5.6). It should be noted that both in Kiata and CS the second band seen in the immunoblots is of lower mobility than the second band in the lines with pattern 1. It may be that an allele of SBE II gene with a structural mutation common to Kiata and CS encodes a larger polypeptide compared to its non mutant allele. However, analysis of a larger number of samples is required to draw any firm conclusion.

5.3.1.3 Discussion on SBE protein analysis

A complex arrangement of genes at SBE I locus in *T. tauschii* is known and two SBE I type genes *wSBE I-D2* and *wSBE I-D4* have been fully characterized (Rahman et al.,

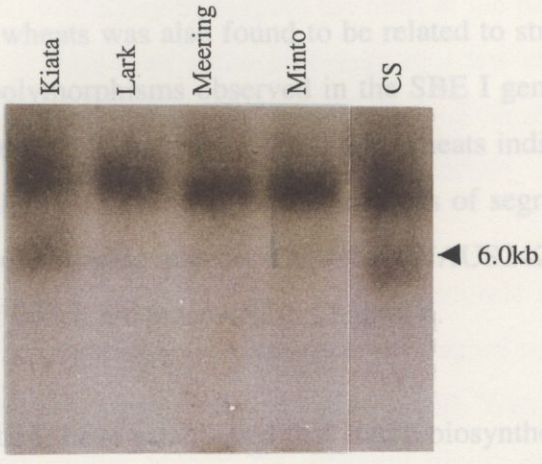


Figure 5.6 Autoradiograph showing polymorphism among hexaploid wheat using the probe sbe 9 specific for wheat SBE II. DNA samples were digested with *Bam*H1.

1997 and 1999). While *wSBE I-D4* encodes an amino acid sequence identical to that determined for the N-terminus of SBE I from hexaploid wheat (Morell, 1997), *wSBE I-D2* appeared to be a transcribed pseudogene as no corresponding SBE protein was found in the wheat endosperm. Although the probe E7.8 used in the molecular analysis of SBE I was a fragment from *wSBE I-D2*, the region is highly conserved within rice SBE I, *wSBE I-D2* and *wSBE I-D4*. It is not clear whether the hybridizing bands obtained in the current study corresponded to *wSBE1-D2* or *wSBE ID4* (or any others). If the major hybridizing bands corresponded to *wSBE I-D4* which encodes the SBE I polypeptides revealed by immunoblotting, then it is possible that the polymorphism observed in the SBE I polypeptides in the *T. tauschii* lines (between AUS24230 and other accessions) is due to structural difference in the gene encoding the SBE I expressed in the endosperm. Structural variation in *wSBE I-D4* between AUS24230 and other lines is also revealed by PCR amplification using primers sr412R and sr406F. Variable mobility in SBE IIa polypeptides in hexaploid wheats was also found to be related to structural varaition in the genes encoding. The polymorphisms observed in the SBE I gene in the *T. tauschii* lines and that observed in the SBE IIa gene in hexaploid wheats indicate allelic variants of single major loci in both the cases, based on the analysis of segregating populations of the crosses between polymorphic parents (AUS24242/AUS24230 and Kiata/Lark respectively), the results of which are presented in Chapter 6.

Studies on GBSS (*Wx* protein) have established that starch biosynthetic enzymes can be produced in variable amounts within the plant. Analyses of *Wx* protein from bread and emmer wheats have established that less *Wx*-A1 protein is produced than *Wx*-B1 protein (Nakamura et al., 1993a, Yamamori et al., 1995). Although the possibility that *Wx*-B1 protein is more extractable than *Wx*-A1 form was suggested, the finding that the loss of *Wx*-B1 protein caused greater reduction in amylose content than the loss of *Wx*-A1 form (Yamamori et al., 1994) supported the statement that the two isoforms are produced in variable amounts. The differential levels of expression of the SBE I genes located on different wheat genomes observed in the study is in agreement with Morell et al., (1997). However, the allelic expression seems to be determined also by the genetic background (if the variable intensities of the same polypeptide band in different genotypes can be taken as an indication of variable expression of the allele). A parallel

observation was made in GBSS where substantial difference in the amount of the two *Wx* proteins *Wx*-A1 and *Wx*-B1 was noticed between bread wheat and emmer wheat and also within certain accessions of emmer wheat (Yamamori et al., 1995b). Variability in the genetic composition and allelic differences at specific loci as in rice (Sano, 1984) are possible explanations. Gene interactions in different genetic background could also have an effect (Singletary et al., 1997). Although molecular analyses have revealed allelic variation in both SBE I and SBE II in relation to varying polypeptide mobilities, no allelic variation, in any of the cases, could be related to the differential expression as indicated by the variable intensities of bands. However it has to be noted that the number of lines/cultivars used for the molecular analyses was limited.

Bands of different intensity can be obtained due to the variable maturity of the endosperms as the pattern of SBE isoforms varied during endosperm development. This effect is minimized in the current study by tagging the head at the time of flowering and freezing the endosperm at 25 days after anthesis, except in a few cases when the exact flowering time was missed and the endosperm was collected on the basis of a visual estimation of the time since anthesis (as in the case of AUS24090).

In general, *T. dicoccoides* and *T. turgidum*, both with a genomic constitution AABB, differed in the mobility of the expressed polypeptides with regard to SBE I and SBE IIa (polypeptide B of SBE I and polypeptides A and B of SBE II). Waxy protein has been a subject of protein structural studies among the different ploidy wheats (Fujita et al., 1996). Comparison of the primary structure of GBSS revealed that the waxy protein encoded by the B genome of *T. turgidum* was identical to that of *T. searsii*, but differed from those of *T. speltoides* and *T. longissimum* by one amino acid substitution. Also, the waxy protein encoded by the B genome of *T. aestivum* differed from that encoded by the B genome of *T. turgidum* by one amino acid substitution. Similar structural differences may be expected in the SBE polypeptides encoded by the same genome from different wheat species giving rise to proteins with varying electrphoretic mobility

Molecular and biochemical analyses of different species of *Triticum* has enabled dissection of the evolutionary relationship of the allohexaploid wheat (*T. aestivum*) to

tetraploid and diploid species. The origin of *T. aestivum* is hypothesised to begin with hybridization between *T. urartu* (*T. monococcum*, AA) and a species related to section *sitopsis* of *Triticum* resulting in *T. turgidum* (AABB). The addition of the D genome derived from *T. tauschii* to a tetraploid wheat likely to be *T. turgidum* var. *dicoccon* resulted in the hexaploid *T. aestivum* with the genome composition of AABBDD (Kimber and Sears, 1987, Talbert et al., 1998). Studies of the extent of shared alleles between wheat and its D genome progenitor *T. tauschii* by sequence comparison of low copy DNA has indicated more than one origin of hexaploid wheat (Talbert et al., 1998). The current study revealed that the *T. aestivum* carries a D genome allele for SBE I like that in the *T. tauschii* line AUS24230 and not that from any of the other lines analysed. Both the biochemical and molecular analysis agree with this notion. PCR analysis using SBE I primers revealed that this line is closer to the *T. tauschii* accession CPI 110799 which was found to be a D genome relative of bread wheat as far as the *Nor-D3* locus was concerned (Lagudah et al., 1991). However in the case of SBE II, AUS24230 seems to carry an allele common to other *T. tauschii* lines. The current observation that not all *T. tauschii* accessions are related to hexaploid wheat is in conformation with the findings of Lagudah and Halloran, (1988) and Lagudah et al., (1991).

5.3.2 Analyses of SBE II variants

5.3.2.1 Number of SBE II type genes in wheat

The probe F2.2 used for Southern blot analysis of SBE II gene/s contained introns 4,5,6,7 and 8 in full and introns 3 and 9 in part, and exons 4,5,6,7,8, and 9 of a wheat SBE II gene, *wSBE II-D1* (Rahman et al., in prep). The *wSBE II-DI* gene encodes the N-terminal of the SBE IIa protein expressed in the wheat endosperm (Morell et al., 1997) and sequence comparisons further confirm the assignment of this gene to the SBE IIa sub-class (Rahman et al., in prep). The *wSBE II-D1* gene has been entirely sequenced by Rahman and co-workers. Hybridisation of *T. tauschii* DNA digested with four restriction enzymes, *Bam*H1, *Dra*1, *Eco*R1 and *Eco*RV, with F2.2 revealed only one

major band in each case, with a few weak bands in certain digests (Figure 5.7). This led to the assumption that the D genome donor of wheat bears only one SBE II gene.

A cDNA fragment containing the sequence corresponding to the exons 5-9 (sbe 9) which represents the mRNA expressed from the *wSBE II-D1* gene (S. Rahman, personal communication) was amplified using the primers DA12/DA13 and used for Southern blot analysis which gave a more complex picture. *Eco*R1 digested *T. tauschii* DNA probed with sbe 9 yielded four bands as against the single band when F2.2 was used as the probe (Figure 5.8). In order to verify the identity of the probe and see whether the PCR amplification product used as the probe contains any non-target sequence, the sbe 9 probe DNA was sequenced. Sbe 9 sequence spanned over a region between ~2.9kb to ~4.1kb of the *wSBE II-D1* gene. Considering the region it targets and the restriction sites of *wSBE II-D1* gene, only one band can be expected from the D genome on *Eco*R1 digestion. The same probe was used to hybridise the *Eco*R1 digested hexaploid wheat DNA (CS), which revealed six clear bands (Figure 5.8). The nulli2D tetra2B (N2DT2B) line of CS lacked the ~1.6 and ~2.5kb bands indicating that these bands are from the D genome. Previous analysis using CS deletion stocks has revealed that the ~3.8kb band is from the A genome and the ~5.4kb band from the B genome (Section 4.3.7.1, Figure 4.6). No evidence was obtained regarding the origin of the two higher molecular weight bands of sizes ~8.5 kb and ~7.4kb. Thus the results of the southern hybridization were unusual in having a more complex picture by hybridization with a cDNA probe than with a corresponding genomic probe.

The primers sr913F and E6R which target the intron 5 region of *wSBE II-DI* gene was used for PCR amplification of *T. tauschii* DNA. Two products of sizes ~200bp and ~230 were amplified (Figure 5.9). The source of the ~230bp product is not the targeted SBE II gene as the expected product size from D genome using these primers is 192bp.

The above results indicate the presence of more than one SBE II type gene *in T. tauschii*. Presence of two different isoforms of SBE II, SBE IIa and SBE IIb, has been reported in maize (Boyer and Preiss, 1978) and Barley (Sun et al., 1998). While the *T. tauschii* DNA exhibited the bands of size ~3.8 and 5.4kb on hybridization with the

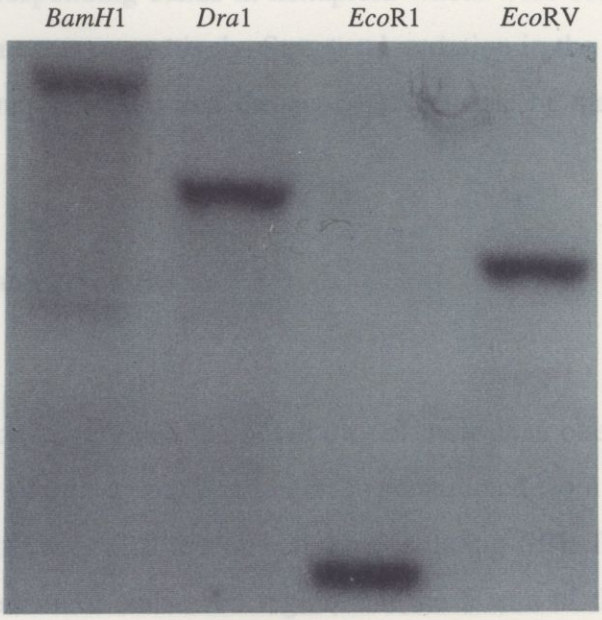


Figure 5.7 Southern blot of *T. tauschii* genomic DNA digested with the restriction enzymes, *Bam*H1, *Dra*1, *Eco*R1 and *Eco*RV using the *wSBE II-D1* probe, F2.2.

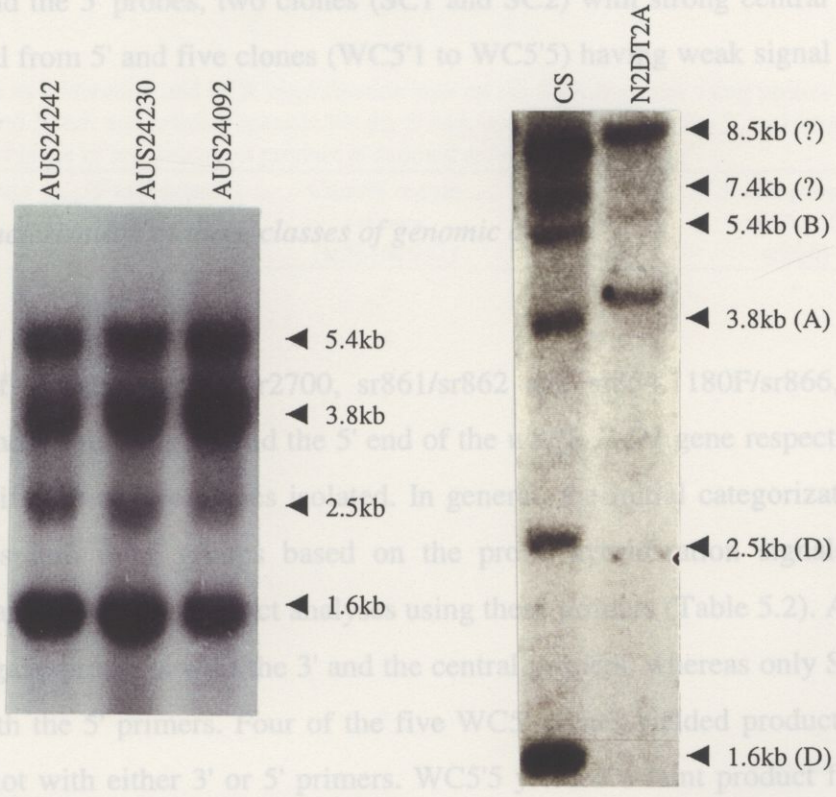


Figure 5.8 Southern blots of *Eco*R1 digested *T. tauschii* genomic DNA compared with that of CS, on hybridization with *wSBE II-D1* probe sbe9. The possible genome source of each band from CS is given in brackets. N2DT2A: Nulli 2D tetra 2A line of CS.

probe sbe 9, the corresponding bands in hexaploid wheat were established to originate from the A and B genomes respectively. Structural variation in the SBE II gene residing in the *T. tauschii* lines analysed and the actual D genome of the hexaploid wheat is likely.

5.3.2.2 Genomic library screening

Since preliminary studies revealed the possibility of more than one SBE II type gene in the D genome donor of wheat, a genomic library constructed from *T. tauschii* (Rahman et al., 1997) was screened with the objective of isolating SBE II type variants. Two probes, targeting the central conserved region and the 5' end of the *wSBE II D1* gene were used for screening the library. Depending on the intensity of hybridising signal using both the probes twelve clones were isolated which came under three categories (Table 5.2). Five clones (SC5'1 to SC5'5) were with strong hybridising signal from both the central and the 5' probes, two clones (SC1 and SC2) with strong central signal but *with no signal from 5' and five clones (WC5'1 to WC5'5) having weak signal from both* central and 5'.

5.3.2.3 Characterization of three classes of genomic clones

Three sets of primers, sr2370/sr2700, sr861/sr862 and sr854.1180F/sr866, designed from the 3' end, central region and the 5' end of the *wSBE II D1* gene respectively were used to amplify the twelve clones isolated. In general, the initial categorization of the twelve clones into three groups based on the probe hybridization signals (Section 5.3.2.2.) remained on PCR product analyses using these primers (Table 5.2). All the five SC5' clones gave products with the 3' and the central primers, whereas only SC5'1 gave a product with the 5' primers. Four of the five WC5' clones yielded products with the central and not with either 3' or 5' primers. WC5'5 yielded a faint product from the 5' primer. Neither SC1 nor SC2 clones amplified with any of the three primers. Although

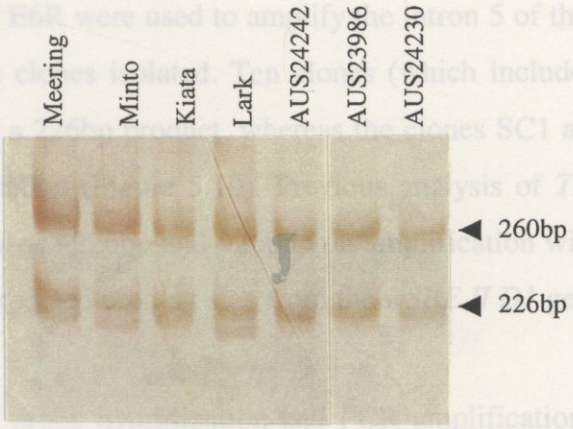


Figure 5.9 Non denaturing PAGE gel of PCR amplified intron 5 region of SBE II gene from *T. tauschii* in comparison with that of hexaploid wheat. The primers sr913F and E6R were used for PCR amplification.

Table 5.2 Probe hybridization and PCR amplification data on the SBE II clones using probes specific for central region and 5'end, and primers specific for the 3' end, central region and the 5, end respectively of wSBE II D1. Presence of amplification product is denoted as '+' and absence as '-

Clone identity	3' end primers (sr2370/sr2700)	Central region primers (sr861/sr862)	Central probe hybridization signal	5' end primers (sr854.1180/ sr866)	5' probe hybridization signal
SC5'1	+	+	strong	+	strong
SC5'2	+	+	strong	-	strong
SC5'3	+	+	strong	-	strong
SC5'4	+	+	strong	-	strong
SC5'5	+	+	strong	-	strong
WC5'1	-	+	weak	-	weak
WC5'2	-	+	weak	-	weak
WC5'3	-	+	weak	-	weak
WC5'4	-	+	weak	-	weak
WC5'5	-	-	weak	+ (weak)	weak
SC1	-	-	strong	-	No signal
SC2	-	-	strong	-	No signal

the clones varied based on the presence or absence of bands, no polymorphism was observed in the amplified products using any of these primers in any of the clones.

The primers sr913F and E6R were used to amplify the intron 5 of the SBE II type genes contained in the twelve clones isolated. Ten clones (which included all the SC5' and WC5' clones) amplified a 226bp product, whereas the clones SC1 and SC2 amplified a larger product of size 260bp (Figure 5.10). Previous analysis of *T. tauschii* DNA has revealed two bands of sizes ~226bp and ~260bp on amplification with the same primers (Section 5.3.2.1). The expected product size from the *wSBE II D1* gene is 192bp.

Based on the results of probe hybridization and PCR amplification, it was tentatively concluded that the SC5' clones contain SBE IIa gene identical to *wSBE II D1*. While SC5'1 may contain a full length SBE IIa gene, SC5'2 to SC5'4 may have the same gene but are missing the 5' end due to the gene residing at the extremity of the cloned DNA. For the WC5' clones the possibilities are either they contain a truncated SBE IIa gene with missing 3' and 5' ends or they represent a distinct subclass of SBE II type genes. PCR analysis of the WC5' clones suggests that these clones contain a gene highly similar or identical to the *wSBEII-D1* gene, however, further analysis is required to investigate why these clones gave weak hybridization signal to the probe targeting the central region of *wSBE II D1* gene. The results clearly reveal that the clones SC1 and SC2 represent a second type of SBE II gene.

5.3.2.4 Characterization of novel SBE II genes

5.3.2.4.1 Restriction analysis of SC clones

*Eco*R1 digested DNA of the SC2 clone was hybridized with a probe targeting the central region of *wSBE II D1* gene and the hybridization pattern was compared with that of a clone F2 containing the *T. tauschii wSBE II D1* gene (Figure 5.11). While clone F2 revealed two bands of sizes 2.5 and 1.8kb, clone SC2 gave only one band of ~3.5kb.

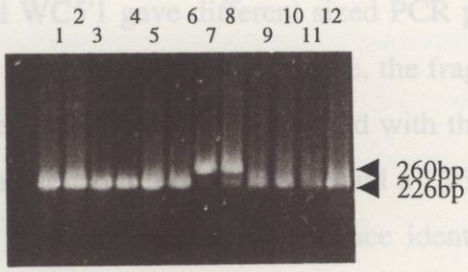


Figure 5.10 PCR amplification of intron 5 region of SBE II using the primers sr913F and E6R from the SBE II clones isolated. Lanes 1. SC5'2, 2. WC5'1, 3. WC5'2, 4. SC5'3, 5. SC5'4, 6. SC5'5, 7. SC2, 8. SC1, 9. WC5'3, 10. WC5'4, 11. WC5'5, 12. SC5'1.

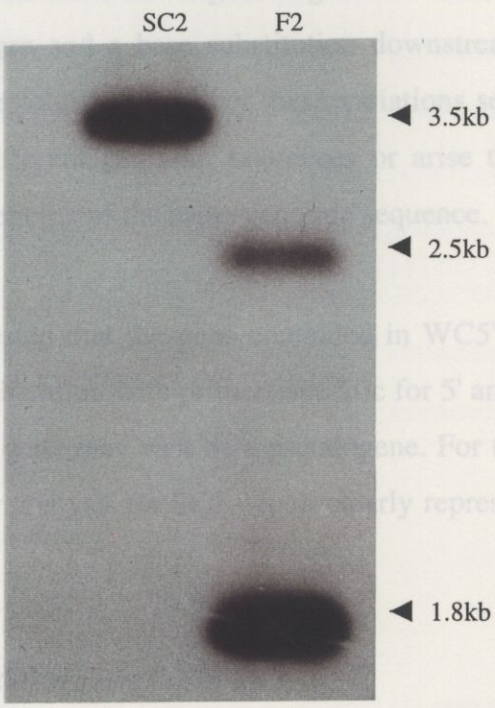


Figure 5.11 Hybridization of *Eco*R1 digested DNA from the SC2 clone with the probe containing the central region of *wSBE II-D1*. F2 is the clone containing the *wSBE II-D1* gene.

The restriction analysis also indicated that the SC2 contains a gene which is different from *wSBE II D1*

5.3.2.4.2 Analysis of intron 5 of SBE II

Since the clones SC1 and WC5'1 gave different sized PCR products using the primers which amplify the intron 5 region of the SBE II gene, the fragments containing intron 5 from these two clones were sequenced and compared with that of *wSBE II D1* gene. A 252bp sequence was obtained from the clone SC2 and a 216bp sequence from WC5'1. While the WC5'1 intron 5 region showed a sequence identity of 99.5% with that of *wSBE II D1*, the overall identity was only 74.3% for the SC2 clone when compared with *wSBE II D1* (Figure 5.12). Between SC2 and WC5'1 the identity was only 71.8%. This supports the assumption that the SBE II gene contained in SC2, designated as *wSBE II D2*, is different from that contained in WC5'1 and *wSBE II D1*. The 216bp fragment sequenced from WC5'1 differed from the aligned region in *wSBE II D1* by having a deletion of four bases upstream and a base substitution downstream of the fragment from WC5'1. It has not been established whether these variations seen between WC5'1 and *wSBE II-D1* represent different genomic sequences or arise through PCR errors during amplification and sequencing of the same genomic sequence.

Preliminary sequencing suggested that the gene contained in WC5'1 is very similar to *wSBE II D1*. The lack of amplification with primers specific for 5' and 3' end of *wSBE II D1* gene is intriguing, but the gene may well be a pseudogene. For these reasons it was decided to concentrate further analysis on SC2 which clearly represented a novel class of SBE II gene.

5.3.2.4.3 Sub cloning and partial sequencing of SC2

*Bam*H1 digested DNA from SC2 was subcloned into pUC19 vector and on transforming *E. coli* competent cells strain JM 101, two subclones, B1 and B4 were isolated.

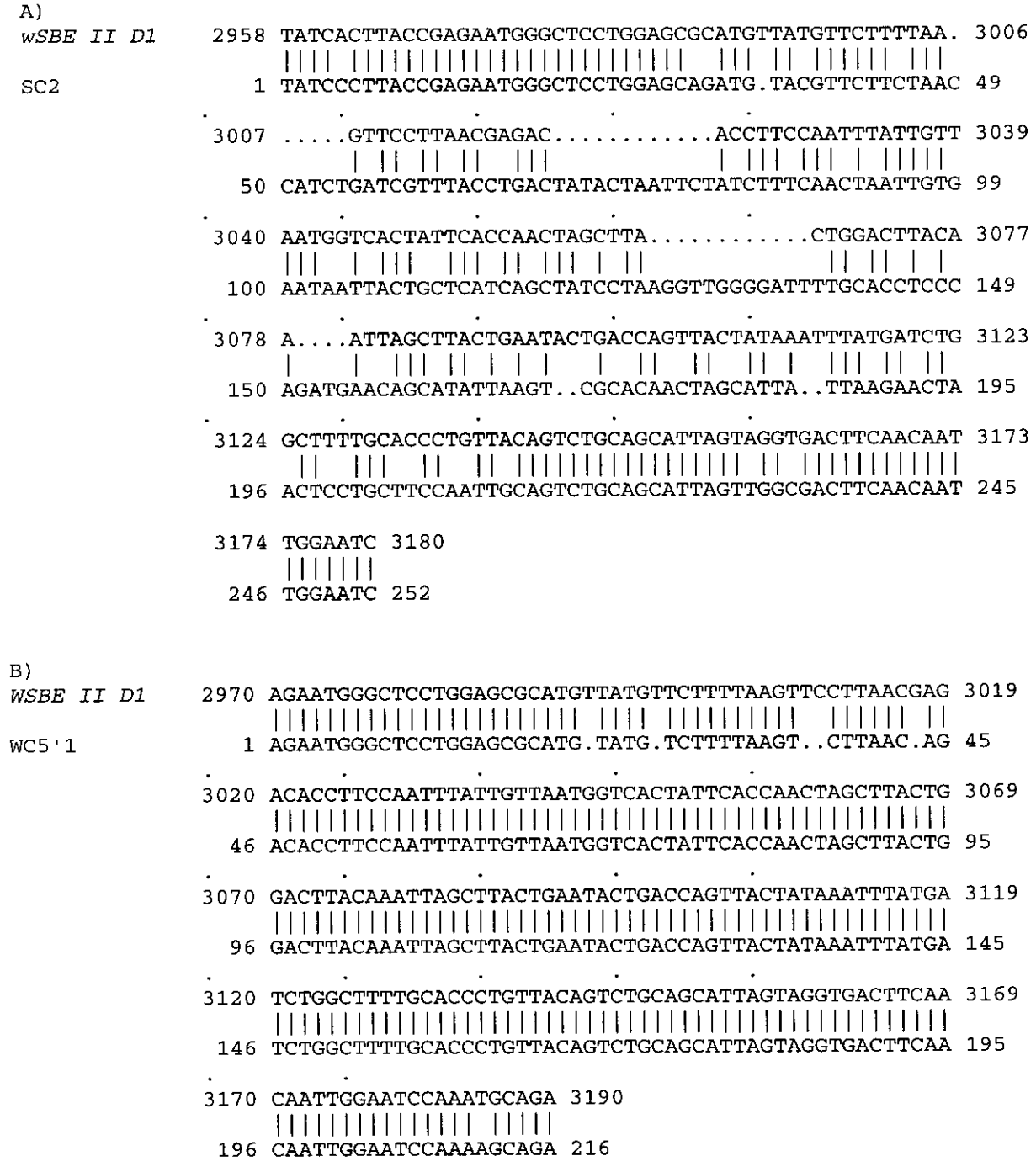


Figure 5.12 Comparison of nucleotide sequences of intron 5 region of A) *wSBE II D1* gene and SC2 clone B) *wSBE II D1* and WC5'1 clone.

Digestion with *Bam*H1 revealed that the subclones B1 and B4 had an insert of size ~2.3kb. Restricting these two clones with enzymes that cut at sites other than the ligation sites (*Pst*1 and *EcoR*1) exposed two fragments each with varying sizes (Figure 5.13). Preliminary sequencing confirmed that B1 and B4 contain the same insert but in reverse orientation. Subclone B1 was fully sequenced and characterized.

5.3.2.4.4 Partial characterization of wSBE II D2

A 2.26kb sequence was obtained from the subclone B1. Sequence alignment with a cDNA encoding a SBE, the sequence of which is most homologous to SBE IIb sequences (Rahman et al., in prep) showed a complete match over a long stretch indicating that *wSBE II D2* gene contained in the clone SC2 is the structural gene encoding SBE IIb. The fragment from subclone B1 (fragment B1) contained the sequence from positions 158 to 887 of the cDNA and it was possible to deduce a partial intron-exon structure of the gene (Figure 5.14). The 2.26kb sequence contained 6 exons (exon 2,3,4 and 5 in full and part of exons 1 and 6) and five introns (introns 1-5 in full). The consensus GT/AG sequences that characterizes the 5' and 3' splice site is present at all exon/intron boundaries. Among the exons detected in full, the length varied from 43bp (exon 5) to 230bp (exon 3). The intron size varied considerably ranging from 74bp (intron 4) to 663bp (intron 2). While a minimal functional length of 70bp has been determined for efficient processing of synthetic introns in higher plants (Goodall and Filipowicz, 1990), most plant introns are reported to be within the size range of 80-139bp (Simpson and Filipowicz, 1996). However, large introns of size over 1kb are described in many instances as in rice *sbe1* (Kawasaki et al., 1993), barley SBE IIb (Sun et al., 1998) and in wheat SS I (Li et al., 1999b). In general, the exons had a higher percentage of G+C content (56.0%) compared to the introns (38.9%). Exons 1 and 2 are considerably GC rich with 76 and 65% respectively compared to 43-53% in the other four exons analysed. These two exons are also characterised by higher frequency of CpG dinucleotide (12-15 per 100bp). Among the introns, intron 1 has a G+C content of 54% and a CpG frequency of 10.2 per 100bp, whereas the other introns are low in GC content (<40%) with 0-1.7 CpG frequency, reflecting their AT- rich nature. In fact, AT

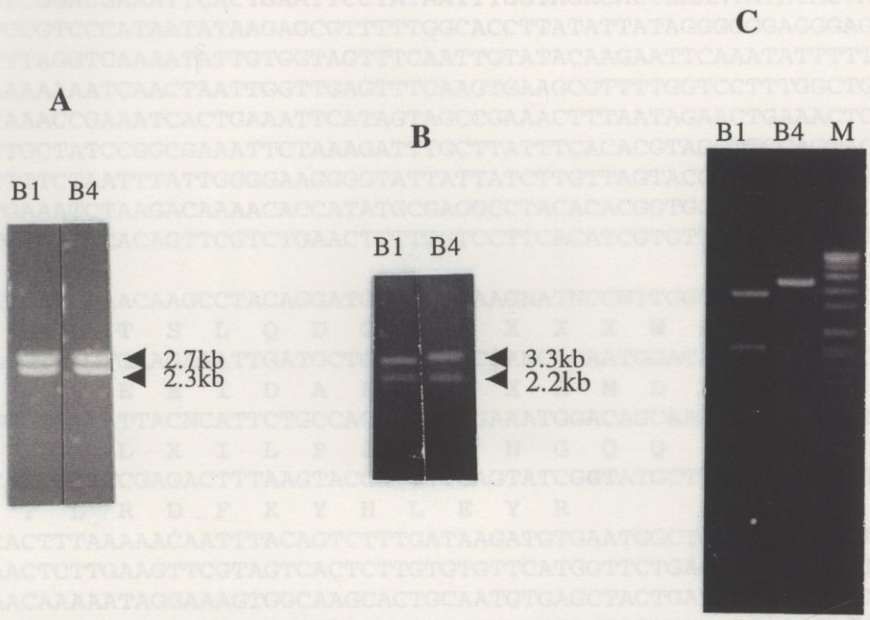


Figure 5.13 Restriction pattern of subclones B1 and B4 using the enzymes A) *Bam*H1, B) *Pst*1, C) *Eco*R1. M: molecular size marker (pUC19/*Hpa*II).

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CGATCCGGCTGCGGCGGCGGCGACGGGATGGCTGCGCCGGCATTCGCAGTTTCCGCGGCG   60
R  S  G  C  G  G  G  D  G  M  A  A  P  A  F  A  V  S  A  A
GGGCTGGCCCGGCCGTCGGCTCCTCGATCCGGCGGGGCAGAGCGGAGGGGGCGCGGGGTG  120
G  L  A  R  P  S  A  P  R  S  G  G  A  E  R  R  G  R  G  V
GAGCTGCAGTCGCCATCGCTGCTCTTCGGCCGCAACAAGGGCACCCGTTCACCCCGTAAT  180
E  L  Q  S  P  S  L  L  F  G  R  N  K  G  T  R  S  P  R
TATTTGCGCCACCTTTCTCACTCACATTCTCTCGTGTATTCTGTCGTGCTCGCCCTTCGC  240
CGACAACGCGTGCCGATTCCGTATCGGGCTGCGGTGTTCAGCGATCTTACGTCGGTTCCC  300
TCCTGGTGTGGTGATGTCTGTAGGTGCCNTCGGCGTCGGAGGTTCTGGATGGCGCGTGGT  360
                          A  X  G  V  G  G  S  G  W  R  V  V
CATGCGCGCGGGGGGGCCGTCCGGGGAGGTGATGATCCCTGACGGCGGTAGTGGCGGAAC  420
 M  R  A  G  G  P  S  G  E  V  M  I  P  D  G  G  S  G  G  T
ACCGCCTTCCATCGACGGTCCCGTTCAGTTCGATTCTGATGATCTGAAGGTAGTTTTTTT  480
 P  P  S  I  D  G  P  V  Q  F  D  S  D  D  L  K
TTTGCATCGATCTGAAGGTACTTGACATATACTACTGTATTACCCTGAGTAAATACTGCC  540
ACCATATTTTTATGGTTCGCTTGAAATACCTGTTTACTTGCTACGGTTTTCACTTTCATT  600
GAGACGTCGGACGAAATTCACTGAATTCCTATAATTTGGTAGACACCGAAATATATACTA  660
CTCCTTCCGTCCCATAATATAAGAGCGTTTTTGGCACCTTATATTATAGGGCGGAGGGAG  720
TACCTTTTAGGTCAAAATATTGTGGTAGTTTCAATTGTATACAAGAATTCAAATATTTTT  780
TTTAAAAAAAAAATCAACTAATTGGTTGAGTTTCAAGTGAAGCGTTTTGGTCCTTTGGCTG  840
AGATGTAAACCGAAATCACTGAAATTCATAGTAGCCGAAACTTTAATAGAACTGAAACTC  900
AAAATCTGCTATCCGGCGAAATTCTAAAGATTTGCTTATTTCACACGTAGGTTGCAGTAC  960
ACCCTCTTTCTAATTTATTGGGGAAGGGGTATTATTATCTTGTTAGTACCTGCCTGCATG 1020
ACAATTGAAATCTAAGACAAAACACCATATGCGAGGCCTACACACGGTGGGTTGGTTTAC 1080
AACTATGTGTGCCACAGTTCGTCTGAACTTTTTGTCCTTCACATCGTGTTAGGTTCCATT 1140
                                                  V  P  F
CATTGATGATGAAACAAGCCTACAGGATGGAGGTGAAGNATNCCNTTGGCTTGGAACGAC 1200
 I  D  D  E  T  S  L  Q  D  G  G  E  X  X  X  W  L  G  T  T
AAATCAGGTTAGTGAAGAAATTGATGCTGAAGACACGANCAGAATGGACAAAGAATCATC 1260
 N  Q  V  S  E  E  I  D  A  E  D  T  X  R  M  D  K  E  S  S
TACGAGGGAGAAATTACNCATTCTGCCACCACCGGGAAATGGACAGCAAATATACGAGAT 1320
 T  R  E  K  L  X  I  L  P  P  P  G  N  G  Q  Q  I  Y  E  I
TGACCCAACGCTCCGAGACTTTAAGTACCATCTTGAGTATCGGTATGCTTCGCTTCTATT 1380
 D  P  T  L  R  D  F  K  Y  H  L  E  Y  R
GTGTGCACTTTAAAAACAATTTACAGTCTTTGATAAGATGTGAATGGCTGCTTGCTGTGA 1440
CACGAAACTCTTGAAGTTCGTAGTCACTCTTGTGTGTTCATGGTTCTGAGGTAACATGGT 1500
AACCGAACAAAAATAGGAAAGTGGCAAGCACTGCAATGTGAGCTACTGATAACCACCCAT 1560
TGTAATTGGGTACACTGATTAATATATATGTCTTCATGGGCTCTATTTTTTTTCAATATC 1620
TATGCCAATTGAACAACAATGCTTTGTGGACGGGTGTTCTTTTACCCTCTTCTTCTATCA 1680
ATAGATGATATGCATACTCATGCGTATCCTACAAAAAATTGAACAACAATGCCACTTTCC 1740
CCCGTGTTGCTTTTGTAAGGATGAAACACATATGTCCAGATCAAACTATACTAGCAGTCT 1800
AACTGTGCCTTAATGGATCAAAAACAGATATAGCCTATACAGGAGAATACGTTCAGACAT 1860
                              Y  S  L  Y  R  R  I  R  S  D  I
TGATGAACACGAAGGAGGCATGGATGTATTTTCCCGCGGTTACGAGAAGTTTGGATTTAT 1920
 D  E  H  E  G  G  M  D  V  F  S  R  G  Y  E  K  F  G  F  M
GCGCAGGTGAAATTTCTTGACTAAATAACTATGTATCTACCTTTTCTTTGTACTCTATCA 1980
 R  S
ACATTCCTCTTCCCATGCAGCGCTGAAGGTATCACTTACCGAGAATGGGCTCCTGGAGCA 2040
                        A  E  G  I  T  Y  R  E  W  A  P  G  A
GATGTACGTTCTTCTAACCATCTGATCGTTTACCTGACTATACTAATTCTATCTTTCAAC 2100
D
TAATTGTGAATAATTACTGCTCATCAGCTATCCTAAGGTTGGGGATTTTGCACCTCCCAG 2160
ATGAACAGCATATTAAGTCGCACAACTAGCATTATTAAGAACTAACTCCTGCTTCCAATT 2220
GCAGTCTGCAGCATTAGTTGGCGACTTCAACAATTNGGATC                     2261
    S  A  A  L  V  G  D  F  N  N  X  D
```

Figure 5.14 Nucleotide and deduced amino acid sequences of B1 fragment of *wSBE II D2* gene. The GT/AG nucleotides corresponding to the intron boundaries are shown in bold.

richness is a common feature of plant pre-mRNA introns and is suggested that AT-richness of introns is essential for their recognition by the splicing machinery and efficient splicing (Simpson and Filipowicz, 1996).

Mapplot programme of ANGIS detected two sites each for the enzymes *Dra*1, *EcoR*1 and *Pst*1 in the fragment of *wSBE II D2* gene sequenced. No sites for the restriction enzymes *Bam*H1, *Eco*RV and *Hind*III were identified (Figure 5.15).

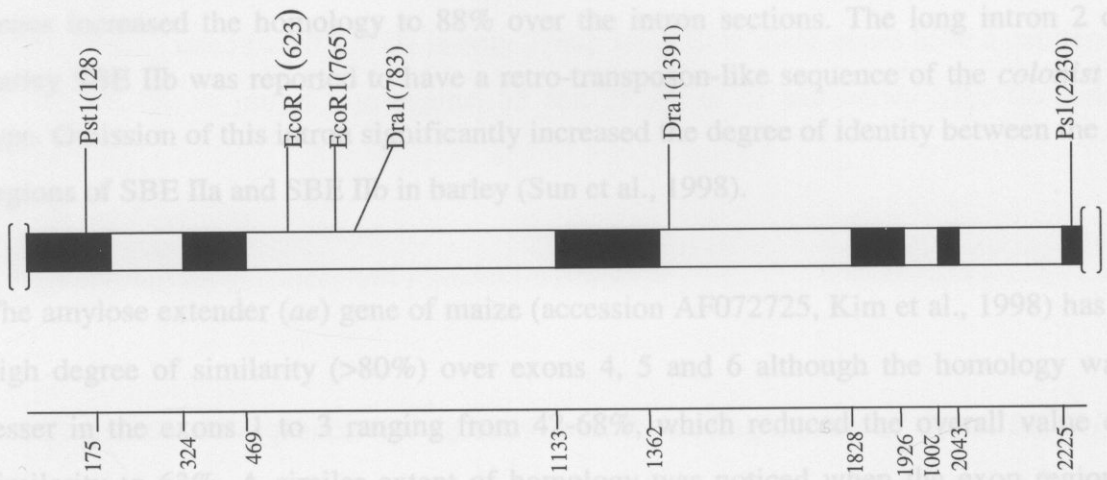


Figure 5.15 Organization of B1 fragment of *wSBE II D2* gene. Exons are indicated by shaded box. The numbers indicated are the nucleotides (bp) considering zero as the start of the fragment. Restriction sites are indicated by the name of respective restriction enzymes. The double bracket on both sides shows that the structure defined is only part of the gene.

5.3.2.4.5 Comparison with other SBE genes

Sequence alignment with other SBE II type genes revealed higher homology of the B1 fragment with SBE IIb genes compared to SBE IIa types indicating that *wSBE II D2* belongs to SBE IIb gene family. Barley SBE IIb (GenBank accession AF064563, Sun et al., 1998) had an overall similarity of 91.6% over the exons. The exon organization of both the genes (up to the exon 6 that was analysed) was very similar with the exons 3, 4 and 5 the same length in both. While the exon 5 of both the genes showed a 100 % identity, a minimum of 86.5% was observed for exon 3. The overall sequence homology was 71% over the introns. The major divergence between the regions of the two genes analysed was the size of intron 2. Barley SBE IIb has an intron 2 of 2064 nucleotides in contrast to 663bp in *wSBE II D2*. Sequence alignment excluding the intron 2 of both the genes increased the homology to 88% over the intron sections. The long intron 2 of barley SBE IIb was reported to have a retro-transposon-like sequence of the *colonist 1* type. Omission of this intron significantly increased the degree of identity between the 5' regions of SBE IIa and SBE IIb in barley (Sun et al., 1998).

The amylose extender (*ae*) gene of maize (accession AF072725, Kim et al., 1998) has a high degree of similarity (>80%) over exons 4, 5 and 6 although the homology was lesser in the exons 1 to 3 ranging from 42-68%, which reduced the overall value of similarity to 63%. A similar extent of homology was noticed when the exon regions were compared with the corresponding sequence from rice branching enzyme 3 mRNA (accession OSBCE3, Mizuno et al., 1993), although the overall identity was higher than the *ae* gene (70.5%). The *ae* gene bears the same number of nucleotides in the exons 4 and 5 as that of *wSBE II D2* and barley SBE IIb genes. The region including the proximal promoter region, first two exons and first intron of *ae* gene was reported to have a higher G+C content and CpG dinucleotide frequency (as observed in the case of first two exons and first intron of *wSBE II D2*). Kim et al., (1998), have related this to the CpG islands found in the mammalian genome, which are usually unmethylated and flanked by methylated regions (Bickmore and Bird, 1992). The intron length varied

between the two genes with the maximum variation noticed in intron 3 which is over 1kb in *ae* compared to 465bp in *wSBE II D2*.

The exon regions of *wSBE II D1* (Rahman et al., in prep) and *wSBE II D2* share an identity of only 56.4% between them. Sequence comparisons suggest that *WSBE II D1* is a SBE IIa type gene (Rahman et al., in prep). It appears that there is a high degree of sequence conservation in the region covering the exons 5 and 6 of SBE IIb and IIa isoforms. This area is revealed to have more than 85% homology when the *wSBE II D2* sequence was compared with *wSBE II D1*, maize SBE IIa mRNA (accession U65948, Gao et al., 1997), barley SBE IIa mRNA (accession AF064560, Sun et al., 1998) and rice SBE 4 mRNA (accession E14723, Baba et al., 1998). With regard to the introns, there was considerable variability both in length and sequence homology between *wSBE II D1* and *wSBE II D2*. While intron 2 of *wSBE II D2* is 663bp in length, the corresponding one in *wSBE II D1* is only 276bp long. Both these genes have a similarity of only 46.4% on intron 5 as against 53 and 75.3% in *ae* and barley SBE IIb genes respectively.

5.3.2.4.6 Analysis of conceptual translation products

A 243 amino acid sequence was deduced from the coding regions of the *wSBE II D2* fragment which included the exons 2, 3, 4, and 5 in full and exons 1 and 6 in part (Figure 5.16). The sequence agreed with those deduced from cDNA2 from wheat (Rahman et al., in prep). Since SBE is coded by a nuclear gene and located in amyloplasts and chloroplast, an appropriate transit peptide can be expected as part of the protein. The SIGCLEAVE application of the GCG package identified a cleavage site for the transit peptide between Ala-23 and Arg-24. However, this programme, based on Heijne, 1986, provides an accuracy of only 75-80% in identifying the site of cleavage. Comparison with the determined N-terminal amino acids of the mature barley SBE IIb (Sun et al., 1998), maize SBE IIb (Fisher et al., 1993) and rice SBE 3 (Mizuno et al., 1993) revealed the more possible cleavage site to be between Arg-73 and Ala-74 (Figure 5.16).

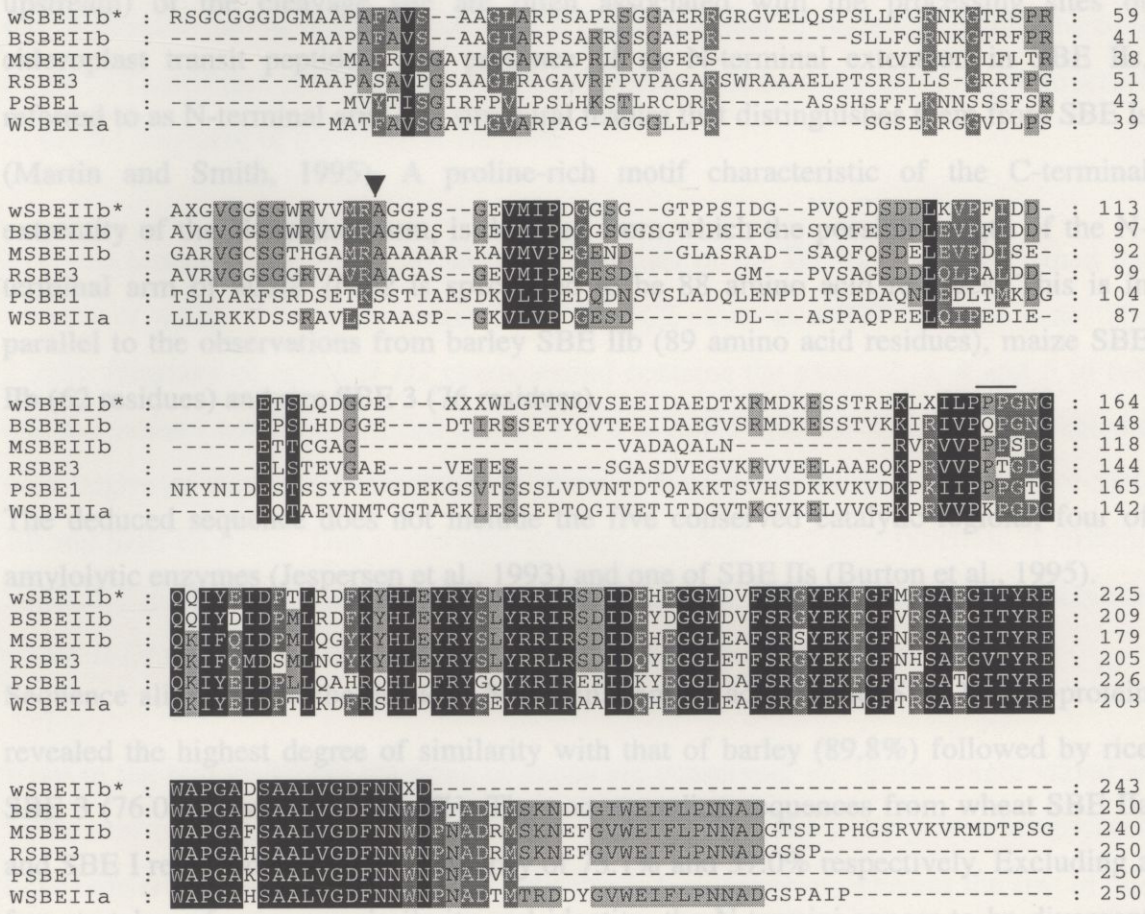


Figure 5.16 Comparison of the deduced amino acid sequence from the coding region of the B1 fragment of *wSBE II D2* gene (represented by wSBEIIb*) and SBE II type isoforms from other plants. The pro-rich motif is indicated by a bar over the sequence. Predicted cleavage site for the wheat SBE IIb transit peptide is indicated by an arrow. Homologous residues are shaded, the intensity ranging from black to white depending on the level of homology. Sources of other SBE II sequences are as follows: BSBEIIb - barley SBE IIb, accession number AAC69754; MSBEIIb - maize SBE IIb, accession number AAC33764; RSBE3 - rice SBE 3, accession number BAA03738; PSBE1 - *P. sativum* SBE I, accession number CAA56319 and WSBEIIa - wheat SBE IIa, accession number CAA72154

(The consensus cleavage site suggested for chloroplast transit peptide is V/L-X-A/C↓A, Gavel and Heijne, 1990). The arginine residues located at the vicinity (-1 and -5 upstream) of the cleavage site are often associated with the processing sites of chloroplast transit peptides. The presence of an N-terminal extension in SBE IIs, referred to as N-terminal arm, is a structural feature that distinguishes them from SBE Is (Martin and Smith, 1995). A proline-rich motif characteristic of the C-terminal extremity of the N-terminal arm, is detected from which the possible length of the N-terminal arm of *wSBE II D2* is speculated to be 88 amino acid residues. This is in parallel to the observations from barley SBE IIb (89 amino acid residues), maize SBE IIb (62 residues) and rice SBE 3 (76 residues).

The deduced sequence does not include the five conserved catalytic regions; four of amylolytic enzymes (Jespersen et al., 1993) and one of SBE IIs (Burton et al., 1995).

Sequence alignment of the deduced N terminal amino acids of the SBE IIb pre-protein revealed the highest degree of similarity with that of barley (89.8%) followed by rice SBE 3 (76.0%) and maize (73.5%). The corresponding sequences from wheat SBE IIa and SBE I recorded an overall similarity of 73.1% and 57.0% respectively. Excluding a few stretches of sequence similarity and identity, the N-termini appear to be divergent (Figure 4.15). Towards the central portion, the sequences shared a high degree of identity. This is in agreement with the report of Sun et al., (1998).

5.3.2.4.7 Discussion on wheat SBE IIb analysis

Morell et al., (1997) reported only one form of SBE II in the soluble phase of wheat endosperm, the N-terminal of which had higher homology to maize SBE IIa than SBE IIb (M. Morell, personal communication). So far no SBE IIb protein has been detected in wheat. The available information suggests that it is either not present in the soluble fraction of wheat endosperm or only weakly expressed relative to SBE IIa (M. Morell, personal communication). Nair et al., (1997) has reported a full length cDNA from wheat which encodes the N-terminal sequence of SBE II as reported by Morell et al.,

(1997). Recently, a SBE II gene (*wSBE II D1*) that encodes the same N-terminal sequence has been isolated and was characterized to be a maize SBE IIa like gene (Rahman et al., in prep). This group have also isolated a second class of SBE II cDNA, the deduced amino acid sequence of which resembled that of barley SBE IIb. The SC2 clone contained this cDNA sequence which helped to deduce the intron-exon structure of the B1 fragment of the *wSBE II D2* gene.

Both nucleotide and amino acid sequence analyses of the B1 fragment confirmed the identity of *wSBE II D2* gene contained in the SC2 clone as a SBE IIb type gene. The 2.26kb fragment of *wSBE II D2* characterized contains the exons 2, 3, 4 and 5 in full and the exons 1 and 6 in part, and introns 1 to 5 in full. Sequence comparison revealed a high degree of sequence conservation in the region covering the exons 5 and 6 of SBE IIb and SBE IIa genes. This is probably an important region of gene function. A striking similarity in the exon structure of the last 17 exons being of identical length between *wSBE II DI* and maize SBE IIb gene was noticed by Rahman et al., (in prep).

Even in the regions where the sizes of exons are conserved, the intron length varied as in the case of wheat and rice SS1 genes (Li et al., 1999b). The waxy gene was observed to be conserved over the exon regions between different species, whereas the size of one intron varied not only between species but also within the three different genomes in wheat (Zhao, 1999). The nucleotide sequence of the 5' UTR intron of microsomal ω-6 desaturase gene, pghD12-1, of *Gossypium* spp was found to be more variable and appeared to have evolved at a relatively higher rate than other regions of the gene. The corresponding intron sequence among *Gossypium*, *Glycine max* and *Arabidopsis thaliana* had diverged to such an extent that no sequence similarities could be identified between them (Liu, 1998). In the case of *wSBE II D2*, the intron regions of wheat are more similar to those of barley than maize. For example, the intron 4 has an identity of 87.8% with that of barley as against 39.4% of maize. The ranking of similarity in introns may reflect the relationship between species. Taxonomically barley and wheat are closely related, both of which coming under the same tribe *Triticeae*.

Studies in maize and rice have disclosed that the amylose extender (*ae*) mutants characterized by an apparently increased content of amylose in storage starches are deficient in SBE IIb isoform activity (Hedman and Boyer, 1982, Mizuno et al., 1993). The isolation of SBE IIb gene from wheat is useful as it can accelerate our efforts to generate a high amylose genotype in this species.

5.4 CONCLUSION

Three subclasses of SBE, SBE I, SBE IIa and SBE IIb, differing in biochemical and enzymatic properties have been identified in cereals such as maize and barley. Independent genetic control of these three subclasses have been established in maize (Gao et al., 1997). In wheat SBE I and SBE IIa genes are located and characterized (Rahman et al., 1997, Rahman et al., 1999). Since no mutants lacking either SBE I or SBE IIa activity have been reported so far in any of the plants, their importance in amylose and amylopectin synthesis has not been clearly elucidated. The current study revealed polymorphisms in SBE I and SBE IIa isoforms within *Triticum* species, and this information has the potential to be useful in understanding the role of these enzymes in starch branching mechanisms.

Amylose extender (*ae*) lines missing SBE IIb activity in maize were characterized by an elevated amylose content and long chained amylopectin molecules. The genes encoding SBE IIb in maize (ae) and barley were recently cloned and characterized (Kim et al., 1998, Sun et al., 1998). The wheat *wSBE II D2* gene isolated and partially characterized in the current study corresponds to SBE IIb gene. In maize the SBE IIb isoform is more highly expressed than the SBE IIa isoform (Gao et al., 1997), whereas in wheat a low expression of SBE IIb isoform was observed in the soluble fraction of endosperm (M. Morell, personal communication). Further analysis is required regarding the expression of SBE IIb in wheat. The isolation of SBE IIb gene in wheat increases the possibility of developing a high amylose genotype in wheat through plant breeding and genetic

engineering techniques. The partial gene sequence obtained from the current study can be used to identify null or altered forms of genes to be useful in plant breeding.

CHAPTER 6

Studies of the Inheritance of Amylose Content in *Triticum* Species

6.1 INTRODUCTION

The use of starch in the food and non-food industry demand specific structural and functional attributes which can be incorporated through various techniques of plant improvement. The discovery of the *waxy* locus encoding the granule bound starch synthase (GBSS), a major protein involved in amylose synthesis has led to the development of zero amylose (waxy) varieties in various crops including wheat (Nakamura et al., 1995, Hoshino et al., 1996, Yasui et al., 1997). The identification of high amylose genes such as *amylose extender* (*ae*) and *amo1* has facilitated the breeding of higher amylose lines in maize and barley respectively. As no genes conferring a high amylose phenotype have been detected in *Triticum* and *Aegelops* species, efforts in this direction are still in their infancy. The narrower range of genetic variability available due to the buffering capacity of the multiple genomes against changes (Mohammadkhani et al., 1999) make the task more tedious. Directional selection towards achieving a high amylose phenotype can be accelerated through creating additional genetic variability. Accumulation of favourable genes through hybridisation of suitable hexaploid donor parents and developing synthetic amphidiploids of *T. durum* (AABB genome) and *T. tauschii* (D genome) are examples of the methodologies that can be adopted to enhance the variability within the population. Hybridisation among the *T. tauschii* lines to create more variability within the D genome for subsequent transfer to the hexaploid wheat is another possibility.

The development and application of molecular markers to wheat has been delayed mainly due to its genome complexity. The techniques used to identify a molecular marker linked to a trait of interest will depend upon the type of trait and the resource and

the marker system available (Langridge and Chalmers, 1998). Information on several features of the trait such as the mode of inheritance; simple or polygenic, and the heritability of the trait is important while devising the most efficient marker development strategy. The results of the breeding programmes initiated to investigate the extend of variability in amylose content that can be created in *Triticum* species and the inheritance studies of the trait are presented in this chapter.

6.2 MATERIALS AND METHODS

6.2.1 Interspecific hybridization

Four tetraploid (*T. dicoccoides*) and two diploid (*T. tauschii*) accessions surveyed in chapter 2 were selected for the study. In order to break the dormancy of *T. tauschii* lines the seed were soaked in water over filter paper in a petri dish and kept at 4°C for 5-7 days and then transfered to room temperature. The germinated seedlings were transferred to pots. Crossing was done in both directions with diploid either as the male or as female parent. The maternal plants were emasculated one to two days before anthesis. Variation in physiological stage of florets is minimized by removing the basal and uppermost florets. Pollinated heads were covered with glassine bags to prevent further pollination. Heads were either collected 2-3 weeks after pollination for embryo culture or were allowed to mature on the plants. In some cases spike culture was performed by detaching the pollinated spikes and growing them in a nutrient solution containing 100mg 2,4-dichlorophenoxyacetic acid (2,4-D) predissolved in 20ml ethanol, 8ml sulfurous acid and 50g sucrose per litre (Ushiyama et al., 1991)

For embryo culture, the collected heads were surface sterilised with 95% ethanol, the embryos were dissected from the immature seeds and placed on the surface of half MS medium (appendix) with 20g sucrose and 1g malt extract per litre. The petri dishes with the embryos were kept at 25°C in the dark by covering with aluminium foil until shoots and roots started emerging.

6.2.2 Development of segregating populations

Seven hexaploid wheat cultivars, Meering, Minto, Kiata, Lark, Vasco, Banks and Sunmist and four *Triticum tauschii* lines viz., AUS24242, AUS24230, AUS23986 and AUS24092 were used to establish intervarietal crosses. The *T. tauschii* lines were germinated in the month of May, considering their photosensitivity. The F_2 population of the selected hexaploid cross was grown in a hydroponic system. The seeds were sown in Jiffy pots with a mixture of 1 part peat moss, 1 part pine bark fines and 1 part rice hulls. The Jiffy pots were then transferred to the hydroponic units. The units were supplied with a nutrient solution made by dissolving a general-purpose hydroponic fertilizer in water (Simple Grow Company). The solution was maintained at pH 5.5-5.8 and replenished weekly. The growth temperature was maintained at 19±2°C and natural light was supplemented by halogen quartz iodide lamps (33-340$\mu Es^{-1}m^{-2}$ light intensity) with a 16h photoperiod. The F_2 population of the selected *T. tauschii* cross was grown in pots in bird-proof cage. Towards maturity, the plants were covered individually with perforated polyethene bags to collect the seeds. The hexaploid F_3 lines were grown in the field.

6.2.3 Amylose estimation

Starch extraction from the F_1 and F_3 seeds was done following the small and large scale extraction protocols respectively described in Section 2.2.2. Amylose content estimation by iodometric and HPLC methods are explained elsewhere (Section 2.2.3).

6.2.4 Genomic DNA Southern blot analysis

Genomic DNAs were isolated from young leaves of hexaploid and diploid parents and their segregating F_2s as described earlier (Section 4.2.3). Twenty microgram of DNA was digested with *Eco*R1 or *Bam*H1 and electrophoresed through an agarose gel (0.8%). The gels were blotted on to Hybond-N+ nylon membrane (Amersham). The filters were

probed with [α-^{32}P]dCTP-labelled DNA fragments specific for starch biosynthetic enzymes. The probes 5CO4B10 and 5CO4AD1 were kindly provided by Dr. T.A. Musket, University of Missouri, Columbia. Dr. S. Rahman, CSIRO Plant Industry, Canberra provided probes E7.8 and Sbe9. Hybridisations were carried out under the standard conditions as mentioned in Section 4.2.4. Following the hybridisation, the filters were washed at moderate stringency conditions, initially twice with 2 x SSC, 0.1% SDS at 65°C followed by two washes in 0.5 x SSC, 0.1% SDS at 65°C prior to autoradiography.

6.2.5 PCR amplification

Primers Sun1F and Sun1R (Shariflou and Sharp, 1999) specific for a microsatellite region tightly linked to the waxy gene were kindly provided by Dr. M. Shariflou, University of Sydney. A 20µl PCR reaction mixture contained 80µM dNTP, 1 x PCR buffer (Advanced Biotechnologies), 2mM $MgCl_2$, 5pmol of each of the primers, 75ng template DNA and 1 unit of *Taq* polymerase. PCR was conducted with an initial denaturation step of 95°C for 3 min followed by 5 cycles at 94°C, 57°C and 72°C each for 1 min, then 25 cycles of 94°C for 1 min, 57°C for 1 min and 72°C for 30 sec and finally holding at 25°C. The PCR products were run on a 6% non denaturing polyacrylamide gel with an acrylamide to bis ratio of 37.5:1.

The primers HVM4L and HVM4R were synthesized based on Maroof et al., (1994). PCR conditions were the same as before except that 200µM dNTP, 1.25mM $MgCl_2$ and 100ng DNA template were used and the annealing temperature was 56C°. The products were analysed on a 0.8% agarose gel. Seven primers specific for starch branching enzymes were provided by Dr S. Rahman and Ms L. Preston, CSIRO Plant Industry, Canberra. PCR using SBE I primers were run using the same conditions as that for the primers HVM4L and HVM4R. The reaction conditions for SBE II primers are described in Section 4.2.5.

6.2.6 Electrophoresis of GBSS

GBSS analysis was done following Zhao and Sharp, (1996). Purified starch granules (approximately 3mg) were mixed with 100µl extraction buffer (62.5mM Tris, pH 6.8, 2.3% SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.05% bromophenol blue) and heated in a boiling water bath for 5 min. The gelled starch was then cooled on ice and centrifuged for 5 min at 13,000g. Supernatant (30µl) was loaded on SDS-PAGE which has a 17% separating gel with an acrylamide:bis-acrylamide ratio of 30:0.1 in 375mM Tris-Cl (pH 7.8), and a 4% stacking gel with an acrylamide:bis-acrylamide ratio of 30:0.8 in Tris-Cl (pH 6.8). Both the gels contained 0.1% SDS. The gel running buffer was 25mM Tris, 400mM glycine and 0.1% SDS (pH 8.0). Electrophoresis was carried out on a vertical electrophoresis unit (Owl Scientific Plastics Inc., U.S.A) of dimension 175mm x 170mm x 0.7mm at 15mA per gel for 16 hours.

After electrophoresis, the gel was fixed for 30 min with 10% acetic acid and 30% ethanol, followed by washing once with 10% ethanol for 10 min and once with deionized water for 5 min. The gel was stained by soaking in 0.2% AgNO3 for 30 min. After rinsing the gel briefly with deionized water, the protein bands were developed in a developing solution containing 3% NaCO3, 0.07% formaldehyde and 2.5 ppm Na2SO3. When the bands were developed well, the gel was placed in 3% acetic acid and 3% glycerol solution for 10 min. The stained gel was finally dried overnight after wrapping with a cellophane sheet.

6.2.7 Statistical analysis

Analyses of variance (ANOVA) and regression analysis were done using the software EXCEL Version 7.0. The heritability estimates were calculated according to Falconer, (1964).

6.3 RESULTS AND DISCUSSION

6.3.1 Interspecific hybridization

With the aim of developing high amylose amphidiploid synthetic wheat lines, interspecific hybridization was attempted with four *T. dicoccoides* (two high amylose (AUS21499 and AUS21401) and two low amylose (AUS21317 and AUS21381) types and two high amylose *T. tauschii* accessions (AUS24242 and AUS24092), selected based on the results of the survey of amylose content (Chapter 2). The following approaches were adopted for hybridization between these tetraploid (AABB) and diploid (D) accessions.

1. Tetraploid as the female parent and diploid as the male parent (cross A) and allowing to set seed.

2. Diploid as the female and tetraploid as the male (cross B) followed by embryo rescue.

3. Cross A using a mixture of viable diploid and dead tetraploid pollens.

4. Cross A, detaching the spikes after pollination and culturing in a nutrient medium containing 2,4-D, followed by embryo rescue.

Tables 6.1 and 6.2 depict the results (in terms of seed/embryo set) of crosses A and B. Although there was a range of seed set varying from 0 to 17% when *T. tauschii* was used as the male parent with a comparatively higher set in the crosses of accession AUS24242, all the seeds were wrinkled in appearance and only a few germinated. The resultant seedlings were malformed with no proper root development. Mixing dead tetraploid pollen and viable diploid pollen did not improve the seed set. One of the major limitations observed in using diploids as the male parent was the inadequate availability of pollen. The reciprocal crosses involving *T. tauschii* as the female parents showed a higher level of crossability as shown by the reasonable number of embryos that could be rescued. However, none of the embryos grew in the media. Pollination

Table 6.1 Crossability of *T. dicoccoides* by *T. tauschii* accessions

Cross combination	No. of florets pollinated	Seed set (%)	Germination of F_1 seeds (No.)	Maturity of F_1 hybrids
AUS21401/AUS24242	85	3.5	0	0
AUS21381/AUS24242	38	0	0	0
AUS21499/AUS24242	37	13.5	0	0
AUS21317/AUS24242	51	9.8	2	0
AUS21401/AUS24092	87	5.7	1	0
AUS21381/AUS24092	48	17.0	0	0
AUS21499/AUS24092	88	0	0	0
AUS21317/AUS24092	83	4.8	2	0

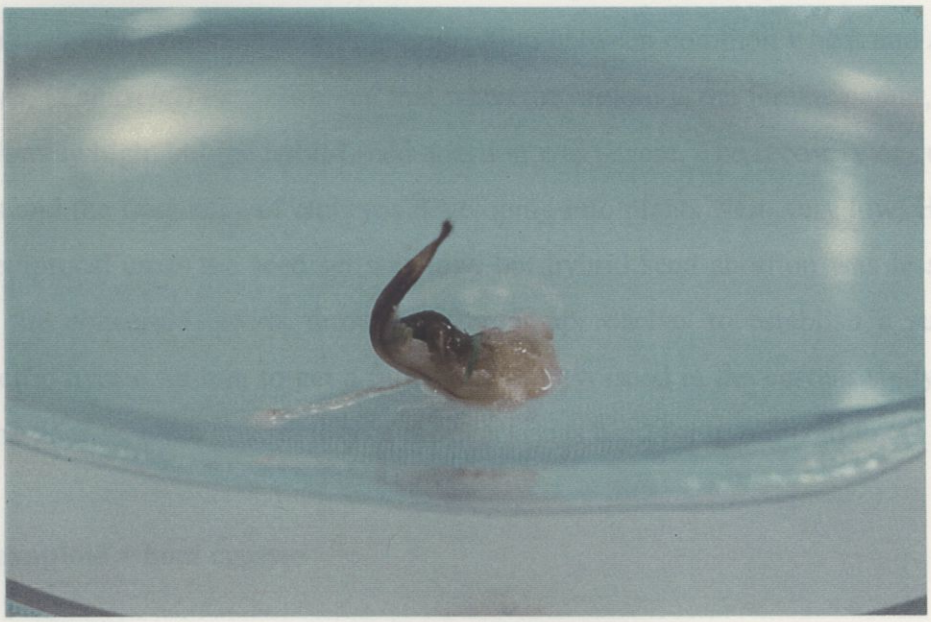
Table 6.2 Crossability of reciprocal crosses between *T. tauschii* and *T. dicoccoides* accessions

Cross combinations	No. of florets pollinated	No. of embryos rescued	No. of plants developed
AUS24242/AUS21401	81	14	0
AUS24242/AUS21381	49	8	0
AUS24242/AUS21499	55	18	0
AUS24242/AUS21317	56	14	0
AUS24092/AUS21401	45	12	0
AUS24092/AUS21381	63	9	0
AUS24092/AUS21499	45	0	0
AUS24092/AUS21317	69	0	0

followed by 2,4-D treatment yielded embryos which showed some signs of growth in the medium, but died eventually (Figure 6.1). In general, no successful outcome was achieved in the interspecific crossing programme.

In most studies of genetic transfer from diploid progenitor species to wheat, *T. turgidum* L. var. d*urum* was used as a bridging species (Sharma and Gill, 1983, Gill and Raupp, 1987). However, crossability of various *Triticeae* species with wheat is reported to vary among species and even among different accessions of a species (Fedak, 1998). The seed set obtained in interspecific or intergeneric hybrids can consist of water filled sacs with no discernable embryo or endosperm formation or a caryopsis with embryo formation only, but rarely a functional embryo plus endosperm. Different techniques are utilized to overcome the barriers of wide hybridization such as incompatability between parent species, inviability of F_1 hybrid and sterility of the F_1 hybrid or its progeny. Application of hormones after pollination to prevent the death of hybrid zygote, to

a) AUS21317/AUS24092



b) AUS21381/AUS24092

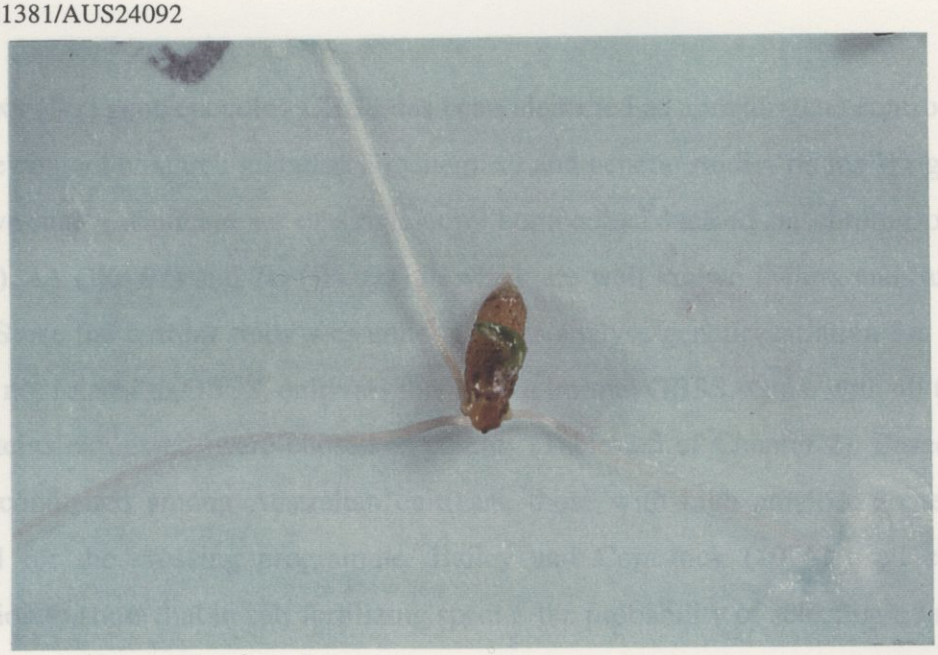


Figure 6.1 Malformed embryo growth in culture medium of interspecific crosses between *T. dicoccoides* and *T. tauschii*.

initiate seed set or to maintain a developing zygote till embryo culture is being possible are reported (Sharma and Gill, 1983, Ushiyama et al., 1991). Gill and Raupp, (1987) suggested that there are advantages and disadvantages in choosing the direction of the cross. In their study on interspecific hybridization between common wheat and *Aegilops squarrosa*, (*T tauschii*) they observed that when the diploid is the female parent, seed set was extremely high, but the hybrid seed abortion was severe. The recovery of culturable embryos and the frequency of embryos developing into plants were very low. However, in the reciprocal cross the seed set was low, but hybrid seed abortion was less severe. Despite the concerted efforts through different approaches to establish a successful interspecific hybrid, failure to get a functional embryo/seed in the current study may be due to the poor inherent crossability of the accessions used for hybridization.

6.3.2 Hexaploid wheat crosses

6.3.2.1 Selection of parents

The waxy (*Wx*) gene encoding GBSS has been identified as a major gene controlling the amylose content of starch granules. Biochemical and genetic studies on the *Wx* genes are extensive and a triplicate set of single-copy homoeoloci located on chromosomes 7A (*Wx-A1*), 4A (*Wx-B1*) and 7D (*Wx-D1*) in wheat are well known (Miura and Sugawara, 1996). Since the current study was undertaken to analyse genetic variation for amylose content not related to GBSS, cultivars that have a normal GBSS status, with all the three *Wx* proteins expressed, were chosen as parents (Table 2.5 of Chapter 2). Based on the survey conducted among Australian cultivars, those with high amylose content were selected for the crossing programme. Bailey and Comstock (1976) used computer simulation to show that in self fertilizing species the probability of selecting a line better than either parent is higher if both parents are equally good (as opposed to one being better than the other). The same conclusion was made earlier by Busch et al., (1974), based on their testing of 28 wheat crosses. However, the good parents must be sufficiently different genetically so that transgressive segregation can occur in their crosses. Since amylose is a quantitative character and the focus of the study was to breed

lines with increased amylose content, high amylose cultivars were used as parents hoping to accumulate favourable genes.

6.3.2.2 Inheritance studies

Initially five crosses were established using seven high amylose cultivars viz., Minto, Meering, Kiata, Lark, Vasco, Banks and Sunmist. The amylose content of the F_1 seeds using the endosperm half of each seed (the embryo half portion kept aside for planting) was analysed (Table 6.3). The crosses Kiata/Lark and Meering/Minto were high in amylose content among the crosses. However, since Kiata and Lark were found to be more polymorphic in several aspects (discussed in Section 6.3.4) than Meering and Minto, the cross Kiata/Lark was carried forward for further inheritance studies. Although it should be noted that other crosses might give better segregants, time constraints enabled only one cross to be further pursued.

Table 6.3 Apparent amylose content of F_1 seeds of hexaploid wheat crosses

Cross	Amylose % (Mean of 3 replicates)
Vasco/Minto	33.4
Meering/Sunmist	28.0
Meering/Minto	36.8
Kiata/Lark	37.1
Vasco/Banks	33.5
Minto	37.1
Rosella	28.0
CD (0.05)	2.5

Three hundred seeds from ten F_1 plants were grown to raise the F_2 population. Since the seeds bearing in a plant is a generation advanced than that of the plant itself, seeds of F_1 plants will represent the F_2 generation. Because each individual F_2 seed will be segregating, the data on F_2 have to be derived from each seed separately in which case it is hard to get starch enough for 3-4 replicate measures of amylose content (as shown in Chapter 2 replicated data are essential for getting reliable values by iodine method). Hence the amylose content of F_3 seeds was analysed which allowed pooling of starch within a plant due to more fixation of the character. Ten seeds from each F_2 plant (F3 generation) were pooled and starch extracted to get 248 starch samples representing a reconstituted F2 generation. The amylose content was estimated iodometrically in three replications. The values ranged from 31.2% to 38.7% with a parental mean of 34.85% (Kiata -. 34.8%, Lark – 34.9%) Figure 6.2 shows the frequency distribution of the F_2 generation. The analysis of variance showed a significant variation among the lines with an $LSD_{(0.05)}$ of 1.8. Since the iodine test gives an estimate of the apparent amylose content only (see Section 2.3.5 of Chapter 2), HPLC analysis of selected F_2 samples was performed to estimate the actual amylose content. Forty lines with high and 40 with low blue values were analyzed by HPLC. The amylose content ranged from 14.9% to 28.5% with a parental mean of 23.8% (Kiata – 24.0%, Lark – 23.7%). The lines differed statistically with $CD_{0.05)}$ of 2.04. The amylose content estimated by HPLC and iodine method showed an insignificant correlation of r=0.093 (Figure 6.3). While the selected lines based on iodometric method fell into two distinct classes (Figure 6.4), the HPLC values of these lines exhibited more or less a continuous distribution pattern, slightly skewed to the high amylose side (Figure 6.5).

Based on the actual amylose content estimated by the HPLC, 20 high and 20 low amylose lines were carried forward to the F3 generation. A narrower range of 19.7% to 26.0% amylose was estimated by HPLC among the F3 progenies (Table 6.4). A correlation of r=0.379 which is significant at the 0.01 level was observed between the F2 and F3 data.

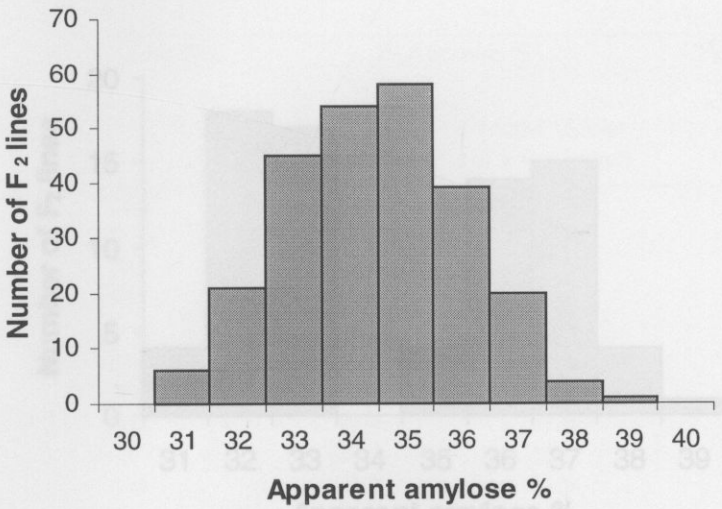


Figure 6.2 Frequency distribution of apparent amylose of F_2 generation of Kiata/Lark

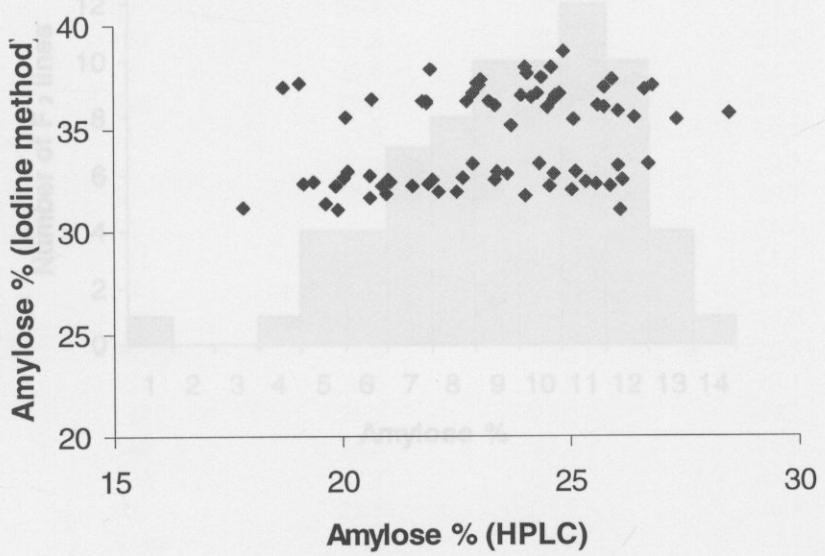


Figure 6.3 Relationship between the amylose content of selected F2 lines of Kiata/Lark estimated by the iodine and HPLC methods

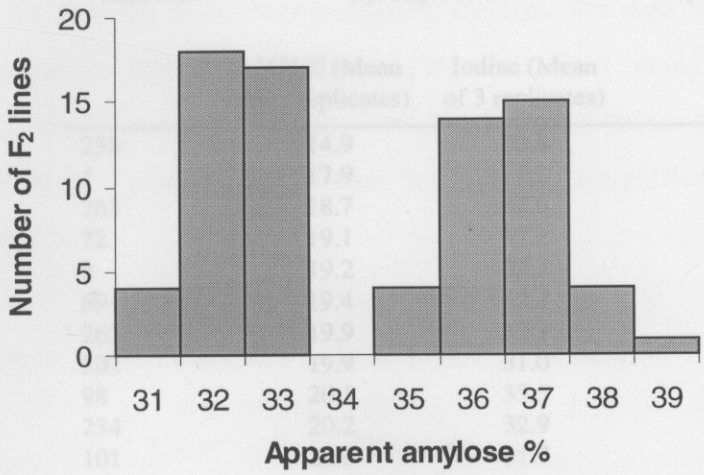


Figure 6.4 Frequency distribution of apparent amylose content of selected F_2 lines of Kiata/Lark

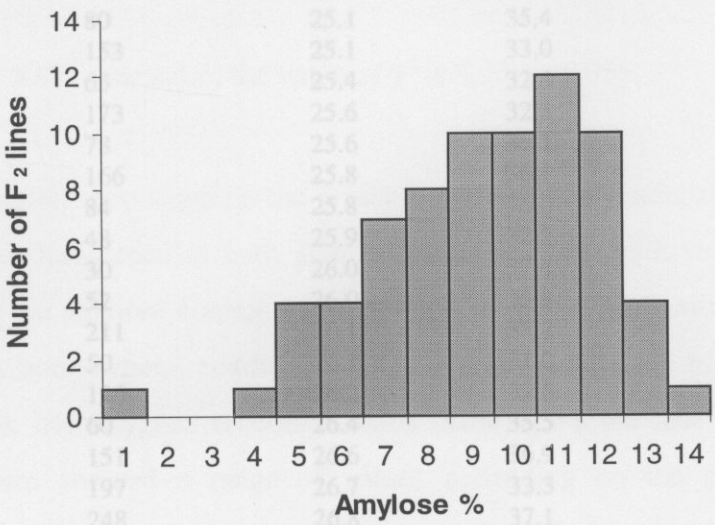


Figure 6.5 Frequency distribution of amylose content measured by HPLC of selected F_2 lines of Kiata/Lark

Table 6.4 Amylose content of selected F_2 and F_3 generations of Kiata/Lark

Line No.	F_2 Amylose %		F_3 Amylose %
	HPLC (Mean of 2 replicates)	Iodine (Mean of 3 replicates)	HPLC
256	14.9	32.8	19.7
1	17.9	31.2	21.3
263	18.7	37.0	18.9
72	19.1	37.2	18.9
2	19.2	32.3	20.2
69	19.4	32.4	20.1
262	19.9	32.3	18.7
202	19.9	31.0	19.7
98	20.1	35.5	22.0
234	20.2	32.9	19.9
101	20.6	31.7	20.1
36	20.9	32.3	23.2
185	21.0	31.9	18.7
104	21.0	32.6	20.1
112	21.1	32.4	20.4
128	21.6	32.3	20.7
73	21.9	37.8	18.6
43	22.0	32.5	20.4
46	22.5	32.0	19.1
261	24.4	37.5	19.0
183	25.1	32.0	20.9
80	25.1	35.4	23.0
153	25.1	33.0	20.3
65	25.4	32.4	20.1
173	25.6	32.3	23.0
78	25.6	36.1	24.3
166	25.8	36.1	19.3
84	25.8	36.0	19.1
48	25.9	32.2	20.7
30	26.0	37.4	20.7
52	26.0	35.8	21.0
211	26.1	33.2	21.5
50	26.1	31.0	24.5
126	26.2	32.5	25.0
60	26.4	35.5	20.5
151	26.6	36.9	18.1
197	26.7	33.3	19.2
248	26.8	37.1	26.0
108	27.4	35.4	25.6
85	28.5	35.7	20.8
Kiata	24.0	34.9	22.1
Lark	23.7	34.8	22.7
LSD (0.05)	2.0	1.8	-*

* Replicated data could not be obtained.

The segregation in the F_2 showed a continuous variation for the apparent amylose content as shown by the frequency distribution graph indicating the simultaneous segregation of many genes affecting the character. Since a continuous variation can also be created for reasons other than polygenic control, the data were examined further, trying to partition the effects. Out of the 248 lines analysed 23 lines showed significantly lower amylose content from the parental mean and 15 lines exhibited significantly higher values. All 210 other lines were not significantly different from the parental means.

The segregation pattern fitted in a ratio of 1:14:1 as follows:

Class	Observed numbers of progeny	Expected numbers of progeny
Group1 (Low amylose)	23	15.5
Group2 (Parental types)	210	217
Group3 (High amylose)	15	15.5

$\chi^2_{(1:14:1)} = 3.871$ (for 2 d.f., the value of χ^2 is 5.99, p=0.05)

The segregation ratio suggests the presence of 2 different independent genes (A and B) with partial dominance at both gene pairs and having additive effect for each allele controlling the amylose content in the population. Thus the dominant alleles of both the genes in a homozygous condition (AABB) gave rise to the high amylose genotype, whereas the homozygous recessive status (aabb) gave the low amylose types.All the heterozygotes showed a range of values depending on the effects of each allele. Nevertheless, the magnitude of the effect of each allele does not seem to be high enough to give significant variation between double and single heterozygotes. For example, the genotypes AABb and AaBb behaved in a similar manner as that of the parents with regard to amylose content. Thus the gene interaction ratio of 1:4:6:4:1 (further partitioned to 1:2:2:1:4:1:2:2:1) for a gene pair with partial dominance and additive effects for each partially dominant gene (Strickberger, 1985) is modified to 1:14:1 in

this case. Since the effect of each gene is not large enough to cause a recognizable discontinuity, the frequency distribution analysis showed a normal distribution pattern. There may be other minor genes also affecting the amylose content as shown by the frequency distribution curve. However, as no transgressive segregants with very high amylose phenotypes were detected in the population, it is more reasonable to state that not many polygenes controlling the amylose content have been cumulated in any of the F_2 lines. Hence the digenic segregation ratio of 1:14:1 gives a more satisfactory fit. However, variation in environmental influences between individuals also operate in producing continuity of measurement for quantitative characters. Strickberger, (1985) has detailed that segregation of a single gene difference with observable quantitative effects would be expected to produce three distinct phenotypes in the F_2 in the absence of dominance, but as the environment effects increases a greater variety of phenotypes are formed until the single gene character gives a normal distribution in the F_2. A broad sense heritability (h^2) was estimated to evaluate the proportion of the actual variance which is due to genetic reasons. An h^2 value of 75% was calculated from the analysis of variance components of the F_2 data. But this value has to be treated with caution since the data was derived from a single location trial. Under the conditions prevailed, the apparent amylose trait exhibited a moderate level of heritability, although the value indicated only the extent of genetic influence and not the selection gain that can be achieved. The narrow sense of heretability based on parent-offspring regression using the F_2 and F_3 HPLC data was estimated to be low (37.9%). However there is a bias in this estimation as the F_2 and F_3 populations were raised in different environments (see Materials and Methods).

6.3.3 Diploid wheat cross

Three high/low amylose crosses of *Triticum tauschii* lines, AUS24092/AUS23986, AUS24242/AUS23986 and AUS24242/AUS24230 were initially established. However due to the difficulty in managing large numbers of *T. tauschii* plants which have quarantine restrictions due to their wild and seed shedding nature, only one cross, AUS24242/AUS24230 was carried forward. This cross has the additional advantage of being more polymorphic at the parental level compared to other combinations, which

will be discussed later in this chapter. The F_1 seed amylose content was not analysed because of the difficulty in enough starch from half seed due to the small seed size. 125 F_2 plants were raised to get the F_3 seeds. The amylose content of the 116 F_2 lines (seeds from F_2 plants pooled the same way as in the hexaploid cross) including the parents was iodometrically analyzed and a wide range of values varying from 21.4% to 41.4% was obtained. The lines varied significantly in amylose content ($CD_{(0.05)} = 3.8$) with the low amylose parent (AUS24230) exhibiting 27.3% and the high amylose parent showing 33.2% amylose content. The mean F_2 value was 28.9%, which was closer to the low amylose parent. Transgressive segregation was observed in both directions with three lines showing amylose content significantly lower than the low amylose parent ($\leq$ 23.5%) and four lines exhibiting amylose content significantly higher than the high amylose parent ($\geq$ 37%). The frequency distribution graph appeared to have three different modes at 26%, 28% and 33% with 28, 58 and 28 individuals under each peak respectively (Figure 6.6). The segregation ratio is a good fit to a 1:2:1 ratio as follows:

Class	Observed numbers of progeny	Expected numbers of progeny
Group 1(Low amylose)	28	28.5
Group 2 (Intermediate)	58	57
Group 3(High amylose)	28	28.5

$\chi^2_{(1:2:1)} = 0.035$ (for 2 d.f., the value of χ^2 is 5.99, $p = 0.05$)

The segregation ratio indicated the possibility of monogenic inheritance with partial dominance and additive gene action. The mean F_2 amylose content being very close to the low amylose parent suggests a slight degree of partial dominance for low amylose. However, it would have been more clear if the F_1 mean amylose content tended to have a low amylose value. The extent to which a gene has a dominant or a recessive effect

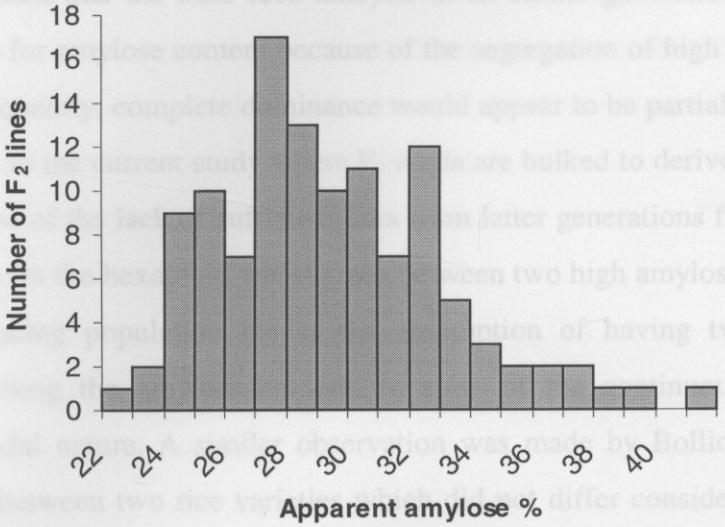


Figure 6.6 Frequency distribution of apparent amylose of F_2 lines of AUS24242/AUS24230.

may also be influenced by modifying genes (Strickberger, 1985). The wide variation observed within each class indicates the possibility of minor genes influencing the amylose content. A heritability value of $h^2 = 78.32\%$ was estimated from the analysis of variance components of the F_2 population. However this is based on a single location trial and hence it depicts the genotypic effect on the trait only on specific conditions that prevailed.

6.3.4 Discussion on the analysis of segregating populations

Numerous reports on inheritance of amylose content in different crops suggested varying types of gene action in controlling the trait. Incomplete dominance of high amylose to low amylose, controlled by one gene of major effects and several modifiers are reported in rice (Bollich and Webb, 1973).

However, Kumar and Khush (1987) demonstrated that the genes for high amylose content were completely dominant over those for low or intermediate amylose content

and stated that the bulk seed analysis in an earlier generation as F_2 would give lower values for amylose content because of the segregation of high and low amylose content. Consequently, complete dominance would appear to be partial dominance. Whether this is true in the current study where F_3 seeds are bulked to derive the F_2 data is not known because of the lack of sufficient data from latter generations for the confirmation of the results. In the hexaploid wheat cross between two high amylose lines, the analysis of the segregating population led to the assumption of having two genes with additivity controlling the amylose content, in spite of the continuous distribution pattern of unimodal nature. A similar observation was made by Bollich and Webb (1973) in a cross between two rice varieties which did not differ considerably in amylose content. Although a relatively symmetrical unimodal form of F2 curve typical of quantitative inheritance involving numerous genes of small and equal effects was obtained, the action of one or two genes were indicated by the recovery of considerable number of parental phenotypes. Genetically, amylose content in rice endosperm seemed to be controlled by one gene of major effect and several modifiers although amylose content often showed continuous variation in segregating populations (Sano, 1985, McKenzie and Rutger, 1983). According to Kumar and Khush, (1987), the lack of bimodal distributions in crosses involving parents with intermediate and high amylose content probably resulted from mainly two genetic reasons: 1) small differences between the amylose content of the parents, and 2) the segregants having one or two doses of gene for high amylose content either had values of amylose content intermediate to the two parents or very close to either of the two parents. Furthermore, segregation of modifiers together with sampling error may reduce the categorical differences among the segregants resulting in a pseudo-unimodal curve in F_2. Analysis of further generations, however, can resolve these uncertainties to some extent. Two dominant complementary genes affecting the amylose content in rice are also reported in the study of McKenzie and Rutger (1983). Thus, wherever several crosses are examined in a single study, different mode of inheritance are detected depending on the individual cross investigated.

Analysis of segregating populations of tetraploid and diploid wheat also indicated different types of gene actions (Mohammadkhani et al., 1999). Segregation ratios of 3:1

and 1:3 are observed in tetraploid wheat crosses indicating the dominance of either high or low amylose respectively. The authors pointed out that the continuous segragation revealed by certain crosses might have been consistent with a 1:2:1 ratio from a major additive gene. In agreement with the present study, one of their diploid wheat crosses showed a 1:2:1 ratio for additive gene action.

The hexaploid parents used in the study, Kiata and Lark, are both normal for GBSS with all the three genes-*Wx*-A1, *Wx*-B1 and *Wx*-D1 present and expressed as shown by the DNA and protein analysis (see Section 6.3.4). Also, both the diploid parents used, AUS24242 and AUS24230, have the waxy gene *Wx*-D1 present and expressed. However allelic variation was observed at this locus in both the cases. (see section 6.3.4). Hence, the segregation indicated in both the crosses may be due to the poymorphism at the waxy locus having an influence on the amylose content through differential activity of the expressed GBSS protein or due to loci other than *Wx*. Although there are reports on allelic variation at the waxy locus affecting the amylose content in wheat through the presence or absence of GBSS protein (Miura et al., 1994, Araki et al., 1999), no literature is available as to the allelic variation within the locus resulting in differential GBSS activity (other than presence/absence) which affects the amylose content. In rice, different waxy alleles- Wx^a and Wx^b appeared to regulate quantitative levels of *Wx* protein and the differential regulation at the waxy locus is one of the major factors controlling the amount of amylose in nonwaxy rice endosperm (Sano, 1985, Sano et al., 1985). The polymorphisms detected at SBE I and SBE IIa loci in Kiata and Lark, and the one detected at SBE I locus in AUS24242 and AUS24230 also might have influenced the segregation of amylose content in these crosses. Further analysis was done to see whether these polymorphisms are linked to amylose content or not which is discussed in Section 6.3.5.

The triploid nature of the endosperm tissue used for the starch extraction results in a segregation pattern different from that of embryo which is diploid. When the gene action is additive and a segregation ratio of 1 aaa:1 Aaa:1AAa;1AAA is expected, Mohammadkhani et al., (1999) postulated that a 1:2:1 ratio (as found in AUS24242/AUS24230) could be explained either by Aaa and AAa being

indistinguishable (or overlapping) in a 1:1:1:1 segregation or by there being a pair of genes, each with a 1:1 type of action. As standard diploid models of biometric genetics being inefficient to study the genetic control in triploid tissues, Pooni et al., (1992) have proposed a comprehensive set of models for triploid tissues. However testing of these models requires data from more generations than studied here.

The satisfactory estimates of broad sense heritability for both the crosses may support the simple genetic control of amylose content in these crosses rather than of a polygenic nature. However the estimates are made on a single location trial. A given population may give different estimates of heritability of a character depending on the environment at the time when the measurements were made. It is important to appreciate that heritability of a quantitative character is not solely the property of the character but of the character in a particular population under particular circumstances (Williams, 1964). The parent–offspring heritability representing the narrow sense heritability estimated for Kiata/Lark was low. The low heritability value could result from limited genetic diversity in the populations (Oliveira et al., 1994). Even though the F_2 of Kiata/Lark showed the possibility of two partially dominant genes with additivity, the effects of these genes are small as seen by the narrow range of values obtained. Also, the veracity of the estimate is doubtful as the estimation was made using data of F2 and F3 which were grown under entirely different environments. Where heritability is estimated based on the correlation between the phenotypes of the relatives, it is important to distinguish between correlations produced by similar genes and similar environment (Strickberger, 1985). Another important point to be noted is that the broad and narrow senses of heritability estimated in the Kiata/Lark population was based on the apparent (iodine method) and the actual (HPLC) amylose content respectively. The amylose content of selected F_2 lines estimated by the iodine method and HPLC showed poor correlation which could possibly be due to two reasons: 1) inaccurate measurement, more likely from the iodine method, and 2) the apparent and actual amylose content among the lines differed indicating some segregation for the amylopectin fraction of the starch. Chances of variation due to the first reason was remote since the iodine method used in this study was well optimized to get a good correlation with the HPLC method (Chapter 2). Frequency distribution graphs of the apparent and the actual amylose content (Figures

6.4 and 6.5) of the selected F_3 lines showed variations in the segregation patterns indicating the influence of the segregating genes on the amylopectin component. Because the boundaries between the amylose and the amylopectin fractions are arbitrary and the genes controlling the amylose content, in many cases, alter the amylopectin fraction also interpretations on the inheritance of amylose content has to be dealt with in caution.

Apart from the few major and many minor genes reported to control the amylose content, the cytoplasm and its interaction with the nuclear genes also found to have pronounced effect on the trait. Consequently, amylose content will be better manipulated if it is treated as a quantitative trait and due consideration is given to the cytoplasmic effects when choosing the parental sources (Pooni et al., 1993, Mohammadkhani et al., 1999). The crosses have been made in the current study only in one direction, and hence the cytoplasmic effects could not be studied.

Within the limitations of the present study, it is apparent that a high /low cross gave more variability and allowed better selection than a high/ high cross. The results are consistent with the findings of Kumar and Khush, (1987) and Mohammadkhani et al., (1999). However, because of the dosage effects in certain cases and the effects of minor genes having small effects, it is important to evaluate the breeding materials in several segregating generations to confirm the gene action and select segregants for desired amylose levels.

6.3.5 Analysis of polymorphisms among the parents

A candidate gene approach was adopted to detect polymorphisms among the parents. Five enzymes involved in starch biosynthesis, ADPG pyrophosphorylase, soluble starch synthase (SS IIb), granule bound starch synthase (GBSS), starch branching enzyme I (SBE I) and starch branching enzyme II (SBE IIa) were targeted for detection of RFLP polymorphisms (Table 6.5). Of the four probes used, E 7.8 specific for SBE I was

Table 6.5 RFLP probes used to detect polymorphisms among the parents under study

Probe	Targeted gene	Restriction enzyme	Polymorphism detected	
			Kiata and Lark	AUS24242 and AUS24230
E7.8	SBE I	Bam H1	+	+
Sbe 9	SBE IIa	Bam H1	+	N
Sbe 9	SBE IIa	Eco R1	-	-
5CO4B10	SS IIb	Bam H1	-	-
AD 1	ADPG pyrophosphorylase	Bam H1	-	-

N: not tested

polymorphic within both sets of parents, Kiata and Lark, and AUS24242 and AUS24230 after *Bam*H1 digestion (figures 6.7and 6.8). The probe sbe 9 specific for a conserved region of SBE IIa gene showed polymorphism between Kiata and Lark when *Bam*H1 was used for restriction (Figure 6.7), whereas these parent were not polymorphic after *Eco*R1 digestion. No polymorphism was observed between AUS24242 and AUS24230 at the SBE IIa locus with the probe sbe 9 on *Eco*R1 digestion. Result on *Bam*H1 digestion using the same probe was not available in the diploid parents. The probes specific for ADPG pyrophosphorylase and SS IIb did not reveal any polymorphism between any of the parents.

Five sets of primers targeting SBE I, two for SBE II and two for the waxy gene were used to detect polymorphisms through PCR (Table 6.6). The primers sr412R and sr406F specific for SBE I revealed polymorphism between AUS24242 and AUS24230 (Figure 6.9). None of the SBE II primers revealed any variation between any of the parents. At the waxy locus, the primers HVM4L and HVM4R did not reveal any polymorphism between Kiata and Lark (Figure 6.10), whereas the primers Sun1F and Sun1R detected polymorphism between these two cultivars (Figure 6.11). The two diploid parents were found to be polymorphic when amplified using the primers HVM4L and HVM4R (Figure 6.12). The primers Sun1F and Sun1R amplify a microsatellite regions at the 3' end of the wheat waxy gene (Shariflou and Sharp, 1999).

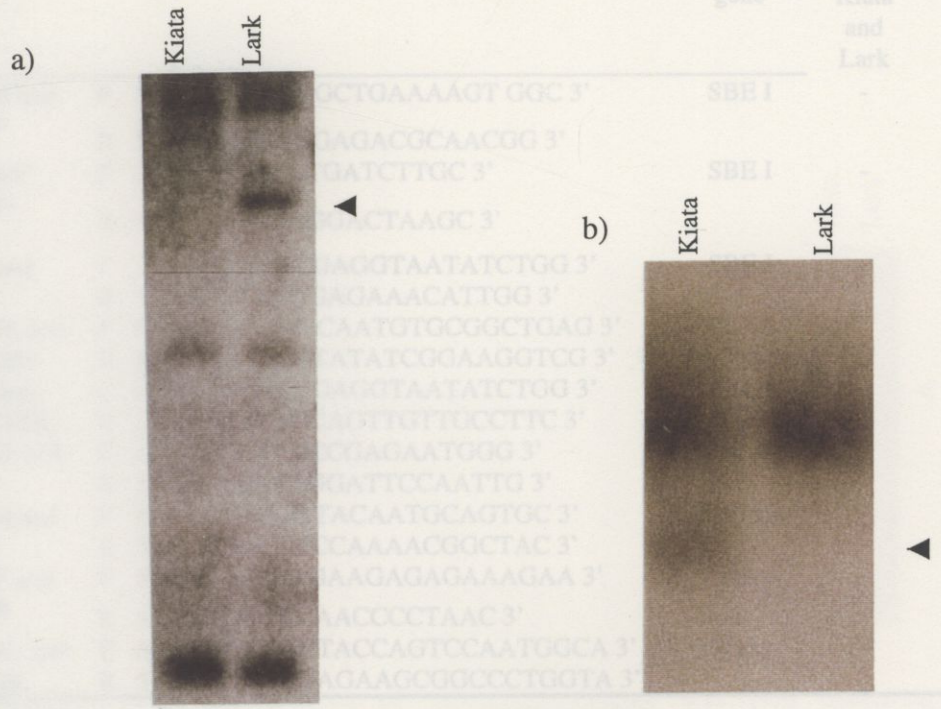


Figure 6.7 Autoradiographs showing polymorphisms between Kiata and Lark in a) SBE I using the probe E7.8 with *Bam*H1 digest, b) SBE II using the probe sbe 9 with *Bam*H1 digest.

Figure 6.8 Autoradiograph showing polymorphism between AUS24242 and AUS24230 in SBE I using the probe E7.8 with *Bam*H1 digest.

Figure 6.9 Agarose gel showing polymorphism between AUS24242 and AUS24230 in SBE I on amplification with the primers Sr412R and Sr406F.

Table 6.6 Primers used to detect polymorphisms among the parents under study

Primer		Sequence	Targeted gene	Polymorphism detected	
				Kiata and Lark	AUS24242 and AUS24230
Sr412R and Sr406F	F	5' TGACGAAGGCTGAAAAGT GGC 3'	SBE I	-	+
	R	5' TGGCGCCGGAGACGCAACGG 3'			
533-540F and 520-F380	F	5' TGTTTACCTGATCTTGC 3'	SBE I	-	N
	R	5' ATCTTCCAGGACTAAGC 3'			
S30R and 538R	F	5' TGGGTAATGAGGTAATATCTGG 3'	SBE I	-	-
	R	5' CATTTACGGAGAAACATTGG 3'			
ZLE15d and ZLBE163	F	5' GGCGGCGGCAATGTGCGGCTGAG 3'	SBE I (intron 2)	-	-
	R	5' CCAGATCGTATATCGGAAGGTCG 3'			
S30R and WBE1E9R	F	5' TGGGTAATGAGGTAATATCTGG 3'	SBE I (intron 8)	-	-
	R	5' CATAACTCCAGTTGTTGCCTTC 3'			
SR913F and E6R	F	5' ATCACTTACCGAGAATGGG 3'	SBE IIa	-	-
	R	5' CTGCATTTGGATTCCAATTG 3'			
da5.seq and E11R	F	5' GGCTTGGATACAATGCAGTGC 3'	SBE IIa	-	-
	R	5' CTGGAGTTCCAAAACGGCTAC 3'			
Sun 1F and Sun 1R	F	5' CGCTCCCTGAAGAGAGAAAGAA 3'	Waxy	+	-
	R	5' ATAGGCACAACCCCTAAC 3'			
HVM4L and HVM4R	F	5' AGAGCAACTACCAGTCCAATGGCA 3'	Waxy	-	+
	R	5' GTCGAAGGAGAAGCGGCCCTGGTA 3'			

N: not tested

At the protein level, GBSS, SBE I and SBE II were targeted to detect polymorphisms among the parents. The parent lines were not polymorphic at the GBSS level, with all the three waxy proteins present in both the hexaploid parents and the 7D isoform present in both the diploid parents (Figure 6.13). Immunoblotting studies using a primary antibody raised against a synthetic peptide designed from the amino acid sequence at N-terminus of wheat SBE I revealed polymorphism between AUS24242 and AUS24230 (Figure 6.14). Kiata and Lark showed variation in the SBE IIa pattern when probed with a primary antibody raised against a synthetic peptide designed from the N-terminus sequence of a wheat SBE IIa (Figure 6.15).

The polymorphisms observed among the parents at the DNA level was analysed in the segregating F_2 populations of Kiata/Lark and AUS24242/AUS24230 crosses. Each

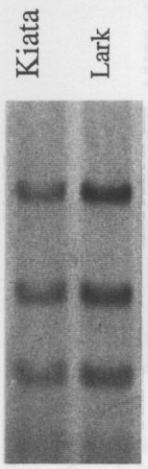


Figure 6.10 Non denaturing PAGE of PCR products of hexaploid wheat parents amplified using the primers HVM4L and HVM4R specific for waxy gene.

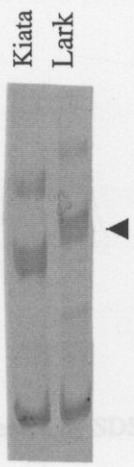


Figure 6.11 Non denaturing PAGE gel showing polymorphism between Kiata and Lark on amplification using the primers Sun1F and Sun1R specific for a microsatellite locus linked to the waxy gene.

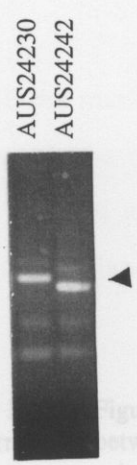


Figure 6.12 Agarose gel showing polymorphism between AUS24242 and AUS24230 at the waxy locus when amplified with HVM4L and HVM4R.

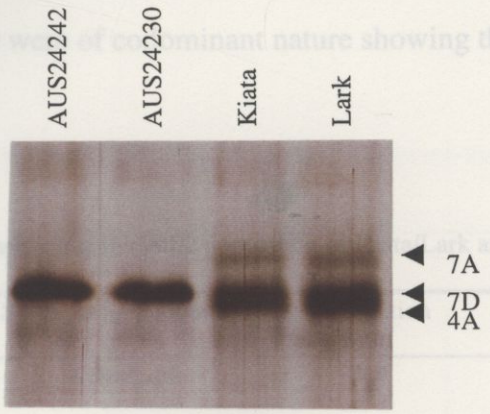


Figure 6.13 GBSS proteins in the hexaploid and the diploid wheat parents as revealed by SDS PAGE. Chromosomal location of each isoform is indicated as per Zhao et al., 1999.

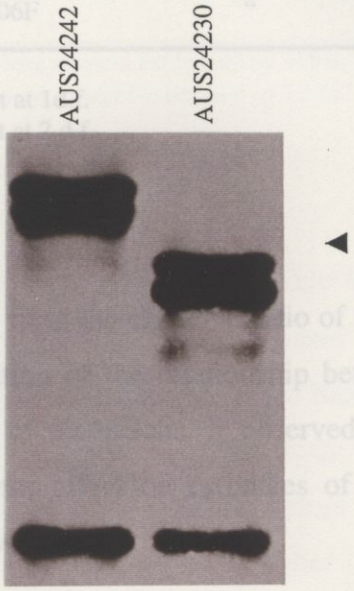


Figure 6.14 Polymorphism detected in SBE I between AUS24242 and AUS24230 on Western blotting using anti- wheat SBE I antibody. Highlighted from figure 5.1 (Chapter 5).

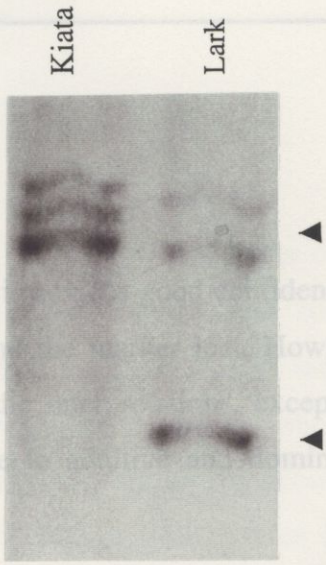


Figure 6.15 Polymorphism detected in SBE II between Kiata and Lark on Western blotting using anti-wheat SBE II antibody. Highlighted from figure 5.5 (Chapter 5).

marker locus is indicated by the probe or primers that detected the polymorphism. Out of the three marker loci scored in the F_2 of Kiata/Lark, two of them fitted a 3:1 segregation ratio indicative of a dominant marker and one the 1:2:1 ratio of a codominant marker (Table 6.7). All the three marker loci scored in the F2 of AUS24242/AUS24230 were of codominant nature showing the expected 1:2:1 ratio.

Table 6.7 Segregation of marker loci in the F2 population of Kiata/Lark and AUS24242/AUS24230

Marker locus	Segregating population	Segregation ratio	Observed numbers	χ^2
E7.8	Kiata/Lark	3:1	57:16	0.367[a]
Sbe 9	"	3:1	54:18	0[a]
Sun 1F and Sun 1R	"	1:2:1	17:34:21	0.667[b]
E7.8	AUS24240/AUS24230	1:2:1	20:29:21	2.086[b]
HVM4L and HVM4R	"	1:2:1	33:52:24	1.704[b]
Sr412R and Sr406F	"	1:2:1	32:48:29	1.69[b]

[a] Non significant at 1d.f.
[b] Non significant at 2 d.f.

The adequate fit in the expected ratio of all the marker loci provides good confidence in the interpretation of the relationship between the trait and the marker loci. However, small degree of distortions, if observed, did not affect the interpretations, except for having a minor effect on estimates of the variance due to additive and dominance contrast (Edwards et al., 1987).

Since amylose can be better manipulated when treated as a quantitative trait (Pooni et al., 1993) a quantitative trait loci (QTL) approach was adopted to see whether any of these marker loci are linked to amylose content. Single factor ANOVA for each pairwise combination of the trait and marker locus was done and *F*-tests were used to determine if significant variation in trait expression was associated with differences in marker-locus genotypic classes (Edwards et al., 1987). Significant F-values were interpreted to

indicate segregation of genotypes at a quantitative trait locus (QTL), which is linked to the marker locus. Table 6.8 shows the genotypic variance due to the different marker classes within each marker locus. In the Kiata/Lark population, separate analyses using the data on amylose content estimated by iodine and HPLC methods were done.

Table 6.8 Analysis of variance (ANOVA) of amylose content between marker classes at various marker loci

Marker locus	Segregating population		Genotypic variance	d.f.	F	P-value
E7.8	Kiata/Lark	I	1.580	1	0.300	0.586
		H	38.361	1	6.359	0.013
Sbe 9	"	I	1.287	1	0.250	0.618
		H	4.053	1	0.610	0.437
Sun 1F and	"	I	2.240	2	0.222	0.801
Sun 1R		H	10.487	2	0.787	0.459
E7.8	AUS24242/AUS24230	I	1.148	2	0.040	0.960
HVM4L and HVM4R	"	I	14.804	2	0.517	0.597
Sr412R and Sr406F	"	I	14.538	2	0.508	0.603

I based on amylose content estimated by iodine method
H based on amylose content estimated by HPLC method

No significant genotypic variance due to marker classes was revealed in either of the populations when the marker classes are grouped based on the amylose content estimated by the iodometric method. Based on the HPLC data, a SBE I locus, as assayed by E7.8 showed statistically significant variation between the marker classes, showing linkage of this locus to HPLC amylose content. The mean value for all the genotypes with the allele from Lark was higher (23.5%) than those with the allele from Kiata (21.7%). In order to estimate the proportion of phenotypic variation explained by this marker locus, the trait value was regressed (y) on to the marker genotype (x) (Kearsey, 1998) to get the R^2 value. In this way it was estimated that E7.8 locus explains 8.2% variation in the amylose content in the population.

6.3.6 Discussion on the analysis of polymorphisms among the parents

Although amylose content is greatly influenced by the *Wx* genes, they do not explain all the variation in this trait as shown by variability observed in the segregating populations in spite of the parents being normal for the *Wx* genes. Analysis of a single chromosome recombinant substitution lines derived from a cross of a normal and null4A GBSS lines revealed more than 70% of the variation accounted by allelic differences at the *Wx-B1* locus (Araki et al., 1999). A single nucleotide polymorphism in a microsatellite linked to the *Wx* gene could explain 79.7% of the variation in the apparent amylose content of 89 non-glutinous rice cultivars (Ayres et al., 1997). However, within the null4A class in wheat with *Wx-B1b* allele, a QTL (*Qamc.ocs-4A.1*) mapped in the 6.2cM *Xbcd1738/Xcdo1387* interval on the short arm of 4A was detected which explained 17% variation. The effect of this QTL was masked in the lines normal for GBSS; an epistatic interaction with *Wx-B1a* appeared to decrease the action of *Qamc.ocs-4A.1* in the presence of *Wx-B1* protein.

No significant QTL effects were observed in any of the populations when iodometric estimates were considered as the trait values. Even at the E7.8 locus where a QTL was detected in the hexaploid population when HPLC estimates were taken as the trait value, use of iodine values failed to detect any effect. The interference of the amylopectin fraction in the iodometric method of amylose estimation might have confounded the effect due to the marker. Hence it is obvious that in attempts to find markers linked to amylose content it is more appropriate to use analytical techniques which give more accurate amylose measurements such a the HPLC method.

The current study detected a QTL in the hexaploid cross at the SBE1 locus which is located on the group 7 chromosomes in wheat (Morell et al., 1997, Rahman et al., 1999). However, this effect was not observed in the *T. tauschii* cross even though the parents had a polymorphism detected by the SBE I probe E7.8. One of the reasons may be that the trait measurement in the diploids was based on the iodometric method. It is also not clear from which genome the probe E7.8 identified the polymorphism between

the hexaploid parents, as aneuploid analysis was not performed. However, the autoradiographs show that the polymorphism detected between Kiata and Lark is different from that between the diploid lines. It is also likely that the polymorphism exhibited in the hexaploid is from the A or B genomes absent from the diploid lines studied. So, it is possible the polymorphism within the hexaploids is associated with a SBE I allele effective in altering amylose content, whereas, that observed in the diploids is not. Previous evidence suggests that alteration of SBE I does not give high amylose but gives subtle alterations in starch structure and functionality (Muller-Rober and Kobmann, 1994, Baga et al., 1998). Failure to detect a QTL for a trait linked to a particular marker locus does not imply that there is no QTL in the region (Edwards et al., 1987). The two parental inbreds may have identical alleles at a linked QTL, which thus escapes detection. Alternatively, the parents may have different alleles at the QTL, which have equivalent expressions for the particular trait. Analysis of SBE I at the protein level did not reveal any polymorphism among the hexaploid parents, whereas it was polymorphic among the diploid parents. However, it is possible that the activity of the expressed proteins vary even though they are not polymorphic. In addition, previous studies have demonstrated that mutant alleles affecting the starch pathway not only have site-specific effects on metabolism, but also influence non-target enzymes. Changes in starch fine structure observed with mutants are a consequence of specific reductions in some enzymes, perhaps in combination with over expression of other enzymes involved in starch biosynthesis (Singletary et al., 1997). Hence, the mutation in the SBE I gene is affecting the amylose content either through altered SBE I activity and/or it is influencing other starch biosynthetic enzymes leading to variation in the amylose content. Polymorphic proteins bearing similar enzymatic activity is also likely as the case may be in the diploid parents with regard to SBE I and in the hexaploid parents with regard to SBE II. The polymorphisms observed at the *Wx* locus did not lead to any difference in expression of the proteins in both diploid and hexaploid parents. The polymorphisms observed in SBE I (AUS24242/AUS24230) and SBE IIa (Kiata/Lark) at the protein level were not analysed in the respective generations due to time constraints.

The current study targeted only certain genes involved in the starch biosynthetic pathway providing very low genome coverage. Amylose content is a complex trait

controlled by several major genes and many modifiers (Mohammadkhani et al., 1999). The ditelosomic analysis reported in Chapter 4 indicated that various chromosomes have an influence on amylose content. While chromosome 5D and 6A showed some major effects, others like group 3 and 1 also showed minor, but significant effects which are not explained by any of the known starch biosynthetic enzymes. The power of a candidate gene approach for detecting QTLs is limited for a complex trait like amylose content which is the product of the concerted effects of many genes. A random marker approach giving a wide coverage over the three genomes coupled with candidate gene approach is suggested to be more potent in capturing QTLs with major and minor effects.

Availability of genetic loci which alter the amylose content even to a small extent will make it feasible to fine-tune genotypes to meet specific requirements (Araki et al., 1999). In this context the QTL for amylose content detected at the E7.8 locus is potentially valuable. Molecular markers for this locus will allow more precise alterations of amylose content than would be possible on the basis of phenotypic selection alone.

CHAPTER 7

General Discussion

The ratio of amylose to amylopectin is an important parameter in characterizing a particular starch. A rapid, but cost-effective, procedure for determining this ratio is required in most investigations of starch. In amylose breeding programmes it is essential to select for very small inherent differences in amylose content and the error in determination should not exceed the selection differential for breeding progress to be made (Fergason, 1994). In such experiments accurate and reproducible methods of amylose estimation provide the basis for a phenotype driven genetic analysis. Three methods of amylose estimation of wheat starch, iodometric, HPLC, and conA precipitation were compared. Due to its efficacy in achieving uniform replicate estimates, the HPLC method was found to be reliable over iodine method. However, wherever the system is not readily available the iodine method followed in the current analysis (Chapter 2) could be adopted due to the strong positive correlation between the amylose values estimated by these methods. However, in breeding experiments aimed at identifying markers linked to amylose content, there is a possibility of complication of the information on marker-amylose linkage due to segregation of genes affecting the amylopectin fraction, through an effect on the estimated amylose content using the iodine method. The HPLC and conA methods, where the influence of amylopectin fraction is lower than in the iodine method provide results focused on amylose content alone.

Information regarding the available genetic variability in amylose content is a prerequisite for initiation of a breeding programme aimed at increasing amylose content. Among cereal starches, a broad range of amylose content is known in diploid species such as barley, maize and rice, with natural mutant lines of phenotypic extremities like waxy (0% amylose) and high amylose (40-90%) reported in each of these plants (Shannon and Garwood, 1984, Fergason, 1994, Salomonsson and Sundberg, 1994). The range observed in wheat was much more narrow (23-39%) when 75 Australian hexaploid varieties were sampled. Examining exotic varieties did not extend the range.

As mentioned earlier, the buffering action of multiple genomes may impede the phenotypic expression of any possible mutations in a polyploid species like wheat. If this is the case, there has to be a higher chance of locating waxy and/or high amylose phenotypes in tetraploid and diploid species of wheat. The current study detected a range of only 17-35% within the limited number of diploid and tetrapolid species of wheat studied. Watanabe et al., (1998) could not pick up any lines with more than 45% amylose content in a more extensive survey of 1,397 accessions of *T. durum* (AABB) and *T. tauschii* (DD). The current survey found considerable non-GBSS variation in amylose content in Australian hexaploid wheat. The material generated in the survey can be utilized in the genetic analysis of non-GBSS variation in amylose content.

Choosing lines with extreme amylose phenotypes as parents for hybridization is found to be more feasible in extending the genetic variability than selecting parents with identical amylose phenotypes. In the cross between high and low amylose *T. tauschii* lines, although a monogenic inheritance with partial dominance and additive gene action was observed, the F2 segregants ranged from 21% to 41% amylose with indication of transgressive segregation typical of quantitative inheritance. Since amylose content of starch is a complex trait influenced by several major and minor factors as shown by the aneuploid analysis and inheritance studies (Chapters 4 and 6), it is advantageous that parents may be selected based on phenotypic expression coupled with molecular and biochemical analyses targeting various known starch biosynthetic enzymes. For an unbiased selection of favourable segregants, providing uniform growth conditions and using seeds of uniform maturity for amylose analysis are extremely important.

Transgressive segregation detected in the *T. tauschii* cross (AUS24242/AUS24230) opens the possibility of selecting a higher amylose genotype from subsequent generations (time constraints did not allow the pursuit of any generation after the F_3 in this study). Following the fixation of the character, the line can be utilized for introgressing into hexaploid wheat through a bridging cross with a suitable tetraploid line. A few interspecific crosses between *T. dicoccoides* and *T. tauschii* were attempted in the current study, but none were successful. Since crossability is species and accession specific, it is worth attempting further crosses in the future as accession

AUS24230, the female parent of the *T. tauschii* cross was not included in the interspecific hybridization experiments. Post pollination application of growth regulators and manipulation of culture conditions could also be tried as these practices are reported to improve crossability and the frequency of embryo germination respectively (Fedak, 1998).

In spite of being comprised of a single building block, glucose and just two linkage types(α 1,4 and α 1,6), starches differ in their properties widely between and within species. Differential organization of starch granules, varying ratio and degrees of branching of amylose and amylopectin molecules, and distinctive levels of inclusion compounds like lipids are some of the factors contributing to the diversity in starch properties. The current examination of structural and functional properties of *T. aestivum* and *T. tauschii* starches has unearthed some significant difference between starches of these two species. In general, *T. tauschii* starches had better gelling ability compared to hexaploid starches indicating the possibility of selecting suitable donors from among *T. tauschii* lines in order to breed for improved starch gelling ability suitable in the food industry. Starch of the *T. tauschii* accession AUS24242 seemed to be interesting in that it had a higher amylose content coupled with higher peak viscosity, as against the commonly established inverse relationship of amylose content and peak viscosity. AUS24242 starch should be further studied to reveal factors influencing pasting properties apart from amylose content. The distinct starch granule distribution patterns observed between the hexaploid and *T. tauschii* starches appear to be of significance in understanding the genetics behind starch granule size distribution in wheat. While the hexaploids revealed a bimodal distribution typical of that of previously reported wheat starches, *T. tauschii* starches exhibited a unimodal type of distribution with ~50% lower proportion by volume of B granules compared to hexaploid starches. Studies by Soulaka and Morrison (1985) revealed little difference in the A and B granules between bread (AABBDD) and durum (AABB) wheat starches. As discussed in Chapter 3, it is possible that the genes controlling the secondary granule initiation in hexaploid wheat reside in A and/or B genomes.

Starch is synthesized in higher plants through the sequential action of at least four known classes of enzymes, ADP glucose pyrophophorylase (AGPase), starch synthase (SS) starch branching enzyme (SBE) and debranching enzyme (DBE). Altered amylose content in association with mutations at loci encoding SS (*Du1* affecting SS III giving an increased amylose content and *wx* affecting GBSS resulting in a reduced amylose content), SBE (*ae* affecting SBE IIb resulting in an increased amylose content) and DBE (*su1* affecting DBE giving an enhanced amylose content) are known (Boyer and Preiss, 1978, James et al., 1995, Nakamura, 1996, Gao et al., 1998). In wheat, chromosomes 7A, 7B and 4A are reported to influence amylose content, mainly through the location of waxy genes encoding GBSS in these chromosomes, although other starch biosynthetic enzymes like SBE1 (Morell et al., 1997, Rahman et al., 1997), AGPase and soluble SSI and SSII (Devos and Gale, 1997, Li et al., 1999a and b) are also localized on Group 7 chromosomes. The analysis of ditelosomic stocks of CS revealed a more complex picture with the deletion of the short and long arms of chromosomes 5D and 6A respectively having effects more pronounced than deletion in any other chromosomes. The lines dt5DL and dt6AS showed around 10% difference in amylose content from euploid CS in the positive and negative directions respectively. As discussed in Chapter 4 interpretation of the results of aneuploid analysis is not straightforward due to the deletion of many genes from any one particular line. Apart from the increased amylose content, dt5DL starch also revealed some significant structural and functional changes. The focus of the current research being dissecting the possibilities of increasing the amylose content in wheat, dt5DL starch provides an opening to be pursued in further research.

Although *in vitro* studies have shown that SBE I transfers longer glucan chains and has higher affinity for amylose than SBE II, the *in vivo* effects of SBE I and SBE IIa on amylopectin synthesis is still unclear due to the absence of mutants lacking these enzymes. Analysis of CS Group 2 chromosome deletion stocks did not reveal any significant effect on amylose content due to the absence of a SBE IIa locus. This is probably due to complementation by the loci on the other two genomes.

The scope of the use of aneupoids in genetic analysis of wheat is limited mainly due to its polyploid nature. Also, the various types of chromosomal aberrations observed in the CS nullisomic-tetrasomic and ditelososmic stocks of wheat might lead to misinterpretation of experimental results. Hence more sophisticated analysis is required in wheat for genetic studies. Identification of all possible genes influencing amylose content is essential in manipulating the phenotype in the desirable direction. Partial characterization of the isolated SBE II genomic clone revealed that it corresponded to SBE IIb type genes with higher similarity to barley SBE IIb than to maize SBE IIb. Phenotypes with increased amylose content in barley were reported to be controlled by the *amo1* gene, which was located in chromosome 5 (1H) (Schondelmaier et al., 1992). More recently the SBE IIb gene in barley was localized on the same chromosome by Sun et al., (1998), which raises the possibility that the *amo1* and SBE IIb genes are one and the same, similar to the maize situation where the *ae* gene encodes for SBE IIb. Preliminary results have revealed that SBE IIb in wheat is located on the chromosome 1 (Rahman et al., in prep), which agrees with the results from barley. So far only one form of SBE II has been detected in the soluble phase of wheat endosperm and the N-terminus of this resembled that of SBE IIa. The expression of SBE IIb in wheat needs to be further investigated. If wheat SBE IIb has an activity corresponding to maize SBE IIb, the lack of which resulting in an amylose extender phenotype, then it may be possible for an amylose extender phenotype to be developed in wheat through plant breeding and genetic engineering techniques.

Molecular markers are now well demonstrated to be of value in the analysis of the inheritance of traits in wheat and understanding genome structure and organization, although the intricacy of the wheat genome has delayed the development and application of molecular markers in this plant. There are now well over 50 loci, largely of disease resistance, that have been tagged with molecular markers in wheat (Landgridge and Chalmers, 1998). Markers linked to quality aspects are also emerging in literature (Blanco et al., 1996, Zhao et al., 1998,). However, there are very few reports of markers associated with amylose content (Ayres et al., 1997, Araki et al., 1999). As the genetic control of amylose is complex as indicated by the current study and earlier reports, the trait is better manipulated as a quantitative trait (Puri et al., 1980, Pooni et al., 1993).

Hence, the identification of individual quantitative trait loci (QTL) will provide insight into the relative contribution of major and minor genes. Selection for linked markers in breeding programmes can avoid multiple evaluation over years and locations along with the laborious process of amylose estimation over large number of segregating samples. A candidate gene approach aiming at various starch related enzymes was fruitful in detecting QTLs linked to amylose content, such as the *Wx-B1* locus accounting for 70% of phenotypic variation in wheat, Qamc.ocs-4A.1 detected within the *Wx-B1b* allele having 17% effect in wheat(Araki et al., 1999), a microsatellite polymorphism linked to *Wx* gene explaining 79.7% of the variation in rice (Ayres et al., 1997) and E7.8 in the SBE1 locus (current study) accounting for 8% of the variation. Considering the complex inheritance of amylose content in wheat which is influenced by different chromosomal regions apart from the known starch biosynthetic enzyme loci (Chapter 4), a high resolution QTL analysis coupling candidate genes and anonymous markers covering all the three genomes is expected to be more effective than focusing the candidate genes alone. Very careful choice of parents in establishing the marker population is important.

A successful interaction of efficient DNA delivery methods, *in vitro* regeneration techniques and suitable gene expression cassettes containing targeted gene has facilitated the development of transgenes in wheat. A survey report on the current status of wheat transformation reveals that manipulation of starch composition by the expression of various biosynthetic enzymes are receiving increased attention, although quite a lot of data remaining confidential (Barcelo et al., 1998). Altered endosperm starch structure following a reduction in SBE 1 activity in the developing wheat kernel by integration of SBE 1 gene expression cassettes in the sense and antisense orientation (Chibbar, et al., 1998) is an achievement of significance. Suppression of SBE IIb activity by sense co-suppression and antisense suppression will be a future direction towards the development of an amylose extender phenotype in wheat. Since antisense RNA technology usually results in the generation of plants showing a range between no and complete inhibition, selection of plants with an optimal degree of inhibition is possible, where the amylose content is maximized with minimum negative effects leading to yield penalties (Muller-Rober and Kobmann, 1994). Further studies are needed to determine whether there are SBE IIb alleles on all the three genomes as in the

case of SBE 1 and SBE IIa. Screening *Triticum* germplasm for partial SBE IIb mutations lacking one or two of the three proteins and using such partial mutations to develop tetra- and hexaploid mutants through hybridization programmes, as in the case of waxy wheat development, can be a parallel strategy for producing a high amylose phenotype in wheat.

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Appendix

MS Regeneration Medium

Ingredients	Concentration (mg/l)
KNO_3	1,900.00
NH_4NO_3	1,650.00
$CaCl_2.2H_2O$	440.00
$MgSO_4.7H_2O$	370.00
$KH_2PO_4.H_2O$	170.00
$MnSO_4.4H_2O$	22.30
$ZnSO_4.7H_2O$	8.60
H_3BO_3	6.20
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CoCl_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.025
$Na_2EDTA.2H_2O$	37.26
$FeSO_4.7H_2O$	27.80
Myo-inositol	100.00
Glycine	2.00
Thiamine-HCl	0.40
Nicotinic acid	0.50
Pyridoxine-HCl	0.50
Sucrose	30,000.00
Agarose (Type 1-A, Sigma)	2,500.00

PH: 5.7-5.8