The Regulation of Starch Biosynthesis in Barley (*Hordeum vulgare*)

by

Zaheer Ahmed

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ABSTRACT

THE REGULATION OF STARCH BIOSYNTHESIS IN BARLEY (*Hordeum vulgare*)

Zaheer Ahmed
Advisor: Dr. Michael J. Emes
University of Guelph, 2014

Starch has enormous uses in the food and non-food industries in different forms. An important form of starch in the food industry is resistant starch (RS). Resistant starches (RS) have potential benefits for human health as a source of low-glycemic carbohydrate and as a prebiotic for the large colon. Such starches are often referred to as high-amylose though this may reflect changes in amylopectin as well the proportion of amylase in the starch granule. This study investigated the relationship between resistant starch (RS) and physical properties of the starch which may contribute to increased RS.

The role of protein phosphorylation, and protein complex formation between enzymes of starch synthesis, was also studied in a range of barley genotypes exhibiting high, low and normal amylose phenotypes. In the final part of the thesis, the relationship between variations in starch physiochemical properties and starch granule proteome from genotypes with no known mutations in starch biosynthetic enzymes was investigated. The results indicate that increased RS is positively associated with increased amylose and B-granule content. Detailed biochemical analysis of barley mutants, with alterations in the starch biosynthesis pathway, revealed that formation of phosphorylation-dependent multi-enzyme complexes among isoforms of starch synthases (SS) and branching enzymes (BE) are potentially important mechanisms of regulating RS biosynthesis. Barley lines down regulated with SBEIIa or SBEIIb, demonstrated distinct patterns of protein-protein interactions compared with a reference genotype, suggesting functional complementation for the loss of either isoform by SBEI and SP (starch phosphorylase). In an *ssiiia* mutant no protein complexes were formed indicating that SSIIa plays an important role in recruiting different components into protein complexes. Detailed biochemical analysis revealed that in some of the mutant lines, different protein complexes are involved in the synthesis of A- and B-granules. These variations in protein
complexes are reflected in the complement of starch synthesizing enzymes detected in starch granules (A- and B-) of different genotypes. The results reinforced the hypothesis that the multi-enzyme complexes play a functional role in biosynthesis of A- and B-granules. Finally, studies of the physiochemical properties of seed and starch in cultivars with no known mutations in starch biosynthetic enzymes, suggest that significant component of variation lie elsewhere, and are independent of the starch granule proteome.
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In the name of ALLAH, the merciful and compassionate, Who bestowed upon me the will and ability to complete this task. Thousands of blessings and clemencies of ALLAH are upon the Holy Prophet Muhammad (Peace Be upon Him), the pride of humanity, who took the responsibility to take the humanity out from the swamp of ignorance and showed us the righteousness.

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Words cannot express the feeling of my love, devotion, thanks and gratitude to my sweet parents. My success is really the fruit of sincerest prayers of my mother, father, brother, sisters and other members of the family. What so ever, I am today is only by the grace of ALLAH Almighty and due to the prayers of my parents they are always very kind to me. Tributes are due for my family, by whom I was always inspired and encouraged for my studies. My deep appreciations are due to my affectionate and loving parents, brother and sisters, who are always praying for my success and brilliant career. I submit my earnest thanks to all of them for their encouragement and moral support which made this possible.

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# TABLE OF CONTENTS

ABSTRACT ...................................................................................................................... ii

ACKNOWLEDGEMENTS ................................................................................................. iv

TABLE OF CONTENTS .................................................................................................. vi

LIST OF TABLES ........................................................................................................ xiii

LIST OF FIGURES ....................................................................................................... xiv

ABBREVIATIONS ......................................................................................................... xvi

Chapter 1: General Introduction ................................................................................. - 1 -

1.1 Overview .............................................................................................................. - 2 -

1.2 Endosperm Development ..................................................................................... - 3 -

- Double fertilization ............................................................................................. - 5 -
- Syncytium formation ........................................................................................... - 5 -
- Cellularization ........................................................................................................ - 6 -
- Differentiation ........................................................................................................ - 7 -
- Embryo surrounding region (ESR) cells ............................................................... - 7 -
- Transfer cells (TCs) .............................................................................................. - 7 -
- Aleurone layer ........................................................................................................ - 9 -
- Starchy endosperm ................................................................................................ - 10 -
- Endoreduplication ................................................................................................ - 12 -
- Maturation ................................................................................................................ - 13 -
- Programmed cell death .......................................................................................... - 13 -
- Desiccation ............................................................................................................... - 13 -

1.3 Starch and its Composition .................................................................................... - 15 -

1.3.1 Molecular structure of starch granule .............................................................. - 18 -

1.3.2 Variations in physiochemical properties of A- and B-granules ....................... - 23 -

1.3.2.1 Uses of starch granules with different properties ........................................ - 25 -

1.3.2.1.1 Uses of starch in food industry ............................................................... - 25 -

1.3.2.1.1.1 Digestion of food ................................................................................ - 26 -

1.3.2.1.1.1.1 What is RS and its health benefits? .................................................. - 26 -

1.3.2.1.1.1.1.1 Types of RS .................................................................................. - 29 -

1.3.2.1.1.1.1.1.1 Factors affecting resistant starch ............................................. - 30 -

1.4 Starch Biosynthesis ............................................................................................... - 33 -
1.4.1 Enzymes involved in starch biosynthesis................................. - 33 -
1.4.1.2 Adenosine 5’ Disphosphate Glucose Pyrophosphorylase (AGPase, EC 2.7.7.27)........................................................................................................ - 33 -
1.4.1.3 Starch Synthases (SS, EC 2.4.1.21)........................................ - 38 -
  1.4.1.3.1 Granule-bound starch synthase (GBSS)......................... - 42 -
  1.4.1.3.2 Starch synthase (SSI)......................................................... - 43 -
  1.4.1.3.3 Starch synthase (SSII).......................................................... - 44 -
  1.4.1.3.4 Starch synthase (SSIII)....................................................... - 45 -
  1.4.1.3.5 Starch synthase (SSIV)....................................................... - 48 -
1.4.1.4 Starch Branching Enzymes (SBEs, EC 2.4.1.18)............... - 48 -
  1.4.1.4.1 Starch branching enzyme I (SBEI)................................. - 49 -
  1.4.1.4.2 Starch branching enzyme II (SBEII)............................... - 51 -
1.4.1.5 Debranching enzymes (DBEs, EC 3.2.1.41 and EC 3.2.1.68)..... - 52 -
  1.4.1.6 Starch Phosphorylase (SP, EC 2.4.1.1)............................... - 56 -
1.4.1.7 Disproportionating enzyme (D-enzyme, E, C. 2.4.1.25)......... - 58 -
  1.4.2 Post-translational modification of starch synthesizing proteins - 59 -
1.4.3 Starch granule-bound proteins................................................ - 63 -
1.5 Experimental Material................................................................ - 67 -
1.6 Hypotheses................................................................................... - 67 -
1.7 Aims and Objectives of the Study.............................................. - 68 -
Chapter 2: Evaluation of physical characteristics of resistant starch from (a range of) barley genotypes ..................................................... - 71 -
  2.1 Introduction ................................................................................ - 72 -
  2.2 Material and Methods ................................................................. - 75 -
    2.2.1 Selection of genotypes......................................................... - 75 -
    2.2.1.1 Selection of genotypes for further detailed analyses........ - 75 -
    2.2.2 Isolation of starch granules................................................ - 75 -
    2.2.3 Measurement of amylose and RS contents........................ - 76 -
    2.2.4 Amylose content determination by gel permeation chromatography ........................................................................................................ - 76 -
    2.2.5 Measurement of amylose by iodine binding....................... - 77 -
    2.2.6 Granule size distribution of different genotypes................. - 78 -
    2.2.7 Granule morphology.............................................................. - 78 -
    2.2.8 Seed characteristics.............................................................. - 79 -
2.2.8.1 Thousand grain weight (TGW) ........................................................ - 79 -
2.2.8.2 Starch content ................................................................................... - 79 -
2.2.9 High-performance anion exchange chromatography .......... - 79 -
2.2.10 Statistical analysis ........................................................................ - 79 -

2.3 Results .................................................................................................. - 80 -
2.3.1 Amylose content ........................................................................ - 80 -
2.3.2 Resistant Starch (RS) Content .................................................... - 82 -
2.3.3 Comparison of different methods for determination of amylose content .................................................................................................. - 84 -
2.3.4 Physical characteristics of seed ................................................... - 84 -
2.3.4.1 Seed characteristics .......................................................................... - 84 -
2.3.4.2 Thousand grain weight (TGW) ........................................................ - 84 -
2.3.4.3 Starch content ................................................................................... - 86 -
2.3.5 Granule size, number and surface area distributions of different genotypes .................................................................................................. - 86 -
2.3.6 Contribution of A-, B- and C-granules in total mass of starch.. - 87 -
2.3.7 Granule morphology ................................................................... - 90 -
2.3.8 Amylopectin chain length distribution ....................................... - 92 -
2.3.9 Principal component analysis (PCA) .......................................... - 97 -

2.4 Discussion ...................................................................................... - 100 -

2.5 Conclusion ..................................................................................... - 104 -

Chapter 3: Post-translational modification, protein-protein interactions among enzymes of starch biosynthesis and their affect on physical properties of starch in high-amylose barley genotypes .......... - 106 -
3.1 Introduction ..................................................................................... - 107 -
3.2 Materials and Methods ................................................................. - 110 -
3.2.1 Plant material ............................................................................ - 110 -
3.2.2 Isolation of starch granules ....................................................... - 110 -
3.2.3 Separation of A- and B-type starch granules ........................... - 111 -
3.2.4 Extraction of amyloplasts and preparation of endosperm whole cell extracts .................................................................................................. - 112 -
3.2.5 Isolation of starch granule-bound proteins .............................. - 112 -
3.2.6 SDS-PAGE and immunoblotting of total starch, A and B granules .................................................................................................. - 113 -
3.2.7 Co-immunoprecipitation ........................................................... - 113 -
3.2.8 Detection of SS and SBE activity following non-denaturing gel electrophoresis ................................................................. - 114 -
3.2.9 Detection of SP activity ............................................................................................................................... - 115 -
3.2.10 Sliver staining .............................................................................................................................................. - 116 -
3.2.11 Pro-Q Diamond phospho-protein staining ................................................................................................. - 116 -
3.2.12 Starch gelatinization ................................................................................................................................. - 117 -
3.2.13 Estimation of amylose and RS content ....................................................................................................... - 118 -
3.2.14 Protein content ........................................................................................................................................... - 118 -
3.2.15 Mass spectrometry ................................................................................................................................. - 118 -
3.2.16 Statistical analysis ..................................................................................................................................... - 118 -

3.3 Results ..................................................................................................................................................................... - 119 -

3.3.1 Physiochemical properties of mutant starches in barley ..................................................................................... - 119 -

3.3.1.1 Starch, amylose and RS content ................................................................................................................ - 119 -
3.3.1.2 Starch gelatinization .................................................................................................................................. - 119 -
3.3.2 Biochemical characterization of different mutants ............................................................................................. - 121 -

3.3.2.1 Amyloplast stromal proteins ...................................................................................................................... - 121 -
3.3.2.2 Detection and estimation of SS activity ...................................................................................................... - 123 -
3.3.2.3 Detection and estimation of SBE activity .................................................................................................. - 126 -
3.3.2.4 Detection and estimation of starch phosphorylase (SP) activity ............................................................. - 126 -
3.3.3 Protein-protein interactions among different proteins of starch biosynthesis .......................................................................................... - 129 -

3.3.3.1 Phosphorylation dependent protein-protein interactions ........................................................................ - 130 -
3.3.4 Analysis of starch granule-associated proteins .................................................................................................. - 133 -

3.3.4.1 Phosphorylation state of the starch granule proteome ................................................................................ - 134 -

3.4 Discussion .................................................................................................................................................................. - 139 -

3.5 Conclusion ............................................................................................................................................................. - 145 -

Chapter 4: Physiochemical and biochemical properties of starch and its relationship to granule size distribution in barley genotypes from diverse genetic back grounds ................................................................................................................................. - 148 -

4.1 Introduction ............................................................................................................................................................. - 149 -

4.2 Material and Methods ............................................................................................................................................... - 154 -

4.2.1 Plant material ..................................................................................................................................................... - 154 -
4.2.2 Isolation of starch granules ............................................................................................................................. - 154 -

4.2.2.1 Separation of A- and B-type starch granules ............................................................................................ - 155 -
4.2.3 Isolation of starch granule-bound proteins .............................. - 156 -
4.2.4 SDS-PAGE and immunodetection of granule-bound proteins - 157 -
4.2.5 Silver staining ............................................................................. - 157 -
4.2.6 Estimation of protein phosphorylation by Pro-Q diamond staining .................................................................................................. ..- 158 -
4.2.7 Measurement of total granule-bound protein content............ - 159 -
4.2.8 In-gel protein quantification ..................................................... - 159 -
4.2.9 Starch gelatinization .................................................................. - 159 -
4.2.10 High-Performance Anion Exchange Chromatography (HPAEC) ......................................................................................................... ...- 160 -
4.2.11 Granule size distribution of different genotypes .......... - 161 -
4.2.12 Granule morphology ............................................................... - 161 -
4.2.13 Thousand grain weight (TGW).................................................. - 162 -
4.2.14 Starch content ........................................................................... - 162 -
4.2.15 RS Content ................................................................................ - 162 -
4.2.16 Amylose content ....................................................................... - 162 -
4.2.17 Mass spectrometric analysis .................................................... - 162 -
4.2.18 Statistical analysis ................................................................. - 163 -

4.3 Results ........................................................................................ - 164 -

4.3.1 Physical characteristics of seed .................................................. - 164 -
4.3.1.1 Seed morphology characteristics .............................................. - 164 -
4.3.1.2 Thousand grain weight (TGW).................................................. - 164 -
4.3.1.3 Starch content ........................................................................... - 164 -
4.3.1.4 Internal seed structures and starch packing within endosperm.... - 164 -
4.3.2 Physiochemical properties of starch ......................................... - 166 -
4.3.2.1 Amylose and resistant starch (RS) contents ....................... - 166 -
4.3.2.2 Granule Size, number and surface area distributions of different genotypes .................................................................................................. - 166 -
4.3.2.3 Contribution of A-, B- and C-granules in total mass of starch..... - 168 -
4.3.2.4 Granule surface morphology .................................................. - 173 -
4.3.3 Starch gelatinization (Thermal properties)............................ - 173 -
4.3.4 Amylopectin chain length distribution (CLD)....................... - 178 -
4.3.5 Biochemical characterization of barley genotypes................... - 184 -
4.3.5.1 Amyloplast stromal proteins (soluble proteins) .................... - 184 -
4.3.5.2 Analysis of starch granule-associated proteins ..................... - 184 -
4.3.5.2.1 Total amount of starch granule-bound protein ...................... - 184 -
4.3.5.2.2 Individual isoforms of starch granule-associated proteins..... - 185 -
4.3.5.2.3 Quantitation of individual granule-bound proteins.............. - 189 -
4.3.5.2.4 Proteomic analysis of C-granules ............................................ - 192 -
4.3.5.3 Phosphorylation of proteins in starch granule .............................. - 192 -

4.4 Discussion .................................................................................. - 196 -

4.5 Conclusion ................................................................................. - 203 -

Chapter 5: General discussion and future work .............................. - 205 -

5.1 General Discussion..................................................................... - 206 -

5.1.1 Analysis of the physiochemical properties of RS as determinant of increased RS genotypes............................................................ - 206 -
5.1.2 Regulation of starch biosynthesis in the barley endosperm is governed by the formation of multi-enzyme protein complexes ...... - 207 -
5.1.3 Formation of multi-enzyme complexes containing starch biosynthetic enzymes in barley endosperm is regulated by protein phosphorylation ................................................................................. - 210 -
5.1.4 Physiochemical properties of seed and starch do not correlate with starch granule proteome ........................................................................ - 210 -

5.2 Future Work ............................................................................. - 212 -

REFERENCES .................................................................................. - 213 -

Appendix 1: Description of genotypes used in the study. ............... - 250 -
Appendix 2: Comparison of three methods used for amylose determination........................................................................................................ - 251 -
Appendix 3: PCA analysis shows association of three methods used for amylose determination........................................................................................................ - 252 -
Appendix 4: Determination of amylose with 6CL-B column .......... - 253 -
Appendix 5: Iodine-staining of barley grains (cross section). .......... - 254 -
Appendix 6: DSC data of different genotypes................................... - 255 -
Appendix 7: Immunological characterization of endosperm amyloplast lysates from different barley mutants. ........................................ - 256 -
Appendix 8: Detection of SS activity and protein......................... - 257 -
Appendix 9: Detection of SBE activity and protein ....................... - 258 -
Appendix 10: Detection of SP protein and activity in different genotypes. ........................................................................................................ - 259 -
Appendix 11: Co-immunoprecipitation of stromal proteins from amyloplasts of different genotypes with SSI, SBEIIa and SBEIIb antibodies. .......................................................... - 260 -

Appendix 12: Summary of novel protein–protein interactions formed between amylopectin-synthesizing enzymes in barley endosperm following either loss of single gene (SSIIa, SBEIIa and SBEIIb) or alteration in single gene (ssiii, amo1 mutant). ........................................... - 263 -

Appendix 13: Starch, A- and B-granules bound proteins. ............... - 265 -

Appendix 14: Granule-bound phospho-proteome analysis and starch, A- and B-granules bound proteins detected by silver staining. ............. - 268 -

Appendix 15: Analysis of starch composition and granule morphology by light microscope (A, B & C) and electron microscope (D & E). ....... - 269 -

Appendix 16: Single nucleotide polymorphisms (SNPs) of barley ssIIIa genomic DNAs from different barley genotypes. .............................. - 270 -

Appendix 17: SNPs of cDNA sequences of barley ssIIIa genomic DNAs from different barley genotypes. ....................................................... - 274 -

Appendix 18: Changes of polypeptide sequences of barley SIIIa protein from different barley genotypes. ....................................................... - 276 -
**LIST OF TABLES**

Table 2.1: Physical characteristics of seed.........................................................85-
Table 2.2: Degree of polymerization (%) of amylopectin from barley genotypes......93-
Table 3.2: Physiochemical properties of starch....................................................119-
Table 3.3: DSC data of different genotypes...........................................................121-
Table 4.4: Physiochemical properties of seed and starch of barley genotypes.....164-
Table 4.2: Thermal properties of starch...............................................................176-
Table 4.5: Amylopectin chain length in different barley genotypes...............179-
Table 4.4: Amount of total starch granule-bound protein (µg/50 mg of starch)........185-
Table 4.5: Correlation between different physiochemical properties of starch determined by principal component analysis.................................196-
LIST OF FIGURES

Figure 1.1: Sucrose metabolism and starch biosynthesis in different cells of plants. ................................................................................................................................... - 4 -
Figure 1.2: Diagrammatic presentation of endosperm cellularization process. ...... - 8 -
Figure 1.3: Presentation of different phases of maize endosperm development.  - 14 -
Figure 1.4: Starch granules morphologies from different cereals. ........................ - 17 -
Figure 1.5: Structural differences between amylose and amylopectin. .......... - 19 -
Figure 1.6: Schematic representation of higher order molecular structure. ....... - 21 -
Figure 1.7: Presentation of different types of crystallinity in starch originated from different sources........................................................... - 22 -
Figure 1.8: Digestion and absorption of different food ingredients in the human digestive tract.............................................................. - 27 -
Figure 1.9: Schematic presentation of starch biosynthesis in the cereal endosperm. ........................................................... - 35 -
Figure 1.10: Comparison of cereal SS domains............................................ - 41 -
Figure 1.11: Diagrammatic representation of the coordinated actions of different enzyme classes in the synthesis of amylopectin. ......................... - 57 -
Figure 1.12: Phosphorylation dependent functional complex formation and entrapment of proteins within starch granule in different maize mutants...... - 66 -
Figure 2.1: % amylose of barley genotypes. .................................................. - 81 -
Figure 2.2: % RS of barley genotypes.............................................................. - 83 -
Figure 2.3A & B: Granule size distribution..................................................... - 88 -
Figure 2.4: Amount of A-, B- and C-granules in total mass of starch. ............... - 89 -
Figure 2.5: Morphology of starch granules from normal and high RS/amylose genotypes observed by SEM......................................................... - 91 -
Figure 2.6: Difference plot of (different genotypes versus wild-type) of amylopectin chain length distribution.................................................. - 95 -
Figure 2.7: Difference plot of (Different genotypes versus waxy) of amylopectin chain length distribution.............................................................. - 96 -
Figure 2.8: PCA analysis shows association of different characters............... - 98 -
Figure 2.9(A & B): Linear correlation between A- and B-granules with % amylose... - 99 -
Initial Heating Summary................................................................................. - 122 -
Figure 3.1: Immunological characterization of endosperm amyloplast lysates from different barley mutants......................................................... - 124 -
Figure 3.2: Detection of SS activity................................................................. - 125 -
Figure 3.3: Detection of SBE activity and protein.......................................... - 127 -
Figure 3.4: Detection of SP protein and activity in different genotypes............ - 128 -
Figure 3.5: Co-immunoprecipitation of stromal proteins from amyloplasts of different barley genotypes with SSI, SBEIIa and SBEIIb antibodies.............. - 132 -
Figure 3.6: Starch, A- and B-granules bound proteins................................... - 136 -
Figure 3.7: Starch granule bound proteome phospho-proteome........................ - 138 -
Figure 3.8: Protein–protein interactions formed between amylopectin-synthesizing enzymes in barley endosperm......................................................... - 147 -
Figure 4.1: Iodine-staining of barley grains (cross section). ............................ - 167 -
Figure 4.2 (A & B): Starch granule size distribution of different genotypes...... - 169 -
Figure 4.3 (A & B): Starch granule number distribution of different genotypes.
................................................................................................................................- 170 -
Figure 4.4 (A & B): Starch granule surface area distribution of different genotypes.
................................................................................................................................- 171 -
Figure 4.5: Starch granule distribution into (A-, B-, and C-) classes. ............... - 172 -
Figure 4.6: Surface morphology of starch granules from different genotypes observed by SEM.......................... - 174 -
Figure 4.7: Difference plot of (other genotypes versus wild-type) for amylopectin chains with different DP.......................... - 181 -
Figure 4.8: Difference plot of (other genotypes versus waxy) for amylopectin chains with different DP. .......................................................... - 182 -
Figure 4.9: Difference plot of (other genotypes versus low amylose) for amylopectin chains with different DP. .......................................................... - 183 -
Figure 4.10: Immunodetection of granule-bound proteins in A- and B-starch granules.......................................................... - 188 -
Figure 4.11: Quantitation of individual granules bound proteins. .................... - 191 -
Figure 4.12: Granule-bound proteins (starch, A- and C-granules) visualized by silver staining.......................................................... - 193 -
Figure 4.13: Analysis of starch composition and granule morphology by light microscope (A, B & C) and electron microscope (D & E).......................... - 194 -
Figure 4.14: Phosphorylation of starch granule associated proteins. .............. - 195 -
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGPase</td>
<td>Adenosine diphosphate glucose pyrophosphorylase</td>
</tr>
<tr>
<td>APase</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>AgNO₃</td>
<td>Silver nitrate</td>
</tr>
<tr>
<td>AMG</td>
<td>Amyloglucosidase</td>
</tr>
<tr>
<td>Ae</td>
<td><em>Amylose extender</em></td>
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<tr>
<td>CaCl₂.2H₂O</td>
<td>Calcium chloride</td>
</tr>
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<td>CLD</td>
<td>Chain length distribution</td>
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<td>CSIRO</td>
<td>Commonwealth scientific and industrial research organisation</td>
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<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>DAP</td>
<td>Days after pollination</td>
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<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
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<td>DP</td>
<td>Degree of polymerization</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ELC</td>
<td>Extra-long unit chains</td>
</tr>
<tr>
<td>GPC</td>
<td>Gel Permeation Chromatography</td>
</tr>
<tr>
<td>GOPOD</td>
<td>Glucose oxidase/peroxidise reagent</td>
</tr>
<tr>
<td>GBSS</td>
<td>Granule-bound starch synthase</td>
</tr>
<tr>
<td>HAG</td>
<td>High amylose glacier</td>
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<td>HPAEC</td>
<td>High-Performance anion exchange chromatography</td>
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<td>HPAEC-PAD</td>
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Chapter 1: General Introduction
1. General Introduction

1.1 Overview

Starch is an important and widely distributed carbohydrate in plants. It is the most significant form of carbon reserve in the majority of species. It is important in terms of the amount made, its distribution among many different plant species and its economic importance. It is an important dietary source of energy for humans and represents 70-80% of the average daily caloric intake (Tetlow, 2006) and also serves as a source of feed, fiber, biofuels and a raw material in many industrial applications. Cereal grains, pulses and tubers are the major sources of starch (Tauberger et al., 2000). As a principal storage compound, starch plays important roles in the life cycle of the plant. In plants, starch is synthesized in plastids, which are derived from proplastids. There are two particularly important types of plastids. Chloroplasts which are present in photosynthetic tissues like leaves, and are responsible for transient starch synthesis: and amyloplasts which are present in non-photosynthetic storage tissues like seeds, tubers and roots and which are responsible for storage starch synthesis (Tetlow, 2006, 2011).

The type of plastid and the plant tissue from which starches are derived are important in determining the functionalities of a particular starch. All the starches which are synthesized in different parts of the plant are degraded at some stage, For example, during the day time transient starches are synthesized in leaves and degraded at night to provide carbon for non-photosynthetic metabolism (Streb and Zeeman, 2012). This supply of carbon from degradation of starch at night is important for normal growth of the plant. By comparison storage starches in developing seeds serve as a long term carbon store for the next generation (Gerard et al., 2001; Tetlow, 2006).
The starch synthesized in chloroplasts during the day time is degraded to maltose during the dark period. The majority of derived hexoses are converted to triose phosphate and transported to the cytosol for sucrose synthesis. To support plant growth this sucrose can readily be transported to non-photosynthetic tissues and then carbon is transported to amyloplasts for starch synthesis. The starting point for the chloroplast pathway of starch synthesis is fructose-6-phosphate, a product of photosynthetic carbon fixation. The starting point for the amyloplast pathway is glucose-1-phosphate, a product of sucrose degradation. In potato, pea, and maize glucose-6-phosphate, in addition to glucose-1-phosphate, can be imported into the amyloplast and can serve as the starting point for starch biosynthesis (Tauberger et al., 2000). Figure 1.1 shows details of the starch biosynthetic pathway of transient and storage starches.

Starch produced in the cereals endosperm is an abundant and renewable source and has many commercial uses (Hannah and James, 2008; Zhang et al., 2008). In the food industry starch is used in cereals and snacks, flavours and beverages. Starch is also utilized in many non-food industries includes textiles, explosives, cosmetic and pharmaceutical, paper, mining and construction industries (Lauro, 1999). The physical and chemical properties of starch granules are important in determining their end use (Lindeboom et al., 2004).

1.2 Endosperm Development

Starch is synthesized in the cereals seeds during endosperm development. Endosperm is the nutritive tissue that surrounds the embryo within seeds of flowering plants. The nutritive endosperm initiated by double fertilization is a unique feature of angiosperms. The endosperm is essential for reproduction because it provides nutrition to
Figure 1.1: Sucrose metabolism and starch biosynthesis in different cells of plants.

TP, triose phosphate; G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; AGPase, adenosine 5' diphosphate glucose pyrophosphorylase; ADPG, adenosine diphosphate glucose; Glc, glucose; Mal, maltose; HXP, hexose phosphate; Open and closed circles, TP and ADPG transporter; Open hexagon, hexose and maltose transporter; closed hexagon, HXP transporter. Figure 1.1 shows that triose phosphate and hexoses are transported to the cytosol of the source via transporters and are converted into hexose phosphate, and then converted into sucrose. This sucrose is transported to different cells. In the cereal endosperm, sucrose is utilized to produce ADP-glucose by cytosolic and plastidial AGPase via some intermediate products (G6P & G1P). ADP-glucose is used by starch synthesizing enzymes to produce starch. (This picture has been transformed from Mitsui et al., 2010).
the germinating embryo. In cereal endosperm, different storage products accumulate during development. Among storage products, starch and proteins are prominent. The great economic and nutritional value of cereals like maize, wheat, rice and barley is due to their starch-enriched grains. In cereals, such as maize, starch represents approximately 75% of the mature seed weight and proteins represent 10% of the total grain weight (Keeling and Myers, 2010). The process of endosperm development in cereals has been divided into several distinct phases, which overlap considerably. These phases are: early development, differentiation and maturation. Early development involves double fertilization, syncytium formation and cellularization.

**Double fertilization**, all angiosperms including maize, wheat and barley (Kiesselbach, 1949; Bennett et al., 1975) show double fertilization in which the fusion of one sperm nucleus with the egg nucleus forms a diploid embryo, and fusion between a sperm nucleus and two polar nuclei giving rise to a triploid endosperm, occur simultaneously. After double fertilization, the triploid endosperm shows a very fast mitotic activity compared to the embryo. The underlying mechanism of this process is not yet known in cereals (Sabelli and Larkin, 2009a).

**Syncytium formation**, the newly fertilized nucleus of the endosperm undergoes a repeated cycle of synchronous divisions in the absence of cell wall formation and cytokinesis, which gives rise to a multi-nucleate structure called a syncytium as shown in Figure (1.2B) (Lopes and Larkins, 1993). Cell division is slower in the zygote, as by the time the zygote has divided for the first time, there are four to eight endosperm nuclei in maize, and an increase up to 512 nuclei occurs within three days after pollination (DAP) (Randolph, 1936). These nuclei arrange themselves at the periphery of the endosperm cell.
as shown in Figure (1.2B). Proliferation of embryonic cells is less compared to endosperm because endosperm nuclei do not involve the synthesis of cytoplasm, cell membranes and cell walls.

**Cellularization**, the formation of the internuclear radial microtubule system (RMS), and an open-ended alveolation process that starts from the periphery of the endosperm and proceeds towards the vacuole, is responsible for the cellularization of the coenocytic endosperm in cereals. Microtubules radiating from the nuclear surface (Figure 1.2C) define the nuclear-cytoplasmic domains, which result in the equal distance lining of the nuclei with the cell wall (Olsen, 2004). Through the deposition of adventitious phragmoplast and the repolarization of microtubules, initiation of cell wall formation takes place anticlinally. This event is followed by the centripetal extension of the cell wall (Figure 1.2D) forming alveoli (open tubular structures in which the inner periclinal cell wall that surrounds each nucleus is absent). After centripetal extension of the cell wall, synchronous and periclinal division of the nuclei occur, immediately followed by cytokinesis. With an overlaying layer of residual syncytial cytoplasm, the alveoli layer displaces farther towards the central cavity (Figure 1.2E). The process of cellularization continues centripetally until the central cavity is filled with cells (Figure 1.2F). In cereals it takes 3-6 DAP. Cellularization is mediated due to interaction between the cell cycle progression machinery and the microtubular cytoskeleton (Olsen, 2001). Cellularization is important because these are the cells from where further differentiation will take place. Cellularization is a metabolically active process and requires energy. During the early phase of development (syncytium formation and cellularization) certain changes are very prominent. These changes include maximum expression of proteins related to actin,
tubulin and cell organization. Similarly, proteins involved in respiration metabolism (glycolysis and Krebs cycle) and protection against reactive oxygen species also have high expression (Prioul et al., 2008). These changes coincide with early development where, due to cell division and cellularization, high metabolic rates exist, requiring more energy, which results in an increase in respiration.

**Differentiation**, during this phase of development, endosperm cells differentiate into four major cell types including embryo surrounding region (ESR) cells, transfer cells (TCs), aleurone layer and starchy endosperm cells, which are the main constituents of the endosperm.

**Embryo surrounding region (ESR) cells**, ESR comprises several cell layers, which completely cover the young embryo at around 4 DAP. With the growth of the embryo, ESR shrinks, and around 12 DAP there are only vestigial remnants of the ESR present at the base of the endosperm (Kiesselbach, 1949). In maize, these cells have dense cytoplasmic contents. Their function is to supply the embryo with sugars through an apoplastic route (Cossegal et al., 2007). An additional function is to protect the embryo against pathogens. These cells are also metabolically active. The mechanism which specifies the fate of ESR is not known.

**Transfer cells (TCs)**, in cereal endosperm, several cell layers near the placenta differentiate into TCs before cellularization is complete. These cells are found at the base of the endosperm, but their position within the caryopsis varies among species (Rost et al., 1984). In maize they are located over the chalazal pad. These cells are characterized by prominent secondary cell wall ingrowths. The plasma membrane of TCs grows, clinging to the cell wall ingrowths, which results in an increase in its superficial area, and
Figure 1.2: Diagrammatic presentation of endosperm cellularization process.

(A), the fertilized triploid nucleus (orange), vacuole (yellow).
(B), multinucleate cell with a large central vacuole.
(C), cellularization, formation of RMS, at the surface of the endosperm nuclei.
(D), cell wall formation, each nucleus surrounded by a tube-like wall structure (alveolus).
(E), continued growth of alveoli.
(F), mitotic division.

This picture has been adapted from Olsen, (2004).
magnification of the surface-to-volume ratio of the protoplast. The morphology of TCs is optimal for absorbing and secreting substances, which facilitate nutrient uptake by the endosperm (Zheng and Wang, 2010). These cells have dense cytoplasm, enriched with spherical mitochondria. The presence of an enormous amount of mitochondria shows that during differentiation of TCs higher metabolic activities are required. TCs produce antimicrobial- resembling proteins, which show that these cells also protect the kernel from potential pathogenic invaders (Magnard et al., 2003).

**Aleurone layer**, aleurone cells form a sheet around the endosperm, except in the transfer cell region. The number of layers of cells in the sheet varies with the species, for example, one in maize and wheat, three in barley and several in rice (Sabelli and Larkins, 2009b). In maize their differentiation occurs between, 6-10 DAP from the outer layers of the endosperm cells. Due to accumulation of spherosomes and protein bodies these cells become cuboidal. Differentiation of aleurone cells depends upon the species (Brown et al., 1994). The presence of numerous small vacuoles with inclusion bodies, termed “aleurone grains”, makes the cytoplasm of aleurone cells dense and granular. These inclusion bodies store protein, lipids and other molecules. These cells possess a well-developed endoplasmic reticulum and a large number of mitochondria. The different range of colors observed in the maize kernels is due to the presence of anthocyanins in mature aleurone cells. This is the only live tissue in endosperm at maturity due to the presence of a specific developmental program that protects it from desiccation (Hoecker et al., 1995). Positional information stored in the periphery of the endosperm, close to the former central cell wall is required for aleurone cell fate specification. The function of the aleurone cells is to synthesize the proteolytic and hydrolytic enzymes, which digest the
endosperm cell wall and remobilize the starch and protein stored in the endosperm, to provide sugars and amino acids for the growing embryo. Production of these enzymes in aleurone cells is stimulated by gibberellic acid (GA) from the imbibed endosperm (Ritchie et al., 2000).

**Starchy endosperm**, the transition from the cell division to the storage phase of endosperm depends upon different signals. One important factor is the glucose/sucrose ratio in the caryopsis. A high glucose/sucrose ratio leads to endosperm cell proliferation, while low glucose/sucrose sucrose ratio will lead to the starch accumulation phase (Sabelli and Larkins, 2009a). Cereal endosperm is one of the most important food sources because it has about 70 % starch by weight. This starch provides more than 80% of daily caloric intake for humans (Keeling and Myers, 2010). Initiation of starch deposition in endosperm varies among species. In wheat it starts soon after cellularization, while in maize it begins around 10 DAP. Starch synthesis is controlled by a series of enzymes which are discussed in this thesis.

The grain filling rate and seed weight are directly related to the number of starch granules in the endosperm. In turn, starch granule number is directly related to the number of cells. This observation shows that initiation and duration of the cell division phase are important in endosperm development and grain yield (Commuri and Jones, 1999). High levels of ATP and high energy states are associated with starch accumulation and granule size, which shows that high metabolic activity is associated with cell expansion and starch accumulation, and these reactions are energy limited (Rolletschek et al., 2005). During endosperm development, cereals store carbohydrates, protein, oils and other compounds. These stored compounds provide nutrition and other necessary
elements for the germination of the embryo. The stored proteins serve as a source of nitrogen and sulphur for the growing seedling. The major stored proteins in cereals are prolamins and globulins. Prolamins are highly hydrophobic and are soluble in organic solvents or denaturing solvents while globulins are soluble in saline solution. Additional minor proteins are also stored in the endosperm (Coleman and Larkins, 1999).

In wheat and barley, two forms of prolamins are present which are closely related. In wheat, the monomeric form is termed gliadin and the polymeric form glutenin, while in barley the polymeric form is hordein (Kreis et al., 1985). In maize, prolamins are called zeins (Coleman and Larkins, 1999). Prolamins are rich in certain amino acids like proline and glutamine, and are deficient in lysine and tryptophan. Prolamins represent 50-60 % of total endosperm stored protein in maize, barley and wheat but in rice accounts for only 5-10 % (Laudencia-Chingcuanco et al., 2007). In the lumen of rough endoplasmic reticulum (RER) both prolamins and globulins form insoluble accretions called protein bodies. In maize and rice, the protein bodies are retained within the RER, the mechanism of which is unknown. In wheat, these accretions are trafficked to vacuoles (Herman and Larkins, 1999; Holding et al., 2007). Prolamins accumulate during middle and late periods of endosperm development. Nucleic acids are also present in starchy endosperm. These are not specific storage molecules but they are present in the starchy cells and remain there in the dead cells. After imbibition, nucleases released by the aleurone degrade starchy endosperm nucleic acid contents (Brown and Ho, 1986). After cellularization, a phase of mitotic cell division occurs that generates the final population of endosperm cells. In central endosperm this phase lasts until 8-12 DAP but in aleurone and subaleurone layers it continues until 20-25 DAP. The mitotic cell division index is
maximum around 8-10 DAP, and after that it declines. Endosperm grows rapidly from 8-10 DAP to fill the entire seed cavity. This growth is correlated with cell division, cell enlargement and endoreduplication (Knowles and Philips, 1988). Different phases of endosperm development have been presented in the Figure (1.3).

**Endoreduplication**, maize endosperm cells around 8-10 DAP switch from a mitotic to an endoreduplication cycle. In this cycle, without chromatin condensation, sister chromatid segregation and cytokinesis, completed and reiterated rounds of DNA synthesis take place (Sabelli and Larkins, 2008). As a result, a gradient of nuclear size is observed in the tissue due to the presence of small nuclei at the periphery and increasingly larger nuclei in the inner central region of the endosperm (Knowles and Philips, 1988). Endoreduplication is also a metabolically active process which requires energy. Endoreduplication seems to be correlated with rapid caryopsis growth, and the synthesis and accumulation of starch and storage proteins. The precise function of endoreduplication is not yet known (Sabelli and Larkins, 2009b). During this phase of development (differentiation) certain changes are prominent, which include the presence of a large amount of proteins which are involved in proteolysis. This turnover in proteins is consistent with a switch from growth and differentiation to storage. During this phase of growth, enzymes related to metabolism are also increased because of endosperm storage filling. The prominent proteins which are expressed at a maximum rate during this stage are chaperones and proteins involve in degradation of metabolites. The presence of the maximum amount of proteins involved in protein folding (chaperone) during this stage is associated with storage protein accumulation in the endosperm. One interesting change at this stage is the decrease in the amount of enzymes involved in the
Kreb’s cycle relative to the glycolytic enzymes. This condition is in agreement with the recent demonstration that starch accumulation in the endosperm takes place under hypoxic conditions (Rolletschek et al., 2005). Due to lack of oxygen, Kreb’s cycle enzymes are not functioning, which influences corresponding enzyme expression. ATP needed for starch synthesis from sucrose is produced in the absence of oxygen by glycolysis and the sucrose synthase UGPase-AGPase cycle. During this stage accumulation of other proteins like those involved in starch synthesis is also increased.

**Maturation**, maturation is comprised of programmed cell death (PCD), dormancy and desiccation (Oslen, 2001). These final steps permit seed dispersal, long term storage and tolerance to harsh environments.

**Programmed cell death**, programmed cell death is an important mechanism in cereal endosperm development which facilitates nutrient hydrolysis and uptake by the embryo at germination (Naguyen et al., 2007). In maize, endosperm PCD starts in two different regions, the central starch endosperm cells and apical cells near the silk scar at around 16 DAP. In this way, the top half of the endosperm becomes dead at around 28 DAP (Young and Gallie, 2000a), while aleurone cells undergo PCD at around 30 DAP (Young and Gallie, 2000b). It is thought that certain proteases and hormones (ethylene) are involved in the progression of PCD (Naguyen et al., 2007). It is thought that abscisic acid (ABA) is also involved in PCD (Young and Gallie, 2000a).

**Desiccation**, desiccation is also important in PCD, because this makes the aleurone responsive to GA. An interesting change which occurs around 21 DAP involves, sudden onset of Pyruvate-Phosphate Dikinase (PPDK). This PPDK plays an important role in the accumulation of storage products. This protein is believed to be involved in regulation of
Figure 1.3: Presentation of different phases of maize endosperm development.  
(A) shows, double fertilization, syncytium formation, and cellularization (3 DAP). The pollen tube and sperm nuclei (yellow), polar nuclei and endosperm (red), The egg cell nucleus and embryo nuclei (green). Endosperm and embryo (red and green outline).  
(B) represents 4-20 DAP, endosperm mitotic division and cell proliferation, followed by endoreduplication (from 8-10 DAP). Programmed cell death (PCD) starts around 16 DAP. The graphs with different C values represent increase in genetic material.  
(C) is presenting parameters of endosperm development, fresh weight, nuclei number, mitotic index, and average DNA content (red, blue, brown and green lines respectively).  
**Abbreviations:** Aleurone (Al); central starchy endosperm (CSEn); embryo (Em); endosperm (En); nucellus (Nu); pericarp (Pe); placentochalaza (Pl); subaleurone layer (SAI); transfer cells (TC). This picture has been adopted from Sabelli and Larkins, (2009a).
storage product synthesis in different ways (Prioul et al., 2008). Other changes at this stage of development are gradual decrease in metabolic activity, which is consistent with PCD.

1.3 Starch and its Composition

Normal starch exists as semi-crystalline insoluble granules varying in size (0.5-100µm) and shape (spherical, elliptical, or polyhedral). The starch granule is composed of two polymers, amylose and amylopectin (Martin and Smith, 1995). Granule size, number and morphology are the characteristics of the organ and species in which they are produced (Jane et al., 1994; Shapter et al., 2008). Based on the size distribution of starch granules, different species have either unimodal (maize) or bi-or trimodal (wheat and barley) granule size distributions (French, 1984). For example, in wheat and barley, bi- or tri-modal granule size distribution is differentiated as (A-, B- and C-) type granules. A-, B- and C-type granules can be distinguished based on size, shape, relative number and the timing of their initiation during seed development. A-granules are lenticellular, varying in size from 10-50 µm in diameter and make up to 70-80 % of the volume but only 10 % of the total number of the starch granules (Hughes and Briarty, 1976; Langeveld et al., 2000). B-granules are spherical, varying in size from 5-10 µm in diameter and represent < 30 % of the volume and > 90 % of the total number of starch granules. Starch granules of size less than 5 µm in diameter are termed as C-granules (Bechtel and Wilson, 2003; Wilson et al., 2006). The small size of C-granules makes it difficult to isolate and quantify them which sometimes lead to their classification as B-granules.

**Timing and location of granule synthesis**, the initiation of A-, B- and C-granules is
developmentally regulated. A-granules are synthesized when endosperm is still actively dividing i.e. between 4-14 days postanthesis (DPA), B-granules are initiated between 10-16 DPA in the evaginations of A-granule-containing plastids (Langeveld et al., 2000; Bechtel and Wilson, 2003) and C-granules start to appear at about 21 DPA (Bechtel and Wilson, 2003). Morphology and size of A- and B-granules from different cereals are shown in Figure (1.4).

Starch is synthesized in plastids in different tissues within the same plant. In developing endosperm these plastids are called amyloplasts. Rice and oats have compound granules in which many granules are formed in a single amyloplast. These granules are small at maturity and are polyhedral in shape due to pressure from the surrounding granules. These polyhedral granules become compressed together to form compound granules which at low magnification appears as a single granule. In Panicoideae, typically a simple, single granule is formed in the individual amyloplasts. However, Triticeae have a unique endosperm bimodal granule size distribution, consisting of large lenticellular A- and small spherical or ovoid B-granules. The A-granules are synthesized in the body of amyloplasts while B-granules are formed independently in the outgrowths of the same cell without appearing as compound granules (Rahman et al., 2000). However, Li et al. (2001) reported the existence of compound granules in certain barley genotypes, which represented clusters of a few granules but with the appearance of a single granule.

There are number of factors involved in the differentiation of the starch granule. These include multiple and complex genetic control and biochemistry which regulate the size and number of plastids, and also environmental conditions during seed development.
Figure 1.4: Starch granules morphologies from different cereals.
This figure illustrates starch granules from the endosperm of cereal seeds using light microscopy and scanning electron microscopy, the methods of which are described in later part of this thesis. (A), light micrograph presenting compound starch granules within amyloplasts of an oat endosperm. Each compound granule is composed of many smaller granules which look like a single granule. (B), scanning electron micrograph is presenting tightly packed A- and B-granules within the barley endosperm cells. (C), scanning electron micrograph of maize endosperm starch. (D), scanning electron micrograph of wheat endosperm starch. Both large A-granules (the arrows indicate equatorial grooves) and small B-granules are clearly visible (Smith, 2010).
1.3.1 Molecular structure of starch granule

Starch is made up of amylose and amylopectin. Amylose and amylopectin are made up of the same basic glucan polymers but with different length and degree of branching (Figure 1.5 A & B). Amylose is essentially a linear molecule with a molecular weight varying between \((10^5 - 10^6)\) Daltons, in which glucose residues are joined via \(\alpha-1, 4\) linkages with very few \(\alpha-1, 6\) linkages and makes up to 20-30% of the starch (Figure, 1.5 A). While amylopectin with a molecular weight of \((10^7 - 10^9)\) Daltons contributes 70-80% share in total starch and contains linear chains of various lengths. Almost 5% of the glucose units in amylopectin are joined by \(\alpha-1, 6\) linkages, which introduce branches in the amylopectin (Davis et al., 2003) (Figure, 1.5 B). In amylose the degree of polymerization of glucose is species dependent (Morrison and Karkalas, 1990). In amylopectin branching of glucan chains exhibits regular periodicity and its length and pattern play a critical role in the proper formation of the granule (Stamova et al., 2009). Intermolecular attraction and association of neighbouring amylose molecules result in unstable aqueous solutions of amylose leading to increase in viscosity, retrogradation and, under specific conditions, precipitation of amylose particles (Hedley, 2002). The characteristics of aqueous solutions of amylopectin include high viscosity, clarity, stability, and resistance to gelling. These properties are important in terms of starch use. For example rehydration of starch depends upon both viscosity and granule size. Similarly, resistance to gelling makes starch stable at high temperature (Hedley, 2002). In the native form of starch, amylose and amylopectin are organised in granules as alternating semi-crystalline and amorphous layers. The ordered regions constituting the semi-crystalline layers are composed of double helices formed by short amylopectin
Figure 1.5: Structural differences between amylose and amylopectin.
The starch granule is composed of two types of glucan polymers; amylose and amylopectin.
(A), Amylose is a relatively less branched polymer with longer chains containing predominant $\alpha(1\rightarrow 4)$ bonds.
(B), Amylopectin is a highly branched and complex glucan polymer with $\alpha(1\rightarrow 4)$ linked chains in which branches are introduced by $\alpha(1\rightarrow 6)$ linkages.
branches, most of which are further ordered into crystalline structures (Hedley, 2002). Amylopectin has a polymodal glucan chain distribution, which allows the condensing of shorter glucan chains and the subsequent development of efficiently packed parallel double helices. These helices coupled with regular branch point clustering give rise to the basis of the organized semi-crystalline nature of the starch granule matrix as shown in Figure (1.6c) (French, 1984; Hizukuri, 1986; Tetlow, 2006). This conserved architecture of amylopectin is responsible for the semi-crystalline, water insoluble starch granule. Granule formation is regulated by the semi-crystalline properties of amylopectin, which are determined by clustering, the length of the linear chains of amylopectin, and the frequency of α-1, 6 linkages (French, 1984; Hizukuri, 1986; Myers et al., 2000). In granules of different sizes the molecular structure of the amylose and amylopectin fractions varies. For barley, it was found that with decreasing granule size, the average degree of polymerization of amylopectin decreased. On the other hand granule size did not affect amylose polymerization (Tang et al., 2001). In amylopectin with large granules, long amylopectin B chains are present in greater number as compared to small granules from the same cultivar (Naka et al., 1985). The crystallites composed of double helices may be densely packed forming an orthogonal pattern, as in cereal starches, and are termed as A-type, or in a less densely packed hexagonal pattern, as in potato starch, when they are termed B-type as shown in (not to be confused with A- and B-starches based on size). The type of crystallinity in cereal and potato is shown in Figure (1.7). The amount and mobility of the structural water contained in both types of crystallites is greater in B type crystallites. Hence, cereal starches are termed A- and potato starches B-type. There are some other species, for example pea, which contain both A- and B-type
Figure 1.6: Schematic representation of higher order molecular structure. (1.6 A), illustrates amorphous and semi-crystalline zones within a starch granule. (1.6 B & C), Enlargement of semi-crystalline growth rings, showing the arrangement of the alternating crystalline and amorphous lamellae. The crystalline lamella is about 6 nm and amorphous lamella is about 3 nm. In a starch granule, both these regions repeat many times to give rise to a well-organized starch granule (Tetlow, 2006).
Figure 1.7: Presentation of different types of crystallinity in starch originated from different sources. 

(A), shows A type crystallinity in cereals, when crystallites composed of double helices are densely packed forming an orthogonal pattern.

(B), shows B type crystallinity in potato, when crystallites composed of double helices are less densely packed forming hexagonal pattern (Ratnayake and Jackson, 2008).
crystallites confined to specific regions of the granule (Hedly, 2002).

1.3.2 Variations in physiochemical properties of A- and B-granules

Previous studies have shown that starches from different barley genotypes vary widely in structure, composition and properties (Kang et al., 1985; Morrison et al., 1993; Lorenz & Collins, 1995; Song and Jane, 2000; Yoshimoto et al., 2000). Besides variations in morphology, size, and origin, wheat and barley large and small starch granules also have differences in characteristics and properties with regard to chemical composition. For example amylose, amylose-lipid complex and phosphorus contents (Raeker et al., 1998; Shinde et al., 2003; Geera et al., 2006; Ao and Jane, 2007), molecular structure (Sahlstrom et al., 2003), resistance to α-amylase digestion (Bertoft and Kulp, 1986), relative granule crystallinity and gelatinization temperatures (Vermeylen et al., 2005; Ao and Jane, 2007) gelatinization temperature and retrogradation (Peng et al., 1999; Singh and Kaur, 2004), granule swelling (Van Hung and Morita, 2005), reactivity to modifying agents (Van Hung and Morita, 2005) and pasting behavior (Geera et al., 2006; Ao and Jane, 2007) may also be different in large and small granules. The isolated and purified B-granules of barley exhibit more susceptibility to cereal α-amylases digestion and acid hydrolysis than the A-granules. This is primarily due to the larger surface area of small granules as compared to large granules (Bertoft and Kulp, 1986; Vasanathan and Bhatty, 1996). Large A-granules of wheat show an increased enthalpy of gelatinization, lower gelatinization temperatures, increased retrogradation (Peng et al., 1999; Singh and Kaur, 2004) and soft textured flours compared to smaller B-granules (Gaines et al., 2000). Similarly, Sandhu et al. (2004) reported that varieties of maize with small starch granules exhibits lowest swelling
power, amylose content, solubility and retrogradation. A study with \textit{waxy} maize and millet and a very low amylose species, amaranth, showed that the small granule size of amaranth was associated with its slower retrogradation (Choi et al., 2004). Wheat starches possessing different granule sizes exhibited different degrees of susceptibility to enzymatic hydrolysis, as well as thermal and pasting properties. Along with granule size, difference in amylose content, protein content, and branch chain length of amylopectin in A- and B-type starch granules, are also major factors responsible for differences in digestibility and other functional properties of starch (Liu et al., 2007). Wheat starch from five different genotypes, four possessing the same amylose content and one lacking amylose (\textit{waxy}) was separated into A-, B- and unfractionated starches. It was found that A-granules had a smaller proportion of short chains of 6-12 DP and a higher proportion of intermediate chains of 25-36 DP than B-granules. The lamellar repeat distance in A-granules was larger than that of B-granules. And the lamellar distances of both A- and B-granules from the \textit{waxy} genotype were smaller than those of non-waxy starches. However, no differences were observed in the crystallinity of either A-, B- or unfractionated starch. \textit{In vitro} digestion kinetics with $\alpha$-amylase of A- and B-granules demonstrated differences. Initially B-granules were digested to a greater extent than A-granules. But after 4 h of incubation, A-granules showed more digestibility than that of B-granules, while \textit{waxy} starch showed similar \textit{in vitro} digestibility of unfractionated and fractionated granules (Salman et al., 2009). Investigation of chemical composition showed that A-granules had lower lipid and higher amylose contents than B-granules. Similarly A-granules also had the highest gelatinization enthalpy and peak and final viscosity, while B-granules had the highest gelatinization temperature and amylose-lipid
complex enthalpy (Dengate and Meredith, 1984; Soulaka and Morrison, 1985; Peng et al., 1999; Sahlstrom et al., 2003; Shinde et al., 2003; Vermeylen et al., 2005).

1.3.2.1 Uses of starch granules with different properties

Because of differences described above, these two types of starch granules are utilized differently, both in food and non-food industries. Along with the above mentioned properties, granule morphology also has an important impact on starch physiochemical properties (Da Silva et al., 1997; Lindeboom et al., 2004) and granule size determines many of the potential food and industrial applications of starch (Ji et al., 2004). For example, small starch granules are suitable as a fat substitute, a carrier material in cosmetics and in paper coating, while large granules are used in the manufacturing of biodegradable plastic film and carbonless copy paper (Lindeboom et al. 2004). Similarly cereal cultivars with different proportion of large and small granules would be very useful to different food and non-food industries (Wei et al., 2010).

1.3.2.1.1 Uses of starch in food industry

As indicated, starch has many important uses in the food industry. Factors such as the ratio of amylose to amylopectin affect its physicochemical properties and end use (Izdorczyk et al., 2000; Hang et al., 2007). For example waxy (amylose free) starch has wide application in the food industry for conferring properties like uniformity, stability, texture, and better freeze-thaw ability (Chibbar and Chakraborty, 2005). Depending upon the % amylose content, barley starch can be classified into normal (25-27 %), waxy (<5 %), and high amylose (>35 %) (Bhatty and Rossnagel, 1992; Bhattay et al., 1998; Izdorczyk et al., 2000; Zheng et al., 1998). These differences in starch composition and structure can be utilized in food applications to reduce the risk of
diabetes and/or digestive tract related diseases like colon cancer which are associated
with increased use of starch rich food.

1.3.2.1.1 Digestion of food

Digestion of food containing starch is a complex process which is influenced by
rate of digestion and absorption in the digestive tract. This process is affected by many
factors including source, components, physical nature and processing methods of food,
and the presence of enzyme inhibitors (O’Dea et al., 1980; Goni et al., 1997). Due to the
high viscosity created by fibre enriched food, the rate of carbohydrate absorption
decreases in the upper digestive tract (Englyst and Kingman, 1990; O’Dea et al., 1991). It
is thought that the fiber component of food excludes the enzymes responsible for
carbohydrate hydrolysis hence lowering the rate of starch hydrolysis (O’Dea et al., 1980).
Starch has been divided into three categories: rapidly digestible starch (RDS), slowly
digestible starch (SDS), and resistant starch (RS). Digestion of food in the human
digestive tract is shown in detail in Figure (1.8).

1.3.2.1.1.1 What is RS and its health benefits?

RS was first identified in 1982 and categorized as part of dietary fibre (Englyst et
al., 1982). Increasing dietary fibre uptake is related to lower rates of obesity,
cardiovascular disease, diabetes and certain cancers (National Health and Medical
Research Council, 2006). Similarly RS resists α-amylase digestion in the small intestine,
and is fermented by the bacteria in the large intestine, producing a variety of end
products, the most important of which are the short chain fatty acids (SCFA) (Englyst et
al., 1996). SCFA primarily consists of butyrate, propionate and acetate and are the
preferred sources of energy for the colonocytes (cells lining the colon). Other beneficial
Food digestion occurs in a series of phases. Starch which has been consumed after gelatinization (cooked), which disrupts the molecular structure of starch and makes glucose chains accessible, is more easily digested by $\alpha$-amylases. Almost all of the starch in food is digested and absorbed in the small intestine. However some starch escapes this digestion process and reached in the large intestine, this starch is called resistant starch (RS). This RS is utilized by the bacteria of the large intestine and different beneficial components including short chain fatty acids are produced. Use of RS and ultimately its consumption in the large intestine has many health-associated benefits, including decreasing the incidence of colorectal cancer.
effects of SCFA include increased colonic blood flow, improvement in the mineral bioavailability, reduction in the growth of pathogenic bacteria by lowering pH in the lumen, and prevention of abnormal colonic cell development (Topping and Clifton, 2001). SCFA, particularly butyrate, also been shown to facilitate other important physiological changes such as, an ability to reverse neoplastic changes in vitro (Ferguson et al., 2000), positive and nutritive effects on the colonic epithelium, and induction of apoptosis (programmed cell death) of damaged cells (Mentschel and Claus, 2003). SCFA help in maintaining healthy viscera (Wei et al., 2010). RS is assumed to be one of the best substrates for butyrate production because RS fermentation produces butyrate at twice the rate of wheat fibre and four times that of pectin (Champ, 2004).

In recent years, the glycemic index (GI) has not only been used as a potentially diet planning tool for diabetic patients, but also as a measure to prevent diabetes, dyslipidemia, cardiovascular disease, and even certain cancers as well (Jenkins et al., 1981). The glycemic index provides a measure of how quickly blood sugar (glucose) levels rise after eating a particular type of food. Glycemic response of an individual is greatly influenced by the digestion of starch present in the food (Liu et al., 2007).

The Commonwealth Scientific and Industrial Research Organisation (CSIRO), has recommended that around 20 g of RS should be consumed per day, this amount is almost four times greater than a typical western diet is currently providing (Baghurst et al., 1996). Studies on a particular type of RS2 (Hi-maize®) showed that 17g/day or more RS is required in the diet for a positive impact on one or more of the accepted parameters of digestive health, (Muir et al., 1995; Phillips et al., 1995; Birkett et al., 1996; Noakes et al., 1996; Muir et al., 2004). Because of the prebiotic properties of RS, it has a symbiotic
effect where it can provide protection to beneficial bifid bacteria in vivo during their travel through the upper gastrointestinal tract (Wang et al., 1999). It has been shown that during diarrhoea and cholera, use of oral rehydration solutions containing RS reduce fecal fluid loss and shorten the duration of disease. This finding can provide further insight into production of oral rehydration solutions which can be used for different purposes. Recently it was shown that fermentation of resistant starch is associated with elevated levels of gut hormones (PYY and GLP-1) which have a role in satiety and potentially long-term energy balance (Keenan et al., 2006). Although this research is still preliminary, the possible link between fermentation products of RS and gene expression of hormones, related to reduced energy intake, is of considerable importance (Higgins, 2004).

1.3.2.1.1.1.1 Types of RS

There are four different types of RS, designated RSI, RSII, RSIII and RSIV. RSI is physically inaccessible starch, as found in partial or intact cereal seeds. α-amylases do not have access to starch as the gastrointestinal tract does not possess enzymes which degrade cellulose, hemicelluloses, lignins, and other constituents of plant cell walls. RSII represents raw (uncooked) starch in its granular form of some plant species, e.g. potato and banana. The phenomenon of raw starch resistance to digestion varies from species to species and depends upon different factors (Leszczyński, 2004). For example in potato, due to large size of granules, limited area is available to enzymes (Ring et al., 1988). Other factors responsible for raw starch resistance include enzyme adsorption on the surface of starch granule (Leloup et al., 1992a), shape, structure of granule, pore size on the surface of granule and crystallinity (Leszczyński, 2004), amylopectin chain length,
occurrence of large blocklets (Kossman and Lloyed, 2000), and amylose contents (Gallant et al., 1997). RS III is retrograded starch. When starch-containing foods are cooked and cooled, a water-insoluble semi-crystalline structure precipitates from starch paste which is resistant to digestion. Retrogradation results in the formation of more thermo-stable structures by amylose than by amylopectin. The amount of RSIII produced by retrogradation is directly proportional to the amylose content of starch (Leloup et al., 1992b; Colquhoun et al., 1995; Leszczyński, 2004; Rahman et al., 2007). RS IV is chemically or physically modified starch in industrially processed food. RSIV includes hydroxypropyl distarch phosphate and acetylated distarch phosphate. The former has two times lower susceptibility to the activity of amylases than native starch (Östergård et al., 1988; Hoover & Zhou, 2003).

1.3.2.1.1.1.1.1 Factors affecting resistant starch

Starch is hydrolyzed by α-amylases, the actions of which are influenced by various physical and structural properties including granule size, phosphorus content, amylose: lipid complexes (Crowe et al., 2000). The molecular structure of starch is also important in determining resistance to digestion; association of amylopectin chains, and the degree of helix formation in amylose and amylopectin (Haralampu, 2000; Miao et al., 2009), occurrence and perfection of crystalline region in both amorphous and crystalline lamellae of the granule, (Eerlingen and Delcour, 1995) and starch crystallinity and packing (Jane et al., 1997) are of significant importance. Other properties, such as hilum and surface channels/pores connection (Kim and Huber, 2008), porosity, degree of integrity and structural inhomogeneity (Copeland et al., 2009), and the presence of α-amylases inhibitors like maltose and maltotriose (Colonna et al., 1988) also contribute in
resistance to α-amylases digestion. Previously, presence of pore or pin holes on the surface of starch granules (Hall and Sayre, 1970; Fannon and BeMiller, 1992) and variation in their distribution within and among different individual species (Fannon et al., 2004) have also been reported to be important factors in determining starch digestibility. In species like sorghum, maize and millet, the presence of pores on the surface of the granule is related to increased rate of enzymatic digestion of the granule. Pores connect the outer surface of the granule to the inner cavities (Huber and BeMiller, 2001; Benmoussa et al., 2006). The presence of such features is important in some cereals like sorghum which has limitations in its end uses because of inherently poor digestibility (Shapter et al., 2008). When starch is heated above a certain temperature, it is gelatinized, which results in the leaching of amylose molecules in the form of coiled polymers from the swollen starch granules. Upon cooling, these coiled polymers associate as double helices and form hexagonal networks (Jane and Robyt, 1984; Haralampu, 2000). In waxy starch, aggregate formation between amylopectin molecules occurs, which is more susceptible to amylases for hydrolysis (Miao et al., 2009). Those factors which make starch inaccessible to amylases contribute to overall resistant starch content. Amylopectin chain length distribution (CLD) and packing play an important role in determining starch digestibility (Asare et al., 2011). For example, the RS content of maize ae-mutant lines was positively correlated with the apparent and the absolute amylose content and the larger proportion of longer glucan chains of amylopectin (Li et al., 2008). The ae-mutant lacks SBEIIb enzyme which is responsible for the addition of branches in the growing amylopectin molecule. Due to absence of SBEIIb, branch frequency decreases significantly, which results in the amylopectin with longer glucan
chains. Because of this structural change, the resultant amylpectin resembles amylose hence this starch is termed as “high amylose”. Analysis of starch from different botanical sources revealed no significant correlation between functional and physical properties such as granule size, shape and apparent amylose content of starch but there was a strong association with chain length distribution of amylpectin (Zhang et al., 2006). The presence of lipids and proteins is also important in determining the digestibility of starch and hence affects RS content (Zhang et al., 2006).

Rapidly digestible starch (RDS) is negatively correlated with amylose content and variations in RS content are significantly influenced by amylose content in meal and pure starch samples. RS is also associated with B-type granules ranging from (5-15 μm) in size and the amylpectin fraction with 19-36 DP (Asare et al., 2011). A study conducted with amyllose extender (ae’), waxy (wx’) and wx ae’ mutants showed that ae’ endosperm starch accumulated an increased amylose content plus long chain amylpectin. There were no significant differences in the unit chain-length distribution of amylpectin or starch granule morphology in ae’ and wx/ae’ starches. While wx/ae’ starch had a higher pasting temperature, higher peak viscosity and higher gelatinization peak temperatures than that of the wild-type starch. The primary structure of the rice wx ae’ amylpectin with high proportion of long chains changes the granular and crystal structure of the starch and increases resistance to in vitro or in vivo digestion by amylases (Kubo et al., 2010). Thus starches from different origins have different physiochemical properties and hence have different applications in food and non-food industries. The factors responsible for variations in starch structure from different biological sources include: amylose to amylpectin ratio, glucan chain length distribution, degree of branching, and granule
size. These are in turn controlled by the activities of different enzymes involved in the synthesis of starch, the subject of which will now be discussed.

1.4 Starch Biosynthesis

1.4.1 Enzymes involved in starch biosynthesis

It is generally accepted that the complex process of starch biosynthesis is catalyzed by a series of biosynthetic enzymes including, ADP-glucose pyrophosphorylase (AGPase), starch synthase (SS), starch branching enzyme (SBE), and starch debranching enzyme (DBE) (Smith et al., 1997; Myers et al., 2000; James et al., 2003; Grimaud et al., 2008; Radchuk et al., 2009). The other enzymes which are also important in starch synthesis are starch phosphorylase (SP) and disproportionating enzyme (D-enzyme) (Li et al., 2003; Leterrier et al., 2008). Across species, highly-conserved families of genes encode these enzymes (Ball & Morell, 2003; Ball & Deschamps, 2009). By studying genetic modifications of starch synthesizing enzymes in numerous plant species, it has been suggested that each enzyme class has a uniquely conserved role in the process of starch synthesis (Peter et al., 2010). A schematic presentation of different enzymes involved in starch biosynthesis is shown in Figure (1.9).

1.4.1.2 Adenosine 5’ Disphosphate Glucose Pyrophosphorylase (AGPase, EC 2.7.7.27)

In higher plants, adenosine 5’ diphosphate glucose pyrophosphorylase (AGPase) is responsible for the catalysis of the first committed step in starch biosynthesis. It controls the synthesis of the nucleotide diphosphate sugar ADP-glucose (ADP-Glc) from glucose-1-phosphate and ATP. This ADP-glucose (ADP-Glc) serves as the soluble precursor and substrate for starch synthases (Tetlow, 2006; Bowsher et al., 2007). The
reaction catalyzed by the AGPase is often considered as the rate-limiting step in starch biosynthesis (Tetlow et al., 2003b). AGPase has a single, highly conserved N-terminal catalytic region and a C-terminal domain made up of a parallel beta helix structure which is involved in allosteric regulation and subunit oligomerization (Jin et al., 2005; McCoy et al., 2007). The AGPase reaction in the majority of plant cells exclusively takes place in plastids. However, an extra-plastidial, cytosolic form of AGPase is also present in the endosperms of cereals and other graminaceous plants (Beckles et al., 2001). A number of studies have revealed that the extra-plastidial form of AGPase is responsible for the majority of AGPase activity in maize, barley, rice, and wheat (Denyer et al., 1996; Thorbjørnsen et al., 1996; Sikka et al., 2001; Tetlow et al., 2003b). The enzyme is largely extraplasmoidal in cereal endosperm in contrast to other cereal tissues and non cereal plants (Giroux et al., 1994; Denyer et al., 1996; Shannon et al., 1996; Thorbjørnsen et al., 1996; Beckles et al., 2001; Comparot-Moss & Denyer, 2009). In wheat, cytosolic AGPase accounts for about 65 %-95 % of the total activity (Tetlow et al., 2003a), and in maize endosperm cytosolic AGPase activity was found to be > 95 % of the total (Denyer et al., 1996). In barley the cytosolic AGPase activity accounts for 85 % of activity (Denyer et al., 1996; Thorbjørnsen et al., 1996; Johnson et al., 2003). Barley mutants lacking cytosolic AGPase (Johnson et al., 2003) had reduced starch synthesis (44 %) even though plastidial AGPase activity was unaffected (Tester et al., 1993).

In cereal endosperm, ADP-glucose synthesized in the cytosol is transported into plastids in exchange for ADP via a small inner envelope protein encoded by the Brittle1 gene (Sullivan, 1995; Mohlmann et al., 1997; Shannon et al., 1998; Emes et al., 2003). It was shown that the maize mutant brittle1 (bt1) accumulated >13% ADP-glucose more
Figure 1.9: Schematic presentation of starch biosynthesis in the cereal endosperm.
In cereal endosperm ADP-glucose (ADPG) is generated by cytosolic and/or plastidial AGPase, and used as a substrate for starch biosynthesis. GBBS is the only enzyme which is involved in amylose biosynthesis. Synthesis of amylopectin is a complex process which is regulated by number of enzyme classes including (SS, SBE, DBE and SP). SSs add linear chains of various lengths in growing amylopectin molecule and branches are introduced by SBEs. Inappropriately attached branches are trimmed by DBE. The exact role of SP in starch biosynthesis is yet not known (Rahman et al., 2007).
than normal, but had reduced starch content (60 % of wild-type) even though activities of starch synthases and starch branching enzymes were unaffected (Shannon et al., 1996). Bowsher et al., (2007) showed that in wheat endosperm, the import of ADP-glucose into amyloplasts is dependent on counter-exchange with the adenylates ADP and AMP. Further, the rate of ADP exported from the amyloplasts was equal to the rate of ADP-glucose utilized by starch synthases, suggesting that ADP is the most likely to be the form of adenylate which exchanges with ADP-Glucose. AGPase is present in all starch-synthesizing tissues of higher plants and involved in the biosynthesis of both transient starch in chloroplasts or chromoplasts and storage starch in amyloplasts. In higher plants, AGPase is heterotetrameric, composed of two large (AGP-L) and two small (AGP-S) catalytic subunits which are highly homologous in both sequence and structure, however each is encoded by different genes (Ballicora et al., 2004; Hannah and James, 2008). In maize, the large and small subunits of AGPase are encoded by *shrunken2* (*sh2*) and *brittle2* (*bt2*) genes respectively (Bhave et al., 1990; Bae et al., 1990). While large and small subunits of the maize plastidial AGPase are encoded by the *Agp1* and *Agp2*, respectively, but no mutants are available (Rosti & Denyer, 2007). Multiple genes encoding the AGPase subunits are differentially expressed in different plant organs which results in variable AGPase subunit composition in different parts of the same plant such as potato (La Cognata et al., 1995), rice (Nakamura and Kawaguchi, 1992), and barley (Villand et al., 1992). The sequences of small subunit of AGPase from various eudicots and monocots vary in exon1 (Hannah et al., 2001).

Different mechanisms have been reported to regulate AGPase activity. First, AGPase is subjected to transcriptional regulation, with elevated (sugar) sucrose causing
an increase in expression (Salanoubat and Belliard, 1989; Sokolov et al., 1998; Muller-Rober et al., 1990) while nitrate and phosphate shown a decrease (Nielsen et al., 1998; Scheible et al., 1997). A second mechanism involves the allosteric regulation of AGPase, being activated by glycerate-3-phosphate (3PGA) and inhibited by inorganic phosphate (Pi) in leaf chloroplasts (Neuhaus and Stitt, 1990), in amyloplasts in cereal endosperm (Tetlow et al., 2003b), and in storage tubers (Tiessen et al., 2003). The level of sensitivity of AGPase to these allosteric effectors seems to be dependent upon tissue, plastid type, the subcellular localization of the enzyme, and the ratios of the allosteric effectors in different species. For example, in wheat endosperm the plastidial AGPase activity is much less sensitive to 3-PGA activation and Pi inhibition compared to potato tubers (Hylton and Smith, 1992; Gomez-Casati and Iglesias, 2002; Tetlow et al., 2003a). Similarly chloroplast AGPase, is highly sensitive to concentrations of allosteric effectors, being activated by micromolar concentrations of 3-PGA and inhibited by Pi (Ghosh and Preiss, 1966).

Fu et al. (1998) also proposed a post-translational mechanism of modification of AGPase involving thioredoxin. They exposed recombinant potato AGPase to oxidized thioredoxin and observed the subsequent formation of disulfide bonds between the N-termini of the small AGPase subunit. In leaves of different plants, starch synthesis is controlled by post-translational regulation of AGPase in response to light and sugar levels. When isolated chloroplasts are illuminated, or sucrose is supplied to leaves in the dark through the petiole, the small subunit of AGPase is rapidly converted from a dimer to a monomer. The reverse happens when pre-illuminated leaves are darkened (Hendriks et al., 2003). AGPase of potato tuber is also subjected to redox-dependent post-
translational regulation, in which an intermolecular cystene (Cys) bridge is formed between the two catalytic small subunits (Tiessen et al., 2002).

The cytosolic localization of AGPase in cereal endosperm may provide an advantage where large amounts of carbon are partitioned to starch when there is a plentiful sucrose available.

1.4.1.3 Starch Synthases (SS, EC 2.4.1.21)

Starch synthases (SSs) produce α-1,4-glucan linkages by transferring sugar moieties from an activated donor molecule (ADP-glucose) to a specific acceptor molecule (growing glucan chain) in a distributive mechanism in which the enzyme dissociates from its substrate during each catalytic cycle (Denyer et al., 1999). Among starch biosynthesis enzymes, SSs has the highest number of isoforms (Fujita et al., 2011). These enzymes are found in the starch granules but are also present in the plastid stroma, leading to their classification as soluble starch synthases, with the exception of granule-bound starch synthase (GBSS), which is exclusively found within starch granules. Five classes of starch synthases are consistently present in higher plants which can be divided into granule-bound starch synthase (GBSS), and soluble starch synthases (SSI, SSII, SSIII and SSIV). These different classes may have multiple isoforms e.g. GBSSI and GBSSII, three SSII isoforms (SSIIa, SSIIb and SSIIc [also defined as, SSII-3, SSII-2 and SSII-1], respectively), two SSIII isoforms (SSIIIa and SSIIIb [SSIII-2 and SSIII-1, respectively]), and two SSIV isoforms (SSIVa and SSIVb [SSIV-1 and SSIV-2, respectively]) (Hirose and Terao, 2004; Fujita et al., 2007). Phylogenetic analyses separate the GBSS, SSI and SSII from SSIII and SSIV classes (Ball and Morell, 2003; Patron and Keeling, 2005; Leterrier et al., 2008).
Sequence alignment comparisons of different isoforms of SSs show that all isoforms of SSs in higher plants and green algae contain a highly conserved core, or catalytic region, of approximately 60 kDa, with a C-terminus similar to that of glycogen synthases (GSs) (Tetlow, 2011). The K–X–G–G–L motif is thought to be responsible for substrate (ADP-glucose) binding in higher plant SSs and in prokaryotic (GSs) (Furukawa et al., 1990, 1993; Busi et al., 2008), and is only present in the C-terminus of higher plants and green algal SSs (Nichols et al., 2000). However, K-X-G-G-L domains are distributed across the GSs protein sequence in prokaryotes (Fukukawa et al., 1990). Glucan primer preference is determined by the presence of lysine in the K–X–G–G–L domain (Gao et al., 2004). Further, in maize SSs, the glutamate and aspartate were found as important residues for catalytic activity and substrate binding (Nichols et al., 2000). In contrast to GS and GBSS all SSs (SSI, SSII, SSIII, and SSIV) have an additional sequence located at N-terminal to the catalytic region; which is sometimes referred as the N-terminal extension. Considerable variation has been found within this region upstream of the catalytic core, and this extension can vary greatly in length from 2.2 kDa in GBSSI to approximately 135 kDa in maize SSIII (Gao et al., 1998). Studies with SSs truncated at N-terminal extension showed that this extension is not required for catalysis (Imparl-Radosevich et al., 1998; Edwards et al., 1999) or glucan affinity (Commuri & Keeling, 2001), however it is involved in determining chain-length specificities of the enzymes or possibly for protein-protein interactions (Hennen-Bierwagen et al., 2008). On the basis of predicted amino acid sequence, the phylogenetic and sequence analyses of plants SSs (Arabidopsis thaliana, wheat and rice) and algal SS and prokaryotic GS isoforms suggests that SSI, SSIIIs and GBSSIs have distinct evolutionary origins as compared to
SSIIIs and SSIVs (Leterrier et al., 2008). In particular, the valine residue present within the highly conserved K-X-G-G-L motif seems to have faced strong evolutionary selection in SSIIIs and SSIVs and in these SSs it may affect primer/substrate binding compared to SSI, SSIIIs and GBSSIs (Leterrier et al., 2008). SSIII and SSIV also have another prominent difference from other SSs, which is the presence of a highly conserved, G-X-G motif near the nucleotide-binding cleft (Leterrier et al., 2008). Sequence comparison of different SS isoforms is shown in Figure (1.10).

Apart from GBSS, other enzyme classes are also found within the starch granule. The partitioning of starch synthases between the soluble plastid stroma and starch granules varies among plant species, plastids type and developmental stage of the plant (Ball and Morell, 2003). In higher plants, a specific group of starch synthesizing enzymes (SSI, SSIIa, and isoforms of SBEII) are consistently found within starch granules as well as the stroma, whereas other enzyme classes (SSIV, SBEI, SP, isoforms of isoamylase, pullulanase-type DBE and D- enzyme) which also play an important role in amylopectin biosynthesis are either absent from the granules or are present in small amounts (Tetlow, 2011). The relative activities of different SSs isoforms are species-, organ- and developmental stage-dependent. For example in maize endosperm, 60 % of the soluble starch synthase activity is contributed by SSI (Cao et al., 1999), in pea embryos SSIIa makes 60 % of the total activity (Denyer and Smith, 1992) and in potato tubers 80 % of soluble starch activity is shared by SSIII (Marshall et al., 1996). These variations may contribute to differences in starch structure in different organs and species. Genetic and biochemical evidence suggest that each SS isoform has different properties and a distinct role in the biosynthesis of amylopectin. This will be discussed below.
Figure 1.10: Comparison of cereal SS domains.
The five known isoforms of SS with their constituent number of amino acids are shown with the name of the corresponding mutant in maize (in parentheses). The Figure shows comparison of SS domain sequences in which C-terminal catalytic domain (including ADP-glucose binding domain) is presented as black. The different SSs have N-terminal domains of varying lengths which is shown as hatched bars. In SSIII particularly a unique N-terminal extension is present which is thought to be involved in controlling protein–protein interactions (Tetlow, 2011).
1.4.1.3.1 Granule-bound starch synthase (GBSS)

Granule-bound starch synthase (GBSS) is different from other SSs because it is exclusively associated with starch granules. There are two isoforms encoded by the waxy locus. GBSS I is confined to storage tissues while GBSS II is encoded by a separate gene and is responsible for transient starch biosynthesis in leaves and other non-storage tissues (Fujita and Taira, 1998; Nakamura et al., 1998; Vrinten and Nakamura, 2000). Studies with different GBSS mutants (amylose free) showed that this is the only enzyme responsible for amylose biosynthesis in maize and other plant species (Denyer et al., 2001). In leaves and endosperm of rice, and presumably other plant species, highly similar GBSS isoforms produced from different genes are also present (Vrinten & Nakamura, 2000). In potato, GBSS also has a significant influence on the granule structure (Fulton et al., 2002). Mutation in the waxy gene leads to loss of GBSS activity, which results in amylose-free (waxy) starches. These waxy starches are still able to form a granule and maintain its semi-crystalline property, which indicates that insoluble granule synthesis does not require amylose (Denyer et al., 1999).

One hypothesis regarding the mechanism, by which GBSSI synthesizes amylose, indicates that GBSSI is stimulated by malto-oligosaccharides (MOS). MOS diffuse into the granule matrix where amylose synthesis by GBSSI takes place by elongating the MOS primers (Denyer et al., 2001; Denyer et al., 1996). It can also elongate the glucan chain within amylopectin. GBSS does not disassociate from the growing glucan chain after addition of glucose units but it remains associated to add new glucose units (Yeh et al., 1981; Hizukuri et al., 1989; Reddy et al., 1993; Maddelein et al., 1994). GBSS absence does not affect granule size distribution significantly (Fujita et al., 1998;
Mangalika et al., 2003), however its activity is related to formation of growth rings in starch granules (Pilling and Smith, 2003). In the early stage of grain filling, soluble SSs could be the predominant enzyme responsible for starch granule size distribution while GBSS may play an important role in the ratio of large to small granules, especially in the late grain filling stage (Chuanhui et al., 2010).

1.4.1.3.2 Starch synthase (SSI)

SSI is responsible for the synthesis of the shortest glucan chains, approximately 10 glucosyl units or less (Commuri and Keeling, 2001), while other SS isoforms are responsible for the further extension of glucan chains. The hypothesis that SSI is involved in the synthesis of short chains comes from the study of *Arabidopsis* (Dauvillée et al., 2005) and rice (Fujita et al., 2006) mutants lacking SSI which show deficiencies in shorter (DP 6–12) glucan chain lengths. In potato, SSI mutants exhibit no detectable changes in starch structure, which suggests that this isoform has only a minor activity in potato tubers (Kossmann et al., 1999). Similarly the SSI mutation did not affect starch contents, size and shape of developing seeds and starch granules significantly (Fujita et al., 2006), but alteration in amylopectin structure has been found where the proportion of chain length of DP 6-12 decreased, however the proportion of chain length of DP 16-19 was increased. Similar results have been reported in *Arabidopsis* transient starch with the SSI mutation (Dauvillée et al., 2005). The catalytic activity of SSI was significantly reduced when longer glucan chains were used as substrate and most of the ADP-glucose was incorporated into shorter chains with DP <10 (Jeon et al., 2010). Thus, smaller glucan chains are extended by SSI up to a certain critical length and then SSI becomes bound to longer amylopectin chains and entrapped.
there as an inactive protein within the starch granule. Further extension of glucan chains must be taken over by other SSs for continued amylopectin biosynthesis (Jeon et al., 2010). In cereals, the complete absence of SSI has no effect on the size and shape of seeds, starch granules and the crystallinity of endosperm starch, which indicates that other SS enzymes are able to compensate for SSI function (Fujita et al., 2006). In contrast to this, in barley, soluble SSs are thought to have a role in determination of granule size. A mutation at the barley shx locus results in reduced SSI activity which leads to reduction in the size of A-granules and transforms the normal bimodal granule size distribution to unimodal (Schulman and Ahokas, 1990; Tyynela and Schulman, 1993; Tyynela et al., 1995). However, Chuanhui et al. (2010) reported that starch granule size distribution in wheat is associated with activities of starch synthases and not specifically SSI. These variations may suggest that impact of SSI on starch synthesis is species dependent.

1.4.1.3.3 Starch synthase (SSII)

Two genes classes (SSIIa and SSIIb) are present in monocots encoding SSII. SSIIa is present in cereal endosperm, while SSIIb is expressed in photosynthetic tissues. In monocots and green algae, SSII is involved in the synthesis of intermediate glucan chains of DP 12-24 by elongating short chains of DP ≤ 10 (Fontaine et al., 1993; Imparl-Radosevich et al., 2003; Morell et al., 2003). SSIIa mutation causes a decrease in the proportion of intermediate chains of DP 12-25 and an increase in short chains of DP 6-10 in amylopectin. The gene encoding SSIIa in the endosperm has been found in many crop species, including maize, wheat, rice, barley and pea (Campbell et al., 1994; Craig et al., 1998; Yamamori, 2000; Umemoto et al., 2002; Morell et al., 2003; Zhang et al., 2004). Although the contribution of SSIIa in the total measureable activity of SSs in the cereal
endosperm is minor, loss/down regulation of this protein has a major impact on the amount and composition of starch (Tetlow, 2011). In *Arabidopsis*, loss of SSII has no affect on growth rate or starch quantity, but causes an increase in amylose content and a decrease in amylopectin (Zhang et al., 2008). In monocots, loss of SSIIa results in reduced starch content, change in granule morphology reduction in amylopectin chain-length distribution, and decreased crystallinity. SSIIa mutants of barley and wheat possess low seed starch content, reduced chain length distribution of amylopectin and crystallinity. Altered granule morphology and amount of amylose in barley starch granules has been significantly increased up to 70 % (Morell et al., 2003; Kosar-Hashemi et al., 2007). The relevant SSIIa mutation in maize is *sugary2*, which results in more short chains of DP 6-10, fewer chains of DP 12-30 and increased levels of amylose up to 40 % (Zhang et al., 2004). This mutation has important applications in the food industry due to changes in the functional properties of starch (Harn et al., 1998). Similar effects have been observed in potato tubers (Edwards et al., 1999; Lloyd et al., 1999) and in *Arabidopsis* leaves (Zhang et al., 2008). In the rice endosperm, abundant transcripts of SSIIa and SSIIIa were found during grain filling, suggesting a crucial role of SSIIa and SSIIIa during starch biosynthesis (Jeon et al., 2010).

### 1.4.1.3.4 Starch synthase (SSIII)

SSIII is involved in the synthesis of longer glucan chains of DP 25-35 or greater (Tomlinson and Denyer, 2003; Zhang et al., 2004, 2005, 2008). SSIII mutants lack longer chains in amylopectin which results in adverse alteration in molecular architecture (Gerard et al., 2009). In rice, SSIII is encoded by two genes, SSIIIa in the endosperm and SSIIIb in leaves (Hirose and Terao, 2004; Dian et al., 2005). After SSI, the second
highest catalytic activity in cereal endosperm (maize and rice) is exhibited by SSIII (Cao et al., 1999; Fujita et al., 2006). SSIII has a primary role in amylopectin synthesis, but the impact of SSIII loss is different within different genetic backgrounds. In an in vitro experiment when glycogen was used as the primer, a SSIIIa purified fraction from rice endosperm generated long chains from DP ≤ 11 chains (Fujita et al., 2006). In ssIIIa mutants of rice, glucan chains with DP 6-8, DP 16-20, and DP ≥ 30 were reduced, whereas glucan chains with DP 9-15 and DP 22-29 were increased (Fujita et al., 2007; Ryoo et al., 2007). This shows that in the rice endosperm SSIIIa contributes to the synthesis of amylopectin chains with DP ≥ 30 in vivo. Interestingly endogenous SSI activity has been seen to increase due to loss of SSIIIa, thereby enhancing the synthesis of glucan chains with DP 9-15 and DP 22-29, respectively (Fujita et al., 2007). Similar observations have been made with maize, where the SSI activity in the SSIIa mutant was higher than wild-type (Cao et al., 1999). In potato, mutation in SSIII causes alteration in glucan chain-length distribution (Abel et al., 1996), whereas in maize, mutation in SSIII produces less significant phenotypic effects, which are detectable only in waxy mutants (Gao et al., 1998). The (SSIII) dull mutants of maize and rice show altered granule morphology and crystallinity and reduced proportion of longer glucan chains of DP ≥ 30, which suggests the role of SSIII in the elongation of these chains (Inouchi et al., 1983; Fujita et al., 2007; Ryoo et al., 2007) white-core, floury endosperm. Similarly, maize ssIIIa mutants show a dull phenotype with a glassy and tarnished endosperm (Gao et al., 1998). In addition to alteration in starch structure and physical properties, in maize endosperm loss of SSIII is associated with pleiotropic effects on other SS and SBE, causing increased activities of SSs (Cao et al., 1999) and reduction in the activity of
SBEIIa (Boyer and Preiss, 1981). But expression of the genes coding different enzymes involved in starch biosynthesis in (ssiii) amo1 appears more or less unaffected at both the transcript and protein levels (Borén et al., 2008) However qualitative analysis with zymogram suggested that the branching enzyme (SBEs) activity differed between amo1 and normal type and starch granule protein content of amo1 was higher than the normal type (Boren et al., 2008). Amol is ssiii- mutant in barley, the same mutation in maize is called dull. Amol mutant does not lack SSIII protein, however it has a leucine to arginine residue substitution in a conserved domain compared to the wild-type protein. This substitution results in reduction in activity of SSIII protein compared to wild-type (Li et al., 2011). Observation with mutants shows that, in addition to catalytic role of SSIII, it also possesses regulatory properties with respect to control over the starch biosynthetic pathway. Several studies have revealed that SSIII is a negative regulator of starch synthesis and mutation in this gene leads to elevated levels of amylose (Li et al., 2011) In Chlamydomonas, mutations in SSIII is associated with increased amount of GBSSI protein and transcript giving rise to more long chains in amylopectin (Ral et al., 2006). Studies with potato single and double mutants for SSII and SSIII showed that these enzymes make distinct contributions towards amylopectin biosynthesis, and they act synergistically, rather than independently, during amylopectin synthesis. Single mutants for both the SSII and SSIII produced minor effects on starch granules, whereas double mutant for SSII and SSIII showed remarkable alteration in starch phenotype (Edwards et al., 1999; Lloyd et al., 1999; Zhang et al., 2008). In Arabidopsis, loss of both SSII and SSIII results in slower plant growth, dramatically reduced starch content and change in amylopectin structure, and their function cannot be substituted by any other conserved
SS, specifically SSI, GBSSI and SSIV (Zhang et al., 2005, 2008) Thus the synergistic effects of the loss of both SSII and SSIII are more severe than the loss of individual isoform, which suggest, partial redundancy with respect to the function these two isoforms in amylopectin biosynthesis (Tetlow, 2011).

1.4.1.3.5 Starch synthase (SSIV)

SSIV is the most recently discovered form of SSs from higher plants (Dian et al., 2005). The precise role of SSIV in starch biosynthesis is yet not well elucidated, but the SSIV mutants of Arabidopsis showed defective granule initiation and it is expressed in the grain during development (Hirose and Terao, 2004; Dian et al., 2005; Roldan et al., 2007). In plants there are two isoforms of SSIV which are differentially expressed in the endosperm (SSIVa) and in leaves (SSIVb) (Leterrier et al., 2008). SSIV mutants of Arabidopsis show a decrease in granule number but an increase in the granule size (Roldan et al., 2007; Zhang et al., 2008). SSIV protein is different from other SS isoforms in the sense that it has an N-terminal extension with two coiled-coil domain and a putative 14-3-3 protein binding site (Leterrier et al., 2008). These features may enable SSIV to interact with other proteins and thus contribute in the granule initiation. In the absence of SSIV, SSIII may be responsible for initiation of single granules since it has a glucosyl transferase domain closely related to SSIV. Double mutants of SSIII and SSIV lack starch in their leaves, although 60 % of the measureable soluble starch synthase activity exists from the remaining isoforms (Szydlowski et al., 2011).

1.4.1.4 Starch Branching Enzymes (SBEs, EC 2.4.1.18)

SBEs catalyze the hydrolysis of an existing α-1,4 linked glucan chain, with subsequent transfer of the cleaved portion of glucan chain with six or more glucose units
to the C$_6$ portion of the same glucan chain (intra chain transfer) or an adjoining glucan chain (inter chain transfer) via an α-1,6 linkage to form the branched structure of amylopectin. There are two major classes of highly conserved SBEs across plant species (SBEI and SBEII) sometimes also referred to as SBEB and SBEA, respectively, and they have a primary role in amylopectin synthesis, although they may also have a role in amylose synthesis which is lightly branched (Keeling and Myers, 2010). There are two isoforms of SBEII namely (SBEIIa and SBEIIb). The functional SBE enzymes have multiple highly conserved regions incorporating two carbohydrate binding sites and an α-amylase domain (Abad et al., 2002). The N-terminal region of SBEs appears to be important for catalysis and structural stability (Guan et al., 1997; Hamada et al., 2007), whereas substrate preference and chain length transfer are determined by both the N- and C-terminus (Kuriki et al., 1997).

1.4.1.4.1 Starch branching enzyme I (SBEI)

The two SBEs classes differ primarily in terms of length of glucan chain transferred *in vitro* and substrate specificities. For example, SBEII transfers shorter glucan chains and has a higher affinity for amylopectin than SBEI which shows higher rates of branching with amylose (Guan and Preiss, 1993; Takeda et al.,1993; Rydberg et al., 2001). SBEI has a lower km for amylose and tends to produce shorter constituent chains, compared to SBEIIa or SBEIIb (Gao et al., 1996). Some of these isoforms are tissue, or developmental-stage specific, in their expression patterns (Yamanouchi and Nakamura, 1992; Gao et al., 1997; Sun et al., 1998; Regina et al., 2005). For example, in maize, SBEI is expressed moderately during middle stages (12–20 DAA) of kernel development and more strongly during the later stages (22–43 DAA), but is only
moderately expressed in vegetative tissues (Kim et al., 1998). The roles of SBEI and SBEIIa are less clear because SBEI mutants of rice have reduced intermediate and long chains (Nakamura, 2002), whereas in maize the chain lengths are unaffected in mutants of SBEI and SBEIIa (Blauth et al., 2001, 2002). SBEI mutants do not show a significant effect on starch structure, but in maize, double mutants of SBEI and SBEIib same noticeable alterations in amylopectin branching pattern which suggests that SBEI has some role in storage starch biosynthesis (Yao et al., 2004). In monocots or dicots, down- or up-regulation of SBEI has minimum effects on starch synthesis and composition in storage and transient starch synthesis (Blauth et al., 2002; Satoh et al., 2003). It was found that loss of enzyme activity was only detected in SBEIIa or SBEIib mutants (Blauth et al., 2002), suggesting either that the lack of SBEI was compensated by other two SBE isoforms or that SBEI does not have a significant role in determining starch quantity or quality in leaves or endosperm (Blauth et al., 2002). Although the precise role of SBEI is not clear, in cereals like wheat and barley, an isoform of SBEI, termed SBEIc, is only found within the large A-granules (Peng et al., 2000). However, in contrast, SBEIc was found in both A- and B-granules of wheat (Bancel et al., 2010). Expression of three functional SBE genes of maize in a yeast strain lacking yeast glucan polymer branching enzymes showed that SBEI was inactive in the absence of either SBEIIa or SBEIib, and that SBEII acts prior to SBEI on precursor polymers (Seo et al., 2002). In plants, SBEI is highly conserved and has been shown to physically interact with other starch biosynthetic enzymes (Liu et al., 2009; Tetlow et al., 2004) which suggest that SBEI plays some function in regulating the starch biosynthetic process. Similarly loss of SBEI in SBEIib-deficient back ground resulted in increased branching, which also
suggests a regulatory role for SBEI in influencing other branching enzymes (Yao et al., 2004).

1.4.1.4.2 Starch branching enzyme II (SBEII)

In monocots, two SBEII gene products (SBEIIa and SBEIIb) are closely related (Rahman et al., 2001). The affect of loss of SBEII on starch phenotype is more pronounced compared to SBEI. Both SBEIIa and SBEIIb are expressed in the developing endosperm of barley and are partitioned between soluble and granule-bound fractions of amyloplasts (Morell et al., 1997; Sun et al., 1998; Rahman et al., 2001). But the expression pattern of both enzymes is different in barley endosperm from other cereals like maize and wheat. In maize, SBEIIa is present 50 times less than SBEIIb. In contrast SBEIIb is present at much lower levels than SBEIIa in wheat endosperm (Gao et al., 1997; Morell et al., 1997; Regina et al., 2005). However, in barley endosperm both proteins are expressed at approximately equal levels (Sun et al., 1998). In maize and rice, loss of SBEIIa produced a clear phenotype of transient starch but no apparent effects on storage starch biosynthesis in the endosperm (Blauth et al., 2001). There was no significant change in kernel phenotype, starch content, starch structure and chain length distribution in the endosperm. This suggest that SBEIIa has a primary role in transient starch biosynthesis but no critical role in storage starch biosynthesis, or that its role is easily compensated by other SBEs (Blauth, et al., 2001; Nakamura, 2002). Based on biochemical studies of the isolated enzyme (Guan et al., 1997; Takeda & Preiss, 1993) and structural analysis of the maize and rice mutants (Nishi et al., 2001; Takeda et al., 1993; Stinard et al., 1993), it was determined that SBEIIb is primarily responsible for transferring longer chains of amylpectin. The maize gene encoding for SBEIIb is
amylose extender (ae), the mutation of which leads to severe alteration in the structure of amylopectin with fewer branches and increased level of apparent amylose up to 50 %, (Garwood et al., 1976; Klucinec & Thompson, 1998). The primary reason of increase in measureable amylose is the synthesis of modified amylopectin, where longer glucan chains are formed with few branches. The resultant amylopectin “resembles” amylose which can be detected by iodine-binding, and hence the resultant phenotype is often termed as “high amylose” although this is misleading. In barley, elimination of either SBEIIB or SBEIIa does not result in a significant alteration in the number of branches in amylopectin or an increase in apparent amylose content although the branch frequency of amylose was increased in sbeiib⁻ (Regina et al., 2010). In wheat, suppression of both genes SBEIIa and SBEIIB by RNAi⁻ is required to produced starch with amylose contents > 70 % (Regina et al., 2006). A double mutant (GBSSI/ SBEIIB) of rice produced starch with much longer chains and few chains with DP ≤ 17, with the greatest decrease in chain length between DP 8-12 (Nishi et al., 2001). This same trend has been observed in a rice ae single mutant. This suggests that SBEIIB plays an important role in the synthesis of amylopectin A chains. In the mutant, SBEI and SBEIIa levels remained unaffected which shows that the extent of change in the chain length profile was related to decreased SBEIIB activity (Tanaka et al., 2004). In an RNAi⁻ generated sbeiib⁻ mutant of barley, increased branching frequency in amylose has been found (Regina et al., 2010).

1.4.1.5 Debranching enzymes (DBEs, EC 3.2.1.41 and EC 3.2.1.68)

Debranching enzymes, also termed isoamylases, are involved in cleaving inappropriately attached glucan chains and contribute to the organized crystalline structure of amylopectin (Ball et al., 1996; Zeeman et al., 1998). This important role of
DBEs has been suggested by observing variations in the amylopectin structure of mutants lacking some of the DBEs. Thus, in addition to SSs and SBEs, isoamylases play an important role in the development of crystalline amylopectin. DBEs are of two types: isoamylase-type and pullulanase-type. The isoamylases hydrolyze α-1,6 linkages in amylopectin and the pullulanases hydrolyze α-1,6 linkages in pullulan, a fungal polymer of malto-triose. The genes encoding three evolutionarily conserved isoamylase-type DBEs and one pullulanase-type DBE have been identified (James et al., 1995; Dinges et al., 2003). The different classes of isoamylases are distinguishable by their amino acid sequence and substrate specificities (Zeeman et al., 2010). The ISO type DBE has three classes (ISO1, ISO2 and ISO3). ISO1 and ISO2 are strongly involved in amylopectin synthesis while ISO3 and pullulanase are primarily involved in starch degradation. In Arabidopsis leaves and potato tuber, ISO1 forms a heteromultimeric enzyme complex with ISO2 (Hussain et al., 2003; Delatte et al., 2005; Wattebled et al., 2005). Whereas, in the endosperm of rice and in other cereals, ISO1 is found as a homomultimeric and as a heteromultimer with ISO2 (Utsumi and Nakamura, 2006). ISO1 has more affinity towards substrate with longer external chains such as solubilised amylopectin, while ISO3 and LDA are more active on substrates with short external chains such as β-limit dextrin. ISO2 appears to be catalytically inactive, and may be a regulatory subunit to ISO1 rather than contributing directly to catalytic activity (Hussain et al., 2003). Transgenic plants or mutants lacking ISO1 or ISO2 type DBEs have reduced amylopectin content, and in its place accumulate large amounts of the water-soluble polysaccharide (WSP), phytoglycogen (Zeeman et al., 1998; Bustos et al., 2004; Wattebled et al., 2005). Genetic alteration in ISO1 activity in maize and rice resulted in significant changes in
starch granule structure (Jane et al., 1994; Kubo et al., 1999) while a barley ISO1 mutant produced compound instead of simple starch granules (Burton et al., 2002). In double ISO1 and ISO2 antisense mutants, potato accumulated a large number of small granules in tubers (Burton et al., 2002; Bustos et al., 2004). These observations and studies with barley mutants and transgenic rice show that isoamylase-type DBE activity plays a crucial role in the initiation of starch granules (Burton et al., 2002; Bustos et al., 2004; Kawagoe et al., 2005). The expression of an isoamylase-type DBE (ISO1) depends upon the stage of development, as it is highest in developing endosperm and undetectable in mature grains (Sun et al., 1991). In maize, a severe phenotype has been observed in a ISO1 mutant known as sugary1 (su1) where reduction in crystalline starch and accumulation of a (WSP) pytaglycogen increased (Dinges et al., 2001; Burton et al., 2002; Fujita et al., 2003; Bustos et al., 2004). These observations suggest that DBEs function in starch synthesis by the selective cleavage of inappropriate branch linkages before crystallization of the molecule (Ball et al., 1996; Myers et al., 2000). Reduction in PUL activity did not produce pleiotropic effects on the other starch synthesizing enzymes in rice (Fujita et al., 2009), However, studies with starch mutants such as rice floury-2 and su1 mutants do induce pleiotropic effects on PUL activity (Kawasaki et al., 1996). A maize PUL mutant zpul-204 was isolated by gene tagging method (Dinges et al., 2003). The structure and composition of endosperm starch from maize zpul-204 were not different to that wild-type; however amylopectin of transient starch contained significantly fewer chains with DP 8-15 than wild-type because of the pleiotropic effect of SBEIIa activity. Similarly, developing endosperm of zpul-204 accumulated more branched malto-oligosaccharides which were not found in the wild-type (Dinges et al.,
In ISO1 (sugl) deficient background zpul-204 showed more accumulation of pytoglycogen in the seeds which was not seen in the wild-type. This indicates that PUL partially compensates for ISO1 deficiency and is important during starch synthesis and degradation (Dinges et al., 2003). PUL function partially overlaps with ISO1, though effects of deficiency in PUL1 are much smaller on amylopectin biosynthesis than that of ISO1, and variations of su1 phenotype are not significantly dependent upon activities of PUL (Fujita et al., 2009). In transgenic rice generated by transforming a wheat ISO1 gene into su1 rice, phytoglycogen synthesis in the endosperm was substantially replaced by starch synthesis (Kubo et al., 2005). These observations suggest that in maize and rice, su1 mutations are caused by deficiency of ISO1 and its activity plays a crucial role in normal amylopectin biosynthesis (Fujita et al., 2009).

Two models have been proposed for the function of DBEs in starch synthesis and phytoglycogen accumulation. The glucan trimming model explains the function of DBEs as removing any branches that would inhibit crystallization and aggregation of amylopectin into an insoluble granular structure (Ball et al., 1996; Myers et al., 2000), as inappropriately attached branches on the surface of the growing starch granules prevent crystallization. Another model suggests a “clearing role” of DBEs and proposes that they are involved in removing soluble glucan not attached to the granule from the stroma (Zeeman et al., 1998). This concept is based on the theory that SSs and SBEs will continue to synthesize glucan polymers if sufficient substrate is present and there will be random synthesis of glucan polymers which could cause accumulation of phytoglycogen, ultimately leading to a reduction in the rate of starch synthesis.

Contribution of different enzyme classes and their isoforms in the synthesis of
organized starch granule is shown in Figure (1.11).

1.4.1.6 Starch Phosphorylase (SP, EC 2.4.1.1)

Starch phosphorylase, a tetramer, is responsible for the reversible transfer of glucosyl units from glucose 1-phosphate to the non-reducing end of a-1, 4-linked glucan chains. Depending upon the concentration of the soluble substrates, SP may work either in a synthetic or a degradative direction. Plastidial SP referred to as Pho1 (or the L-form) has higher affinity towards amylopectin than glycogen (Mu et al., 2001). Based upon properties or timing of expression, SP from maize endosperm can be separated into different forms (Tsai and Nelson, 1969). SPI (Pho1) is present during all stages of endosperm development and at germination as well. However, SPII and SPIII are present during the period of rapid starch synthesis and are absent during germination and can initiate glucan chain synthesis without a primer. SP consists of N-terminal and C-terminal, where C-terminal domain shares significant similarity with the nucleotide-binding domain of SS (Buschiazzo et al., 2004). In developing rice endosperm, 96 % of the total phosphorylase activity is controlled by Pho1 (Satoh et al., 2008). In potato SP is classified as high (SP-H) and low (SP-L) isozymes, according to their affinity for glucans (Mori et al., 1993). An extra 80–amino acid insertion is present in the plastidic form, which is absent from cytosolic Pho2. The plastidic form has a high affinity towards low molecular weight malto-oligosaccharides (MOS) and amylose, while the cytosolic isoform (Pho2 or Pho-H) has a high affinity towards glycogen (Yu et al., 2001). Studies with Arabidopsis mutants clearly indicate that SP is not required for starch degradation (Zeeman et al., 2004). Activity of the plastidial isozyme (L-form) in sweet potato roots is regulated by proteolysis of a 78-amino acid peptide. Digestion of this peptide by
Figure 1.11: Diagrammatic representation of the coordinated actions of different enzyme classes in the synthesis of amylopectin.

The Figure demonstrates that in growing amylopectin molecules, linear chains are added by the SS. When these chains are extended to a particular length, SBE cleaves additional glucan chains and adds branches to the growing amylopectin molecule. During this process some branches are inappropriately attached which can hinder the formation of proper crystalline structure. Thus these inappropriately attached branches are trimmed by DBE, which facilitates proper crystallization of the molecule.
endogenous protease results in an increase in catalytic activity of SP in the phosphorolytic direction (Chen et al., 2002). In wheat, three variable phosphorylase activity forms (P₁, P₂, P₃) of SP have been identified. P₁ and P₂ are cytosolic in younger leaves, whereas mature leaves have only the plastidic form (P₃) (Schupp, 2004). SSIV mutants of Arabidopsis showed increased activity of SP, but starch structure or the amylose/amylopectin ratio remained unaffected in these mutants. However, in the dark, starch accumulation was decreased in two eco-types as compared to mutant leaves with a significant influence on granule size (Roldan et al., 2007).

1.4.1.7 Disproportionating enzyme (D-enzyme, E, C. 2.4.1.25)

D-enzyme or disproportionating enzyme is present in the soluble fraction of plastids in different starch synthesizing organs of plants (Takahashi et al., 1993; Lin et al., 1988). D-enzyme catalyses transfer of two glucose units from malto-triose to a longer glucan chain, making them available for β-amylosis and the remaining glucose monomer becomes available for export from the plastids. An Arabidopsis mutant of D-enzyme showed reduced rates of nocturnal starch degradation which indicates role of D-enzyme in the pathway of chloroplast starch degradation (Critchley et al., 2001). Some studies suggested that D-enzymes may work together with SP, contributing to starch synthesis by a phosphorolytic SP reaction (Takahashi et al., 1998). This model, is based on the “glucan-trimming” model. According to this model, short-chain malto-oligosaccharides (MOS) liberated by the trimming action of DBEs are converted to longer-chain glucans by D-enzyme, which in turn become substrates for phosphorolysis by SP, liberating G-1-P, being used to synthesize ADP-glucose by plastidial AGPase (Takahashi et al., 1998). It was shown by Colleoni et al. (1999) that phosphorolytic SP
reaction in *Chlamydomonas reinhardtii*, can be stimulated by the presence of D-enzyme.

**1.4.2 Post-translational modification of starch synthesizing proteins**

Some of the enzymes involved in starch biosynthesis are subjected to post-translational modification involving protein phosphorylation, allosteric and redox modification. Similarly there is increasing evidence that starch synthesis does not take place in a simple, linear fashion, as described in previous sections, but involves protein-protein interactions, i.e. formation of protein complexes coordinates the multiple actions of different proteins involved in the synthesis of starch polymer. Thus, synthesis of highly organized crystalline starch granule is due to the coordinated action of starch synthases (SSs), starch branching enzymes (SBEs), together with starch debranching enzymes (DBEs) (Ball and Morell, 2003). Allosteric and redox modification of certain proteins to control their activities have already been described in the previous section (see above). However, other important mechanisms of post-translational modification involve phosphorylation of certain proteins as described by Tetlow et al. (2004). Activities of different stromal isoforms of SBEII and SSIIa and the association of SBEI with SBEIIb and SP were found to be regulated by phosphorylation (Tetlow et al., 2004). When intact plastids from wheat were incubated with $\gamma^{32}\text{P}-\text{ATP}$, three isoforms of the SBE’s (SBEI, SBEIIa, and SBEIIb) were found to be phosphorylated on serine residues (Tetlow et al., 2004). This suggests that phosphorylation is also directly involved in the regulation of enzyme activity and in the formation of protein complexes (Tetlow et al., 2004, 2008; Liu et al., 2009). Evidence that protein phosphorylation is directly involved in protein complex formation came from experiments with wheat endosperm
amyloplasts, in which SP and SBEIIb could be co-immunoprecipitated with SBEI when phosphorylated within the soluble protein fraction (Tetlow et al., 2004). Conversely, dephosphorylation with alkaline phosphatase caused disassembly of this protein complex.

Based on the analysis of enzyme kinetics and mutations in the starch biosynthetic pathway there is evidence which suggests that enzymes involved in starch biosynthesis have physical and functional coordination (Yao et al., 2002, 2004; Colleoni et al., 2003; Seo et al., 2002; Dinges et al., 2001, 2003; Nishi et al., 2001; Beatty et al., 1999; Gao et al., 1998; Boyer and Preiss, 1979, 1981; Hawker et al., 1974). Most recently, biochemical analysis of endosperm soluble extracts provided direct evidence of protein-protein interactions among enzymes involved in starch synthesis (Liu et al., 2009; Hennen-Bierwagen et al., 2008, 2009; Tetlow et al., 2004, 2008). The idea of heteromeric enzyme complex formation is further supported by the observation of numerous pleiotropic effects on SSs, SBEs, and AGPase resulting from mutations in genes encoding specific enzymes of the starch biosynthesis pathway (Singletary et al., 1997; Boyer & Preiss, 1981). In maize, mutations which affect both a pullulanase-type DBE (zpu1-204) and an isoamylase-type DBE (ISO1), results in loss of SBEIIa activity, although the amount of SBEIIa protein is unchanged (Dinges et al., 2001; Dinges et al., 2003; James et al., 1995). However this effect was not observed when catalytically inactive SU1 was present, which suggests that SU1 has both enzymatic and non-enzymatic effects and different enzymes involved in starch biosynthesis may form protein complexes (James et al., 2003). Similarly in the maize sugary2 (su2−) mutant, loss of SSIIa caused decreased association of SSI, BEIIb, and SSIII within the starch granule, and SSI levels increased in the amyloplast lysate (Grimaud et al., 2008). In an ae− mutant
of maize lacking SBEIIb, binding of SBEI to starch granules was significantly increased, which suggests that loss of SBEIIb might unmask a binding site for SBEI in a multisubunit complex (Grimaud et al., 2008; Hennen-Bierwagen et al., 2008). In developing endosperm of sex6 mutant of barley, which lacks SSIIa, the mutation also loses the binding of SSI, SBEIIa and SBEIIb to the starch granules, despite the fact that there is no detectable alteration in their expression levels in the soluble fraction (Morell et al., 2003). Similarly, in wheat and rice endosperms, loss of SSIIa results in reduction of amylopectin synthesis and loses the presence of SSI, SBEIIa, and SBEIIb within the starch granules (Yamamori, 2000; Umemoto and Aoki, 2005). These pleiotropic effects are consistent with a central role for SSIIa in mediating protein-protein interactions. In maize, the interdependency of starch synthase IIa (SSIIa), SSIII, starch branching enzyme IIb (SBEIIb), and SBEIIa was tested by assessing assembly into multi-subunit complexes. It was found that mutations that eliminated one of these proteins also prevented the other proteins from assembling into a high molecular complex of almost 670 kDa. Similarly, in high molecular weight fractions of developing maize and wheat endosperm, different enzymes complexes, containing SSI, SSIIa, SSIII, SBEIIa, and/or SBEIIb, in various combinations have been reported (Hennen-Bierwagen et al., 2008, Tetlow et al., 2008). In maize kernel extracts, SSI activity was stimulated by the addition of purified SBEI or SBEII (Boyer and Preiss, 1979; Seo et al., 2002) suggesting some interaction. In barley SSI, SSII, and SBEIIa are able to bind with amyloplast 14-3-3 proteins in a phosphorylation-dependent manner. This may suggest a potential mechanism for assembly of the SS and SBEII proteins via protein phosphorylation and plastidial 14-3-3 proteins (Alexander and Morris, 2006). In maize, SSIII is involved in
protein-protein interactions with other enzymes (Hennen-Bierwagen et al., 2008, 2009) and, in addition, possesses sequences that confer a glucan binding function (Palopoli et al., 2006; Senoura et al., 2007; Valdez et al., 2008). Hennen-Bierwagen et al. (2009) reported a novel protein complex in maize endosperm containing SSIII, SSIIa, SBEIIa, SBEIIb, large and small subunits of AGPase, and Pyruvate Phosphate Dikinase (PPDK). Existence of these novel protein complexes could have a broader metabolic significance because proteomic analyses of maize endosperm have given rise to the hypothesis that PPDK is a key factor in regulating the partitioning of carbon flux between starch and protein (Mechin et al., 2007). The concept that different proteins involved in the same metabolic pathway are interacting with each other in stable complexes suggests that these interactions are of significant physiological importance. Recently Liu et al. (2009) also reported that in \( \text{ae}^- \) mutant of maize due to loss of SBEIIb, a novel protein complex containing (SSI, SSIIa, SBEI, SBEIIa and SP) forms, as opposed to wild-type in which a protein complex containing only SSI, SSIIa and SBEIIb was observed. The formation of these protein complexes was found to be phosphorylation-dependent because following dephosphorylation by alkaline phosphatase, no protein complex was observed in both genotypes. However, re-phosphorylation with ATP resulted in the restoration of protein complex formation in both genotypes. Similarly, Liu et al. (2009) also reported that SBEIIb present in the wild-type complex and SBEI and SP present in the \( \text{ae}^- \) mutant complex were phosphorylated. Formation of phosphorylation-dependent complex and phosphorylation of individual proteins are shown in Figure (1.12).

Although existence of protein complexes among starch biosynthetic enzymes has
been reported in different plant species, it is not clear whether different starch synthesizing organs in the same plant have similar protein complexes because protein complexes found in the amyloplasts of wheat endosperm were not found in the chloroplasts of starch synthesizing leaves of wheat (Tetlow et al., 2004).

1.4.3 Starch granule-bound proteins

The starch biosynthetic enzymes described earlier are distributed between the soluble fraction of the plastids and the insoluble starch granules to varying degrees (Ball and Morell, 2003; Tetlow et al., 2004, 2011; Grimaud et al., 2008; Dai et al., 2009). Thus, other than GBSS, proteins are also found within the starch granule. The proteins entrapped within the starch granules are involved in the synthesis of glucan chains of short to intermediate length which form clusters, resulting in semicrystalline lamellae. The amylopectin biosynthesis model proposed by French, 1984 and Hizukuri, 1986 states that gradual and periodic synthesis of amylopectin clusters joined by amorphous lamellae result in entrapment of proteins involved in starch biosynthesis within starch granules. However, detailed kinetic analysis of some of SSs isoforms suggest that increased affinity of these proteins for longer glucan chains during catalysis may be a cause for their association with the starch granules (Commuri and Keeling, 2001). But the mechanism by which these proteins become granule-bound is not well understood. However, Liu et al. (2009) reported that different enzymes of starch synthesis form functional protein complexes (described earlier) and these complexes become entrapped in the starch granule. Similarly it was also reported that different components of a protein complex are phosphorylated. Thus, from these observations it can be speculated that these phosphorylated proteins are coordinated as a protein complex and become
entrapped within the starch granule. This idea is further supported by evidence which shows that some of the proteins found in the starch granules are phosphorylated. In wheat, GBSS, SSI and SSII were phosphorylated in their starch granule-bound state (Bancel et al., 2010). Genetic analyses of the maize mutant lacking BEIIb, the ae mutant shows significant increases in BEI, BEIIa, SSIII, and SP in the granule, without affecting SSI or SSIIa (Grimaud et al., 2008). Staining the internal granule-associated proteins with phospho-protein-specific dye showed that at least three proteins, GBSS, SBEIIb, and SP, are phosphorylated in the starch granules (Grimaud et al., 2008; Liu et al., 2009). The idea that starch biosynthetic enzymes form functional protein complexes and these complexes become entrapped within the starch granule is further supported by two independent studies by Liu et al. (2012a, b). In a maize mutant, ae 1.2, which was expressing SBEIIb enzyme lacking glucan binding ability due to single nucleotide mutation. However, this protein was found within starch granule because this protein was found as part of a protein complex which was trafficked to the starch granules. Similarly, in a su2 mutant of maize which was expressing a catalytically inactive SSIIa, no protein was observed in the granule.

Apart from these proteins, other proteins which are not involved directly in starch biosynthesis or degradation, and do not have a assigned function, have also been found within the starch granules, such as plastid-localized starch granule-associated glycogen synthase kinase (GSK-3)-like protein kinase in Medicago and Arabidopsis. Over-expression of GSK-3-like kinase results in accumulation of starch in leaves under saline conditions (Kempa et al., 2007).

Similarly granule associated phosphatases have also been found which play an
important role in starch catabolism by priming the polymer for degradation via phosphorylation of glucose units (Comparot-Moss et al., 2010; Kotting et al., 2009; Kerk et al., 2006; Niittyla et al., 2006; Sokolov et al., 2006; Fordham-Skelton et al., 2002).

Although substantial progress has been made in understanding the function of different starch synthesizing enzymes, it is still not clear how these enzymes (SSs, SBEs and DBEs) are coordinated and how different structures of amylopectin arise. There is increasing evidence that heteromeric protein complexes act as functional assemblies to improve polymer construction efficiency (Tetlow, 2006).
Figure 1.12: Phosphorylation dependent functional complex formation and entrapment of proteins within starch granule in different maize mutants.

(A), shows WT amyloplast stroma in which protein complex formation is shown to be phosphorylation dependent and this phosphorylated protein complex becomes entrapped with in starch granule.

(B), shows that in ae mutant of lacking SBEIIb, phosphorylation dependent novel protein complex containing (SSI, SSIIa, SBEI, SBEIIa and SP) is formed in which SBEI and SP are phosphorylated and this protein complex also becomes entrapped within starch granule.

(C), Su2 mutant expressing a catalytically inactive SSIIa protein due to a mutation which resulted in the loss of SSIIa glucan binding ability. This mutation did not affect ability of SSIIa to coordinate with SSI and SBEIIb in a trimeric protein complex. Thus due to loss of glucan binding ability of SSIIa, other proteins (SSI and SBEIIb) were not able to bind to starch granule. This Figure also explains that SSIIa plays a major role in trafficking other proteins into to the starch granule (Liu et al., 2009, 2012).
1.5 Experimental Material

In this study, barley (*Hordeum vulgare*) was used as the experimental material. Barley is among the most widely cultivated and utilized cereals after maize, rice and wheat, with the ability to provide valuable nutrients necessary for humans and domestic animals (Zeeman et al., 2010). But this cereal is seldom for human consumption and hence referred as poor man’s food in underdeveloped countries (Asare et al., 2011). However, in the recent past there is renewed interest in barley as a food because of the potential health benefits associated with constituents of its grain, including dietary fibre (β-glucans), phenolic compounds and vitamins (Jadhav et al., 1998). Barley grain constitutes of carbohydrates (60-80 %), water (10-15 %), protein (9-13 %), crude fibre (5-6 %), ash (2-2.5 %), fat (1-2 %) other (5-6 %) (Chibbar et al., 2004). Although starch is the major carbohydrate in barley grain ranging from (62-77 %) of the grain dry weight (Bhatty and Rossnagel, 1998), it has not been studied to the same extent as in other cereals such as corn, wheat and rice. As experimental material, barley has other attractions because of the large collections of genetic variants available, and because of its diploid nature, it is easier to study relative to wheat.

1.6 Hypotheses

Starch is a renewable biopolymer which has numerous food and industrial applications which mostly depend upon physiochemical properties of starch. The diverse physiochemical properties of starch are either controlled by complex genetic variations or by a process which is regulated by the coordinated interaction of different enzymes and their isoforms involved in starch biosynthesis. There is increasing evidence that these enzymes co-operate physically, forming functional proteins complexes. Thus mutations in
these enzymes can also alter other starch synthesizing enzymes pleiotropically. The present study is based on an investigation of the following hypotheses:

I. Increased RS is associated with distinctly altered physiochemical properties of starch, which could be used as a diagnostic tool to determine high RS genotypes.

II. The relationship between the granule proteome and starch properties from barley mutants with defined mutations can be related to variation in protein-protein interactions in the stroma.

III. Variation in the granule proteome of A- and B-granules can be used to understand the relationship between granule type, its proteome and mechanisms underpinning granule size and morphology.

IV. Some variations in starch physiochemical properties do not always occur due to defined mutations in starch biosynthetic enzymes, but are result of complex interaction between genetic background and biochemical processes.

1.7 Aims and Objectives of the Study

This research project focused on the following objectives,

I. The first objective of the study was to analyze starch physical properties including (amylose and RS contents, granule size, number and surface area distributions, granule morphology, amylopectin chain length distribution and thermal properties etc.) in a wide range of barley cultivars to determine characteristics of resistant starch genotypes (Chapter 2).

II. The second objective of the study was to analyze soluble and starch granule associated proteins from different high RS/amylose genotypes to determine relationships among partitioning of enzymes between the stroma and granule and RS
content (Chapter 3).

**III.** The third objective of this study was to investigate the composition of different soluble protein complexes, the mechanisms involved in heteromeric complex formation in different mutants, and binding of different proteins to starch granule and also to determine whether different protein complexes contribute to the formation of A- and B- type granules in barley (Chapter 3).

**IV.** The fourth objective of this study was to investigate barley genotypes with known variations in the physiochemical properties of starch but for which there is no characterized mutation, and to relate variations in starch biosynthetic enzymes, and the granule proteome to the resulting starch phenotype (Chapter 4).
Chapter 2 is prepared as a research article to be submitted for publication
Chapter 2: Evaluation of physical characteristics of resistant starch from (a range of) barley genotypes
2.1 Introduction

Starch is the most important and widely distributed storage polysaccharide in plants and the major storage biopolymer in cereal endosperms providing more than 75% of daily caloric intake and is a major source of feed, fiber, biofuels and a raw material in many industries (Stamova et al., 2009). It occurs in a well-organized granular form composed of linear and highly branched glucan polymers called, respectively, amylose and amylopectin. Amylose and amylopectin are found in semicrystalline, water insoluble lamellae within the granule. Amylose is essentially a linear molecule with predominantly α-1,4 linked glucose units with a low frequency (< 1%) of α-1,6 linked branches, whereas, amylopectin is a much larger and highly branched molecule with a high frequency (~ 5%) of α-1,6 linkages (Myers et al., 2000). In amylopectin, branching of glucan chains exhibits regular periodicity (Davis et al., 2003), and its length and pattern play a critical role in the proper formation and organized structure of the starch granule and the resisting physiochemical properties (Liu, 2005). The size, shape and number of starch granules vary within and among species (Shapter et al., 2008).

In Festucoid grasses, including wheat and barley, starch granules can be distinguished as A-, B- and C-granules, based on size, shape, relative number and the timing of their initiation during seed development (Wei et al., 2010). A-granules are lens shaped, varying in size from 10-50 μm in diameter and make 70-80% of the volume and normally account for approximately 10% of the total number of the starch granules (Langeveld et al., 2000; Hughes and Briarty, 1976). B-granules are spherical, varying in size from 5-15 μm in diameter and represent < 30% of the volume and > 90% of the total number of starch granules. The starch granules of size < 5 μm in diameter are
termed C-granules (Wilson et al., 2006; Bechtel and Wilson, 2003). Wheat and barley starches with different granule sizes show different degrees of amylolysis and thermal and pasting properties (Liu et al., 2007). Along with granule size, differences in amylose content, protein content, and branch chain length of amyllopectin in A- and B- type starch granules, could be important factors responsible for differences in digestibility and determining the end use of starch (Liu et al., 2007).

Starch has many uses in food industries and, depending upon nutritional classification, starch has been divided into three categories: rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS). RS was first identified in 1982 by Englyst et al. (1982), and defined as the starch that resists $\alpha$-amylase digestion in the small intestine and is fermented by the bacteria in the large intestine, producing a variety of end products, the most important of which are short chain fatty acids (SCFA) (Leszczynski, 2004). RS can be classified into different types: RSI is physically inaccessible starch as found in partial or intact cereal seeds. RSII is the raw starch in its granular form in some plant species, e.g. potato and banana. RSIII is retrograded starch, which occurs when starch-containing foods are cooked and cooled and RSIV is chemically or physically modified starch, or processed food (Leloup et al., 1992a; Colquhoun et al., 1995; Leszczynski, 2004; Rahman et al., 2007).

The Commonwealth Scientific and Industrial Research Organisation (CSIRO) in Australia, has recommended that around 20 g of RS should be consumed per day, but the consumption level of RS in a typical western diet is only about 25 % of the recommended amount (Baghurst et al., 1996). To meet this deficiency, the best approach is to develop crop genotypes high in RS and barley is one of the best candidates for this purpose.
Barley (*Hordeum vulgare*) is the fourth most widely cultivated cereal after maize, rice and wheat. Interest in the use of barley as a food component with the ability to provide valuable nutrients necessary for humans and domestic animals has increased because of the potential health benefits associated with constituents of its grain, including dietary fiber, phenolic compounds and vitamins (Jadhav et al., 1998; You and Izydorczyk, 2007). Barley grain is composed of carbohydrates (60-80 %), water (10-15 %), protein (9-13 %), crude fibre (5-6%), ash (minerals) (2-2.5 %), fat (1-2 %) and other (5-6 %) (Fahrenholz, 1998; Chibbar et al., 2004). Starch is the major carbohydrate in barley grain ranging from (62-77 %) of the grain dry weight (Bhatty and Rossnagel, 1998). Although starch is the major component of barley grain, it has not been studied to the same extent for human consumption as the starch of corn, wheat and rice. Barley has many advantages for use as a model plant which includes its relative simple genome compared to wheat and corn, and the large number of morphological and biochemical mutants available. This study was conducted using barley and tested the hypothesis that increased RS is associated with distinct physical properties of starch, which could be used as a diagnostic tool to determine high RS genotypes. Comprehensive analyses were used to investigate which physical parameters of barley starch can be used to improve the selection of high RS genotypes.
2.2 Material and Methods

2.2.1 Selection of genotypes

In this study 33 genotypes of barley have been used. These genotypes were selected based on diverse agronomic and physiological characteristics from different genetic back grounds and different geographical origins. These genotypes include covered and hulless, two and six rowed varieties or advance lines in different combinations. Similarly, different mutants with known mutations in starch biosynthetic enzymes such as, granule bound starch synthase (GBSS), starch synthase (SSIII or amo1), starch branching enzymes down regulated lines (sbeiia and sbeiib) and lines derived from amo1 are also included. Detailed description of the genotypes used in this study has been given in the Appendix 1.

2.2.1.1 Selection of genotypes for further detailed analyses

From the initial 33 screened genotypes, 12 genotypes either high in amylose or RS were selected for further detailed study. The rationale for selecting these genotypes from the wider pool, were based on the following, the reason behind increased amylose/RS content in different genotypes may be different. It is may be due to mutation in specific genes, different genetic background or geographical origin of different genotypes. All these variations can give a better understanding that how different physical parameters under different conditions are important in producing starch of the desired RS phenotype. Along with these genotypes, one normal genotype was also used as reference.

2.2.2 Isolation of starch granules

Mature dry barley grains (≈ 55 g) were completely ground to flour with a
Retsch® MM301 homogenizer in liquid nitrogen. Equal amounts of flour (≈ 50 g) were suspended in 150 ml of buffer containing 100mM Tricine-KOH, pH 7.8, 1mM Na₂-EDTA, 1mM DTT and 5mM MgCl₂ at 4°C (Liu et al., 2009). The suspension was mixed thoroughly by vortexing for 5-10 minutes and then was left for 5-10 minutes on ice. The suspension was filtered through six layers of cheese cloth to remove debris and bran. Buffer solution was used to rinse traces of starch from the cheese cloth. The sieved milky suspension (≈ 450-500) ml was centrifuged at 16000 g for 15 min. The supernant was discarded and pellet was washed in ≈ 100 ml buffer containing 50 mM TRIS-acetate, pH 7.5, 1 mM Na₂-EDTA, and 1 mM DTT. The pellet was suspended in the same washing buffer and was centrifuged at 5000 g for 5 min. This washing step was repeated 5-7 times until only a thick yellow layer of debris was left on the top of starch. The yellow layer containing very small pieces of debris was completely removed with spatula attempting to avoid any starch losses. The starch was again washed twice in buffer and centrifuged at 5000 g for 5 min. Purified starch was washed three times with acetone followed by three washes with 2% (w/v) sodium dodecyl sulphate (SDS), and three washes with water and then dried with speed vacuum (Eppendorf, Vacufuge™) at 25 °C for 3 h.

2.2.3 Measurement of amylose and RS contents

The apparent amylose and RS from raw starch were measured with Megazyme amylose/amylopectin assay kit (K-AMYL 07/11) and resistant starch assay kit (K-RSTAR 08/11) respectively according to manufacturer instructions using three biological and two technical repeats.

2.2.4 Amylose content determination by gel permeation chromatography

To measure the amylose content using Gel Permeation Chromatography (GPC),
10 mg of acetone-washed dried granular starch was used with three biological and two technical repeats. In 4 ml glass sampling bottles, starch was dissolved in 200 µL of 90% DMSO with continuous stirring (50 rpm) on a magnetic stirrer overnight. 1600 µL of boiling HPLC grade water was added to the dissolved starch. The dissolved starch was cooled down from 90°C to room temperature and 200 µL of 0.01N sodium acetate buffer (pH 5.5) was added. To debranch the starch, 3 µL of isoamylase enzyme (59000U/mg of protein) (Hayashibara, Japan) was added and the mixture was stirred over night at 50 rpm at room temperature. The enzyme was inactivated by placing the sampling bottles in a hot water bath (90-95 °C) for 5 min. The mixture was cooled down at room temperature and 200 µL of 5M NaOH was added and mixed well. The completely debranched starch mixture was filtered through a 0.45 µm nylon filter (Sarstedt). 1 ml of the filtered sample was injected into the CL 6B column (concentration of the starch solution was ~5 mg starch/ml) and 1 ml fraction was collected in glass test tubes. 1 ml of 5% phenol and 5 ml of 37 % sulphuric acid were added to each test tube, mixed, and the samples cooled. Absorbance was measured at 490 nm on a Beckman Coulter spectrophotometer.

2.2.5 Measurement of amylose by iodine binding

Apparent amylose content of starches was determined with three biological and two technical repeats by the method proposed by Chrastil (1987), with certain modifications. In 20 mg acetone dried starch, 1M NaOH (2 ml) and distilled water (4 ml) were added in a screw cap tube. The tube was capped and heated in a water bath at 95 °C for 30 min with occasional mixing on a vortex mixer (Eppendorf®). The solution was cooled to room temperature and an aliquot of 0.2 ml in duplicates was added to 5 ml of 0.5 % trichloroacetic acid in a separate glass test tube. The solutions were mixed
thoroughly and 0.1 ml of 0.1N I₂-KI solution (1.27 g of I₂ and 3 g of KI/L) was added to each tube and mixed immediately. The resulting blue color was measured at 620 nm on a Beckman Coulter spectrophotometer, after 30 min against a reference prepared without starch. The amylose content was calculated using the standard curve prepared using mixtures of pure potato amylose and amylopectin (over the range 0–100 % amylose).

2.2.6 Granule size distribution of different genotypes

Granule size, number and surface area of starch from different genotypes were measured by means of laser scattering using the Master Sizer (Malvern Mastersizer 2000, Malvern Instruments Ltd., UK). For each measurement, starch (± 100 mg) was weighed into glass tubes and suspended with 10 ml of distilled water. Sample concentrations were within equipment recommendations and the refractive indices of 1.31 for water and 1.52 for starch were used as the standard and distribution was measured as both the percentage volume and percentage number.

2.2.7 Granule morphology

To study starch granule (A-, B- and C-) morphology in detail, SEM was performed on a field emission scanning electron microscope (S-4500, Hitachi, Tokyo, Japan) as described by (Liu et al., 2007), equipped with Quartz PCI digital image acquisition software (Quartz Imaging, Vancouver, BC, Canada). The acetone-dried barley starch samples were sprayed on a metal plate covered with double-sided adhesive tape. The samples were coated with gold using a Polaron SC500 sputter coater (Quorum Technologies, East Sussex, UK). The samples were examined at 10 kV accelerating voltage and representative micrographs were taken for each sample at different magnifications according to requirement.
2.2.8 Seed characteristics

2.2.8.1 Thousand grain weight (TGW)

1000 grains of each genotype were counted and weighed to 0.1 mg using a tabletop electrical balance (Denver Instrument, Bohemia, NY, USA). The mean of three replicates was used in statistical analysis (de Rocquigny, 2011).

2.2.8.2 Starch content

The amount of starch in barley seeds from the different genotypes using three biological repeats was determined as described for wheat endosperm (Tetlow et al., 1994).

2.2.9 High-performance anion exchange chromatography

To measure the chain length distribution of barley starch, High-Performance Anion Exchange Chromatography (HPAEC) was used as described by (Liu et al., 2007).

2.2.10 Statistical analysis

For one way analysis of variance (ANOVA) and least significant difference (LSD), Statsoft’s Software, Statistica (http://www.statsoft.com) was used. For principal component analysis (PCA), XLSTAT (http://www.xlstat.com/en/) was used and biplots were generated.
2.3 Results

2.3.1 Amylose content

Barley starch can be classified into normal (25-27 % amylose), waxy (< 5 % amylose), and high (> 35 %) amylose (Bhattay and Rossnagel, 1992; Bhattay and Rossnagel, 1998; Izdorczyk et al., 2000). However in this study, depending upon amylose content, the genotypes were divided into four groups (I), waxy (< 5 %), (II), low amylose (5-20 %), (III), normal (20-27 %) and (IV), high amylose (> 30 %), as shown in the Figure (2.1). Significant variation in amylose content was observed among and within different groups of genotypes as shown in Figure (2.1). Group (I) contains all the waxy genotypes which either lack or have inactive (GBSS), the enzyme which is solely involved in amylose biosynthesis. Groups II & III (with low and normal amylose, respectively) include normal established varieties, advanced lines and breeding material. Group (IV) consists of mutants with mutations in starch synthase (SS) III (ssiii), starch branching enzyme (SBE) IIa and IIb (sbeiia- and sbeiib-) or a mutation at the sexl locus (Appendix 1). Figure (2.1) shows all the genotypes in group IV have increased amylose content compared to reference genotype. The sbeiia- and sbeiib- have similar amounts (30 %) of amylose. The genotypes with mutation at the sexl locus (083611-118 sexl, 083611-124 sexl, 020113-385 sexl) also possessed high (35%) amylose content. The amo1 mutants (HAG amo1 and SB94983 amo1) exhibited (40 and 30 %) amylose content respectively. The genotypes of series 081011-928 - 081011-932 originated from the same amo1 parent but these genotypes showed variation in their amylose content. For example, in genotypes 081011-928, 081011-929 and 081011-932, amylose content was similar (± 45 %), however genotypes 081011-930 and 081011-931 possessed
Figure 2.1: % amylose of barley genotypes.
The amylose content was measured by Megazyme amylose/amylopectin assay kit using acetone washed starch. Different groups of genotypes based on % amylose content are presented with different bars representing standard error mean. Waxy, low amylose and normal genotypes have amylose content varying between 1-27 %. While all other genotypes with amylose content >27% are included in high amylose group.
significantly less (39 and 28 %) amylose at $p < 0.05$, respectively.

### 2.3.2 Resistant Starch (RS) Content

RS was measured for genotypes and divided into three different groups: (I), Normal, genotypes having RS content < 5 % and < 27 % amylose. (II), High amylose-Low RS, genotypes having higher amylose > 30 % but with RS content < 5 %. (III), High amylose-High RS, genotypes having high amylose > 27 % with RS content > 5 %, as shown in Figure (2.2). The rationale for dividing the genotypes into different groups is primarily based on the amount of RS, the lowest amount of RS in a high amylose genotype was 5.6 %, all genotypes in this group have an amylose content > 27 % (Figure 2.1).

Group (I) includes *waxy*, normal barley and experimental material, all these genotypes have RS content < 5 %, but apparent amylose content higher than expected. The genotypes Neopolis, CDC Fibar and CDC Rattan are *waxy* genotypes (Figure 2.1) but possess higher RS than indicated by amylose content alone (Figure 2.2) demonstrating that amylopectin may also contribute to the total RS contents. The amount of amylose is positively related to RS but the above mentioned *waxy* genotypes show that in addition to the amylose, some features of amylopectin may also be important in determining the proportion of RS. Genotypes in group (II) are apparently high in amylose (35%, Figure 2.1) but low (1.4 %) in measureable RS (Figure 2.2). In group III, all the high amylose genotypes (Figure 2.1) are also high in RS (Figure 2.2). The genotypes in group III exhibit varying content of high amylose which correlate positively ($r = 0.80, p < 0.05$) with increased RS, though the relationship is not strictly proportionate. Two genotypes 081011-930 and 081011-931 were of particular interest as they represent
Figure 2.2: % RS of barley genotypes.

The RS content measured by Megazyme resistant starch assay kit using acetone washed starch. Different groups of genotypes based on % RS content are presented. The normal group includes genotypes which have amylose content < 27 % and RS content < 5 %. High amylose- Low RS group include those genotypes which have amylose content > 27 % but RS content < 5 %. High amylose- High RS group includes all those genotypes which have amylose content > 27 % and are also high in RS > 5 %.
different selections from the same cross involving an *amo1* parent. The genotype 081011-930 had an intermediate level of RS (6.5 %) whilst 081011-931 was a “normal” type with (3 % RS).

2.3.3 Comparison of different methods for determination of amylose content

Three different methods were used to measure the amylose content i.e. biochemical assay, iodine binding and GPC. PCA showed that the three methods correlated well (Megazyme and iodine binding, $r = 0.94$, $p < 0.05$; Megazyme and GPC, $r = 0.67$, $p < 0.05$; iodine binding and GPC, $r = 0.60$, $p < 0.05$), (Appendices 2 and 3).

2.3.4 Physical characteristics of seed

2.3.4.1 Seed characteristics

Seed characteristics including seed length, width and thickness were measured. Different genotypes showed significant differences in different seed parameters. Seed length varied from 7.1 to 9 mm among different genotypes. All high amylose genotypes showed a higher seed length ($\geq 7.5$ mm) than normal genotype (7.4 mm), except *sbeia* which possessed 7.1 mm seed length. In the case of seed width, the down regulated lines of branching enzymes IIa and IIb, one *amo1* mutant (HAG *amo1*) and all the *sex1* mutants showed lower seed width ($\leq 3.6$ mm) compared with wild-type (3.7 mm) while all other high amylose/ RS genotypes had greater seed width ($\geq 3.8$ mm) than the wild-type (Table 2.1). Normal genotype had the highest seed thickness (2.9 mm). All the *sex1* mutants with shrunken endosperm had lowest (2 mm) seed thickness (Table 2.1).

2.3.4.2 Thousand grain weight (TGW)

The genotypes showed significant variation in grain weight. The genotype OAC Baxter had a TGW of 63.1 g. Other genotypes exhibited lower seed weight than the
Table 2.6: Physical characteristics of seed

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Type</th>
<th>Rows</th>
<th>Seed Characteristics (mm)</th>
<th>Length: Width</th>
<th>TGW</th>
<th>% Starch Content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>SL</td>
<td>SW</td>
<td>ST</td>
<td></td>
</tr>
<tr>
<td>OAC Baxter</td>
<td>C</td>
<td>6</td>
<td>7.4&lt;sup&gt;i&lt;/sup&gt;</td>
<td>3.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.9&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.0&lt;sup&gt;j&lt;/sup&gt;</td>
</tr>
<tr>
<td>sheila&lt;sup&gt;*&lt;/sup&gt;</td>
<td>C</td>
<td>2</td>
<td>7.1&lt;sup&gt;g&lt;/sup&gt;</td>
<td>3.6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.6&lt;sup&gt;ad&lt;/sup&gt;</td>
<td>2.0&lt;sup&gt;j&lt;/sup&gt;</td>
</tr>
<tr>
<td>sheib&lt;sup&gt;*&lt;/sup&gt;</td>
<td>C</td>
<td>2</td>
<td>7.5&lt;sup&gt;e&lt;/sup&gt;</td>
<td>3.6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.6&lt;sup&gt;ad&lt;/sup&gt;</td>
<td>2.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>083611-118 sexI</td>
<td>H</td>
<td>6</td>
<td>8.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.4&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.0&lt;sup&gt;l&lt;/sup&gt;</td>
<td>2.4&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>083611-124 sexI</td>
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<td>2.5&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>HAG amoI</td>
<td>C</td>
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<td>8.6&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.4&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>2.5&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
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<td>H</td>
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<td>8.6&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.7&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>2.2&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>2.2&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
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<td>2</td>
<td>8.6&lt;sup&gt;ab&lt;/sup&gt;</td>
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<td>2.7&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>2.2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
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<td>H</td>
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<td>8.5&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>3.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.7&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>2.2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>081011-931</td>
<td>C</td>
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<td>7.8&lt;sup&gt;e&lt;/sup&gt;</td>
<td>3.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.0&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>081011-932</td>
<td>H</td>
<td>2</td>
<td>8.4&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>3.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.8&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>2.2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

SL= Seed length, SW= Seed width, ST= Seed thickness, TGW= Thousand grain weight

Table 2.1, shows physical characteristics of seed, type indicates whether seed is covered (C) or hulless (H). Rows represent rows of fertile spikelets on the barley spike. % starch content of wild-type (OAC Baxter) was used as a reference and taken as 100 % to which the genotypes were compared. Data present means of three replicates. Different letters in columns following each mean value indicate whether genotypes are significantly different at (p<0.05), genotypes sharing same alphabetical letter for a given character are not significantly different. For seed length, width and thickness three biological repeats with hundred independent observations for each character were taken.
normal genotype and all \textit{sex1} lines (both covered and hulless) had the lowest TGW at < 45 g (Table 2.1).

\textbf{2.3.4.3 Starch content}

Starch is an important constituent of cereal seed and final yield. Different genotypes showed significant variation in their starch contents. The starch content of the reference genotype OAC Baxter was used as a reference and taken as 100\% to which the other genotypes were compared. The shrunken mutants (083611-118 \textit{sex1}, 083611-124 \textit{sex1}, 020113-385 \textit{sex1}) possessed lowest starch content < 48 \% compared to reference genotype. The \textit{sbeiia}, \textit{sbeiib} and HAG \textit{amo1} mutants were lower (95.8, 84.4 and 93.9 \%, \textit{p} < 0.05) in starch content compared to wild-type. However, SB94983 \textit{amo1} and \textit{amo1} derived lines: 081011-928, 081011-929, 081011-930, 081011-931 and 081011-932 exhibited between 104.0 - 118.0 \% starch content compared to the reference genotype (Table 2.1). It should also be noted that these lines were hulless and so the starch was not diluted by hull except 081011-931 genotype which had covered seed.

\textbf{2.3.5 Granule size, number and surface area distributions of different genotypes}

The reference genotype, OAC Baxter, possesses a normal bimodal granule size distribution with median granule size of 3.6 and 20.5 \textmum for B- and A-granules, respectively (Figure 2.3A), whereas all high amylose genotypes with variable amylose/RS contents have a unimodal granule size distribution (Figure 2.3A & B). The \textit{sbeiia}, \textit{sbeiib} and \textit{sex1} mutants (083611-118 \textit{sex1}, 083611-124 \textit{sex1}, 020113-385 \textit{sex1}) exhibited average (6.6 -7.5 \textmum) size for B granules (Figure 2.3A). The \textit{amo1} mutants (HAG \textit{amo1} and SB94983 \textit{amo1}) possessed average B-granule size varying between 5.7 - 6.9 and 6.8 - 7.7 \textmum, respectively. The \textit{amo1} derived lines 081011-928, 081011-929
and 081011-932 exhibited average (5.2 - 6.7 µm) size for B granules, while two other lines, 081011-930, 081011-931 have (5.9 - 7.2 and 7.5 - 8.7 µm) average B granule size, respectively (Figure 2.3B). For large A-granules, the *sbeiia*, SB94983 *amo1*, 083611-118 *sex1* and 081011-930 exhibited the same average (17-19 µm) size (Figure 2.3A & B). The *sbeiib* and 081011-931 have average size (17.5-19.5 µm) for A-granules. While, HAG *amo1*, 083611-124 *sex1* and 020113-385 *sex1*, 081011-928, 081011-929 and 081011-932 exhibited the same average (16-18 µm) size A-granules.

The reference genotype OAC Baxter showed bimodal granule number distribution in which B- and C-granules contributed almost 98 % to total granule number and A-granules constituted only 2 % of total granule number. The reference genotype OAC Baxter also showed bimodal starch granule surface area distribution. However, all high amylose/RS genotypes exhibited unimodal granule number and surface area distributions (data not shown).

**2.3.6 Contribution of A-, B- and C-granules in total mass of starch**

Amount of different types of granules (A-, B- and C-) is important in characterizing starch from different mutants. In this study different genotypes showed noticeable differences in the amount of different types of granules (Figure 2.4). The reference genotype OAC Baxter has a higher amount (11 %, p < 0.05) of C-granules while all high amylose/RS mutants have very small or no C-granules. In the case of B-granules, reference genotype and genotype 081011-931 have lower amount (24 and 37 %, p < 0.05) of B-granules and higher amount (66 and 63 %, p < 0.05) of A-granules, respectively, in contrast high amylose/RS genotypes have higher amount (> 50 %) of B- and lower amount (< 50 %) of A-granules (Figure 2.4). In some genotypes (083611-124
Figure (2.3A & B): Granule size distribution.
Granule size distribution was measured by means of laser scattering using the Mastersizer (Malvern Mastersizer 2000, Malvern Instruments Ltd., UK). Acetone washed starch (± 100 mg) was suspended in 10 ml of distilled water and used according to manufacturer instructions.
Figure 2.4: Amount of A-, B- and C-granules in total mass of starch.
Determination of A-, B- and C-granules using a Mastersizer. Acetone washed starch (± 100 mg) was suspended in 10 ml of distilled water and used according to manufacturer instructions (Malvern Instruments Ltd., UK).
sex1, HAG amo1, 081011-928, 081011-929 and 081011-932) amount of B-granules exceeded 70% as shown in Figure (2.4).

2.3.7 Granule morphology

The starch granules from different barley mutants not only vary in size but also in shape, which was revealed by scanning electron microscopy. Generally, A-granules are disc or lenticular, B-granules spherical and C-granules irregular shaped (Wei et al., 2010). In this study starch granules of reference genotype showed the expected (reported) morphology. All high amylose/RS genotypes exhibited altered granule morphology (Figure 2.5). For example, in the sbeiia’ and sbeiib’ down regulated lines, round A-granules with tunnels were observed. In amo1 mutants and lines derived from amo1 parent, irregular A-granules with bumps were found in abundance. The sex1 mutants exhibited round and flat A-granules with small ditches at the surface. In all the above mentioned mutants morphology of B-granules did not change, however average size of B-granules increased compared to reference genotype. The genotype (081011-931) which has comparatively less amylose, exhibited bigger B-granules which resemble A-granules, while its A-granules were not severely deformed compared to its counterparts. In most of the high RS/amylose genotypes C-granules were either absent or present in very small amounts. The accumulation of very high amount of B-granules in all high amylose/RS genotypes shows that amount of amylose/RS is positively correlated (r = 0.89, p < 0.01) to B-granules while negatively correlated (r = -0.80, p < 0.01) to A-granules as shown in the Figure 2.9 A & B.
Figure 2.5: Morphology of starch granules from normal and high RS/amylose genotypes observed by SEM.

For SEM analysis acetone dried barley starch samples were sprayed on a metal plate covered with double-sided adhesive tape. The samples were gold coated with Polaron SC500 sputter coater (Quorum Technologies, East Sussex, UK). The samples were examined at 10 kV accelerating voltage and representative micrographs were taken for each sample at same magnification. Scale bar for electron micrograph is given under each panel. Each type of granule is presented by a colored arrow.
2.3.8 Amylopectin chain length distribution

The measured degree of polymerization (DP) of glucan chains in amylopectin among different genotypes ranged from 6-50 DP with varying proportions of different DP classes. The observed chain length distribution was divided into four groups as shown in Table (2.2) (Liu et al., 2007). In group I, only two genotypes showed significant differences (p < 0.05) for glucans in the range DP 6-12; genotype, 081011-931 has highest proportion (46.3 %) and the genotype sbeiib- has lowest proportion (42.3 %), (Table 2.2). There were no significant differences among genotypes for intermediate chains with DP 13-24. A small proportion of longer chain glucans with DP 25-36 was found in all genotypes with highest amount (8.8 %) in OAC Baxter and lowest (7.1 %) in 081011-931 (Table 2.2). The proportion of glucans chains with length of DP 37-50 was lowest in all genotypes. However, different genotypes exhibited significant differences (p < 0.05) relative to the reference genotype with some exceptions. For example, sbeiia- and sbeiib- were not significantly different (p < 0.05) from waxy and the reference genotype, respectively, but were significantly different from one another (Table 2.2).

To understand differences in individual classes of glucans DP among different genotypes, difference plots of normal barley and mutants were produced (Figure 2.6). A difference plot of (mutants - wild-type) showed that all genotypes exhibited differences in glucan chains with DP 6-20. However, the extent of difference in particular glucan chain groups in all genotypes compared to reference genotype was not very high. In the case of longer chains with DP > 20, proportion of difference among different genotypes compared to reference genotype was not statistically significant (Figure 2.6).
Table 2.2: Degree of polymerization (%) of amylopectin from barley genotypes

<table>
<thead>
<tr>
<th>Genotype/chain length</th>
<th>DP 6-12</th>
<th>DP 13-24</th>
<th>DP 25-36</th>
<th>DP 37-50</th>
<th>Avg. DP</th>
</tr>
</thead>
<tbody>
<tr>
<td>OAC Baxter</td>
<td>43.3&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>43.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.36</td>
</tr>
<tr>
<td>CDC Fibar (waxy)</td>
<td>45.2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>43.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.5&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>3.6&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>sbeiiia</td>
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<td>43.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.0&lt;sup&gt;bc&lt;/sup&gt;</td>
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<td>20.18</td>
</tr>
<tr>
<td>HAG amo1</td>
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<td>45.9&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>18.79</td>
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Table 2.2, different groups of chain length with varying DP (s) have been presented. Different letters in columns following each mean value indicate significance at (p<0.05), genotypes sharing same alphabetical letter for a given group of chain length are not significantly different.
The difference plot between different genotypes and \textit{waxy} showed differences in amylopectin chain length distribution among different high amylose genotypes, \textit{sbeiiA-}, HAG \textit{amo1}, SB94983 \textit{amo1} and 081011-929 relative to the amylose free genotype, CDC Fibar (Figure 2.7). All genotypes exhibited a lower proportion of chain length with DP \leq 10 and a higher proportion of chain length with DP 10-20 compared to \textit{waxy} with some exceptions. In the case of chain length with DP > 20, no significant variations between the proportions of different chain lengths were observed in all genotypes compared to the \textit{waxy} genotype (Figure 2.7).
Figure 2.6: Difference plot of (different genotypes versus wild-type) of amylopectin chain length distribution.

Amylopectin chain length distribution of barley genotypes was measured by HPAEC. The chain length distribution of wild-type OAC Baxter was deducted from the chain length distribution of different genotypes. The positive value shows that different genotypes exhibited higher proportion of that specific glucan chain. While negative value shows that OAC Baxter exhibited a higher proportion of that specific glucan chain. X-axis is representing degree of polymerization (DP) between 6-50 and Y-axis represents % moles of carbohydrates.
Figure 2.7: Difference plot of (Different genotypes versus \textit{waxy}) of amylopectin chain length distribution.

Amylopectin chain length distribution of barley genotypes was measured by HPAEC. The chain length distribution of \textit{waxy} CDC Fibar was deducted from the chain length distribution of different genotypes. The positive value shows that different genotypes exhibited higher proportion of that specific glucan chain. While negative value shows that CDC Fibar exhibited a higher proportion of that specific glucan chain. X-axis is representing degree of polymerization (DP) between 6 - 50 and Y-axis represents % moles of carbohydrates.
2.3.9 Principal component analysis (PCA)

PCA, along with simple correlation analysis (Figures 2.8 & 2.9) was performed to determine relationships among different physiochemical parameters of starch and seed. PCA illustrates more specific relationships among different parameters as compared to linear correlation. Simple linear correlation shows that the amount of amylose/RS is negatively correlated ($r = -0.80, p < 0.01$) with the proportion of A-granules of size 15-45 µm and B-granules (1.2-15 µm) are positively correlated ($r = 0.89, p < 0.01$) with amylose/RS (Figure 2.9 A & B). Large granules of size (39-45 µm) and chain length of DP 6-12 appear to be closely associated with seed thickness. Other seed parameters such as, seed width are closely associated with % starch content and seed weight while seed length and L/W ratio are not associated with any of the measured characters. The type of the seed (covered or hulless) and rows of fertile spikelets on the spike (2 or 6 rows) do not show an association with measured starch characters (data not shown).

Three different methods were used to measure the amylose content. PCA showed that all the three methods are closely associated (Appendices 2 and 3). PCA was also used to examine relationships among molecular structure and other physiochemical properties of starch and it was revealed that overall the three methods used for amylose determination, amylose content, RS content, amount of B-granule, B-granules of size (5-15 µm), and chain length of DP 13-24 are closely associated. Amylopectin content, amount of A-granules and granules of size (15-30 µm) are also closely associated. PCA also demonstrates that the amount of A- and B-granules is closely related to 15-30 µm and 5-15 µm granules, respectively which means that these two granule sizes are in higher proportion and contribute more to the total mass of starch.
Figure 2.8: PCA analysis shows association of different characters. The name of each character is given in the PCA while different genotypes are presented by (+). Different characters present in close vicinity are associated while different characters opposing each other are negatively related. Genotypes have been clustered into different groups depending upon their association with different characters and each cluster is presented by a different circle: black, normal barley; Light green, experimental lines; red, waxy; blue; Dark green, high amylose.
Figure 2.9(A & B): Linear correlation between A- and B-granules with % amylose. % amylose was measured by using Megazyme amylose/ amylopectin assay kit and GPC. Amount of A- and B-granules was determined by the Mastersizer (Malvern Mastersizer 2000, Malvern Instruments Ltd., UK). Correlation between amylose and large (15-45 µm) and small (1-15 µm) granules was determined by Microsoft Excel.
2.4 Discussion

The foods we eat significantly affect our health. Type-2 diabetes, obesity, heart disease and stroke were once thought to be caused by single gene mutations, but there is now growing evidence attributing these conditions to a network of biological dysfunctions and the food we eat is an important factor in that dysfunction (Vorster, 2009). Foods rich in resistant starch (RS) provide health benefits by preventing/reducing certain diseases like diabetes, obesity, colon cancer, and cardiovascular diseases (Sharma et al., 2008). RS-rich foods show low glycaemic index, and also reduce colorectal cancer risk by promoting bowel health (Asare et al., 2011). Cereal grains are the best source of different nutrients in balanced ratios and are also the best source of RS given the volume of consumption in most diets. In the present study, a range of barley genotypes from different sources were selected to screen for RS content and analyze different seed and starch physicochemical characteristics to determine their contribution to RS. A group of genotypes was identified which showed increased RS and that certain physical characteristics of the seed and starch were important determinants of RS. All the high RS genotypes (Figure 2.2) were also high in amylose (Figure 2.1), meaning that the increase in amylose is directly related to increase in RS. Previously it was also reported that starch with increased amylose is more resistant to α-amylase digestion (Crowe et al., 2000; Emami et al., 2010). This correlation was true for all genotypes except two sex1 mutants (083611-118 sex1 and 083611-124 sex1) which were high in amylose but low in RS (Figures 2.1 & 2.2). The reason for apparent discrepancy is not known. However, it could be argued that the possible reason of discrepancy of these two genotypes may arise from the molecular structure of the starch because with respect to other physicochemical
characteristics these two genotypes were similar to other sex1 mutant and high amylose genotypes. This finding also shows that in some cases, increase in amylose is not the only factor in increased RS but the molecular structure of the starch and interplay between amylose and amylopectin also plays an important role in determining RS. In addition to amylose: amylopectin ratio, structure, the packing of amylopectin within the granule (Benmoussa et al., 2007) amount of lipids and proteins (Copeland et al., 2009), and complexes with long chain fatty acids (Putseys et al., 2010) are also important contributors in RS. Similarly, structural aspect such as starch crystallinity (Jane et al., 1997), and occurrence and perfection of crystalline region in both amorphous and crystalline lamellae of the granule (Zhang et al., 2006) are also important characteristics in determining the starch digestibility and significantly contribute to the RS. It should also be noted that in barley these starches, high amylose, do gelatinize at near to normal temperatures and that in the gelatinization process the RS is lost (but potentially gained back with retrogradation). Different waxy genotypes showed higher RS than expected from their amylose content, which also indicates that, apart from amylose, characteristics of amylopectin are important in determining RS content. Other possible explanations of high RS content in waxy genotypes may be due to presence of β-glucans. Barley is rich in β-glucans and waxy genotypes accumulate even more β-glucans (Ajithkumar et al., 2005) which may be adding to the RS content of the genotype.

To examine the contribution of different physical characteristics of seed in the increased RS, PCA was used and showed that hulless or covered seed and barley types such as two rows or six rows do not associate with either amylose/RS or any other seed starch characters. Both of these characters were independent of different physiochemical
properties of starch. A negative relationship between amylose and starch content has been reported previously (Morell et al., 2003; Boren et al., 2008; Regina et al., 2010; Asare et al., 2011) which was also true for genotypes in the present study except 081011-928, 081011-929, 081011-930 and 081011-932 which were derived from amo1 mutant and have more starch and amylose/RS than the reference genotype. These genotypes are of significant importance in overcoming the problem of high amylose content associated with low starch/ and grain yield. Other physical characteristics of seed such as seed length (SL) was independent of all other characteristics measured, but seed thickness (ST) and seed width (SW) were positively related to starch content and seed weight. These characters contribute significantly to yield and some lines with increased amylose/RS have increased seed width and seed thickness and may have higher yield than the normal type as mentioned earlier. The sex1 mutants behaved differently in terms of different seed and starch characters because all these mutants have shrunken endosperm (starch mutants) and store more protein and have a different protein profile (Bosnes et al., 1992).

In the present study it was found that granule size is negatively related to % amylose/RS while granule number is positively related to % amylose/RS. All the high amylose genotypes accumulated more small granules of size (5-15 µm). Reduction of granule size in high amylose genotypes was related to unimodal granule size distribution which shows that amylose also affects composition of starch granules. Two amo1 lines 081011-930 and 081011-931 derived from the same amo1 parent have lower amylose than their counterparts, 081011-928, 081011-929 and 081011-932 (Figure 2.1). The genotype 081011-930 has lower amylose, larger granule size (34 µm) and less altered
granule morphology than its counterparts (081011-928, 081011-929 and 081011-932). The genotype 081011-931 has even lesser amylose than 081011-930, and in this genotype granule size increased up to 39 µm and the proportion of granules with regular morphology also increased (Figure 2.5). These observations suggest that amylose: amylopectin are significantly correlated with granule size and granule size can therefore be used as a diagnostic tool for the screening of high amylose genotypes.

To examine differences in the molecular structure of amylopectin from different genotypes, chain length distribution of normal, waxy and increased amylose/RS genotypes was determined. In maize and rice, high amylose/ RS contents are related to longer modified amylopectin chains (Yoshimoto et al., 2002; Kubo et al., 2010) for example in the $ae^-$ mutant of maize (Hilbert and MacMasters, 1946; Banks et al., 1974; Klucinec and Thompson, 2002). However, in this study a higher proportion of long chains in amylopectins from high amylose genotypes was not observed compared to waxy and normal genotypes (Table 2.2). It shows that higher amylose content in barley does not necessarily affect the structure of amylopectin at level of chain length distribution. Therefore, amylopectin from different genotypes did not have much variation which has also been reported by various authors (Macgregor and Morgan, 1984; Tester and Morrison, 1990; Czuchajowscka et al., 1998; Schulman et al., 1995; Takeda et al., 1999; Yoshimoto et al., 2000; Yoshimoto et al., 2002). Three different methods have been used to measure amylose content and it was revealed that three methods were in agreement (Appendices 2 and 3). This correlation may suggest that amylopectin from different barley genotypes with varying amylose may be similar because differences in these methods depend upon variations within amylopectin structure.
By observing different physical properties of seed and starch it is suggested that variation in granule size was more consistent in all increased amylose genotypes and this character could be used as to identify high amylose genotypes.

2.5 Conclusion

The present study was focused on identifying genotypes high in RS/amylose and their potential use in human diet. This study shows that in high amylose/RS genotypes, starch exhibits a unimodal granule size distribution. Distinct division of granules into A-, B- and C-, based on size is not possible because of decreased granule size, increased amount of intermediate granules with altered granule morphology. Percentage RS is positively related to the proportion of small granules. Amylose content and starch granule size related strongly with some seed dimensions and physiochemical properties. In general, production of starch with desired characteristics such as RS leads to reduction in starch content and yield (Asare et al., 2011). The available high RS barley mutants have reduced starch contents with increased amounts of fibre, lipids and phosphate (Morell et al., 2003; Boren et al., 2008; Regina et al., 2010). Genotypes 081011-928, 081011-929 and 081011-932 are potentially useful for further breeding programs to develop genotypes high not only in RS but also having increased seed size, starch content and yield equal to or in some cases higher than normal or wild-type.
Chapter 3 is prepared as a research article to be submitted for publication
Chapter 3: Post-translational modification, protein-protein interactions among enzymes of starch biosynthesis and their affect on physical properties of starch in high-amylose barley genotypes
3.1 Introduction

Starch accumulates in cereal endosperm as an energy reserve for the next generation and is used globally as a human food, livestock feed as well as numerous important industrial applications including biofuels. Starch is found in higher plants, mosses, ferns and some microorganisms (Keeling and Myers, 2010) and is an insoluble polyglucan composed of two polymers of glucose, amylose and amylopectin. Amylose is, essentially, a linear molecule with a molecular weight varying between \((10^5-10^6 \text{ Da})\), in which glucose residues are joined via \(\alpha-1, 4\) linkages with very few \(\alpha-1, 6\) linkages, and typically constitutes up to 20-30 % of total starch. Amylopectin has a molecular weight of \((10^7-10^9 \text{ Da})\) constitutes 70-80 % of total starch, containing linear chains of various degree of polymerization. Almost 5 % of the glucose units in amylopectin are joined by \(\alpha-1, 6\) linkages, which introduce branches in the amylopectin in a non-random fashion. Starch exists in the form of structurally well organized granules in which amylopectin exhibits non-random distribution of linear chains and a clustered arrangement of branch linkages which gives rise to a high degree of structural organization. This conserved architecture of amylopectin is responsible for the semi-crystalline water insoluble starch granule (James et al., 2003). Amylopectin is required for normal size and shape of the granules, whereas granules with varying low, or no, amylose retain the same shape and size as granules with normal amylose content (Keeling and Myers, 2010). In Festucoides, such as wheat, oats and barley, granules can be divided into B (1-15 \(\mu\text{m}\), round) and A-granules (> 15 \(\mu\text{m}\), lenticular) (Evers, 1973; Bechtel et al., 1990; Bechtel and Wilson, 2003; Wilson et al., 2006), synthesis of which is developmentally regulated (Langeveld et al., 2000; Bechtel and Wilson, 2003). The amylose: amylopectin ratio, and the size and
shape of granules, are important parameters which impact the end use of starch (Wei et al., 2010).

The organization of the starch granule is a complex process involving several classes of enzyme, each with isoforms, which include adenosine diphosphate glucose pyrophosphorylase (AGPase), granule bound starch synthase (GBSSI and GBSSII), soluble starch synthases (SSI, SSIIa, SSIII, SSIV), starch branching enzymes (SBEI, SBEIIa and SBEIIb), starch debranching enzymes (DBEs) such as isoamylases (ISAI, ISAII and ISA III), pullulanase (PU), and starch phosphorylase (SP) (Tsai and Nelson, 1969; James et al. 1995; Ball and Morell, 2003; Patron and Keeling, 2005; Leterrier et al., 2008). These different enzymes and their isoforms are differentially distributed in the soluble and starch granule fractions of plastids (Tetlow et al., 2011).

Recent evidence has demonstrated that many enzymes involved in starch biosynthesis are subjected to post translational modification by protein phosphorylation, and also interact to form heteromeric protein complexes (Hennen-Bierwagan et al., 2008; Tetlow et al., 2008; Liu et al., 2009, 2012b). In wheat GBSS, SSI and SSIIa are phosphorylated in their starch granule bound state (Grimaud et al., 2008; Tetlow et al., 2008a; Bancel et al., 2010). In wheat amyloplasts phosphorylation of SBEIIb, SSIIa and SP has also been reported by radioactive labelling of amyloplasts with $\gamma^{32}$P-ATP (Tetlow et al., 2004).

Co-immunoprecipitation of SBEIIb, SBEI, and SP provided direct evidence of multi-enzyme complex formation in soluble extracts of wheat endosperm (Tetlow et al., 2004), dependent upon the phosphorylation of target proteins. Other enzyme complexes, containing SSI, SSIIa, SSIII, SBEIIa, and/or SBEIIb, in various combinations have also
been reported (Hennen-Bierwagen et al. 2008, Tetlow et al., 2008b). Hennen-Bierwagen et al. (2009) also reported a novel complex in maize endosperm containing SSIII, SSIIa, SBEIIa, SBEIIb, large and small subunits of AGPase, and pyruvate phosphate dikinase (PPDK). Liu et al. (2009) reported that, in maize, a null mutant of SBEIIb (ae-) contained a novel protein complex comprising SSI, SSIIa, SBEI, SBEIIa and SP which became entrapped in the starch granule. Another ae- allele was described which expressed a catalytically inactive SBEIIb protein. Although this protein was not able to bind glucan substrate, it was still found as a granule associated protein, as a result of being able to form a heteromeric protein complex leading to the entrapment of inactive protein within the starch granule (Liu et al., 2012a). In related work (Liu et al., 2012b) reported that a point mutation in SSIIa in a su2- mutant of maize, led to loss SSI and SBEIIb, as well as SSIIa from starch granule. These observations suggest that single gene mutations affect partitioning of several proteins between the soluble and granule bound fractions of amyloplasts and impact amylopectin fine structure. They further imply that alteration in the protein fingerprint of the granule may reflect variations in protein-protein interactions in the stroma. Such changes in the granule proteome arising from allelic variations also give rise to variation in granule structure and composition (Liu et al., 2012a). The present study examines the relationship between the granule proteome and starch properties in several varieties of barley with defined mutations, and relates this to variation in protein-protein interactions in the stroma. Variation in the granule proteome of A- and B-granules was also investigated with a view to understanding the relationship between granule type, its proteome and mechanisms underpinning granule size and morphology.
3.2 Materials and Methods

3.2.1 Plant material

The genotypes used in this study included, wild-type, starch branching enzyme (sbeiia and sbeiib) down regulated lines, ssiia (sex6) mutant, amo1 (ssiii) mutant and a waxy (amylose free) mutant lacking GBSS (Table 3.1). The sources of mutant seed were, sbeiia and sbeiib (Regina et al., 2010), sex6 (Morrell et al., 2003), HAG amo1 (Banks et al., 1971). The seeds for OAC Baxter (reference genotype) and Neopolis (waxy) were obtained from Plant Agriculture, University of Guelph. The plant material was grown in the glasshouse at the University of Guelph under conditions previously described for growing wheat (Tetlow et al., 2008).

3.2.2 Isolation of starch granules

Mature dry barley seeds weighing \( \approx 55 \) g were completely ground to flour with a Retsch\textsuperscript{®} MM301 homogenizer in liquid nitrogen. 50 g of flour was suspended in 150 ml buffer containing 100 mM Tricine-KOH, pH 7.8, 1 mM Na\textsubscript{2}-EDTA, 1mM DTT and 5 mM MgCl\textsubscript{2} at 4 °C. The suspension was vortexed (Eppendorf) for 5-10 minutes to make a uniform suspension and left for 5-10 minutes on ice. The well-mixed suspension was sieved through six-layers of cheese cloth to remove debris and bran. Buffer was added to wash traces of starch from the cheese cloth. The sieved milky suspension, 450-500 ml, containing starch, fine pieces of debris and bran was centrifuged at 16000 g for 15 min. The supernant was discarded and the pellet resuspended in 100 ml buffer containing 50 mM TRIS-acetate, pH 7.5, 1 mM Na\textsubscript{2}-EDTA, and 1 mM DTT (wash buffer). The resuspended pellet was centrifuged at 6000 g for 5 min. This washing step was repeated 5-7 times until a thick yellow layer of debris was left on the top of starch. The yellow
layer, containing very fine pieces of debris and bran, was completely removed with a spatula whilst minimizing loss of starch. After removing debris the starch was again washed and centrifuged twice (see above) in wash buffer. The purified starch was washed three times, each with 45 ml of 99 % acetone (termed acetone-washed starch) followed by three washes, each with 45 ml of 2 % (w/v) sodium dodecyl sulphate (SDS) in water, to remove proteins bound to the surface of starch granule. This was followed by 3 washings with 45 ml distilled water and the starch finally dried under vacuum (Eppendorf, vacufuge™) at 25 °C for 3 h.

3.2.3 Separation of A- and B-type starch granules

A- and B- starch granules were separated based on a method previously described by Peng et al. (1999). Approximately 0.5 g of the acetone-washed starch was suspended in 5 ml of dH₂O. This starch suspension was then carefully laid on top of 10 ml of 70 % (v/v) Percoll in dH₂O in a 15 ml tube, followed by centrifugation at 10 g for 10 min at room temperature. The larger A-granules were centrifuged through the Percoll pad and precipitated at the bottom of the tubes, whereas the smaller B- granules remain in suspension. After centrifugation, the supernatant (containing B- granules) was carefully removed. The pellet containing A-granules was washed twice with dH₂O, by resuspension and centrifugation at 4000 g for 5 min. The resulting pellet was resuspended in 5 ml dH₂O and laid on top of 10 ml of 70 % (v/v) Percoll. Centrifugation through Percoll with subsequent washing in dH₂O was repeated 3 times. A-granules were then centrifuged 3 times through 100 % Percoll for 10 min at 10 g. All supernants from each Percoll centrifugation step were pooled and centrifuged at 4000 g for 5 min. The resulting pellet
was washed twice with dH2O, resuspended and centrifuged at 4000 g for 5 min, and comprised B- granules.

3.2.4 Extraction of amyloplasts and preparation of endosperm whole cell extracts

Barley endosperm amyloplasts were isolated as described by Tetlow et al. (2008b). Endosperm whole cell extracts were prepared as described previously (Tetlow et al., 2003).

3.2.5 Isolation of starch granule-bound proteins

The isolation of protein bound within the internal matrix of the starch granules (as opposed to proteins present on the surface of starch granules, which can be removed by extensive washing with SDS) was carried out as previously described by (Tetlow et al., 2004 and Liu et al., 2009). The acetone and SDS-washed starch, either from mature seed, fresh amyloplast lysate, or whole cell extract, was washed twice with 1 % (w/v) SDS, to remove any traces of proteins attached to the surface of starch granules. To extract the starch granule-bound proteins, equivalent amounts of starch (50 mg) were boiled in 1 ml SDS loading buffer containing 62.5 mM TRIS-HCl, pH 6.8, 2 % (w/v) SDS, 10 % (w/v) glycerol, 5 % (v/v) β-mercaptoethanol and 0.001 % (w/v) bromophenol blue. Boiled samples were centrifuged at 13,000 g for 10 min and the supernatant used to determine total granule bound protein content (see below), and for SDS–PAGE. To determine the equivalence of starch, 20 mg of starch was washed twice with 1% SDS, and 5 times with 50 mM CH3COONa, pH 4.8. Following washing, the starch was boiled in 250 µl sodium acetate (pH 4.8) buffer at 95 °C for 6-7 min, and gelatinized starch was left at room temperature to cool. 25 µl (containing 15 U) of amyloglucosidase (AMG) and α-amylases (Sigma-Aldrich) prepared in acetate buffer was added to the gelatinized starch and left at
37 °C overnight. Following digestion the sample was centrifuged at 13,000 g for 15 min. Glucose was assayed using hexokinase and glucose-6-phosphate dehydrogenase coupled to NADPH production measured at 340 nm (Deeg et al., 1980).

3.2.6 SDS-PAGE and immunoblotting of total starch, A and B granules

Equivalent amounts of starch, A- and B- granules (see above) were used to extract protein as described earlier. For SDS–PAGE and immunodetection, either SDS gels with 10 % (w/v) acrylamide or pre-cast NUPAGE Novex 4–12% BISTRIS acrylamide gradient gels (Invitrogen Canada, catalogue No. NP0335BOX) were used following the manufacturer’s instructions. Gradient gels were run at room temperature in a MOPS-based running buffer prepared according to the manufacturer’s instructions (Invitrogen). For immunodetection, following electrophoresis, gels were transblotted onto nitrocellulose membranes (Pall Life Science), and blocked at room temperature in 1.5 % (w/v) BSA for 15 min with gentle shaking. Different antibodies to wheat proteins were used according to specifications described earlier (Li et al., 1999; Rahman et al., 2001; Morell et al., 2003; Regina et al., 2005; Bresolin et al., 2006; Tetlow et al., 2008). In this study antibodies to wheat proteins were used because these antibodies cross reacted with barley target proteins, having a higher degree of amino acid sequence homology.

3.2.7 Co-immunoprecipitation

Co-immunoprecipitation experiments were performed as described by Liu et al. (2009), with some modification. SSI, SSIIa, SBEIIa, and SBEIIb antibodies were used for the co-immunoprecipitation experiments with amyloplast lysates (1 ml, 1-1.25 mg. ml⁻¹ protein) and endosperm whole extract (1 ml, 1.5-2 mg. ml⁻¹ protein) from different genotypes. The antibody and amyloplast lysate mixture was incubated at room
temperature on a rotator for ~1 h. Immunoprecipitation was performed by adding 50 µl of protein A-Sepharose beads (Sigma-Aldrich), pre-made as a 50 % (w/v) slurry with phosphate-buffered saline (PBS) containing 137 mM NaCl, 10 mM Na$_2$HPO$_4$, 2.7 mM KCl, 1.8 mM KH$_2$PO$_4$, pH 7.4 at room temperature for 50 min. The protein A-Sepharose-antibody-protein complex was centrifuged at 1000 g for 5 min at 4 °C in a refrigerated micro-centrifuge. Each supernatant was stored to check the presence of unbound proteins. Pellets were washed five times (1.4 ml each) with PBS, followed by four times washing with (HEPES) buffer containing 10 mM HEPES-NaOH, pH 7.5, and 150 mM NaCl. Washed pellets from all genotypes were boiled in SDS loading buffer and separated by SDS-PAGE by loading equal amounts on a 10 % gel, followed by immunoblot analysis with different antibodies. In order to exclude the possibility that different proteins (SSs, SBEs and SP) co-immunoprecipitated together as a result of binding to the same glucan chain, plastid lysates and endosperm whole cell extracts used for co-immunoprecipitation were pre-incubated with glucan-degrading enzymes such as amyloglucosidase (EC 3.2.1.3, Sigma product number A7255, from Rhizopus) and α-amylase (EC 3.2.1.1, Sigma product number A2643, from porcine pancreas), 5 U each for 20 min at 25 °C. Following amyloglucosidase/α-amylase digestion of glucans, released glucose was measured as described previously (Tetlow et al., 1994). To check the phosphorylation dependent complex formation, amyloplast lysates from different genotypes were prepared according to protocol described by Liu et al. (2009)

3.2.8 Detection of SS and SBE activity following non-denaturing gel electrophoresis

To locate SS and SBE activities following non-denaturing gel electrophoresis, zymograms were run as described previously (Liu et al., 2012; Tetlow et al., 2004, 2008)
with some modifications For each zymogram, protein samples were mixed with a buffer containing 62.5 mM Tris-HCl, pH 6.8, 10 % (w/v) glycerol, and 0.001 % (w/v) bromophenol blue. 300 µg protein was loaded into each well of 5 % (w/v) polyacrylamide gels in (375 mM Tris-HCl, pH 8.8, containing 10 mg of Acarbose (Prandase, Bayer) the α-amylase inhibitor). Gels for determination of SS activity contained 0.3 % (w/v) amylopectin (Sigma-Aldrich) as primer in the gel. Following electrophoresis gels were incubated for 48–72 h in a buffer containing (50 mM glycylglycine, pH 9.0, 20 mM DTT, 100 mM (NH₄)₂SO₄, 0.5 mg ml-1 BSA, and 4 mM ADP-Glc (Sigma-Aldrich). To detect SBE activity, the gel contained 0.2 % (w/v) maltoheptaose (Sigma-M7755) and 1.4 U of rabbit muscle extracted phosphorylase a (Sigma-Aldrich, catalogue No. P-1261). Following electrophoresis, gels were washed three times each for 15 min with buffer containing (20 mM MES-NaOH, pH 6.6 and 100 mM Na-citrate) followed by incubation in a buffer containing (20 mM MES-NaOH, pH 6.6, 100 mM Na-citrate, 45 mM Glc-1-P, 2.5 mM AMP, 1 mM Na₂-EDTA and 1 mM DTT) for 2–3 h at 28 °C in a shaking incubator. After incubation gels were washed with water and were developed with Lugol’s solution and visualized immediately (Liu et al., 2009).

3.2.9 Detection of SP activity

For detection of SP activity 3.75 % (w/v) polyacrylamide (stacking gel) and 12.5 % (w/v) polyacrylamide (resolving gel) were used. The non-denaturing gels were prepared according to the protocol given above, except the resolving gel contained 0.05 % glycogen. Following electrophoresis, gels were incubated in buffer containing 100 mM Na-citrate, pH 6.5, 0.1 % glycogen, 20 mM glucose-1-phosphate (sigma, G-7000), 2.5
mM AMP and 1 mM DTT. After incubation the activity bands were visualized by Lugol’s solution.

3.2.10 Silver staining

Silver staining was performed as described (Mortz et al., 2001) with some modification. After electrophoresis, the SDS gel was kept in well-washed glass plates in 50 ml fixing solution (50 % Methanol [v/v], 5 % Acetic acid [v/v]) for 20 min on a shaker, followed by washing buffer containing 50 % Methanol [v/v] for 10 min on a shaker. The gel was then kept in distilled water at least for 1 h or overnight with occasional changes of water. Sensitizing buffer (0.02 % Na$_2$S$_2$O$_3$ [w/v]) was added for 1 min, followed by two washes in distilled water for 1 min each. The gel was incubated in ice-cold silver nitrate buffer (0.1 % AgNO$_3$ [w/v]) for 20 min at 4 °C followed by two washings with distilled water, each 1 min. The gel was immersed in developing solution (2 % Na$_2$CO$_3$ [w/v], 0.04 % formalin [v/v]) for 3-5 min and then fresh developing buffer (given above) was added, and the gel stained until the proteins bands were visualized. Staining was stopped by adding 5 % acetic acid [v/v] for 5 min, and the gel transferred to distilled water.

3.2.11 Pro-Q Diamond phospho-protein staining

Equal amounts of starch were loaded onto 4–12 % acrylamide gradient gels and run according to the manufacturer’s instructions. Following electrophoresis, gels were fixed in 50 % methanol, 10 % acetic acid overnight in dark. The gels were then washed 3 times with ddH$_2$O and then incubated in Pro-Q Diamond stain according to the manufacturer’s instructions (Molecular Probes) in the dark for 90 min. Gels were then incubated 3 times each, for 30 min, in destaining solution containing 20 % acetonitrile,
50 mM sodium acetate, pH 4.0 and washed with water 2 times each 5-7 min. Proteins were visualised using a Typhoon scanner (Amersham Biosciences) with an excitation wavelength of 530 nm and emission filter at 580 nm. A phospho-protein marker was used as standard.

3.2.12 Starch gelatinization

Thermal analyses were performed as described by (Liu et al., 2007) using a differential scanning calorimeter (2920 Modulated DSC, TA Instruments, New Castle, DE). For gelatinization and retrogradation of starches, this system was equipped with a refrigerated cooling system (RCS). Samples of starch granules were weighed into high volume pans. A micropipette was used to add distilled water to make suspensions with 70% moisture content. Approximately 20 mg of starch sample was used. Sealed pans were equilibrated overnight at room temperature before heating in the DSC. Measurements were taken at a heating rate of 10 °C/min from 5 to 180 °C. Calibration of the instrument was done using indium and an empty pan as reference. To measure the enthalpy ($\Delta H$) of phase transitions from the endotherm of DSC, thermograms based on the mass of dry solid, software (Universal Analysis, v.2.6D, TA Instruments) were used. Peak temperature ($T_p$) of endotherms was also measured from DSC thermograms. For retrogradation, the heated starch from the above procedure was cooled to 5 °C. Once the temperature has reached 5°C, the sample was immediately stored at 5 °C. After two weeks, stored samples were heated from 5 to 180 °C at 10 °C/min and, based on dry solid mass, the enthalpy ($\Delta H$) and peak temperature ($T_p$) of the endotherm were measured from DSC thermograms.
3.2.13 Estimation of amylose and RS content

Amylose and RS content of different genotypes have been measured with gel permeation chromatography (GPC) as described (Chen and Christine, 2007) and Megazyme RS assay kit according to manufacturer instructions with the procedure earlier described (Goni et al., 1996). For each measurement three biological repeats were used.

3.2.14 Protein content

Protein content of amyloplasts lysate or whole cell extracts was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Canada) according to the manufacturer’s instructions and using BSA as a standard.

3.2.15 Mass spectrometry

In-gel digestion with trypsin and peptides for MS was performed according to the protocol described by Tetlow et al. (2008). Using a hybrid Q-TOF spectrometer (Micromass), interfaced to a Micromass CapLC capillary chromatograph, tandem electrospray mass spectra were recorded previously described by (Tetlow et al., 2004).

3.2.16 Statistical analysis

For one way analysis of variance (ANOVA) and least significant difference (LSD), Statsoft’s Software, Statistica (http://www.statsoft.com) was used. For principal component analysis (PCA), XLSTAT (http://www.xlstat.com/en/) was used and biplots were generated.
3.3 Results

3.3.1 Physiochemical properties of mutant starches in barley

3.3.1.1 Starch, amylose and RS content

All the mutants used in this study had lower starch content than the wild-type genotype (Table 3.1). Neopolis is a waxy (amylose free) genotype. All the other mutants in this study possess increased amylose and RS content compared to wild-type (Table 3.1).

3.3.1.2 Starch gelatinization

Gelatinization of starch is an important parameter which contributes significantly in determining different uses of starch and is an important diagnostic of alterations in starch structure/architecture (Bhattacharyya et al., 2004). Gelatinization of starch from different high RS genotypes was measured by DSC, demonstrated by two endothermic peaks. Table 3.2 shows differences among genotypes with respect to onset (To) gelatinization temperature. The highest onset gelatinization temperature (59.7 °C) is shown by the waxy genotype, while the reference barley genotype (OAC Baxter) demonstrated the lowest (55.8 °C) onset temperature. For (To) all mutants with altered amylose content, exhibited values between waxy and normal barley. In the case of peak temperature (Tp), genotypes also exhibited significant differences. The waxy genotype exhibited the highest value (67.8 °C) of (Tp) while the reference genotype the lowest (62.4 °C) Tp. For completion temperature (Tc), all genotypes presented significant variation compared to reference genotype. In contrast to (To and Tp) the highest Tc (78.9 °C) was shown by the high amylose barley genotype, HAG amo1, whilst the reference genotype exhibited the lowest value (73.4 °C, Tc). Variations were also observed.
Mutations in genes involved in starch/amylopectin biosynthesis (SBEIIa, SBEIIb, SSIIa and SSIII) are as indicated. OAC Baxter was used as a reference genotype. Neopolis is a waxy genotype lacking GBSS. % amylose and % RS contents were measured by GPC and Megazyme RS kit respectively. Starch content (means of three replications, ± indicates standard deviation). Starch content of reference genotype OAC Baxter was taken as 100 % and other mutants were compared to reference genotype. Alphabetical letters in each starch content column represent existence of significant differences at (p < 0.05).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Predicted mutation</th>
<th>% amylose</th>
<th>% RS</th>
<th>Starch content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OAC Baxter</td>
<td>Normal</td>
<td>24.3</td>
<td>1.9</td>
<td>100 ±1.20</td>
</tr>
<tr>
<td>Neopolis</td>
<td>Waxy</td>
<td>2.0</td>
<td>1.3</td>
<td>92 ±1.20</td>
</tr>
<tr>
<td>sbeiia</td>
<td>sbeiia</td>
<td>30.7</td>
<td>7.3</td>
<td>94 ±1.95</td>
</tr>
<tr>
<td>sbeiiib</td>
<td>sbeiiib</td>
<td>31</td>
<td>12.6</td>
<td>83 ±2.00</td>
</tr>
<tr>
<td>sex6</td>
<td>ssiia</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HAG amo1</td>
<td>ssiii</td>
<td>35.6</td>
<td>5.9</td>
<td>95 ±1.10</td>
</tr>
</tbody>
</table>
regarding transition enthalpy (\(\Delta H\)) for different genotypes. The \textit{waxy} barley showed maximum \(\Delta H\) while high amylose genotypes exhibited significantly lower \(\Delta H\) compared to normal barley except \textit{sbeiia} which did not show a significant difference compared to the reference genotype. In the second phase of gelatinization where disruption of amylose–lipid complex occurs, the \textit{sbeiia} genotype exhibited the highest onset temperature (96.3 °C) \(T_o\). However, different genotypes with variation in amylose content did not show significant differences. A similar trend was observed for \(T_p\) among the different genotypes investigated. For \(T_c\) and \(\Delta H\), HAG \textit{amo1} exhibited the highest values, while \textit{sbeiib} exhibited lowest (\(T_c\)) values, and normal barley exhibited the lowest \(\Delta H\) respectively. The \textit{waxy} genotype did not show any value for different phases of disruption of the amylose–lipid complex, indicating that \textit{waxy} starch has a very low amount of amylose-lipid complex (Yoshimoto et al., 2002). In the second phase of gelatinization of retrograded starch no significant variations were observed for various transition temperatures between different genotypes.

3.3.2 Biochemical characterization of different mutants

3.3.2.1 Amyloplast stromal proteins

Amyloplasts from developing barley endosperm from the various genotypes were isolated (18-25 DAP). Amyloplast lysates were used to detect the presence of different proteins involved in starch biosynthesis in the amyloplast stroma using various peptide-specific antibodies. Immunological analysis of amyloplast lysates from different mutants showed that some did not express the protein responsible for the mutation. The \(\Delta sbeiia\) exhibited reduced expression of SBEIIa protein (Figure 3.1). In the \(\Delta sbeiib\) a small amount of expressed SBEIIb protein was also detectable. Similarly,
Table 3.8: DSC data of different genotypes

**Initial Heating Summary**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Starch Gelatinization</th>
<th>Melting of amylose-lipid complex</th>
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<tr>
<td></td>
<td>To (Co)</td>
<td>Tp (Co)</td>
</tr>
<tr>
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<td>62.4&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
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<td>56.7&lt;sup&gt;d&lt;/sup&gt;</td>
<td>65.3&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>HAG amo1</td>
<td>57.4&lt;sup&gt;e&lt;/sup&gt;</td>
<td>66.4&lt;sup&gt;e&lt;/sup&gt;</td>
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**Re-Heating Summary**

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<th>Genotype</th>
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<th>Melting of amylose-lipid complex</th>
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<td>53.4&lt;sup&gt;a&lt;/sup&gt;</td>
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Thermal analyses of starch from different genotypes were performed as described by (Liu et al., 2007) using a differential scanning calorimeter (2920 Modulated DSC, TA Instruments, New Castle, DE). In this analysis Neopolis was used as waxy genotype. Alphabetical letters in each column represent significant differences among genotypes for a given character at (p<0.05).
immunological analysis revealed that the sex6 mutant lacks SSIIa protein in the soluble fraction (Figure 3.1). The amo1 (ssiii) mutant (HAG amo1) exhibited similar amount of SSIII protein to that of the reference genotype. The amo1 mutant (HAG amo1) does not lack SSIII protein. However, molecular analysis of this mutant indicates the mutant ssiii has a leucine to arginine residue substitution in a conserved domain compared to the wild-type protein (Li et al., 2011) resulting in a reduction of SSIII activity compared to wild-type. Although immunoblotting is not quantitative, it can be used to provide a qualitative impression of the amount of different proteins among different genotypes. Immunological characterization of amyloplast lysates revealed that all other proteins (SSI, SSIIa, SSIII, SSIV, SBEI, SBEIIa, SBEIIb, ISO1, AGPase large subunit, AGPase small subunit) other than known mutations were present in comparable amounts to the reference genotype (Figure 3.1). Based on immunological detection of SDS-denatured proteins variation was found in the content of different proteins in the soluble fraction of certain genotypes. For example, the sex6 mutant had comparatively more SSI protein than the reference genotype (Figure 3.1). The branching enzyme mutants, sbeiiα- and sbeiiβ- appeared to possess less soluble SP compared to the reference genotype.

### 3.3.2.2 Detection and estimation of SS activity

SS activity was visualized on zymograms and compared with the reference genotype. Among all soluble SSs, SSI appears to exhibit highest detectable activity on zymograms (Figure 3.2A). The identity of different SS’s was determined by immunoblotting similar zymogram gels with SS isoform specific antibodies (Figure 3.2B-E). Significant variations were not observed in other SS activities which were far less pronounced than SSI. It is noticeable that in branching enzyme mutants (sbeiiα- and
Figure 3.1: Immunological characterization of endosperm amyloplast lysates from different barley mutants.

Amyloplast lysates (~1.3 mg protein/ml) were prepared from developing barley endosperms at 18–25 DAP. Aliquots of soluble (stromal) proteins were separated on 10% polyacrylamide gels and electroblotted onto nitrocellulose membranes. Immunoblots were developed with peptide-specific anti-wheat antibodies as described in Methods. Left hand side indicate cross-reactions of each of the antibodies with its corresponding target protein, and the name of each genotype is given. The approximate molecular mass for each protein, based on its SDS–PAGE migration and predicted mass, is given on right hand side.
Figure 3.2. Detection of SS activity.
Non-denaturing electrophoresis was performed in 5% (w/v) polyacrylamide gels containing 0.3% (w/v) amylopectin. Approximately 300 µg protein from whole cell extracts of developing endosperm was loaded onto each lane. Following electrophoresis, gels were incubated for 48–72 h at 30 °C as described (Methods). Activity of SS was visualized by staining gels with I$_2$–KI (A). The identity of SSI was determined by coupling non-denaturing PAGE with immunoblotting, and protein detected by using specific antibodies (B). Arrows indicate the activity band corresponding to SSI protein in zymogram (2A) and presence of SSI in immunoblot incubated with SSI antibody (2B). C, D and E represent immunoblots incubated with SSIIa, SSIII and SSIV antibodies respectively.
sbeiib'), the activity of the major SS band (SSI) is significantly reduced (absent) compared to the reference genotype (Figure 3.2A) even though the proteins is readily detected (Figure 3.2B).

The sex6 mutant exhibited higher SSI activity compared to the reference genotype (Figure 3.2A), and is associated with an increase in the amount of detectable soluble SSI protein (Figure 3.2B).

### 3.3.2.3 Detection and estimation of SBE activity

SBE activity was detected by zymogram analysis (Figure 3.3A) and isoforms identified by using peptide-specific antibodies (Figure 3.3B-D). SBE mutants did not show clear changes in SBE activity compared to reference genotype. However, an activity band of SBE corresponding to the loss of SBEIIb protein is clearly lost in the sbeiib− mutant (Figure 3.3A). In the sex6 mutant an activity band corresponding to SBEIc was significantly increased compared to wild-type (Figure 3.3A). The putative SBEI activity was only visible when higher concentration of (phosphorylase a) was used and gels were incubated in Lugol’s solution > 15 min (data not shown). On the zymogram, activity of SP (Pho1) can also be seen as a blue band, and the results suggest that activity of soluble SP has been significantly reduced (absent) in the sbeiib− mutant (Figure 3.3A).

### 3.3.2.4 Detection and estimation of starch phosphorylase (SP) activity

Given the previous observation with the sbeiib− mutant (Figure 3.3A), the effects on SP activity were studied directly. In sbeiib, negligible Pho1 activity was detected on zymograms (Figure 3.4A). However, Pho1 protein is clearly present in the soluble fraction of (sbeiib−) amyloplasts (Figure 3.4B). By contrast, activity and protein content of Pho1 remained unaffected in the sbeiia− mutant (Figure 3.4A). Thus mutation in either
Figure 3.3: Detection of SBE activity and protein.

Non-denaturing electrophoresis was performed in 5% (w/v) polyacrylamide gels containing 0.2% (w/v) maltoheptaose and 1.4 U of rabbit muscle extracted phosphorylase a. Approximately 300 µg protein from whole cell extracts was loaded onto each lane. Following electrophoresis, gels were incubated for 3 - 3.5 h at 28°C. Activity of SBE was visualized by staining gels with I₂-IK. Activity of SP is detected as dark blue bands. Migration of SBE isoforms was determined by immunoblotting with specific antibodies (3B, SBEIIa; 3C, SBEIIb; 3D, SBEI and SBEIc). Name of each genotype shown. Arrows indicate specific activity band in zymogram and presence of respective proteins in immunoblots incubated with different antibodies.
Figure 3.4: Detection of SP protein and activity in different genotypes.

(A) Activity of SP (Pho1) is visualized as a dark blue band. (B) Immunodetection of SP (Pho1 and Pho2). (C) Identification of PHO1 in amyloplasts extract. Non-denaturing electrophoresis was performed in 3.75 % (w/v) polyacrylamide (stacking gel) and 12.5 % (w/v) polyacrylamide (resolving gel containing 0.05 % glycogen). Approximately 300 µg protein from whole cell or amyloplasts extracts of developing endosperm was loaded onto each lane. Following electrophoresis, gels were incubated according to methods and the activity bands were visualized by Lugol’s solution.
SBEIIa or SBEIIb gives rise to different pleiotropic effects on SP activity. In HAG *amo1* and *sex6*, activity of Pho1 was readily detected and comparable to the reference genotype OAC-Baxter (Figure 3.4A). Interestingly, in HAG *amo1* and *sex6* mutants Pho1 protein could not be detected in immunoblots of non-denaturing gels (Figure 3.4B), even though it was readily detected following denaturation and SDS-PAGE (Figure 3.1). Cytosolic SP (Pho2) is found in small amounts in barley endosperm (Figure 3.4B), though activity is barely detected in any genotype under the conditions employed (Figure 3.4A). Confirmation of the identity of plastidial SP (Pho1) was determined in amyloplast lysates in which activity of Pho1 only was observed (Figure 3.4C).

### 3.3.3 Protein-protein interactions among different proteins of starch biosynthesis

Co-immunoprecipitation was used to determine physical interactions among enzymes involved in starch biosynthesis in the different barley mutants studied here, using antibodies to SSI, SSIIa, SBEIIa and SBEIIb.

Immunoprecipitation of SSI in OAC Baxter (reference genotype) was accompanied by co-precipitation of SSIIa, SBEIIb and small amount of SBEIIa (Figure 3.5A). A similar pattern of co-immunoprecipitation was observed in the branching enzyme mutant *sbeiia*'. However, in the *sbeiib*' mutant, SBEIIa more clearly co-immunoprecipitated with SSI and SSIIa. In the *amo1* mutant (HAG *amo1*) the SBEIIa and SBEIIb could both be co-precipitated with SSI and SSIIa. However, in the *sex6* mutant, the SSI antibody did not result in co-immunoprecipitation of the other proteins (Figure 3.5A). Other proteins SSIII, SSIV, SBEI, SBEIc, Isoamylases and SP were not immunoprecipitated by anti-SSI in any genotypes studied.
Protein complexes from different genotypes were also co-immunoprecipitated with SBEIIa antibodies. In the reference genotype, OAC Baxter, SSI, SSIIa and SBEIIb co-precipitated with SBEIIa antibody (Figure 3.5B). In the sbeiiα mutant, not surprisingly, the SBEIIa antibody did not co-precipitate other proteins. In the sbeiiβ mutant, SBEIIa antibody precipitated SSI, SSIIa, SBEI, SP and SBEIIa (Figure 3.5B). In the amo1 mutant (HAG amo1) the co-immunoprecipitation pattern of SBEIIa was similar to that seen with anti-SSI in this mutant. In the sex6 mutant, the SBEIIa antibody did not result in co-immunoprecipitation of other proteins, unlike the situation observed in other genotypes (Figure 3.5B).

The co-immunoprecipitation study was further extended by using an antibody to SBEIIb. In the reference genotype SSI, SSIIa and SBEIIa were co-precipitated by anti-SBEIIb (Figure 3.5C). In the sbeiiα mutant, SBEIIb antibody precipitated SSI, SSIIa, SBEI, SP and SBEIIb. Consistent with the reduced expression of SBEIIb protein, in sbeiiβ no other proteins were co-precipitated by SBEIIb antibody. In HAG amo1 a similar pattern of co-immunoprecipitation was observed to that seen with SSI and SBEIIa antibodies. In the sex6- mutant, the SBEIIb antibody did not co-precipitate other proteins (Figure 3.5C). Other proteins SSIII, SSIV and Isoamylases were not found as a part of immunoprecipitated protein complexes.

3.3.3.1 Phosphorylation dependent protein-protein interactions

Previously it was reported that the formation of protein complexes is dependent upon phosphorylation of different components of a protein complex (Tetlow et al., 2004; Tetlow et al., 2008; Hennen-Bierwagen et al., 2009; Liu et al., 2009). To study similar phenomenon in barley, ATP and alkaline phosphatase (APase) treatments were employed.
Figure 3.5: Co-immunoprecipitation of stromal proteins from amyloplasts of different barley genotypes with SSI, SBEIIa and SBEIIb antibodies.

Aliquots (0.75-1 ml) of amyloplast lysates (0.8–1.3 mg protein ml/ml) prepared from endosperms of different genotypes at 18–25 DAP were incubated with specific anti-SSI (5A), anti-SBEIIa (5B) and anti-SBEIIb (5C) antibodies at 25 °C for 1h, and then immunoprecipitated with protein A–Sepharose beads. The protein A–Sepharose–antibody–antigen complexes were washed several times to remove non-specifically bound proteins, boiled in 200 µl of SDS loading buffer, and 25 µl was loaded in each lane of 10 % polyacrylamide gels. Following electrophoresis, gels were electroblotted onto nitrocellulose, and developed with various anti-barley antibodies as indicated. D), Phosphorylation dependent protein complex formation in the reference genotype of...
barley. Amyloplast lysates were pre-treated with 1 mM ATP (for phosphorylation) or APase (for dephosphorylation) and co-immunoprecipitation performed as indicated. E), Amyloplast lysates from sbeiia\(^{-}\) and \textit{sbeiib}\(^{-}\) were treated with 1 mM ATP and APase and co-immunoprecipitation was performed with anti-SSI antibody.

In co-immunoprecipitation experiments similar to those described in the previous section. In all treatments with ATP or APase the ability of the respective antibody to precipitate its target protein was not affected (Figure 3.5D). In amyloplast lysates of the reference genotype, interaction between SSI, SSIIa, SBEIIa and SBEIIb as described earlier with SSI, SBEIIa and SBEIIb antibodies were enhanced by pre-treatment of lysate with 1 mM ATP (Figure 3.5D). Similarly these interactions were undetectable following dephosphorylation with APase (Figure 3.5D). In the reference genotype, SP and SBEI were not co-precipitated by any of the antibodies used (Figure 3.5) whether treated with ATP and APase or not. Identical ATP and APase treatments were also used in co-immunoprecipitation experiments with endosperm amyloplast lysates from \textit{sbeiia}\(^{-}\), \textit{sbeiib}\(^{-}\), \textit{sex6} and \textit{amo1} mutants. In these mutants, protein-protein interactions were enhanced by the presence of ATP, but no differences were observed in composition of different protein complexes based on their ability to be co-immunoprecipitated. Anti-SSI co-immunoprecipitation data for \textit{sbeiia}\(^{-}\) and \textit{sbeiib}\(^{-}\) mutants illustrates no difference in the pattern of other co-precipitated proteins with ATP or APase treatments compared to the reference genotype (OAC Baxter) (Figure 3.5E).

\textbf{3.3.4 Analysis of starch granule-associated proteins}

Following extensive washings with buffer, SDS and acetone, granule associated proteins were extracted (see methods). In barley, starch is composed of two types of
starch granules large A-type and small B-type granules, which were separated using the method described of Peng et al. (1999), and proteomic analysis was performed by immunodetection (Figures 3.6A, B & C). The purity of A- and B- granules was confirmed by light and electron microscopy. As an illustration the micrograph of the reference genotype OAC Baxter are presented in the Appendices (15). Granule proteome analysis revealed that in the reference genotype and in HAG amo1: SSI, SSIIa, SSIII, SBEIIa and SBEIIb were all present in total starch, and independently in A and B granules (Figures 3.6A & B). Other than these proteins, an additional polypeptide of approximately 110 kDa was also observed in all genotypes except sex6 (Figure 3.7A). This 110 kDa protein was identified as SP in the sbeiiα and sbeibβ mutants. This SP was also only present in A granules (and therefore total starch) (Figures 3.6A & B). In the sbeiiα and sbeibβ mutants, along with SP, SBEI was also detected in total starch. Fractionation of granules shows that SSI, SSIIa, SSIII, and whichever SBEII protein is expressed, are present in A- and B-granules, whereas SBEI and SP are only present in A-granules (Figures 3.6A, B & C). In all genotypes, SBEIc was only detected in A-granules (and therefore, also, total starch). In the sex6 mutant, little or no protein was detected in granules apart from GBSS the latter being present in all genotypes in both types of granules.

3.3.4.1 Phosphorylation state of the starch granule proteome

Previously it has been shown that protein complex formation is dependent upon protein-phosphorylation (Tetlow et al., 2004; Liu et al., 2009, 2012b) and some proteins entrapped with in starch granules are also phosphorylated (Grimaud et al., 2008; Liu et al., 2009, Bancel et al., 2010). To investigate the phosphorylation state of different granule-
Starch granules were isolated from mature barley grains by grinding seeds and preparing starch granules as described Materials and Methods. The purified, acetone washed starch was used to separate large A and small B granules with Percoll gradient centrifugation method from all genotypes. The separated A, B granules and starch were washed extensively to remove proteins loosely bound to the granule surface. A 50 mg aliquot of purified starch, A and B granules were boiled in 1 ml of SDS loading buffer, and 40 µl of the supernatant from the boiled sample loaded onto 4–12 % acrylamide gradient gels and 10 % acrylamide SDS gels. Following electrophoresis, gels were electroblotted to nitrocellulose membrane and developed with various specific anti-wheat antibodies as indicated (A): SSI, SSIIa, SSIII, SSIV, (B): SBEI, SBEIIa, SBEIIb, (C): SP, ISO1 and GBSS. Each Figure is labelled with respective antibody and type of granules used either starch, A- or B-. 

Figure 3.6: Starch, A- and B-granules bound proteins.
bound proteins, Pro-Q Diamond staining was used. GBSS is the most abundant protein in the starch granule and this protein showed very strong staining for protein phosphorylation in all genotypes including, surprisingly, the *waxy* genotype (Figure 3.7B) which has significantly reduced amount of GBSS (Figure 3.7A). In barley, granule bound SSIIa, SBEIIa and SBEIIb migrate similarly on SDS gels (Figure 3.7A) and all appear to be phosphorylated (Figure 3.7B) which makes it difficult to identify individual phosphorylated proteins. However, study of branching enzyme mutants (*sbeiia* and *sbeiib*) showed that SSIIa and SBEIIb are phosphorylated in their granule bound states. Similarly SSI also appeared to be phosphorylated (Figure 3.7B). In the branching enzyme mutants *sbeiia* and *sbeiib*, SP is also granule localized and phosphorylated (Figure 3.7B). Other proteins SSIII and SBEIc were entrapped in the granule (Figure 3.7A) but did not appear to be phosphorylated. Physiochemical and biochemical data of remaining *amo1* and *sex1* mutants are presented in Appendices.
Figure 3.7: Starch granule bound proteome phospho-proteome.
To determine granule bound phospho-proteome equal amounts of starch from all genotypes were run onto 4–12% acrylamide gradient gels, and stained with Pro-Q Diamond. Following Pro-Q Diamond staining the same gel (B) was subject to silver staining (A) to visualize all bands. The bands visualized with silver stain are numbered as (1, SSIII; 2, SBEIc; 3, SP/unknown; 4, 5, 6, SBEIib; SSIIa; SBEIIa; SBEI 7, SSI and 8, GBSS).
3.4 Discussion

This study presents a detailed biochemical characterization of different barley genotypes possessing mutations in isoforms of starch synthesizing enzymes resulting in high-amylose starches and compared to a reference and a waxy genotype. Barley starch is composed of two types of granules (A- and B-) and the results suggest that association of different proteins within the growing starch granules is a reflection of the formation of stromal functional heteromeric protein complexes, involved in amylopectin biosynthesis, which become granule-localized. Furthermore, as will be discussed, there is evidence that different protein complexes are partitioned into A and B granules differentially.

All the mutants have increased “apparent amylose” compared to reference genotype and waxy (Table 3.1). Interestingly genotypes with similar high amylose content showed differences in RS content, with the sbeiib’ mutant exhibiting significantly higher RS than sbeiia’. In maize lack of SBEIIb leads to longer internal chains length and less frequently branched outer chains in amylopectin compared to wild-type starches (Hilbert and MacMasters, 1946; Banks et al., 1974; Klucinec and Thompson, 2002), giving rise to starches which are characterized as “high amylose” even though the modification is in amylopectin. In barley, elimination of either SBEIIa or SBEIIb does not result in a significant alteration in the number of branches in the amylopectin molecules nor in higher proportions of longer glucan chains (Ahmed et al., 2013, manuscript in preparation). Interestingly, however, the branch frequency of amylose was found to be increased in sbeiib’ (Regina et al., 2010). The apparent reason for these differences between cereals may be the expression pattern of SBE isoforms which are different in barley endosperm than other cereals like maize and wheat. In maize,
expression of SBEIIb has been estimated as 50 times higher than SBEIIa (Gao et al., 1997). In contrast SBEIIb is present at much lower levels than SBEIIa in wheat endosperm (Gao et al., 1997; Morell et al., 1997; Regina et al., 2005). However, in barley endosperm both proteins are expressed at approximately equal levels (Sun et al., 1998). Therefore it is suggested that in barley loss of either of SBEII isoform is, to some degree, compensated by the expressed isoform. Yao et al. (2004) and Regina et al. (2010) suggested an inhibitory role of SBEIIb upon SBEI, and in the absence of SBEIIb, SBEI potentially adds more branches onto amylose. It is possible that this branched amylose with longer glucan chains is more resistant to α-amylase digestion and consequently \( sbeii^{-} \) exhibits increased RS content compared to \( sbeia^{-} \), as observed in the present study. In the \( sbeia^{-} \) mutant, amyllopastidic SP (Pho1) has normal activity while in \( sbeib^{-} \) its activity is undetectable. These variations did not affect starch granule phenotype drastically, although the starch content of \( sbeib^{-} \) was significantly reduced compared with the \( sbeia^{-} \) mutant (Table 3.1).

Our results indicate that the reference genotype has lower (\( T_{o} \)) and higher (\( \Delta H \)) than high amylose genotypes, with the exception of \( sbeia^{-} \) which has higher (\( \Delta H \)) than reference genotypes. Previously You and Izydorczyk, (2007) reported that (\( \Delta H \)) values were associated with the amount of double helical domains of amylopectin and single helical structure (amylose–lipid complexes). Similarly others reported that (\( \Delta H \)) values reflect the loss of double helical order rather than loss of X-ray crystallinity (Zheng et al., 1998; Morrison et al., 1993; Cooke and Gidley, 1992; Li et al., 2001; Yoshimoto et al., 2000; You and Izydorczyk, 2007). Tester and Morrison, (1990) proposed that (\( \Delta H \)) provides an estimate of crystallinity, that (\( T_{p} \)) can be used to measure the crystallite
quality, and that (To) has a significant correlation with average branch chain length (Jane et al., 1999; Yuan et al., 1993; Wang et al., 1993 and Shi and Seib, 1992). The apparent loss of soluble SSI activity in SBEII mutants, even though the protein is present, may have contributed to a reduction in the proportion of short chains in amylopectin, leading to a reduction in crystallinity and consequently smaller (ΔH) values compared to reference genotype. In this regard the effect of loss of SBEIIb was more severe than SBEIIa with respect to (ΔH) (Table 3.2). These observations suggest that SBE mutations in barley are having different effects on starch structure and organization compared to the reference genotype.

Western blot analysis of whole cell extracts, amyloplast stroma and starch granules showed that the sex6 mutant lacks SSIIa protein consistent with previous results (Morrell et al., 2003). Similarly sbeiia- demonstrates reduced expression of SBEIIa protein while sbeiib- has small amount of expressed SBEIIb protein. It has previously been reported that HAG amo1 does not lack SSIII protein but has a leucine to arginine residue substitution in a conserved domain at amino acid 1480 compared to the wild-type protein (Li et al., 2011). This substitution results in reduction in the activity of SSIII protein compared to wild-type (Li et al., 2011). The waxy genotype is not a null mutant and has significantly reduced expression of GBSSI. Mutations in SBEIIa and SBEIIb appear to result in intriguing pleiotropic effects with respect to the measureable activities of SP (Pho1) and SSI.

Both sbeiia- and sbeiib- mutants appear to possess less soluble plastidial SP protein compared to reference genotype (Figure 3.1). Previously (Liu et al., 2009) observed a decrease in SP activity in soluble extracts of a maize ae- mutant lacking
SBEIIb and wild-type. In SBEII mutants, activity of SSI was not detected (Figure 3.2A) even though the SSI protein was present in similar amounts to the reference genotype (Figure 3.2B). This suggests that SSI activity is in some way dependent on the presence of both SBEII isozymes, since each genotype is mutated in only one or other isoform. In the case of branching enzyme activities, the activity of SBEIIb appeared reduced in the sbeii\textsuperscript{b} mutant on zymograms, but the effect on SBEIIa activity in the sbeii\textsuperscript{a} mutant was less obvious (Figure 3.3). In sbeii\textsuperscript{b} plants, negligible activity of plastidial SP (Pho1) was found (Figure 3.4A) even though plastidial SP protein was present (Figure 3.4B). Whereas, in the sbeii\textsuperscript{a} mutant activity of SP (Pho1) remained unaltered (Figure 3.4A). This suggests that the presence of SBEIIb is required for SP (Pho1) activity. In previous reports of ae- mutants of maize and rice, a decrease in soluble SS activity, particularly SSI, from whole cell extracts compared to wild-type has been observed (Nishi et al., 2001; Nakamura et al., 2012). However, in contrast to these observations Liu et al. (2009) reported increased SS activities from amyloplast stromal proteins of the ae- mutant of maize compared to wild-type, and a decrease in plastidial SP (Pho1) activity from ae- mutant. Nishi et al. (2001) observed no change in SP activity in the endosperm of rice ae- mutants compared to wild-type. In amo\textsuperscript{l} and sex\textsuperscript{6} plants, pleiotropic affects on SP are intriguing since in these mutants SP activity was readily detected on non-denaturing gels (Figure 3. 4A), where as the protein itself was not detectable (Figure 3.4B) even though the presence of the SP protein was confirmed under denaturing conditions (Figures 3.1 & 3.6). This could arise from conformational changes or perhaps by association with other proteins leading to the antigenic epitopes being masked under non-denaturing conditions.
Given the pleiotropic effects observed on enzymes activities, we investigated whether any differences in protein-protein interactions occurred in the genotypes studied. Co-immunoprecipitation experiments with different antibodies resulted in purification of different types of protein complexes. For example, in the reference genotype, a protein complex containing SSI, SSIIa, and either of SBEIIa or SBEIIb could be precipitated with SSI, SBEIIa and SBEIIb antibodies (Figure 3.5). It is argued that either SBEIIa or SBEIIb can interact with SSI and SSIIa, resulting in the formation of two distinct protein complexes in the reference genotype. In the branching enzyme mutants sbeii\textsuperscript{a} and sbeii\textsuperscript{b}, co-precipitation of SSI, SSIIa, SBEI and SP was observed when using anti-SBEIIb or anti-SBEIIa antibodies (Figure 3.5). Importantly it was found that SBEI and SP were also granule-localized in these two mutants and were present only in A-granules and not in B granules (Figure 3.6). In these two mutants, a protein complex comprising of SSI, SSIIa and whichever SBEII is expressed, was purified with anti-SSI antibody. Anti-SSI antibody did not co-precipitate SBEI and SP (as mentioned earlier) in these mutants, suggesting that two types of enzyme complexes are formed. One complex comprising SSI, SSII, and whichever of the SBEII proteins is available is found in A- and B-granules. A second multi-enzyme complex comprising SBEI and SBEIIb (or IIa) and SP, similar to that previously reported in wheat (Tetlow et al., 2004), is only found in A-granules. This is distinct from the SSI/SSII/SBEII complex, consistent with the observation that SSI antibodies do not precipitate SP or SBEI. SP/SBEI containing complexes became entrapped in the A-granules but not B-granules (Figure 3.6). The observation that the two types of stromal multi-enzyme complexes described are differentially partitioned between the two types of starch granules implies that they have
distinctive roles in the formation of A- and B- granules at least in the branching enzyme mutants.

In the amo1 mutant, a protein complex containing SSI, SSIIa, SBEIIa and SBEIIb was co-precipitated with anti-SSI, anti-SBEIIa and anti-SBEIIb antibodies. As anti-SBEIIa precipitates SBEIIb protein and vice versa, so it could be argued that may be SBEIIa and SBEIIb form a dimer which is precipitated with either of the SBEII antibodies in addition to distinct SSI/SSIIa/SBEIIa or SSI/SSIIa/SBEIIb protein complexes. There is also a possibility that SBEIIa and SBEIIb form a larger protein complex with SSI and SSIIa, which was co-precipitated with anti-SSI, anti-SBEIIa and anti-SBEIIb antibodies. In the sex6 mutant, none of the proteins appear to interact and none are detected (apart from GBSS) in the granule, or are present only at much lower amounts (Figures 3.5 & 3.6).

Analysis of granule bound proteins revealed that SSI, SSIIa, SBEIIa, SBEIIb and GBSSI are granule localized in different barley mutants. In SBEII mutants, it appears the remaining SBEII isoform which is expressed is found in higher amounts in the granule in comparison to the reference line possibly indicating its role as a “substitute” for the missing protein or as a part of complex with SBEI and SP (Figure 3.6). In sbeii\textsuperscript{b} plants, more SP appears to be partitioned to the granule than for sbeii\textsuperscript{a} barley (Figure 3.6C). This may contribute to loss of detectable soluble SP (Pho1) activity in zymograms of sbeii\textsuperscript{b} mutants (Figure 3.4). SBEI\textsubscript{c} was found only in the A-granules in all genotypes (as shown previously Peng et al., 2000). SSI, SSIIa, SBEIIa, SBEIIb and GBSSI are also routinely found within the starch granules of other cereals like maize, wheat, barley and rice (Rahman et al., 1995; Morell et al., 2003; Boren et al., 2004; Regina et al., 2005;
Umamoto and Aoki, 2005; Liu et al., 2009, 2012a, 2012b). A small amount of SSIII and an unknown protein of 110 kDa were also found in the starch granules of wild-type and the mutants studied here. All of these starch granule associated proteins, which are involved in starch biosynthesis, are also components of identified soluble protein complexes in wheat and maize amyloplasts (Tetlow et al., 2008; Hennen-Bierwagen et al., 2008; Liu et al., 2009, 2012a, 2012b). In the sex6 mutants, due to lack of SSIIa, no soluble protein complex was detected (Figure 3.5A) and therefore none of the above mentioned proteins was found in the granule except GBSSI. Thus it is suggested that SSIIa plays an important role in trafficking other proteins to the granule by forming protein complexes. Similar results have been reported recently by Liu et al., (2012b) in the sugary2 (su2) mutant of maize. A summary of different protein complexes and their association with granules is presented in Figure (3.8).

3.5 Conclusion

This study has provided a detailed biochemical analysis of barley mutants with mutations in key amylopectin synthesizing enzymes and their relationship to altered physiochemical properties of starch. In different mutants, amylopectin synthesizing enzymes form phosphorylation-dependent functional protein complexes in various combinations in the amyloplast stroma compared to the reference genotype. These protein complexes become entrapped in the starch granules and are reflected as alterations in the starch granule proteome. Due to loss of either SBEII isoform SP and SBEI become part of a heteromeric protein complex. This protein complex containing SP and SBEI is arguably involved primarily in the synthesis of A-granules, reflected in the A-granule proteome, and not B-granules. Such alterations may also affect the
physiochemical properties of starch, since \textit{sbeii\textsubscript{b}} mutants have significantly higher RS content than \textit{sbeii\textsubscript{a}} even though the apparent amylose content is similar. This could be due to an inhibitory role of SBEII\textsubscript{b} on SBEI. In the \textit{amo1} mutant HAG \textit{amo1} protein complex comprising SSI, SSII\textsubscript{a}, SBEII\textsubscript{a} and SBEII\textsubscript{b} was co-precipitated. In this mutant starch with altered physiochemical properties was observed compared to reference genotype. Similarly mutation in SSII\textsubscript{a} prevented formation of heteromeric protein complexes which is reflected in the lack of other amylopectin synthesizing enzymes in the granule proteome of this mutant.
Figure 3.8: Protein–protein interactions formed between amylopectin-synthesizing enzymes in barley endosperm.

In the reference genotype (A), the major forms of SBEII (SBEIIa and SBEIIb) form distinct, phosphorylation-dependent protein complexes with SSI and SSIIa. In the branching enzyme mutants (B & C), two distinct protein complexes consisting of SSI/SSIIa and expressed SBEII, and SP/SBEI and expressed SBEII are formed and became entrapped in granules. SP/SBEI/SBEII is partitioned to A-granules only. Loss of SSIIa leads to the absence of other amylopectin synthesizing enzymes from the granules (D). In amo1 mutant (E) protein complex consisted of SSI, SSIIa, SBEIIa and SBEIIb was co-precipitated. It is possible that SBEIIa and SBEIIb form distinct complexes with SSI and SSIIa as described for reference genotype. It is possible that SBEIIa and SBEIIb form a dimer which is co-precipitated along with the above mentioned distinct complexes, or that SBEIIa and SBEIIb form a larger complex with SSI and SSIIa. A- and B- granules are presented as grey circles. In the sex6 mutant severely deformed A-granules are presented. Coloured dots present different protein complexes and their distribution in A- and B- granules. GBSS is present in both A- and B- granules and not represented here.
Chapter 4: Physiochemical and biochemical properties of starch and its relationship to granule size distribution in barley genotypes from diverse genetic back grounds
4.1 Introduction

The semicrystalline, water-insoluble starch granule is of significant agricultural and commercial importance. This water-insoluble, osmotically inactive granule confers advantages to plants for short- and long-term carbon reserves. This stored starch not only provides energy reserves for the next generation but is also used as food, feed, biofuels and raw material for many industries. Starch is made up of two glucan polymers: amylose and amylopectin. Amylose is sparsely branched and contributes only 20-30 % of the total starch and makes the amorphous portion of the granule while amylopectin is highly branched and the major constituent (70-80 %) of starch granules forming partially semicrystalline structures (Hizukuri, 1996; Lemke et al., 2004). In amylopectin, the distribution of glucan chains and branch point clustering allow short linear chains to pack together efficiently as parallel, left-handed, double helices. The organized array of clusters is the basis of the semicrystalline nature of much of the starch granule, resulting in a water-insoluble granule. The size, shape and number of starch granules vary within and among species (Shapter et al., 2008).

In cereals, such as wheat and barley, starch granules can be distinguished based on size and shape as, A-type (15-45 µm, lenticular), B-type (5-15 µm, round) and C-type (1-5 µm, polygonal). Along with other features, granule morphology has an important impact on starch physiochemical properties (Da Silva et al., 1997; Lindeboom et al., 2004) and granule size determines most of the potential food and industrial applications of starch (Ji et al., 2004). Starch with a greater proportion of small granules is suitable for use as a fat substitute, paper coating and as a carrier in cosmetics (Lindeboom et al., 2004). However, starch with predominantly large granules can be used in the
manufacture of biodegradable plastic films, carbonless copy paper and in other industries (Lindeboom et al., 2004). Starches extracted from different barley genotypes vary widely in structure, composition and properties (Kang et al., 1985; Morrison et al., 1993; Lorenz & Collins, 1995; Song & Jane, 2000; Yoshimoto et al., 2000). Besides variations in morphology, size, and origin, large and small starch granules in wheat and barley also show differences in characteristics and properties with regard to chemical composition. These include amylose content, amylose-lipid complex and phosphorus contents (Raeker et al., 1998; Shinde et al., 2003; Geera et al., 2006; Ao and Jane, 2007), molecular structure (Sahlstrom et al., 2003), gelatinization temperature, and retrogradation (Peng et al., 1999; Singh and Kaur, 2004). Large A-granules of wheat show an increased enthalpy of gelatinization, lower gelatinization temperatures, increased retrogradation (Peng et al., 1999; Singh and Kaur, 2004) and softer textured flours compared to smaller B-granules (Gaines et al., 2000). Wheat starches possessing different granule sizes exhibited different degrees of susceptibility to enzymatic hydrolysis, as well as thermal and pasting properties (Morrison & Gadan, 1987; Peng et al., 1999; Liu et al., 2007; Raeker et al., 2007). Along with granule size, differences in amylose content, protein content, and branch chain length of amylopectin in A- and B-type starch granules are also major factors responsible for differences in digestibility and other functional properties of starch (Liu et al., 2007).

The biosynthesis of starch is controlled by different enzyme classes: adenosine diphosphate glucose pyrophosphorylase (AGPase), five classes of starch synthases and two classes of starch branching enzymes. Starch synthases can be distinguished as granule-bound starch synthase (GBSS) and soluble starch synthases (SSI, SSII, SSIII and
Different SS classes have different isoforms, two GBSS isoforms (GBSSI and GBSSII), three SSII isoforms (SSIIa, SSIIb and SSIIc) two SSIII isoforms (SSIIIa and SSIIIb) and two SSIV isoforms (SSIVA and SSIVb) (Hirose and Terao, 2004; Fujita et al., 2007). Starch branching enzymes (SBEs) in cereals include SBEI, SBEIIa and SBEIIb. In addition, starch debranching enzymes (DBEs) such as isoamylases (ISOI, ISOII and ISOIII) and pullulanase (PU), and starch phosphorylase (SP [Pho1 and Pho2]) are also involved in starch metabolism (Ball et al., 1996; Zeeman et al., 1998).

GBSS is primarily involved in amylose biosynthesis, while other classes of enzymes such as SSs, SBEs, and DBEs are primarily responsible for amylopectin biosynthesis. The relative amounts of SS and SBE isoforms differ among different organs of the same plant or among species (Ball and Morell, 2003; Li et al., 2003; Patron and Keeling, 2005; Leterrier et al., 2008). The integrated activities of these different enzymes result in semicrystalline water insoluble starch granule. The interaction between starch synthesizing enzymes is a complex process, partly due to cyclic substrate-product relationships. For example, SSs produce linear glucan chains which become substrates for SBEs to create branches in amylopectin, while shorter glucan chains of DP 6-7, produced during branch formation, become substrates for SSs (Zeeman et al., 2007; Liu et al., 2009). Similarly the product of SBE activity is also a substrate for DBEs (Szydlowski et al., 2011). Apart from their interdependence, these proteins are also subjected to post-translational modifications such as, protein phosphorylation and protein complex formation (Tetlow et al., 2004, 2008; Liu et al., 2009, 2012a). Previously, it was suggested that during granule formation these complexes become entrapped as functional protein complexes (Liu et al., 2009). The above observations illustrate that starch
biosynthesis is a complex process which is controlled at multiple levels of organization. Thus, it is important to understand the biochemical basis of starch physiochemical properties, and the regulation of its biosynthesis in the endosperm, as key steps to improve and modify starch properties for a wider variety of applications (Stamova et al., 2009). An understanding of the genetic and biochemical bases of multimodal starch granule size distribution is of great interest, in crop species like wheat and barley because each type of granule has varied physiochemical properties and consequently different end uses (Sahlstrom et al., 1998; Lindeboom et al., 2004; Yonemoto et al., 2007). There are a number of factors involved in the differentiation of starch granules, including multiple and complex genetic controls and biochemistry, size and numbers of plastids, environmental conditions during seed development, availability of malto-oligosaccharides and the integrated effect of all these factors (Shapter et al., 2008).

Recently, understanding the synthesis of barley starch has gained more attention due its ability to replace other starches and because of its physiochemical properties and potential to be used in different industries (Asare et al., 2011). Although much information is available regarding differences in physiochemical properties of starches with varied granule sizes, little is known about the underlying biochemical processes. In this study, barley genotypes with different proportions of large and small granules, possessing different physiochemical properties, have been used to examine the relationship among the starch granule proteome and the physiochemical properties of starch. As described earlier, from a wide range of genotypes from different genetic backgrounds, amylose/RS contents were measured (Chapter 2). From these genotypes, a group of high amylose mutants with known mutations in amylopectin synthesizing
enzymes was selected for detailed biochemical analyses (Chapter 3). The remaining genotypes with no apparent mutations in amylopectin synthesizing enzymes, but with variations in starch physiochemical properties were selected in this study for detailed physiochemical analyses of starch and analysis of granule proteome.
4.2 Material and Methods

4.2.1 Plant material

Nineteen different barley genotypes from different genetic backgrounds were employed. Table (4.1) outlines the different characteristics/description of the barley genotypes. Different barley genotypes were grown at the University of Guelph under glasshouse conditions in a soil medium containing, Turface soil (Profile Products), Turface MVP (Profile Products), lime, peat moss, and Nutricote (14-14-14; Morton’s Horticultural Products) in a ration of 3:1:0.01:1:0.01 (w/w). Plant growth conditions were maintained at a temperature of 15-25 °C. Barley was also grown at the University of Guelph fields, Elora, under natural conditions on a clay loam soil. Each year, planting of barley started in the first week of May.

4.2.2 Isolation of starch granules

Mature dry barley seeds weighing ≈ (55) g were completely ground to flour with a homogenizer (Retsch® MM301) in liquid nitrogen. Equal amounts of flour (= 50 g) were suspended in 150 ml of rupturing buffer containing 100mM Tricine-KOH, pH 7.8, 1mM Na₂-EDTA, 1mM DTT and 5mM MgCl₂ at 4°C. The suspension was vortexed (Eppendorf, vortex) for 5-10 minutes to make a uniform suspension and left for 5-10 minutes on ice. The well mixed suspension was sieved through six-layers of cheese cloth to remove debris and bran. Excess buffer was added to wash traces of starch from the cheese cloth. The sieved milky suspension ≈ 450-500 ml containing starch, fine debris and bran was centrifuged at 16000 g for 15 min. The supernant was discarded and pellet was washed in ≈ 100 ml wash buffer containing 50 mM TRIS-acetate, pH 7.5, 1 mM Na₂-EDTA, and 1 mM DTT. The resuspended pellet was centrifuged at 6000 g for 5 min.
This washing step was repeated 5-7 times until a thick yellow layer of debris was left on the top of starch. The yellow layer, containing very fine pieces of debris and bran, was completely removed with a spatula minimizing starch losses. After removing debris, the starch was again washed and centrifuged twice with washing buffer. Purified starch was washed three times with acetone, followed by three washes with 2 % (w/v) sodium dodecyl sulphate (SDS), 3 times with distilled water, and then dried using a speed vacuum (Eppendorf, vacufuge™) at 25 °C for 3 h.

4.2.2.1 Separation of A- and B-type starch granules

A- and B-/C-type starch granules were separated using a method previously described by Peng et al. (1999). Approximately 0.5 g of the dried, acetone washed starch was suspended in 5 ml of dH2O. This starch suspension was then carefully laid on top of 10 ml, 70 % (v/v) Percoll in a 15 ml tube, avoiding precipitation through the underlying Percoll, followed by centrifugation at 10 g for 10 min at room temperature. The larger A-granules passed through the Percoll gradient and precipitated at the bottom of the tubes (along with some B-granules). The majority of the smaller B-/C-granules remained in suspension. After centrifugation, supernatant (containing B-granules) was removed to fresh tubes. The pellet, containing A- and some of B-granules, was washed (twice) with dH2O by resuspension and centrifugation at 4000 g for 5 min. After centrifugation the supernatant was discarded and the pellet was resuspended in 5 ml of dH2O and laid on top of 10 ml of 70 % (v/v) Percoll. This process of A- and B-/C-granule separation (70 % (v/v) Percoll gradient centrifugation at 10 g for 10 min, and washing of the pellets in dH2O) was repeated for 3 cycles, and supernants from each cycle were pooled. The pellet containing predominantly A-granules was then further purified by centrifugation through
100 % (v/v) Percoll as described for 70 % Percoll, to produce a homogeneous A-granule population in the resultant pellet. Similarly, the supernant from this step was pooled with the supernant from 70 % Percoll gradient separation and centrifuged at 4000 g for 5 min, and the supernatant discarded. The pellet was washed (twice) with dH2O by resuspension and centrifugation at 4000 g for 5 min. This pellet comprised B-/C-granules. To separate C-granules of size < 5 µm, sufficient amount (∼1 g) of partially purified B-granules were suspended in 50 ml of water and left for ∼40-50 min to precipitate A-granules and most of the B-granules, so that very small C-granules (<5 µm) remained in the supernatant. The supernant from this suspension was collected in a separate tube, centrifuged and washed in the same way as described earlier. These three types of granules were used for further experiments.

4.2.3 Isolation of starch granule-bound proteins

The isolation of protein bound within the internal matrix of the starch granules (as opposed to proteins present on the surface of starch granules, which can be removed by extensive washing with SDS) was carried out by the method previously described by Tetlow et al. (2004) and Liu et al. (2009). The acetone and SDS washed starch, either from mature seed, amyloplast lysates, or whole cell extracts was washed with 1 % (w/v) SDS. This washing step was repeated to remove any traces of proteins attached to the surface of starch granules. To extract the starch granule-bound proteins, an equivalent amount of starch (∼50 mg) was boiled in 1000 µl SDS loading buffer containing, 62.5 mM TRIS-HCl, pH 6.8, 2 % (w/v) SDS, 10 % (w/v) glycerol, 5 % (v/v) β-mercaptoethanol and 0.001 % (w/v) bromophenol blue. Boiled samples were centrifuged at 13 000 g for 10 min and the supernatant used to determine total granule-bound protein.
content, as well as for SDS–PAGE and immunodetection of granule-bound proteins.

**4.2.4 SDS–PAGE and immunodetection of granule-bound proteins**

For proteomic analysis of starch A- and B-granules, equivalent amounts of starch, A- and B-granules were used to extract protein in the same way as described in section (4.2.3). For SDS–PAGE and immunoblotting, either SDS gels with 10 % (w/v) acrylamide or pre-cast NUPAGE Novex 4–12 % BISTRIS acrylamide gradient gels (Invitrogen Canada, catalogue No. NP0335BOX) were used, following the manufacturer’s instructions. At room temperature, gradient gels were run in a MOPS-based running buffer prepared according to the manufacturer’s (Invitrogen) instructions. For immunoblotting, following electrophoresis, gels were transblotted onto nitrocellulose membranes (Pall Life Science), and blocked at room temperature in 1.5 % (w/v) BSA for 15 min with gentle shaking. The SSI, SSIIa, SSIII, SSIV, SBEIc, SBEIIa, SBEIIb, SP, ISO1 and GBSS wheat-antibodies were used at dilutions as follows: anti-SSI 1:2000, anti-SSII 1:2000, anti-SSIII 1:2000, anti-SSIV 1:2000, anti-SBEI 1:5000, anti-SBEIIa 1:5000, anti-SBEIIb 1:5000, anti-SP 1:1000 and anti-ISO 1:1000 in 1.5 % (w/v) BSA. Different antibodies to wheat proteins were used according to specifications described earlier (Li et al., 1999; Rahman et al., 2001; Morell et al., 2003; Regina et al., 2005; Bresolin et al., 2006; Tetlow et al., 2008). Alkaline phosphatase (APase)-conjugated goat anti-rabbit IgG (Sigma) was used as a secondary antibody.

**4.2.5 Silver staining**

Silver staining was performed according to a procedure described earlier Mortz et al. (2001). Following electrophoresis, the gel was kept in a well-cleaned glass plate in 50 ml fixing solution buffer containing, 50 % methanol (v/v), 5 % acetic acid (v/v) for 20
min on a shaker, followed by washing in washing buffer (50 % methanol [v/v]) for 10 min on a shaker. Following washing, the gel was kept in distilled water for at least 1 h, or overnight, with occasionally changing water. The gel was then immersed in sensitizing buffer containing 0.02 % Na$_2$S$_2$O$_3$ (w/v) for 1 min and washed twice in distilled water for 1 min each. The gel was incubated in ice-cold silver nitrate buffer (0.1 % AgNO$_3$ [w/v]) for 20 min at 4°C followed by two washings with distilled water 1 min each at room temperature. Finally, the gel was developed by adding developing solution containing 2 % Na$_2$CO$_3$ (w/v), 0.04 % formalin (v/v) for 3-5 min and then fresh developing buffer was added and gel was stained until the proteins bands were visualized. Staining was stopped by adding a solution containing 5 % acetic acid [v/v] for 5 min and the gel was transferred to distilled water.

4.2.6 Estimation of protein phosphorylation by Pro-Q diamond staining

Equal amounts (1.5 mg) of starch from all genotypes were loaded in each lane onto 4–12 % acrylamide gradient gels (Invitrogen) and were separated by SDS-PAGE according to manufacturer’s instructions. Following electrophoresis, gels were fixed in 50 % methanol, 10 % acetic acid overnight in dark. The gels were then washed three times with ddH20 and then incubated in Pro-Q Diamond stain according to the manufacturer’s instructions (Molecular Probes, Life Technologies) in the dark for 90 min. Gels were then destained three times each for 30 min in distaining solution containing 20 % acetonitrile, 50mM sodium acetate, pH 4.0 and then washed with water two times each 5-7 min. Gels were visualised using a Typhoon scanner (Amersham Biosciences, Artisan Technology Group, IL, USA) with an excitation wavelength of 530 nm and emission filter at 580 nm.
A phospho-protein marker was used as standard and phosphorylated bands appeared black.

4.2.7 Measurement of total granule-bound protein content

To measure total granule-bound protein equivalent amounts (50 mg) of acetone-washed starch were used. Protein was extracted by boiling starch in 2 % (w/v) SDS for 5 min followed by centrifugation at 13500 g for 10 min (see above). Total granule-bound protein in the supernatant from three biological repeats was quantified with Pierce® BCA protein assay kit (Thermo Scientific, catalogue No. 23227) according to manufacturer instructions, using BSA as a standard.

4.2.8 In-gel protein quantification

The amount of individual protein from starch, A- and B-granules was measured following silver staining by using Molecular Imager® Gel Doc™ XR+ and Chemi Doc™ XRS+ Systems with Image Lab™ Software, version 3 (BioRad Laboratories Inc.) according to manufacturer’s instructions, using protein markers (Bio Rad). In-gel protein content was determined using 100-1500 ng BSA to generate a linear standard ranges for calibration. Following electrophoresis of triplicate samples, gels were silver stained and the intensity of each band determined by imaging and protein content calculated.

4.2.9 Starch gelatinization

Thermal analyses of starches were performed as described by Liu et al. (2007) using a differential scanning calorimeter (DSC) (2920 Modulated DSC, TA Instruments, New Castle, DE, USA). For gelatinization and retrogradation of starches, this system was equipped with a refrigerated cooling system (RCS). Samples of starch granules were weighed into high volume pans. A micropipette was used to add distilled water to
produce suspensions with 70 % moisture content. Approximately 20 mg of starch was used per sample. The sealed pans were equilibrated overnight at room temperature before heating in the DSC. Measurements were taken at a heating rate of 10°C/min from 5°C to 180°C. Calibration of the instrument was performed using indium and an empty pan as reference. To measure the enthalpy ($\Delta H$) of phase transitions from the endotherm of DSC, thermograms based on the mass of dry solid, software (Universal Analysis, v.2.6D, TA Instruments) were used. Peak temperature ($T_p$) of endotherms was also measured from DSC thermograms. For retrogradation, the heated starch from the above procedure was cooled to 5°C. Once the temperature reached 5°C, sample was immediately removed from the DSC and stored at 5°C. After two weeks, stored samples were heated from 5 to 180°C at 10°C/min and, based on dry, solid mass, the enthalpy ($\Delta H$) and peak temperature ($T_p$) of the endotherm were measured from DSC thermograms.

4.2.10 High-Performance Anion Exchange Chromatography (HPAEC)

To measure the chain length distribution of barley starch, HPAEC was used as described by (Liu et al., 2007). Isoamylase-debranched starch granules were dispersed in 2 ml of 90 % (v/v) DMSO (5 mg/ml) by stirring in a boiling water bath for 20 min. The sample was left to cool to room temperature, followed by addition of 6 ml methanol, mixing and incubation in an ice bath for 30 min. The suspension was centrifuged (1,000 × g for 12 min), and the pellet dissolved in 2 ml, 50 mM sodium acetate buffer (pH 3.5) by continuous stirring in a boiling water bath for 20 min. The sample was removed and equilibrated at 37 °C, after which 5 μL isoamylase (EN102, 68,000 U/mg of protein, Hayashibara Biochemical Laboratories, Okayama, Japan) was added and digested for 22 h. To inactivate the enzyme, the sample was boiled for 10 min. The sample was cooled
and 200 μL of debranched sample was diluted with 2 ml of 150 mM NaOH. The diluted sample was filtered (0.45 μm nylon syringe filter) and injected into the HPAEC-PAD system (50 μL sample loop). The HPAEC-PAD system consisted of a Dionex DX 600 equipped with an ED50 electrochemical detector with a gold working electrode, GP50 gradient pump, LC30 chromatography oven, and AS40 automated sampler (Dionex Corporation, Sunnyvale, CA, USA).

4.2.11 Granule size distribution of different genotypes

Granule size, number and surface area of starch from different genotypes were measured by means of laser scattering using the Mastersizer (Malvern Mastersizer 2000, Malvern Instruments Ltd., Worcestershire, UK). For each measurement, acetone washed starch (≈ 100mg) was weighed into glass tubes and suspended with 10 ml of distilled water. Sample concentrations were within equipment recommendations and the refractive indices of 1.31 for water and 1.52 for starch were used as standards and distribution was measured as both the percentage volume and percentage number.

4.2.12 Granule morphology

To study starch granule morphology in detail, SEM was performed on a field emission scanning electron microscope (S-4500, Hitachi, Tokyo, Japan) equipped with Quartz PCI digital image acquisition software (Quartz Imaging, Vancouver, BC, Canada) as described by Liu et al. (2007). The acetone dried barley starch samples were sprayed on a metal plate covered with double-sided adhesive. The samples were coated with gold using a Polaron SC500 sputter coater (Quorum Technologies, East Sussex, UK). The samples were examined at 10 kV accelerating voltage and representative micrographs were taken for each sample at different magnifications, according to requirement.
4.2.13 Thousand grain weight (TGW)

1000 grains of each genotype were counted and weighed to 0.1 mg using a tabletop electrical balance (Denver Instrument, Bohemia, NY, USA). The mean of three biological replicates was used in statistical analysis (de Rocquigny, 2011).

4.2.14 Starch content

The amount of starch in barley seeds from the different genotypes using three biological replicates was determined as described by Tetlow et al., (1994) for wheat endosperm.

4.2.15 RS Content

RS was measured by using, 100 mg acetone washed starch from three biological replicates with Megazyme resistant starch assay kit (K-RSTAR 08/11, Megazyme International, Ireland) following manufacturer’s instructions.

4.2.16 Amylose content

The apparent amylose content was measured by using, 25 mg acetone washed starch from three biological replicates with Megazyme amylose /amylopectin assay kit (K-AMYL 07/11, Megazyme International, Ireland) following manufacturer’s instructions.

4.2.17 Mass spectrometric analysis

In-gel digestion of protein bands was carried out with trypsin and peptides for MS were prepared according to the protocol described by Tetlow et al., (2008). Using a hybrid Q-TOF spectrometer (Micromass) interfaced to a Micromass CapLC capillary chromatograph, Tandem electrospray mass spectra were recorded according to the method previously described (Tetlow et al., 2004).
4.2.18 Statistical analysis

For one way analysis of variance (ANOVA) and least significant difference (LSD), Statsoft’s Software, Statistica (http://www.statsoft.com) was used. For principal component analysis (PCA), XLSTAT (http://www.xlstat.com/en/) was used and biplots were generated.
4.3 Results

4.3.1 Physical characteristics of seed

4.3.1.1 Seed morphology characteristics

Seed characteristics, including seed length, width, thickness and length to width ratio were measured. Different genotypes showed significant differences in different parameters of seed. Seed length varied from 7.3 to 9.4 mm among different genotypes with lowest seed length exhibited by McGwuire and highest by genotype 083211-122, respectively. Seed width of different genotypes varied between 3.4 to 4.4 mm. Similarly, differences in seed thickness among different genotypes were observed. Details of different seed parameters are shown in Table (4.1).

4.3.1.2 Thousand grain weight (TGW)

Significant differences were observed among genotypes for thousand grain weight Table (4.1). The genotype 083211-120 possessed highest 72.8 g TGW while genotype Emperor had the lowest, 40.9 g TGW. Both waxy genotypes (CDC Fibar and CDC Rattan) had lower TGW than the reference genotype (OAC-Kawartha).

4.3.1.3 Starch content

Starch content of wild-type (OAC Kawartha) has been taken as a reference and all other genotypes were compared to it. All genotypes possessed higher starch content than wild-type except 083311-104, which contained less starch. Genotype 083511-118 possessed highest (139.8 %) starch (Table, 4.1).

4.3.1.4 Internal seed structures and starch packing within endosperm

To observe internal seed structures and starch packing within endosperm, cross sections along seed length from all genotypes were made and stained with iodine (Figure
Table 4.1: Physiochemical properties of seed and starch of barley genotypes.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>T</th>
<th>R</th>
<th>Amylose (%)</th>
<th>RS (%)</th>
<th>Seed Characteristics (mm)</th>
<th>1000 grain weight (g)</th>
<th>% Starch content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>SL</td>
<td>SW</td>
<td>ST</td>
</tr>
<tr>
<td>OAC Kawartha</td>
<td>C</td>
<td>6</td>
<td>25.0 ±1.1</td>
<td>1.8 ±0.5</td>
<td>7.9 ±0.7</td>
<td>3.8 ±0.1</td>
<td>2.9 ±0.2</td>
</tr>
<tr>
<td>McGuire</td>
<td>H</td>
<td>2</td>
<td>18.1 ±1.0</td>
<td>1.8 ±0.4</td>
<td>7.3 ±0.4</td>
<td>3.7 ±0.2</td>
<td>2.9 ±0.1</td>
</tr>
<tr>
<td>GB992033</td>
<td>C</td>
<td>2</td>
<td>25.3 ±0.6</td>
<td>1.6 ±0.4</td>
<td>7.5 ±0.3</td>
<td>3.7 ±0.1</td>
<td>2.9 ±0.1</td>
</tr>
<tr>
<td>Sloop</td>
<td>C</td>
<td>2</td>
<td>16.0 ±1.0</td>
<td>1.9 ±0.4</td>
<td>7.6 ±0.4</td>
<td>3.9 ±0.1</td>
<td>2.6 ±0.2</td>
</tr>
<tr>
<td>CDC Fibrar</td>
<td>H</td>
<td>2</td>
<td>1.0 ± 0.3</td>
<td>1.9 ±0.5</td>
<td>7.6 ±0.7</td>
<td>3.6 ±0.3</td>
<td>2.8 ±0.2</td>
</tr>
<tr>
<td>CDC Rattan</td>
<td>H</td>
<td>2</td>
<td>2.3 ± 0.6</td>
<td>1.3 ±0.4</td>
<td>7.8 ±0.6</td>
<td>3.7 ±0.2</td>
<td>2.7 ±0.1</td>
</tr>
<tr>
<td>Soft Barley</td>
<td>H</td>
<td>2</td>
<td>12.0 ±1.1</td>
<td>1.0 ±0.1</td>
<td>7.5 ±0.5</td>
<td>3.6 ±0.1</td>
<td>2.7 ±0.1</td>
</tr>
<tr>
<td>Hard Barley</td>
<td>H</td>
<td>2</td>
<td>24.2 ±1.3</td>
<td>2.2 ±0.6</td>
<td>8.7 ±0.6</td>
<td>3.6 ±0.2</td>
<td>2.6 ±0.1</td>
</tr>
<tr>
<td>AC Metcalfe</td>
<td>C</td>
<td>2</td>
<td>23.3 ±1.5</td>
<td>1.6 ±0.5</td>
<td>8.2 ±0.4</td>
<td>3.9 ±0.2</td>
<td>2.8 ±0.2</td>
</tr>
<tr>
<td>083211-120</td>
<td>C</td>
<td>2</td>
<td>21.3 ±1.5</td>
<td>2.0 ±0.7</td>
<td>8.9 ±0.4</td>
<td>4.1 ±0.2</td>
<td>2.9 ±0.2</td>
</tr>
<tr>
<td>083211-122</td>
<td>C</td>
<td>2</td>
<td>23.3 ±0.6</td>
<td>1.8 ±0.6</td>
<td>9.4 ±0.3</td>
<td>4.3 ±0.1</td>
<td>2.8 ±0.1</td>
</tr>
<tr>
<td>083311-104</td>
<td>C</td>
<td>6</td>
<td>26.3 ±0.6</td>
<td>2.1 ±0.8</td>
<td>7.7 ±0.6</td>
<td>3.8 ±0.2</td>
<td>2.8 ±0.1</td>
</tr>
<tr>
<td>083411-113</td>
<td>H</td>
<td>6</td>
<td>22.0 ±1.3</td>
<td>2.3 ±0.6</td>
<td>8.2 ±0.6</td>
<td>3.8 ±0.2</td>
<td>2.7 ±0.2</td>
</tr>
<tr>
<td>AC Alberte</td>
<td>H</td>
<td>2</td>
<td>20.0 ±2.4</td>
<td>2.0 ±0.7</td>
<td>8.5 ±0.3</td>
<td>3.4 ±0.2</td>
<td>2.5 ±0.2</td>
</tr>
<tr>
<td>AC Bacon</td>
<td>H</td>
<td>6</td>
<td>27.2 ±1.8</td>
<td>2.5 ±0.7</td>
<td>8.6 ±0.5</td>
<td>3.4 ±0.2</td>
<td>2.6 ±0.2</td>
</tr>
<tr>
<td>Emperor</td>
<td>H</td>
<td>2</td>
<td>22.7 ±1.5</td>
<td>2.0 ±0.7</td>
<td>8.9 ±0.7</td>
<td>3.4 ±0.1</td>
<td>2.4 ±0.2</td>
</tr>
<tr>
<td>Sunderland</td>
<td>C</td>
<td>2</td>
<td>18.1 ±1.8</td>
<td>2.2 ±0.6</td>
<td>8.1 ±0.4</td>
<td>3.6 ±0.1</td>
<td>2.8 ±0.1</td>
</tr>
<tr>
<td>083511-109</td>
<td>H</td>
<td>2</td>
<td>21.3 ±1.6</td>
<td>2.0 ±0.6</td>
<td>8.5 ±0.6</td>
<td>4.1 ±0.2</td>
<td>2.8 ±0.2</td>
</tr>
<tr>
<td>083511-118</td>
<td>H</td>
<td>2</td>
<td>22.0 ±1.4</td>
<td>2.6 ±0.9</td>
<td>8.2 ±0.4</td>
<td>4.4 ±0.2</td>
<td>3.2 ±0.1</td>
</tr>
</tbody>
</table>

T = Type, C = Covered, H = Hulless, R = Rows, SL:SW = Seed length to seed width ratio
SL = Seed length, SW = Seed width, ST = Seed thickness, TGW = Thousand grain weight

Table 4.1: Physical characteristics of seed and physiochemical characteristics of starch. Type (T) indicates whether seed is covered (C) or hulless (H). Rows (R) represent number of rows (2 or 6) of fertile spikelets on the barley spike. Amylose and resistant starch (RS) contents were measured using Megazyme kits. % starch content of the reference genotype, OAC-Kawartha wild-type was taken as 100 %. Data represent means of three biological replicates for amylose (%), RS (%) and % starch content. For SL, SW, ST and SL: SW data from hundred independent observations with three biological replicates are presented. For 1000 grain weight, data from thousand independent observations with three biological replicates are presented. ± standard deviation.
4.1). In all genotypes, seeds were fully stained internally showing uniform distribution of starch within the seed except \textit{waxy} genotypes CDC Fibar and CDC Rattan and low-amylose genotype Soft Barley (12 % amylose) which did not stain due to reduced iodine-binding (Figure 4.1). Most of the genotypes possessed thin pericarp except genotypes Sloop, AC Alberte (hulless) and AC Bacon (hulless) which possessed comparatively thicker pericarp.

4.3.2 Physiochemical properties of starch

4.3.2.1 Amylose and resistant starch (RS) contents

Differences with respect to amylose content were observed among different genotypes. OAC Kawartha (which represents normal, wild-type barley) has 25 % amylose. Most of the genotypes possessed normal amylose content with certain variations compared to wild-type as shown in the Table (4.1). The Soft Barley genotype has 12 % amylose and is characterized as a low amylose genotype. Two genotypes (CDC Fibar and CDC Rattan) possessed < 5 % amylose and are characterized as \textit{waxy} genotypes. All genotypes showed < 5 % RS content.

4.3.2.2 Granule Size, number and surface area distributions of different genotypes

Differences among the barley genotypes were observed for granule size, number and surface area distributions. The reference genotypes OAC Kawartha and McGwuire exhibited bimodal granule size, number and surface area distributions (Figures 4.2, 4.3 & 4.4A & B). However in all other genotypes, unimodal distribution was observed. In all genotypes maximum granule size was upto 45 \( \mu \text{m} \), except in genotypes, Sloop and Emperor in which maximum granule sizes upto 39 \( \mu \text{m} \) and 34 \( \mu \text{m} \) were observed respectively. Figures (4.3A & 4.5) show that in normal genotypes granules
To observe internal structure and starch packing within seed from different genotypes, cross sections along the length of seed were made and incubated in Lugol’s solution.
of size $< 5\mu m$ in diameter represents $> 90\%$ of total granule number but contributes $< 15\%$ to total starch mass.

4.3.2.3 Contribution of A-, B- and C-granules in total mass of starch

Barley starch granules can be divided into three major classes based on size and shape which include C-type ($< 5\ \mu m$, polygonal), B-type (5-15 $\mu m$, round) and A-type (15-45 $\mu m$, lenticular). Figure (4.5) shows that genotypes OAC Kawartha and McGwuire possess considerable amounts of C-granules unlike the other genotypes studied. However, based on size distribution all genotypes contain considerable amount of B-granules in which genotypes sloop and CDC Fibar possessed highest amount of B-granules compared to wild-type. A-granules constituted the major portion of total starch mass in all genotypes and among all genotypes 083411-113 possessed the highest amount at 89.76 $\%$ of A-granules (Figure, 4.5).
Figure 4.2 (A & B): Starch granule size distribution of different genotypes.
Granule size distribution in normal, low amylose and waxy starches from various genotypes. Granule size distribution was measured by means of laser scattering using the Mastersizer (Malvern Mastersizer 2000, Malvern Instruments Ltd., UK). Starch (≈100 mg) was suspended in 10 ml of distilled water and used according to manufacturer instruction.
Figure 4.3 (A & B): Starch granule number distribution of different genotypes.
Granule number distribution in normal, low amylose and waxy starches from various genotypes. Granule number distribution was measured by means of laser scattering using the Mastersizer (Malvern Mastersizer 2000, Malvern Instruments Ltd., UK). Starch (∼100 mg) was suspended in 10 ml of distilled water and used according to manufacturer instruction.
Figure 4.4 (A & B): Starch granule surface area distribution of different genotypes. Granule surface area distribution in normal, low amylose and waxy starches from various genotypes. Granule surface area distribution was measured by means of laser scattering using the Mastersizer (Malvern Mastersizer 2000, Malvern Instruments Ltd., UK). Starch (≈100 mg) was suspended in 10 ml of distilled water and used according to manufacturer instruction.
Figure 4.5: Starch granule distribution into (A-, B-, and C-) classes.
A-granules (15-45 µm); B-granules (5-15 µm) and C-granules (< 5 µm).
4.3.2.4 Granule surface morphology

Granule surface morphology of different genotypes was observed using scanning electron microscopy (SEM). In all genotypes, no differences were observed in the morphology of small granules up to 15 µm in size. However differences were observed in the morphology of A-granules from different genotypes. In all genotypes A-granules were lenticular except; Hard Barley, 083411-113, AC Alberte, AC Bacon and Sunderland. In these genotypes, A-granules with altered morphology were observed (Figure, 4.6). The extent of alteration was different in these genotypes, for example, in genotype 083411-113, A-granules were severely deformed and lost lenticular shape (Figure, 4.6). However in all other genotypes, with altered granule morphology (Hard Barley, AC Alberte, AC Bacon and Sunderland), the lens shape of A-granules was retained although grooves or ditches were observed in the granules. In waxy genotypes CDC Fibar and CDC Rattan, no difference was observed in the starch granule morphology compared to the reference genotype.

4.3.3 Starch gelatinization (Thermal properties)

To measure the thermal properties of starch, six genotypes were selected from above mentioned genotypes based on certain parameters. The selected genotypes included OAC Kawartha (reference genotype); CDC Fibar (waxy); Soft Barley (low amylose, with normal amount of B-granules of size, 5-15 µm); Hard Barley (normal amylose, with normal amount of B-granules of size, 5-15 µm); AC Metcalfe and 083411-113 (normal amylose, with significantly reduced amount of small granules of size, 1-10 µm). To measure the thermal properties of starch derived from selected genotypes, differential scanning calorimetry (DSC) was employed. Table (4.2) shows that significant
Figure 4.6: Surface morphology of starch granules from different genotypes observed by SEM.

For SEM analysis acetone dried barley starch samples were sprayed on a metal plate covered with double-sided adhesive tape. The samples were gold coated with Polaron SC500 sputter coater (Quorum Technologies, East Sussex, UK). The samples were examined at 10 kV accelerating voltage and representative micrographs were taken for each sample at same magnification. Scale bar for electron micrograph is given under each panel. Each type of granule is presented by a colored arrow.
differences were present among different genotypes with respect to onset (To) gelatinization temperature where highest gelatinization temperatures (59.3 and 59.1°C) were exhibited by waxy genotype CDC Fibar and low amylose genotype Soft Barley, respectively. Genotype 083411-113 exhibited lowest (54.7 °C) onset gelatinization temperature (To). Wild-type, Hard Barley and AC Metcalfe exhibited lower (To) than waxy genotype. In the case of peak temperature (Tp), genotypes also exhibited significant differences with highest (Tp) possessed by CDC Fibar and lowest by Hard Barley. The Soft Barley genotype also possessed higher (Tp) than wild-type. Similarly genotypes CDC Fibar and 083411-113 possessed highest completion temperature (Tc) of starch gelatinization. Genotype 083411-113 exhibited significantly higher (Tc-To) of 21.6 °C compared to wild-type, Soft Barley and Hard Barley. Significant variations were also observed regarding transition enthalpy (∆H) for different genotypes in which highest (∆H) 13.3 and 13.2 J/g was exhibited by CDC Fibar and 083411-113, respectively, and lowest (∆H) 9.8 J/g was exhibited by Soft Barley (Table, 4.2). In the second phase of gelatinization where disruption of amylose-lipid complex took place, different genotypes showed interesting variations, for example, Hard Barley and AC Metcalfe possessed highest and wild-type showed lowest onset temperature (To) of melting amylose-lipid complex. Hard barley also exhibited highest peak temperature (Tp) and genotypes Soft Barley and AC Metcalfe possessed lowest (Tp) values indicative of melting amylose–lipid complex. Soft Barley has highest and OAC Kawartha, AC Metcalfe and 083411-113 exhibited lowest completion temperatures (Tc). Genotype Soft Barley exhibited highest (Tc-To) values indicative of degree of branching of amylopectin compared to all other genotypes. Different genotypes also exhibited significant variation in transition enthalpy.
CDC Fibar and 083411-113 possessed highest values for transition enthalpy ($\Delta H$) (13.3 and 13.2 J/g), respectively. Low amylose genotype Soft Barley exhibited lowest ($\Delta H$) 9.8 J/g. The waxy genotype (CDC Fibar) did not show any value for all transition phases of melting amylose-lipid complex, indicating waxy starch has very low amount or no amylose-lipid complex as previously shown by Yoshimoto et al. (2000).

In the 2nd phase of gelatinization of retrograded starch, no significant variations have been observed for different phases of gelatinization. However, the genotype Soft Barley, which is a low amylose genotype, did not show any value for melting of retrograded starch as was also observed for the waxy genotype CDC Fibar. Interestingly, Soft Barley showed comparable values to that of other genotypes for different phases of melting of the amylose-lipid complex ($T_0$, $T_p$, $T_e$) of retrograded starch. However, Soft Barley showed a very low value of $\Delta H$ compared to other genotypes (Table, 4.2).
Table 4.2: Thermal properties of starch.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Starch Gelatinization (°C)</th>
<th>Melting of amylose-lipid complex (°C)</th>
<th>(\Delta H) (J/g)</th>
<th>Re-Heating Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>To</td>
<td>Tp</td>
<td>Tc</td>
<td>(Tc-To)</td>
</tr>
<tr>
<td>OAC Kawartha</td>
<td>56.4(^c)</td>
<td>62.7(^c)</td>
<td>73.2(^{ab})</td>
<td>16.9(^b)</td>
</tr>
<tr>
<td>CDC Fibar</td>
<td>59.3(^a)</td>
<td>67.4(^b)</td>
<td>77.4(^a)</td>
<td>18.2(^{ab})</td>
</tr>
<tr>
<td>Soft Barley</td>
<td>59.1(^a)</td>
<td>65.4(^b)</td>
<td>73.5(^{ab})</td>
<td>14.4(^b)</td>
</tr>
<tr>
<td>Hard Barley</td>
<td>55.8(^d)</td>
<td>60.9(^c)</td>
<td>70.7(^b)</td>
<td>14.9(^b)</td>
</tr>
<tr>
<td>AC Metcalfe</td>
<td>57.5(^b)</td>
<td>62.9(^c)</td>
<td>75.2(^b)</td>
<td>17.6(^{ab})</td>
</tr>
<tr>
<td>083411-113</td>
<td>54.7(^c)</td>
<td>61.7(^{d})</td>
<td>76.3(^a)</td>
<td>21.6(^a)</td>
</tr>
</tbody>
</table>

Table 4.2, Thermal analyses of starch from different genotypes. DSC was performed according to the method described by Liu et al. (2007) using a differential scanning calorimeter (2920 Modulated DSC, TA Instruments, New Castle, DE). In this analysis CDC Fibar represents a genotype *waxy* and Soft Barley a low amylose genotype. The data are presenting mean of two replicates. Values in the same column followed by the same letter are not significantly different at \(p < 0.05\).
4.3.4 Amylopectin chain length distribution (CLD)

Amylopectin structure and the proportion of chain lengths with different degrees of polymerization (DP) (chain length distribution) are important for determining the functionality of starch. These features contribute to texture, stability, uniformity and freeze-thaw properties of starch (Chibbar et al., 2005). Amylopectin chain length distribution of selected genotypes, OAC Kawartha, Sloop, CDC-Fibar, 083211-122 and 083411-113 (Section, 4.3.3) was measured. The measurable degree of polymerization in different genotypes ranged from 6 DP to 50 DP with varying proportions of different DP. The measured DP has been divided into four groups as shown in Table 4.3. Interestingly, all genotypes showed no significant differences in first three DP ranges (6-12, 13-24 and 25-36) as shown in Table (4.3). However, in case of group IV which is represented by chains of DP > 37, genotypes showed significant differences. Interestingly, the \textit{waxy} genotype CDC Fibar possessed the highest amount of longer chains, while low amylose (Soft Barley) and normal amylose genotypes (Hard Barley and 083411-113) exhibited the lowest amount of longer chains of DP > 37. There was no significant difference in average chain length distribution for any genotype.

To understand differences in individual DP among different genotypes, difference plots of: different genotypes versus “reference genotype” OAC-Kawartha; different genotypes versus \textit{“waxy genotype”} CDC Fibar; and different genotypes versus “low amylose genotype” Soft Barley, have been produced. Compared to reference genotype (OAC-Kawartha) all other genotypes studied have a lower proportion of DP 6, except Hard Barley, which has a higher proportion of short chains with DP 6. CDC Fibar has a relatively higher proportion of chains with DP 7-9 and long chains with DP > 37 and a
lower proportion of chains with DP 10-17 and DP 29-33. Soft Barley has lower proportion of chains with DP 6-8 and intermediate and long chains beyond DP 21 and has higher proportion of chains with DP 9-16 compared to wild-type. Genotype AC Metcalfe has greater proportion of chain lengths with DP 7-10 and lower proportion of some chains with DP > 11 (Figure 4.7). Genotype 083411-113 also exhibited interesting results in which proportion of chains with DP 7-11 has increased and the proportion of all chains with DP > 12 decreased (Figure 4.7).

A second set of difference plots: different genotypes versus waxy was generated, and it was found that all genotypes have a relatively higher proportion of chains with DP 6 compared to waxy except Soft Barley. OAC Kawartha (WT) has lower proportion of chains with DP 7-9, all other chains with different DP were either higher in proportion or exhibited minor differences compared to waxy. Soft Barley also has a smaller proportion of chains with DP 7-9 and chains with DP > 33 and a higher proportion of chains with DP 10-33, with some exceptions (Figure 4.8). Similarly, Hard Barley, 083411-113 and AC Metcalfe have lower proportion of longer chains and higher proportion of small to intermediate chains compared to waxy (Figure 4.8).

Difference plot of different genotypes versus low amylose genotype showed that, compared to the low amylose genotype, all other lines exhibited variations in chains with DP 6-18. All of the genotypes had a higher proportion of small chains with DP < 10 and a lower proportion of chains with DP 10-18 compared to the low amylose variety. With reference to chains with DP > 18 all genotypes exhibited minor differences compared to the low amylose genotype (Figure 4.9).
Table 4.3: Amylopectin chain length in different barley genotypes.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>DP</th>
<th></th>
<th></th>
<th>Avg. CL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6-12</td>
<td>13-24</td>
<td>25-36</td>
<td>37-50</td>
</tr>
<tr>
<td>OAC Kawartha</td>
<td>44.2 ±2.6</td>
<td>44.4 ±2.0</td>
<td>8.1 ±0.7</td>
<td>3.2ab ±0.1</td>
</tr>
<tr>
<td>CDC Fibar</td>
<td>45.2 ±2.2</td>
<td>43.7 ±0.8</td>
<td>7.5 ±1.2</td>
<td>3.7a ±0.2</td>
</tr>
<tr>
<td>Soft Barley</td>
<td>44.9 ±2.3</td>
<td>45.1 ±1.9</td>
<td>7.5 ±0.3</td>
<td>2.6c ±0.2</td>
</tr>
<tr>
<td>Hard Barley</td>
<td>46.9 ±1.2</td>
<td>44.2 ±1.1</td>
<td>6.7 ±0.5</td>
<td>2.3c ±0.3</td>
</tr>
<tr>
<td>AC Metcalfe</td>
<td>45.8 ±1.3</td>
<td>44.0 ±1.1</td>
<td>7.5 ±0.6</td>
<td>2.7bc ±0.4</td>
</tr>
<tr>
<td>083411-113</td>
<td>47.8 ±1.5</td>
<td>43.1 ±1.1</td>
<td>6.6 ±0.3</td>
<td>2.5c ±0.1</td>
</tr>
</tbody>
</table>

Table 4.3, different groups of chain length with varying DP (s) are presented in four different classes. The data are presented as % moles of carbohydrate. Values in the same column followed by the same letter are not significantly different at p < 0.05. ± standard deviation.
Figure 4.7: Difference plot of (other genotypes versus wild-type) for amyllopectin chains with different DP.

Amylopectin chain length distribution of barley genotypes was measured by HPAEC. The chain length distribution of wild-type OAC Kawartha was deducted from the chain length distribution of other genotypes. The negative value shows that reference genotype exhibited a higher proportion of that specific glucan chain, while positive value shows that other genotypes exhibited a higher proportion of that specific glucan chain.
Figure 4.8: Difference plot of (other genotypes versus waxy) for amyllopectin chains with different DP.

Amylopectin chain length distribution of barley genotypes was measured by HPAEC. The chain length distribution of waxy genotype CDC Fibar was deducted from the chain length distribution of other genotypes. The negative value shows that waxy genotype exhibited a higher proportion of that specific glucan chain, while positive value shows that other genotypes exhibited a higher proportion of that specific glucan chain.
Figure 4.9: Difference plot of (other genotypes versus low amylose) for amyllopectin chains with different DP.

Amylopectin chain length distribution of barley genotypes was measured by HPAEC. The chain length distribution of low amylose genotype Soft Barley was deducted from the chain length distribution of different genotypes. The negative value shows that low amylose genotype exhibited a higher proportion of that specific glucan chain, while positive value shows that other genotypes exhibited a higher proportion of that specific glucan chain.
4.3.5 Biochemical characterization of barley genotypes

4.3.5.1 Amyloplast stromal proteins (soluble proteins)

Amyloplasts from developing barley endosperm were isolated (18-25 DAP) from selected genotypes, OAC Kawartha, Sloop, CDC-Fibar, 083211-122 and 083411-113 (Section 4.3.3). Amyloplast lysates were used to detect the presence of different proteins involved in starch biosynthesis in the amyloplast stroma using a range of peptide specific antibodies, which include SSI, SSIIa, SSIII, SSIV, SBEI, SBEIc, SBEIIa, SBEIIb, ISO1, SP, AGPase large subunit and AGPase small subunit. These proteins were observed at their expected sizes on SDS-gels based on known sequences. Immunological analysis of amyloplast lysates from these genotypes showed that similar amounts of SSI, SSIIa, SSIII, SSIV, SBEI, SBEIc, SBEIIa, SBEIIb, ISO1, SP, AGPase large subunit, AGPase small subunit are present when compared to wild-type (data not shown).

4.3.5.2 Analysis of starch granule-associated proteins

Granule associated proteins are those which remain entrapped within the starch granule after extensive washing with, buffer, SDS and acetone.

4.3.5.2.1 Total amount of starch granule-bound protein

The total amount of starch granule-associated protein (µg of protein/50 mg of starch) was measured. Genotype 083511-118 exhibited highest amount (109 µg) while waxy genotype CDC Rattan exhibited lowest amount (26 µg) of total granule-bound protein compared to reference genotype OAC Kawartha (91 µg). All other genotypes studied exhibited minor differences compared to reference genotype (Table 4.4).
4.3.5.2.2 Individual isoforms of starch granule-associated proteins

The starch granule proteome of different genotypes was analysed. Barley starch has trimodal granule size distribution comprising of A-, B- and C-type granules. These A-, B- and C-granules were separated (Methods 4.2.2.1) from all genotypes using a method described by Peng et al. (2000) and differences in the proteome of these three types of granules have been determined. To isolate starch granule-bound proteins, buffer, SDS and acetone washed starch, A-, B- and C-granules were used and starch granule-bound proteins were extracted (see methods). These proteins were analysed by silver staining and immunoblotting using antibodies against various enzymes of the biosynthetic pathway. Along with other previously described granule-bound proteins, such as GBSS, SSI, SSIIa and SBEIIb other proteins were also found to be associated with starch granules, such as SSIII, SBEIc, SBEIIa. Immunoblot analysis revealed that all genotypes under study have comparable amounts of granule-bound SSI, SSIIa, SSIII, SBEIc, SBEIIa, SBEIIb and GBSS, except genotypes CDC Rattan and Soft Barley which have reduced amount of GBSS (Figure 4.10). Although different proteins have been found to be granule-associated, other isoforms of some proteins like SSIV, SBEI, Isoamylases and SP, were not detected within the starch granule (Figure 4.10). No qualitative differences have been found in the starch granule proteome of all genotypes except CDC Rattan and Soft Barley. However, detailed analyses of the proteome of A- and B-granules revealed that B-granules lack SBEIc protein, and is only present in A-granules (Figure 4.10). In certain genotypes AC Metcalfe, 083411-113, AC Bacon, 083511-109 and 083511-118, due to presence of low amounts of B-granules, immunoblot analysis was not performed. Similarly in all genotypes immunodetection of C-
Table 4.4: Amount of total starch granule-bound protein (µg/ 50 mg of starch).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Protein</th>
<th>Genotype</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>OAC Kawartha</td>
<td>91\textsuperscript{b} ± 5.5</td>
<td>083211-122</td>
<td>89\textsuperscript{b} ± 9.6</td>
</tr>
<tr>
<td>McGwuire</td>
<td>89\textsuperscript{b} ± 3.7</td>
<td>083311-104</td>
<td>90\textsuperscript{b} ± 5.0</td>
</tr>
<tr>
<td>GB992033</td>
<td>87\textsuperscript{c} ± 11.6</td>
<td>083411-113</td>
<td>88\textsuperscript{b} ± 6.5</td>
</tr>
<tr>
<td>Sloop</td>
<td>90\textsuperscript{b} ± 4.4</td>
<td>AC Alberte</td>
<td>86\textsuperscript{bc} ± 7.0</td>
</tr>
<tr>
<td>CDC Fibar</td>
<td>74\textsuperscript{c} ± 6.6</td>
<td>AC Bacon</td>
<td>88\textsuperscript{bc} ± 8.2</td>
</tr>
<tr>
<td>CDC Rattan</td>
<td>26\textsuperscript{d} ± 4.2</td>
<td>Emperor</td>
<td>89\textsuperscript{b} ± 11.2</td>
</tr>
<tr>
<td>Soft Barley</td>
<td>31\textsuperscript{d} ± 6.7</td>
<td>Sunderland</td>
<td>88\textsuperscript{bc} ± 10.6</td>
</tr>
<tr>
<td>Hard Barley</td>
<td>83\textsuperscript{bc} ± 8.7</td>
<td>083511-109</td>
<td>88\textsuperscript{bc} ± 11.0</td>
</tr>
<tr>
<td>AC Metcalfe</td>
<td>82\textsuperscript{bc} ± 6.2</td>
<td>083511-118</td>
<td>109\textsuperscript{a} ± 12.4</td>
</tr>
<tr>
<td>083211-120</td>
<td>91\textsuperscript{b} ± 8.9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.4. Total granule-bound protein was determined from 50 mg of acetone-washed starch using Pierce® BCA protein assay kit. The data for each genotype represent means of three biological replications. Values in the same column followed by the same letter are not significantly different at p < 0.05. ± standard deviation.
Figure 4.10: Immunodetection of granule-bound proteins in A- and B-starch granules. Starch granules were isolated from mature barley grains, the purified, acetone washed starch was used to separate large A- and small B-granules. 50 mg aliquot of purified starch, A- and B-granules were boiled in 1 ml of SDS loading buffer, and 40 µl of the supernatant from the boiled sample loaded onto 4–12% acrylamide gradient gels and 10% acrylamide SDS gels. Following electrophoresis, gels were electroblotted to nitrocellulose membrane and developed with various specific anti-wheat antibodies. Each figure is labelled with respective antibody and predicted molecular weight of protein it is cross reacting with (right) and type of granules (left). N/A B-granules were not available.
granules proteome was not performed due to lower amount of these granules.

4.3.5.2.3 Quantitation of individual granule-bound proteins

To measure the amount of individual protein entrapped within A- and B-granules of starch, silver stained gels were used and the intensity of each band was measured (see Methods). The identity of each band was confirmed by immunoblotting similar gels with different antibodies (see above). Silver staining revealed that all genotypes have a distinct protein band of approximately (110 kDa) size. However this protein was not identified by immunoblotting with antibodies available. Interestingly this 110 kDa protein was only present in A-granules (Figure, 4.12 a & b). Quantitative analysis revealed that GBSS was the most abundant protein in A- and B-granules of all genotypes, except CDC Rattan and Soft Barley (Figure, 4.11). In genotypes, CDC Rattan and Soft Barley small amounts of GBSS were present compared to the reference genotype SSIIa was the next most abundant protein in all genotypes. However certain proteins like SSIII and unknown protein of 110 kDa were present in small amounts in all genotypes.
Figure 4.11: Quantitation of individual granules bound proteins.
Starch granules were isolated from mature barley according to the method described earlier (Peng et al., 2000). Large A- and small B-granules were separated and granule-bound proteins were extracted. Equal amounts of starch, A- and B-granules were loaded onto 4–12% acrylamide gradient gels. Following electrophoresis gels were visualized by silver staining. Different proteins were identified by immunoblotting using specific antibodies and some of proteins were also identified by Q-TOF-MS analysis. Amount of individual protein was determined by measuring the intensity of each band with Molecular Imager® Gel Doc™ XR+ and Chemi Doc™ XRS+ Systems with Image Lab™ Software, version 3 (BioRad laboratories Inc.). The measured intensity was compared with known amount of BSA protein (100-1500 ng) which was run as standard in each gel. Y-axis is presenting the amount of protein in (ng) while X-axis represents genotypes in number form which are as follow, (1, OAC Kawartha; 2, McGwuire; 3, GB992033; 4, Sloop; 5, CDC Fibar; 6, CDC Rattan; 7, Soft Barley; 8, Hard Barley; 9, AC Metcalfe; 10, 083211-120; 11, 083211-122; 12, 083311-104; 13, 083411-113; 14, AC Alberte; 15, AC Bacon; 16, Emperor; 17, Sunderland; 18, 083511-109; 19, 083511-118. Protein quantity in C-granules was not determined because of very low amount of proteins in these granules.
4.3.5.2.4 Proteomic analysis of C-granules

In order to determine the proteome of C-granules, purified B-granules were used to obtain C-granules (see Section 4.2.2.1). The size of these granules was determined by light and electron microscopy. In Figure (4.13) OAC Kawartha is presented as an illustration for size determination of different types of purified granules. Silver staining revealed that C-granules exhibited significant qualitative and quantitative differences compared to A- and B-granules. Particularly the amount of individual proteins was decreased in C-granules of all genotypes compared to A- and B-granules (4.12c).

4.3.5.3 Phosphorylation of proteins in starch granule

Pro-Q diamond staining was employed to study phosphorylation of different granule-bound proteins and it was found that some of the starch granule proteins were phosphorylated. GBSS is the most abundant protein in the starch granule and this protein showed very strong indication of protein phosphorylation in all genotypes (Figure 4.14). After GBSS, the second most abundant protein in barley granule was SSIIa and the data suggest that SSIIa in the starch granules is also phosphorylated in all genotypes (Figure 4.14). The other major granule-bound protein which showed evidence of phosphorylation was SBEIIb (Figure 4.14). SSI, which is also found within the starch granules, showed evidence of phosphorylation. In all genotypes a band of 110 kDa was observed but this band was not found to be phosphorylated. In the barley starch granules along with the above mentioned proteins, other proteins (SSIII and SBEIIa) were also present, but these proteins were not phosphorylated. Overall, GBSS1, SSI, SSIIa, and SBEIIb were found to be phosphorylated in their granule-bound state.
Figure 4.12: Granule-bound proteins (starch, A- and C-granules) visualized by silver staining.

Figure 12, Detection of individual starch synthesizing enzyme bound with in starch, A- and C-granules from different genotypes of barley. No differences were observed between A- and B-granules, therefore, silver stained gels of B-granules are not presented. Equal amounts (1.5 mg) of starch, A- and C-granules were loaded onto 4–12 % acrylamide gradient gels. Following electrophoresis, proteins were visualized by silver staining. Protein markers, molecular mass (left hand side) kDa. Right hand side-different bands are indicated by numbers, (1, SSIII; 2, SBEic; 3, unknown; 4, 5, 6, SSIIa, SBEIIa,
SBElb; 7, SSl; 8, GBSS; 9, degraded GBSS and SSs). SSIIla was present in small amounts in starch and A-granules in all genotypes.

Figure 4.13: Analysis of starch composition and granule morphology by light microscope (A, B & C) and electron microscope (D & E). Separation of A- and B-/C-granules was performed according to method described earlier (Peng et al., 2000). Total starch containing, A-, B- and C-granules (A). Purified A-granules (B). Purified B-/C-granules (C). Electron micrograph of total starch, with A-, B- and C-granules (D). Purified B-/C-granules (E). The reference genotype, OAC Kawartha is presented as an illustration for A- and B-/C-granules purity confirmation in all genotypes studied here. The scale bar on panels A, B and C represents 20 µm. Scale bar for electron micrograph is given under each panel. Each type of granule is presented by a colored arrow.
Figure 4.14: Phosphorylation of starch granule associated proteins.

To determine phosphorylation of granule-bound proteins equal amounts (1.5 mg) of starch from all genotypes were run onto 4–12% acrylamide gradient gels. Following electrophoresis the gels were stained with Pro-Q Diamond according to the manufacturer’s instructions (Molecular Probes) in the dark for 90 min. Following destaining and washing, the gels were visualised using a Typhoon scanner (Amersham Biosciences) with an excitation wavelength of 530nm and emission filter at 580nm. After Pro-Q Diamond staining the same gels were subject to silver staining to visualize all bands. The bands visualized with silver stain are numbered as (1, SSIII; 2, SBEIc; 3, unknown; 4, 5, 6, SSIIa, SBEIIa, SBEIIb; 7, SSI; 8, GBSS).
4.4 Discussion

The present study investigated the physiochemical properties and the biochemical markers of starch biosynthesis from various barley genotypes in an attempt to identify biochemical characteristics responsible for useful starch phenotypes. To determine possible interactions between different physiochemical properties of the seed and starch, Principal Component Analysis (PCA) was performed (Table 4.5). It was found that seed characteristics and type such as: hulless or covered seed; two rows or six rows; seed length, width, thickness and seed length; and width ratio, did not correlate with different physiochemical properties of starch, e.g. amylopectin chain length distribution and gelatinization temperature. However, seed length, width and thickness showed a positive correlation with thousand grain weight, in which seed width showed a higher correlation (r = 0.78, p < 0.01) with TGW. PCA also demonstrates that RS (r = -0.79, p < 0.01) and amylose (r = -0.52, p < 0.01) contents are negatively related to starch content, while RS is positively (r = 0.59, p < 0.01) related to amylose content. Similar results have been reported earlier in barley (Asare et al., 2011). Analysis of different sizes of granules demonstrates that A-granules are a major contributor to the total mass of starch, by weight, while B-granules contributed in total granule number. Previously, it was reported by Evers et al. (1973) that small size starch granules account for > 90 % of total granule number but contribute < 30 % in total starch weight of wheat endosperm. Raeker et al. (1998) also reported that small granules of size 1–10 µm make up to 99 % of total granule number in wheat. PCA also demonstrated that starch content was negatively (r = -0.57, p < 0.01) correlated with total granule-bound protein. In waxy or low amylose genotypes, lower iodine binding was observed due to lack of amylose resulting in the
Table 4.5: Correlation between different physiochemical properties of starch determined by principal component analysis.

<table>
<thead>
<tr>
<th>Variables</th>
<th>SL (mm)</th>
<th>SW (mm)</th>
<th>ST (mm)</th>
<th>L:W</th>
<th>TGW</th>
<th>Starch Content</th>
<th>% B Granules</th>
<th>% A granules</th>
<th>RS</th>
<th>Amylose</th>
<th>TGBP</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL (mm)</td>
<td>1</td>
<td>0.22</td>
<td>-0.26</td>
<td>0.64</td>
<td>0.44</td>
<td>-0.46</td>
<td>-0.39</td>
<td>0.39</td>
<td>0.37</td>
<td>0.41</td>
<td>0.32</td>
</tr>
<tr>
<td>SW (mm)</td>
<td>0.22</td>
<td>1</td>
<td>0.71</td>
<td>-0.60</td>
<td>0.78</td>
<td>-0.27</td>
<td>-0.19</td>
<td>0.19</td>
<td>0.19</td>
<td>0.15</td>
<td>0.32</td>
</tr>
<tr>
<td>ST (mm)</td>
<td>-0.26</td>
<td>0.71</td>
<td>1</td>
<td>-0.72</td>
<td>0.51</td>
<td>-0.08</td>
<td>-0.14</td>
<td>0.14</td>
<td>0.14</td>
<td>0.05</td>
<td>0.27</td>
</tr>
<tr>
<td>L:W</td>
<td>0.64</td>
<td>-0.60</td>
<td>-0.72</td>
<td>1</td>
<td>-0.25</td>
<td>-0.19</td>
<td>-0.17</td>
<td>0.17</td>
<td>0.16</td>
<td>0.22</td>
<td>0.01</td>
</tr>
<tr>
<td>TGW</td>
<td>0.44</td>
<td>0.78</td>
<td>0.51</td>
<td>-0.25</td>
<td>1</td>
<td>-0.59</td>
<td>-0.26</td>
<td>0.26</td>
<td>0.40</td>
<td>0.34</td>
<td>0.40</td>
</tr>
<tr>
<td>Starch Content</td>
<td>-0.46</td>
<td>-0.27</td>
<td>-0.08</td>
<td>-0.19</td>
<td>-0.59</td>
<td>1</td>
<td>0.33</td>
<td>-0.33</td>
<td>-0.79</td>
<td>-0.52</td>
<td>-0.57</td>
</tr>
<tr>
<td>% B Granules</td>
<td>-0.39</td>
<td>-0.19</td>
<td>-0.14</td>
<td>-0.17</td>
<td>-0.26</td>
<td>0.33</td>
<td>1</td>
<td>-1.00</td>
<td>-0.39</td>
<td>-0.35</td>
<td>-0.14</td>
</tr>
<tr>
<td>% A granules</td>
<td>0.39</td>
<td>0.19</td>
<td>0.14</td>
<td>0.17</td>
<td>0.26</td>
<td>-0.33</td>
<td>-1.00</td>
<td>1</td>
<td>0.39</td>
<td>0.36</td>
<td>0.14</td>
</tr>
<tr>
<td>RS</td>
<td>0.37</td>
<td>0.19</td>
<td>0.14</td>
<td>0.16</td>
<td>0.40</td>
<td>-0.79</td>
<td>-0.39</td>
<td>0.39</td>
<td>1</td>
<td>0.59</td>
<td>0.84</td>
</tr>
<tr>
<td>Amylose</td>
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<td>0.15</td>
<td>0.05</td>
<td>0.22</td>
<td>0.34</td>
<td>-0.52</td>
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<td>0.36</td>
<td>0.59</td>
<td>1</td>
<td>0.69</td>
</tr>
<tr>
<td>TGBP</td>
<td>0.32</td>
<td>0.32</td>
<td>0.27</td>
<td>0.01</td>
<td>0.40</td>
<td>-0.57</td>
<td>-0.14</td>
<td>0.14</td>
<td>0.84</td>
<td>0.69</td>
<td>1</td>
</tr>
</tbody>
</table>

SL= Seed length, SW= Seed width, ST= Seed thickness, L:W= length to width ratio, TGW= Thousand grain weight, RS= Resistant starch, TGBP= Total granule-bound protein

Principal Component Analysis (PCA) was used to determine relationship between different physiochemical properties of starch and biplots and correlation table were generated. But only correlation table with selected variables are shown in the Table (4.5).
dilution of dark blue color (Figure, 4.1). However, in the low amylose genotype, Soft Barley relatively dark blue color compared to waxy genotypes was observed which indicates the presence of a certain amount of amylose or extra-long unit chains (ELCs) in this genotype, even though GBSS is low or absent (Figure 4.1).

PCA also shows that different gelatinization temperatures are positively correlated with amylopectin chain length with different DP. Starch gelatinization is demonstrated by two endothermic peaks. The first endothermic peak shows the transition phase of starch upon heating from an ordered granule structure to a random coil state in the presence of excess water. The second peak represents the disruption of the amylose-lipid complex (Kugimiya, 1980). Similarly, for retrograded starch, the transition phase is represented by two endothermic peaks. The first peak shows the retrogradation of retrograded starch to a random coil state upon heating, and the second peak shows disruption of amylose-lipid complex. The results indicate that the waxy genotype has a higher $\Delta H$ and $T_0$ than normal amylose genotypes. In contrast, amylose extender (ae) and waxy amylose extender (wx ae) of rice and maize showed higher gelatinization peak temperature ($T_p$) and transition enthalpy ($\Delta H$) than wild-type, and waxy because of their many long branch-chain of ae amylopectin (Kubo et al., 2010; Shi et al., 1995; Cooke and Gidley, 1992). Our results indicate that, in barley, amylopectin chain length distribution from different genotypes possessing varying proportions of amylose does not exhibit significant variations in chain length with DP 6-36, compared to the reference genotype or waxy cultivars (Tables 4.1 & 4.3). This is consistent with previous reports showing that the amylopectins of different barley genotypes, possessing varied amylose content are similar in structure (Yoshimoto et al., 2002, Schulman et al., 1995; Takeda et
al., 1999; Yoshimoto et al., 2000 Czuchajowska et al., 1998; MacGregor & Morgan, 1984; Song and Jane, 2000; Tester and Morrison, 1990). Thus similar amylopectin structure appeared to be characteristic for barley cultivars with altered amylose content. A difference plot (waxy -WT) shows that long amylopectin glucan-chains are in higher proportion in waxy barley compared to reference genotype and other genotypes (Figure 4.8). Previously, You and Izydorczyk, (2007) reported that ΔH values were associated with the amount of double helical domains of amylopectin and single helical structure (amylose–lipid complexes). Similarly, others (Zheng et al., 1998; Morrison et al., 1993; Cooke and Gidley, 1992; Li et al., 2001; Yoshimoto et al., 2000; You and Izydorczyk, 2007) reported that (ΔH) values reflect the loss of double helical order rather than loss of X-ray crystallinity. Tester and Morrison (1990) and Li et al. (2001) reported that ΔH is an estimation of crystallinity and Tp can be used to measure the crystallite quality, and To has a significant correlation with average branch chain length (Jane et al., 1999; Yuan et al., 1993; Wang et al., 1993 and Shi and Seib, 1992). Thus, the higher ΔH of the waxy line is probably due to the higher proportion of amylopectin, because higher amylopectin content will lead to greater crystallinity, and hence more energy will be required to break these interactions. Similarly, waxy variety CDC Fibar exhibits the highest onset temperature of gelatinization. This indicates that CDC Fibar has higher amounts of crystallites and double helical domains than starches from other genotypes, since the onset temperature (To) corresponds to the melting of crystallites of starch, mainly derived from amylopectin. Based on the results it is argued that, in barley, increased amylopectin provide more stability to the starch granule against gelatinization, as a result of increased crystalline organization as oppose to other cereals. Different genotypes
showed significant differences in Tc–To in line 083411-113, showed a wider Tc–To value than other genotypes. Previously Biliaderis et al. (1980) proposed that the degree of branching of amylopectin influences Tc–To i.e. the greater the degree of branching, the wider the melting temperature range. It is also possible that Tc–To may also indicate the degree of heterogeneity of the starch crystallites. Thus it can be speculated that 083411-113 has more branched amylopectin, with a higher proportion of short glucan chains of DP 6-12, as shown in Figures (4.7, 4.8 & 4.9). Li et al. (2001) reported that Tc–To is influenced to a large extent by the proportion of small granules. For example, starches with a high proportion of small granules exhibited the wider Tc–To (Li et al., 2001). In contrast, our results show that 083411-113 has a significantly reduced proportion of small granules (Figure 2B) and a wider “Tc–To” (Table 2) compared to wild-type and waxy, (which have considerable amounts of B-granules but narrower Tc–To). Thus it can be hypothesized that Tc-To is mainly influenced by chain length distribution.

Starch granule-associated proteins from all genotypes (starch, A- and B-granules) were detected by immunoblotting with different antibodies (see Methods). Analysis of granule-bound proteins did not reveal significant variation in the granule-associated proteome of starch, A- or B-granules from different genotypes compared to normal reference genotype, with the exception of waxy and low amylose genotypes (CDC Fibar and Soft Barley) which have significantly reduced amount of GBSS. In all genotypes, a core group of granule-associated proteins was detected including SSI, SSIIa, SBEIIa, SBEIIb and GBSSI. These proteins (SSI, SSIIa, SBEIIb and GBSSI) are also routinely found within the starch granules of other cereals like maize, wheat, barley and rice (Rahman et al., 1995; Morell et al., 2003; Boren et al., 2004; Regina et al., 2005;
Along with these proteins, other proteins (SSIII, SBEIc, unknown protein of 110 kDa) were also found in all genotypes. SBEIc and an unknown protein of 110 kDa were absent from B-granules. The amount of individual starch granule proteins involved in starch biosynthesis in the granules was also measured from total starch, A- and B-granules from all genotypes (see methods). Only minor differences were observed in the amount of different proteins. Previously it was reported that alteration/mutation in specific starch biosynthetic enzymes results in the alteration of starch granule proteome of mutants (Morell et al., 2003; Grimaud et al., 2008; Li et al., 2011; Liu et al., 2012a, 2012b; Chapter, 3). However, in this study such variations have not been found in the starch granule proteome, which suggests that these genotypes do not have mutations in the major isoforms of different proteins involved in starch biosynthesis (other than GBSS). Evidence suggests that most of these starch granule-associated proteins are components of previously identified, soluble protein complexes, and that the formation of these protein complexes is phosphorylation dependent (Tetlow et al., 2008; Hennen-Bierwagen et al., 2008; Liu et al., 2009, 2012a, 2012b; Chapter 3). These functional protein complexes become entrapped within starch granules, resulting in soluble proteins becoming granule-bound (Liu et al., 2009). Thus, association of different proteins within the growing starch granules is, at least partially, a reflection of the formation of stromal functional heteromeric protein complexes involved in amylopectin biosynthesis. Analyzing the granule proteome of various genotypes, may provide an indication of the existence of protein complexes in the stroma. Our results show that GBSS, SSI, SSIIa, and SBEIIb are phosphorylated in their granule-bound state. It was also reported previously that in wheat,
GBSS, SSI and SSII were phosphorylated in their starch granule-bound state (Bancel et al., 2010). Similarly phosphorylation of GBSS, SBEIIb, and SP in maize and wheat was reported by Grimuad et al. (2008) and Tetlow et al. (2008). It is also possible that some granule-bound proteins are phosphorylated and granule-associated as free, non-complexed forms.

Barley has a bimodal starch granule size distribution in which granules can be divided into large A- and small B-granules or C-granules. The biochemical processes underlying synthesis of A- and B-granules is not well understood. Peng et al. (2000) reported that, in wheat, one peak of granule nuclei formation occurs before 15 DPA and another after 15 DPA. In the second stage of development, A-granules grow larger than 10 µm, while B-granules lack this ability. The underlying reason for this is not known. In barley, soluble SSs are thought to have a role in determination of granule size. For example, a mutation at the barley shx locus results in reduced SSI activity and a reduction in the size of A-granules, transforming the normal bimodal granule size distribution to unimodal (Schulman and Ahokas, 1990; Tyynela and Schulman, 1993; Tyynela et al., 1995). There is evidence that, along with starch biosynthetic enzymes, synthesis of A- and B-granules is controlled by other molecular factors. Stamova et al. (2009) reported that the rate of the change in volume (%) of the A-granule was correlated with the expression profiles of two unknown genes (BE422634, r = 0.832 and BE438268, r = 0.922), a putative DEAD box RNA helicase (r = 0.849), and chitinase2 (r = 0.811). The number and volume distributions of B-granules, the ratio of B- to A-granules in terms of their volume and number predominantly correlated with the changes in transcriptional expression of storage protein genes (Stamova et al., 2009). The percent number of A-
granules has been shown to be correlated with the expression profiles of genes encoding several transcription factors and other proteins involved in carbohydrate metabolism such as, Glucose-6-phosphate dehydrogenase and GBSSII (Stamova et al., 2009). In this study, variation in granule size distribution and (%) amount of small granules in total mass of starch in different barley genotypes have been found. However, no significant differences have been observed in the granule proteome analysis of A- and B-granules from different genotypes. Similarly, phospho-proteome analysis from different genotypes did not reveal significant variations. Thus, it can be deduced that granule size distribution and % amount of A-, B- and C-granules may be regulated by different factors other than enzymes which become entrapped within the granule. Our results also show that very small C-granules have significantly reduced amounts of SSI, SSIIa, SBEIIa and SBEIIb proteins (Figure, 4.12c) per unit (1.5 mg) of starch. The possible reason for this reduction in the amount of protein may be related to large surface area to volume ratio of very small granules compared to large granules. However, more detailed molecular and biochemical analyses are required to understand the regulation of synthesis of A- and B-granules.

4.5 Conclusion

This study provides detailed physiochemical and biochemical analyses of barley genotypes with varying proportions of A- and B-granules and amylose content. Different physical and morphological properties of the seed did not correlate with physiochemical and biochemical properties of starch. The detailed biochemical analyses of granule-bound proteins did not show significant differences, except that B-granules lacked SBEIc and an unknown granule protein of 110 kDa. The phospho-proteome
analysis showed that GBSS, SSI, SSIIa, and SBEIIb are phosphorylated in their granule-bound state in A- and B-granules. The ratios of different granule-bound proteins with respect to SSI, SSIIa, SBEIIa and SBEIIb were not altered in different genotypes.
Chapter 5: General discussion and future work
5.1 General Discussion

In this thesis, detailed analyses of the physiochemical properties of resistant starch, and potentially important biochemical mechanisms, of RS biosynthesis, have been investigated in barley. RS has significant beneficial effects on human health (Asare et al., 2011). There are different types of RS (reference to Section, 1.3.2.1.1.1.1). RSII is most important because it is naturally occurring starch in its granular form which is resistant to digestion by $\alpha$-amylase. The natural occurrence of RSII makes it possible to study variations in the physiochemical properties of RS, as well as alterations in the biochemical pathway of starch biosynthesis which give rise to the formation of RS. Barley has proved a very useful model system of starch biosynthesis given its diploid nature, ease of transformation, and large number of starch mutants and genotypes available. Given the similarities in granule structure between wheat and barley, barley serves as an ideal model system for studying cereal starch biosynthesis.

5.1.1 Analysis of the physiochemical properties of RS as determinant of increased RS genotypes

To determine the relationship between seed and starch physicochemical properties, and resistant starch, a wide range of barley genotypes from different sources were selected to screen for their RS content. Analysis of these genotypes showed that there are certain physico-chemical properties of the seed and starch which can be used as an indication of RS content. A correlative analysis suggested that increased RS is associated with higher amylose content. Previously it was also reported that amylose is the principal component in the formation of RSII (Sajilata et al., 2006; Asare et al., 2011). Previous reports suggested that during cooking when starch is gelatinized amylose
leaches out of the swollen starch granules as coiled polymers which upon cooling associate as double helices and form hexagonal networks (Jane and Robyt, 1984; Haralampu et al., 2004). In waxy starches this network does not form where instead, aggregate formation occurs which is more susceptible to α-amylase hydrolysis (Miao et al., 2009). The results presented in Chapter 2 indicated that increased amylose is associated with an alteration in proportion of small granules to large granules. All the genotypes with increased amylose exhibited a higher proportion of small granules (Figure 2.4). Similarly in all the high amylose genotypes starch granules with altered morphology were observed compared to waxy and reference genotypes (Figure 2.5). The data suggest that these two characters can be used as physical markers for high amylose genotypes. The major challenge in the development of high amylose genotypes is reduced yield. The known high amylose mutants of barley, sex6 and sbeiia/sbeiib double mutant (> 60 % amylose) exhibited reduced starch content (Morrell et al., 2003: Regina et al., 2010). Similarly our data suggest that amylose content is negatively related to starch content and seed size. None the less, during this study certain genotypes, 081011-928, 081011-929 and 081011-930, were identified which exhibited significantly higher amylose content than the reference genotype while starch content and TGW were comparable to the reference genotype (Table 2.1). It is proposed that these lines can be used for further breeding programs to develop high amylose barley genotypes without loss of yield.

5.1.2 Regulation of starch biosynthesis in the barley endosperm is governed by the formation of multi-enzyme protein complexes

In the recent past, several studies have shown the existence of physical interactions between enzymes of starch biosynthesis. In a study by Tetlow et al. (2004a)
with wheat endosperm, it was shown that SBEI, SBEIIb and SP form phosphorylation-dependent multi-enzyme complexes. Similarly in the maize endosperm, the existence of physical interactions among major isoforms of starch biosynthetic enzymes in the form of protein complexes has also been reported (Hennen-Bierwagen et al., 2008; Liu et al., 2009, 2012a, 2012b). These studies were further developed by determining the existence of possible protein complexes in the barley endosperm (Chapter, 2, 3 & 4). Similar to maize and wheat, protein-protein interactions between SSs and SBEs were also observed in barley, suggesting that protein-protein interactions between starch biosynthetic enzymes are a common feature in cereal endosperm.

Analysis of defined barley mutants revealed novel protein-protein interactions due to single gene mutations. In the SBEII mutants, SBEI, SP and the expressed form of SBEII, were clearly observed forming multi-enzyme complexes. However, since anti-SSI antibody did not co-precipitate SBEI and SP, it can be argued that in the absence of one or other isoform of SBEII, SBEI and SP interact with the remaining expressed form of SBEII. Liu et al. (2009) also reported a similar protein complex in the \textit{ae} mutant of maize (lacking SBEIIb protein) in which SBEI, SBEIIa and SP were clearly observed, although in that case they appeared to form multi-enzyme complexes with SSI and SSIIa which is not the case here. In the \textit{amo1} mutant, both SBEIIa and SBEIIb were co-immunoprecipitated with SSI and SSIIa proteins. A number of possibilities might account for this observation. It is possible that SBEIIa and SBEIIb were co-purified together as a result of forming a dimer, or that SBEIIa and SBEIIb interact with SSI and SSIIa independently. In the \textit{sex6} mutant (lacking SSIIa protein) interactions among different isoforms of starch biosynthetic enzymes were not observed reinforcing the centrality of
SSIIa in forming protein complexes (Liu et al., 2012b).

The importance of interactions among different enzymes of starch biosynthesis pathway was revealed by the study of different mutants. For example, in either of the branching enzyme II mutants studied, SSI activity was significantly decreased (Chapter 3). This may indicate that association, or presence, of both SBEII proteins is required for SSI activity. Similarly, SP activity was not observed in the sbeiib− mutant (Chapter 3), suggesting that in vivo association of SBEIIb with SP might confer a positive regulatory effect on SP activity. Similarly, in the sex6 mutant (lacking SSIIa), SSI activity increased, suggesting that SSIIa possibly has a negative regulatory effect on SSI activity. Detailed biochemical analysis of granule proteins revealed that in the SBEII mutants, different protein complexes are involved in the synthesis of A- and B-granules. It is argued that a complex containing SSI/SSIIa and either form of expressed SBEII is involved in the synthesis of A- and B-granules, whereas protein complexes containing SBEI and SP are involved only in the synthesis of A-granules. This hypothesis was supported by detailed biochemical analysis of starch granules in which SBEI and SP were only present in the A-granules.

Functional association of key enzymes involved in starch biosynthesis within multi-enzyme complexes may enhance the efficiency of amylopectin construction. For example, products of SSs become the substrates of SBEs, and the products of SBEs in turn become the substrates of SSs. Previously, abnormal amylopectin structures were observed in the mutants lacking key starch biosynthetic enzymes (Yuan et al., 1993; Shi and Seib, 1995; Klucinec and Thompson, 2002; Morell et al., 2003; Zhang et al., 2004;
Delvalle et al., 2005; Regina et al., 2010; Liu et al., 2009, 2012a), which suggests a higher level of organization indicative of multi-enzyme coordination.

5.1.3 Formation of multi-enzyme complexes containing starch biosynthetic enzymes in barley endosperm is regulated by protein phosphorylation

The evidence suggests that the physical interactions between isoforms of starch synthases (SSs) and branching enzymes (SBEs) in cereal endosperm amyloplasts are driven by protein phosphorylation (Hennen-Bierwagen et al., 2008; Liu et al., 2009, 2012; Tetlow et al., 2004a, 2008). In barley, co-immunoprecipitation experiments indicated that conditions that would be expected to favour protein phosphorylation (+ ATP) lead to an increase in protein complex formation. It also appears that SSI, SSIIa, SBEIIb, SP and SBEI are phosphorylated in their granule-bound state arguing that it is complexes carrying phosphorylated proteins which become entrapped in the starch granule.

5.1.4 Physiochemical properties of seed and starch do not correlate with starch granule proteome

As described earlier, using a wide range of genotypes from different genetic backgrounds, amylose/RS contents were measured (Chapter 2). From these genotypes, detailed biochemical analyses of high amylose mutants with known mutations in amylopectin synthesizing enzymes were performed (Chapter 3). Similarly, detailed physiochemical analyses of starch and analysis of granule proteome of the remaining genotypes where the source of variation was unknown was also performed (Chapter 4). The results presented indicate that these genotypes exhibited variations in physiochemical properties of seed and starch. However, analysis of the starch granule proteome did not
reveal variation among different genotypes, except for waxy genotypes which could account for this (Chapter 4). From these genotypes, OAC Kawartha, CDC- Fibar, Soft Barley, Hard Barley, AC Metcalfe and 083411-113 were further selected on the basis of parameters described in section (4.3.3). Analysis of soluble proteins (involved in starch biosynthesis) from amyloplast lysates of these genotypes also did not reveal significant variation. The starch granule proteome of genotypes with no characterized mutation in amylopectin synthesizing enzymes did not correlate to variations in the physiochemical properties of seeds and starch. This argues that the source of variation in physiochemical properties of seed and starch for these cultivars lie elsewhere and are independent of starch granule proteome. Results from chapter (2, 3 & 4) indicate that starch granule proteome is not always indicative of variation in starch physiochemical properties. However, the starch granule proteome can be used to identify mutations in starch biosynthetic enzymes and variations in starch physiochemical properties, when mutations are in the pathway of starch biosynthesis (Chapter 3).

This study also showed that in high amylose/RS genotypes, starch exhibits unimodal granule size distribution due to an increased proportion of small granules compared to large granules and % RS/amylose was positively related to the proportion of small granules (Banks et al., 1971; You and Izydorczyk, 2002: Asare et al., 2011). Amylose content and starch granule size related strongly with some seed dimensions and physiochemical properties. Production of starch with desired characteristics such as high RS is often associated with a reduction in starch content and yield. As stated earlier, this study recommends genotypes, 081011-928, 081011-929 and 081011-932 for further
breeding programs to develop genotypes high not only in RS, but also having starch content comparable to normal or reference genotype.

5.2 Future Work

In this study an effort has been made to study the biochemical processes involved in the synthesis of RS. Future work should be aimed at understanding the involvement of post-translational modifications, particularly protein-protein interactions in the synthesis of RS. For example, what protein kinases and phosphatases are involved and how would their modification affect starch synthesis and the type of starch produced. Similarly, experiments which analyse the physiological significance of the observed multi-enzyme complexes in relation to the synthesis of A- and B-granules, are needed. Finally use of transgenic plants with modifications which prevent specific protein-protein interactions, and consequently the synthesis of particular classes of granule in vivo, would be a powerful tool to study the physiological functions of protein complexes.


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Appendices
## Appendix 1: Description of genotypes used in the study.

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<th>comments</th>
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<td>sex1 rob (orange lemma)</td>
</tr>
</tbody>
</table>

1. Dr. Duane Falk, Cereal Breeder, Department of Plant Agriculture, University of Guelph, Guelph, Ontario
2. Dr. Mathew Morell, Cereal Chemist, CSIRO, Black Mountain, Canberra, ACT, Australia
3. Dr. Brian Rossnagel, Cereal Breeder, Crop Development Centre, University of Saskatchewan, Saskatoon, Saskatchewan

- 250 -
Appendix 2: Comparison of three methods used for amylose determination.

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<th>GPC</th>
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Amylose content measured by three different methods; Megazyme amylose/amylopectin assay kit, iodine binding and GPC.
Appendix 3: PCA analysis shows association of three methods used for amylose determination.

**Figure S1:** The name of each character is given in the PCA while different genotypes are presented by (+). Characters present in close vicinity are associated. Genotypes have been clustered into different groups depending upon their association with different characters and each cluster is presented by a different circle: black, normal barley; Light green, experimental lines; red, waxy; blue, shrunken (sex1); Dark green, high amylose.
Appendix 4: Determination of amylose with 6CL-B column.

Amylose eluded in early fractions and amylopectin in late fractions. Each genotype with different symbol has been presented in the legends.
Appendix 5: Iodine-staining of barley grains (cross section).

To see the internal structure and starch packing within seed from each genotype, cross sections along the length of seed were made and incubated in the Lugol's solution. Name of each genotype is given under each section of figure.
Appendix 6: DSC data of different genotypes.

Initial Heating Summary

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Re-Heating Summary

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Thermal analyses of starch from different genotypes were performed as described by (Liu et al., 2007) using a differential scanning calorimeter (2920 Modulated DSC, TA Instruments, New Castle, DE).
Appendix 7: Immunological characterization of endosperm amyloplast lysates from different barley mutants.

Amyloplast lysates (~1.3 mg/ml) were prepared from developing barley endosperms at 18–25 DAP. Aliquots of soluble (stromal) proteins were separated on 10 % polyacrylamide gels and electroblotted onto nitrocellulose membranes. Immunoblots were developed with peptide-specific anti-wheat antibodies. Left hand side indicate cross-reactions of each of the antibodies with its corresponding target protein, and the name of each genotype is given below. The approximate molecular mass for each protein, based on its SDS–PAGE migration, is given on right hand side.
Appendix 8: Detection of SS activity and protein.

Non-denaturing electrophoresis was performed in 5% (w/v) polyacrylamide gels containing 0.3% (w/v) amylopectin. Approximately 300 µg protein from whole cell extracts was loaded onto each lane. Following electrophoresis, gels were incubated for 48–72 h at 30°C. Activity of SS was visualized by staining gels with I₂–KI (A). Arrows indicate activity band corresponding to different SS protein in zymogram (A). The identity of SS has been made by coupling non-denaturing PAGE with immunoblotting, and proteins were detected by using specific antibody (B, C, D, E).
Non-denaturing electrophoresis was performed in 5% (w/v) polyacrylamide gels containing 0.2% (w/v) maltoheptaose and 1.4 U of rabbit muscle extracted phosphorylase a. Approximately 300 µg protein from whole cell extracts was loaded onto each lane. Following electrophoresis, gels were incubated for 3 - 3.5 h at 28°C. Activity of SBE was visualized by staining gels with I$_2$-IK. Activity of SP is detected as dark blue bands (A). Migration of SBE isoforms was determined by immunoblotting with specific antibodies (B, SBEIa; C, SBEIb; D, SBEI and SBEIc). Name of each genotype shown. Arrows indicate specific activity band in zymogram and presence of respective proteins in immunoblots incubated with different antibodies.
Appendix 10: Detection of SP protein and activity in different genotypes.

(A) Activity of SP (Pho1) is visualized as a dark blue band. (B) Immunodetection of SP (Pho1 and Pho2).
Appendix 11: Co-immunoprecipitation of stromal proteins from amyloplasts of different genotypes with SSI, SBEIIa and SBEIIb antibodies.

A) Immunoprecipitation with anti-SSI antibody

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(A)
B) Immunoprecipitation with anti-SBEIIa antibody

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Arrows indicate the following molecular weights:
- SSI: 70 kDa
- SSIIa: 87 kDa
- SBEI: 88 kDa
- SBEIIa: 83 kDa
- SBEIIb: 93 kDa
- SP: 112 kDa
Aliquots (0.75-1 ml) of amyloplast lysates (0.8–1.3 mg protein ml/ml) prepared from endosperm of different genotypes at 18–25 DAP were incubated with specific anti-SSI (A), anti-SBEIIa (B) and anti-SBEIIb (C) antibodies at 25°C for 1h, and then immunoprecipitated with protein A–Sepharose beads. The protein A–Sepharose–antibody–antigen complexes were washed several times to remove non-specifically bound proteins, boiled in 200 µl of SDS loading buffer, and 25 µl was loaded in each lane of 10 % polyacrylamide gels. Following electrophoresis gels were electroblotted onto nitrocellulose, and developed with various anti-barley antibodies including (SSI, SSIIa, SSIII, SSIV, SBEI, SBEIc, SBEIIa, SBEIIb, SP and ISO). However immunoblot incubated with SSI, SSIIa, SBEI, SBEIIa, SBEIIb and SP antibodies are presented.
Appendix 12: Summary of novel protein–protein interactions formed between amylopectin-synthesizing enzymes in barley endosperm following either loss of single gene (SSIIa, SBEIIa and SBEIIb) or alteration in single gene (ssiii, amo1 mutant).

In reference genotype (A), the major form of SBEII (SBEIIa and SBEIIb) form phosphorylation-dependent protein complex with SSI and SSIIa independently. In the
branching enzyme mutants (B & C), possibly two distinct protein complexes consisting of SSI/SSIIa and expressed SBEII, and SP/SBEI and expressed SBEII were purified. In which protein complex, SP/SBEI/ expressed SBEII partitioned to A-granules not B-granules. Loss of SSIIa was more significant (D) where due to absence of SSII no protein complex was formed and consequently no protein was found within the starch granule. In case of amo1 mutant protein complex consisted of SSI, SSIIa, SBEIIa and SBEIIb were purified. However, there is a possibility of existing of other complexes in different combinations such as, SSI/SSIIa/SBEIIa and SSI/SSIIa/SBEIIb. For each of the genotype, the components of the protein complexes found in the plastid stroma are also found in the respective A and B starch granules but in sex6 mutant where no protein complex was formed, no protein was found in the starch granules. Inverted arrows between A and B granules show possible involvement of same protein complex in A and B granules synthesis.
Starch granules were isolated from mature barley grains by grinding seeds and preparing starch granules as described Materials and Methods. The purified, acetone washed starch was used to separate large A and small B granules with Percoll gradient centrifugation method from all genotypes. The separated A, B granules and starch were washed extensively to remove proteins loosely bound to the granule surface. A 50 mg aliquot of purified starch, A and B granules were boiled in 1 ml of SDS loading buffer, and 40 µl of the supernatant from the boiled sample loaded onto 4–12 % acrylamide gradient gels and 10 % acrylamide SDS gels. Following electrophoresis, gels were electroblotted to nitrocellulose membrane and developed with various specific anti-wheat antibodies (SSI, SSIIa, SSIII, SSIV, SBEI, SBEIIa, SBEIIb, SP, ISO1 and GBSS). Each section is labelled with respective antibody and type of granules used either starch, A- or B-.
Appendix 14: Granule-bound phospho-proteome analysis and starch, A- and B-granules bound proteins detected by silver staining.

To determine granule-bound phospho-proteome equal amounts of starch from all genotypes were run onto 4–12 % acrylamide gradient gels, and stained with Pro-Q Diamond. Following Pro-Q Diamond staining the same gel (Phospho-proteome) was subject to silver staining (total starch) to visualize all bands. The bands visualized with silver stain are numbered as (1, SSIII; 2, SBEIc; 3, SP/unknown; 4, 5, 6, SSIIa; SBEIIa; SBEIIb; 7, SSI; 8, unknown; and 9, GBSS). In A-granules two additional bands, 10 and 11 present degraded GBSS and SS respectively. However all these bands were not found to be phosphorylated and among above mentioned bands only GBSS1, SSI, SSIIa and SBEIIb were found to be phosphorylated in their granule-bound state (Phospho-proteome).
Appendix 15: Analysis of starch composition and granule morphology by light microscope (A, B & C) and electron microscope (D & E).

Separation of A- and B-/C- granules was performed according to method described earlier (Peng et al., 1999). Total starch containing, A-, B- and C- granules (A). Purified A-granules (B). Purified B-/C- granules (C). Electron micrograph of total starch, predominant with A- and C- granules (D). Purified B-/C- granules (E). The reference genotype, OAC Baxter is presented as an illustration for A- and B-/C- granules purity confirmation in all genotypes studied here. The scale bar on panels A, B and C represents 20 µm. Scale bar for electron micrograph is given under each panel. Each type of granule is presented by an arrow.
Appendix 16: Single nucleotide polymorphisms (SNPs) of barley \textit{ssIIIa} genomic DNAs from different barley genotypes.
The Names of barley genotypes are labeled on the left hand side. The number of nucleotide residues is labeled above. The dots indicate the same nucleotide residues as the first line. The substituted nucleotide residues are indicated at position 330, 2101 (for 083611-124 sex1), 2693, 5610, 8338 and 860 (for HAG amo1, SB94983 amo1, 081011-928, 081011-929, 081011-930, 081011-931 and 081011-932) and 3273 and 6323 (for HAG amo1, SB94983 amo1, 081011-928, 081011-929, 081011-930 and 081011-932).
Appendix 17: SNPs of cDNA sequences of barley \textit{ssIIIa} genomic DNAs from different barley genotypes.

![SNP Diagram]
The Names of barley genotypes are labeled on the left hand side. The number of nucleotide residues is labeled above. The dots indicate the same nucleotide residues as the first line. The substituted nucleotide residues are indicated at position 1084 (for 083611-124 sex1), 1676 and 4439 (for HAG amo1, SB94983 amo1, 081011-928, 081011-929, 081011-930, 081011-931 and 081011-932) and 2256 (for HAG amo1, SB94983 amo1, 081011-928, 081011-929, 081011-930 and 081011-932).
Appendix 18: Changes of polypeptide sequences of barley SSIIIa protein from different barley genotypes.
The Names of barley genotypes are labeled on the left hand side. The number of nucleotide residues is labeled above. The dots indicate the same nucleotide residues as the first line. The substituted amino acid residues are indicated at position 362 (for 083611-124 sex1) and 559 and 1480 (for HAG amo1, SB94983 amo1, 081011-928, 081011-929, 081011-930, 081011-931 and 081011-932).