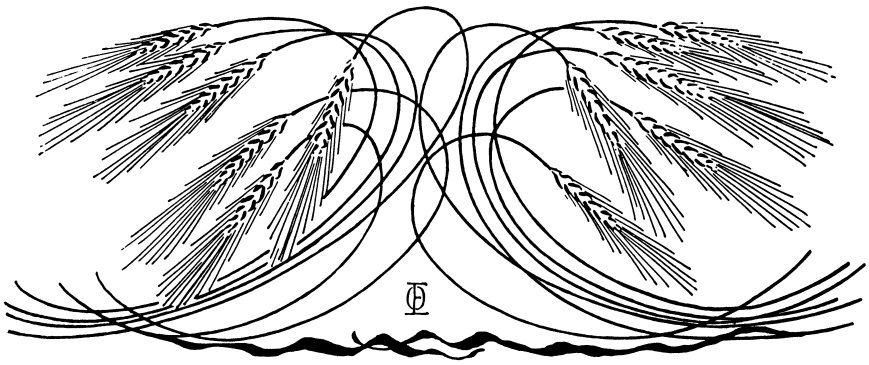
Proteomics of Barley Starch Granules

Mats Borén
Faculty of Natural Resources and Agricultural Sciences
Department of Plant Biology and Forest Genetics
Uppsala



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Abstract

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Starches in various forms are an important part of the human diet. Furthermore, the use of starch as a renewable and biodegradable raw material for various industrial applications is becoming increasingly attractive. Non-food applications of starch make new demands on quality and type of starches produced. Ideally these new demands should be met by tailoring starches within the plant. A number of such *in planta* produced specialty starches exist today, *e.g.* high-amylose starch.

Our aims with this Ph.D. project have been to increase the knowledge of starch synthesis and caryopsis development. We have attempted to do this primarily by investigating the proteins entrapped within the starch granule. This has been done using proteomic techniques. Our main focus has been on characterizing a barley mutant with high amylose starch, *amo1*. Apart from proteomic characterization of granule proteins we have also done comparative starch characterizations and assayed for starch branching activity in mutant and wild type seeds. We also investigated the occurrence of a caspase-like protease activity, a VEIDase, during caryopsis development.

A protocol for extracting proteins from starch granules has been developed and a 2D proteomic reference map of integral granule proteins established. The molecular mechanism of a fragmentation of granule proteins induced during sample preparation has been found. An alternative sample preparation procedure has been established that reduces fragmentation considerably. A developmentally regulated caspase-like proteolytic activity, a VEIDase, with possible connection to developmental programmed cell death, has been characterized and found to be chiefly active during early stages of caryopsis development. The VEIDase activity has been localised to small vesicles in starchy endosperm cells.

Keywords: barley, starch, endosperm, starch granule, proteomics, protein, 2D PAGE, VEIDase, developmental PCD, acid induced hydrolysis, Asp-Pro, fragmentation

Author's address: Mats Borén, Department of Plant Biology and Forest genetics, Box 7080, SLU, SE-750 07 Uppsala, Sweden. E-mail: mats.boren@vbsg.slu.se

Contents

Abbreviations

List of selected abbreviations used in the text:

2-D	2-Dimensional separation of proteins (Isoelectric point and mass)
3-PGA	3-PhosphateGlycerate
ADP	Adenosine DiPhosphate
AFLP	Amplified Fragment Length Polymorphism
AGPase	ADP-Glucose Pyrophosphate
BAC	Bacterial Artificial Chromosome
BSA-AFLP	Bulk Segregant Analysis Amplified Fragment Length Polymorphism
Cys	Cystein
DP	Degree of Polymerisation
Dpa	Days Post Anthesis (Days after pollination)
EST	Expressed Sequence Tag
FMK	Fluoro Methyl Ketone
GBSS	Granule Bound Starch Synthase
Glc	Glucose
LSU	Large SubUnit of AGPase
PCD	Programmed Cell Death
PPDK	Pyruvate orthoPhosphate DiKinase
PP_i	PyroPhosphate, inorganic
SBE	Starch Branching Enzyme
SDS-PAGE	Sodium Dodecyl Sulphate PolyAcrylamide GelElectrophoresis
SS	Starch Synthase
SSS	Soluble Starch Synthase
SSU	Small SubUnit of AGPase
TUNEL	(TdT)-mediated dUTP Nick End Labelling
VEID	Valine-Glutamic acid-Isoleucine-Aspartic acid
wt	wild type

Appendix

Papers I-IV

I. Borén, M., Larson, H., Falk, A., Jansson, C. 2004. The barley starch granule proteome – internalized granule polypeptides of the mature endosperm. *Plant Science* 166, 617-626.

II. Borén, M., Bozhkov, P., Höglund, A., Falk, A., Jansson, C. 2005 Developmental regulation if a VEIDase caspase-like proteolytic activity in barley caryopsis. (under revision in *J. Exp. Botany*)

III. Borén, M., Larson, H., Falk, A., Jansson, C. 2005 Acid hydrolysis causes fragmentation of intrinsic starch granule proteins during extraction. (submitted)

IV. Borén, M., Glaring, M., Ghebremedhin, H., Blenow, A., Falk, A., Jansson, C. Molecular Characterisation of the High Amylose Barley Mutant *amo1*. (manuscript)

Introduction

Starch is part of everyday life although most people rarely think about it. There is starch in a lot if everyday foodstuff and we eat it everyday. It takes in fact quite an effort to avoid eating starch. For example whenever you eat bread, pasta, rice, or French fries you are eating starch prepared in different ways from several different sources. Apart from the products above, where starch is the major ingredient, starch is usually added to many foodstuffs in order to attain certain properties. Surface improvement of deep fried foods, to improve consistency and replace fat in light products and to solidify gum candy, are a few examples where starch is used to improve food. Starch also has a range of industrial applications. Some of the more well known applications are in adhesives, cosmetic powders, and in the coating of paper. Starches from different biological sources, including various starch mutants, have different properties making them ideally suited for different applications. The range of starch applications can be further expanded through different, usually chemical, modifications of the starch (Tomasik & Schilling, 2004). The isolation of special natural starches as well as chemical modifications of starch can sometimes be difficult, expensive and/or hazardous to the environment. Ideally, the desired type of starch should be produced in an agronomically well suited plant species and the need for post harvest processing should be minimized by introducing the modifications already in the plant. In order to make the rational design of starches possible, a full understanding of the molecular mechanisms underlying seed and tuber development and starch synthesis in particular must be obtained.

History of barley and its potential in research

Barley has played an important role throughout human history. The oldest archaeological remains indicating the use of wild barley, *Hordeum spontaneum*, are from as early as 17000 BC (Zohary & Hopf, 1994). When prehistoric man turned to farming, barley was among the first crops to be domesticated. There is relatively little difference between the early domesticated form and the wild forms and it is believed that both were farmed by early settlers. The main difference between the wild form of barley and the early cultivated forms is the ability to retain grains on the ear after maturity. This characteristic is very advantages in a farming-system and is believed to have arisen soon after agricultural use of wild barley was initiated around 8000 BC (Zohary & Hopf, 1994). Based on genetic markers it is believed that barley was domesticated in the Fertile Crescent somewhere in the Israel-Jordan area (Badr *et al.*, 2000). Along with the practice of agriculture the use of barley spread from the Fertile Crescent throughout the rest of the known world. Due to an initial large genetic variation together with new mutations and recombinations barley successfully adapted to new environmental conditions.

Today, barley is still one of the most important cereal crops and is cultivated in one of the widest climatic ranges, from sub-arctic regions via the tropics to arid

semi-deserts. Due to its importance for human food, especially for brewing, and animal feed, large efforts have been made in plant breeding and collecting of germplasm from all over the world. Currently, there are some 378 000 barley accessions in the world's genebanks (van Hintum & Menting, 2003). A large number of those are believed to be duplicates but a large collection of unique entries of landraces, modern and obsolete varieties, mutants as well as wild *Hordeum* relatives still remains.

As research interests shift towards molecular characterisation of genes and proteins, this resource, especially the mutant collections, has proven very valuable. In one of the largest collections of barley mutants, at the Nordic Genebank in Alnarp, some 10 000 accessions are stored, many of them have been extensively morphologically characterized. If the mutational site can be identified, mutants provide an opportunity to identify the functional role of genes discovered by genome sequencing. A number of mutants known to be affected in genes involved in starch synthesis have been characterized and contributed to the understanding of starch synthesis. Most mutants affected in various aspects of grain development and starch deposition have so far not been molecularly characterized but once they are, valuable new insights will be gained.

The *amo1* mutant is one of the first isolated mutants that are affected in starch deposition. *Amo1* was originally discovered in an effort to find a high-amylose barley variety better suited for making whisky malt (Merritt, 1967). Unfortunately it was soon proven that high-amylose barley was poorly suited for making whisky (Ellis, 1976). High-amylose starch from other sources have however found a multitude of other uses such as in thickeners of jellies and gum candies, coating of photographic films, coating of deep fried snacks and as a source of resistant starch for human consumption (Slattery, Kavakli & Okita, 2000, Topping *et al.*, 2003). Despite being discovered nearly 40 years ago the mutation site and molecular mechanism of the *amo1* mutation have not yet been determined.

There are several good reasons for using barley as a cereal model plant:
1. It is the forth larges crop in the world, grown virtually everywhere.
2. Large germplasm collections exist with a range of well characterized mutants.
3. Homozygous lethal mutants can be studied as seedlings due to the large seed reserves.
4. Barley genetic resources such as transformation, microarray, and BAC libraries are available.
5. A large amount of EST sequences have been generated which partly make up for the lack of full genome sequencing.
6. Barley is diploid and naturally self pollinating which makes genetics simpler and facilitates the isolation of induced mutants.
7. It is closely related to and exhibits a high degree of synteny with other economically important grasses enabling interspices comparison.
8. Easy to sample various tissues and get enough material for analysis.

There are of course some notable drawbacks to using barley as a model crop. Most notable are the huge genome and rather long generation time, at least compared to *Arabidopsis*.

Development of the barley caryopsis and the role of PCD in the process

Proper orchestration of programmed cell death (PCD) is essential for correct development of the caryopsis (Young & Gallie, 2000). The mature cereal caryopsis consists of two main parts, the embryo and the endosperm, each resulting from independent fertilizations. This dual parallel beginning is called double fertilization (Faure, 2001). The double fertilization is initiated by a pollen grain germinating on the stylus and extending the pollen tube down through the stylus. The pollen grains of grasses contains three cells, one vegetative and two generative. All three have diploid nuclei and the two generative nuclei result from a mitotic division of a haploid precursor (McCormick, 1993). The three nuclei follow the pollen tube until it reaches the female gametophyte. Once there, one of the generative cells fuses with the egg cell, to form what will develop into the embryo, and following that initial fertilization the second generative cell fuse with the two polar haploid nuclei, the three cells fuse together to form the start of the endosperm (Engell, 1989). The egg cell and the polar nuclei are the result of rounds of mitotic division of a haploid nucleus. The initial double fertilization results in a diploid ovule and a triploid endosperm, both with the same haploid nuclei mixture.

The embryo develops into the reproductive part of the seed. The embryo suspensor cells are the first cells of the embryo to show signs of PCD, starting 14 days post anthesis (dpa) in corn. In late embryonic development, *ca.* 27 dpa in corn, a few cells in the scutellar node are still TUNEL positive, as a result of vascular differentiation (Giuliani *et al.*, 2002). Towards the end of caryopsis development the embryo develops desiccation tolerance and remains alive in the mature seed. After fertilization the endosperm undergoes rapid syncytial enlargement forming a coenocyte, a sphere-like multinucleated cell with a single layer of nuclei evenly spaced around a central vacuole (Figure 1 A). In barley, cellularization of the coenocyte occurs between 3 and 5 dpa, during which cell division is halted (Olsen, 2004). Cell walls form between the nuclei surrounding the vacuole but no cell wall is formed towards the vacuole. When cell division resumes, new cells are formed in a column, called an alveolus, inwards towards the endosperm centre (Figure 1 B & C). During these very early developmental stages, PCD is first observed in the maternal nucellar and pericarp tissues as early as five dpa in wheat (Dominguez, Moreno & Cejudo, 2001). The maternal tissues are progressively degraded, to supply nutrients and provide space, until the last specialized transfer cells in the nucellar projection finally denucleate around 18 dpa in wheat (Dominguez, Moreno & Cejudo, 2001) and 24 dpa in corn (Kladnik *et al.*, 2004).

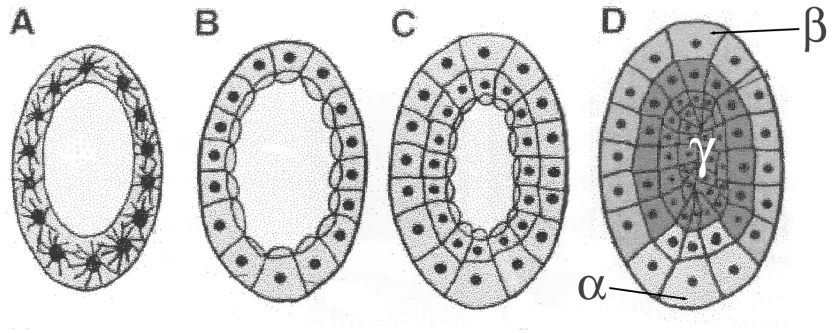

Figure 1 Early endosperm development

A-Coenocyte B & C-Cellularization from the periphery with open ended alveoli D- Three of the main cell types of the triploid endosperm after cellularization: α – transfer cells β – aleurone layer and γ – starchy endosperm (After Olsen (2004))

The endosperm differentiates into four main tissues: the starchy endosperm develops in the central region, three peripheral cell layers form the aleurone layer, and an embryo-surrounding region develops between embryo and endosperm and finally the transfer cells, which develop along the vascular bundle where they facilitate solute transportation into the endosperm (Figure 1 D). The number of cells of the starchy endosperm continues to increase both through cell divisions, until 14 dpa, and through conversion of newly formed aleurone cells, until 21 dpa (Kvaale & Olsen, 1986). Cells of the aleurone and starchy endosperm that no longer divide undergo endoreduplication. During endoreduplication the level of ploidity increases drastically without cell or nuclear division. The exact role of endoreduplication is not known but it has been speculated that it enables higher metabolic rate through higher transcriptional and translational activity and allows an increase in cell size (Larkins *et al.*, 2001).

In mature barley seeds, all cells in the starchy endosperm are dead; however it is not clear when PCD is initiated. The onset of PCD depends on which cell death indicators are used. According to ultra structural studies of membranes and nuclear integrity in rice (Lan *et al.*, 2004) and loss of membrane integrity in wheat (Golovina, Hoekstra & van Aelst, 2000) it is believed that PCD initiates almost immediately after completion of cellularization. More classical markers of cell death, such as TUNEL and Evan's blue staining can be seen from 12 dpa in rice (Lan *et al.*, 2004) and 16 dpa in wheat and corn (Young & Gallie, 1999). In barley, wheat and rice, cells entering into PCD seem randomly distributed throughout the starchy endosperm in contrast to corn where the older central cells first show signs of PCD. As early as during the coenocyte stage, small starch grains can be seen but it is not until cellularization of the endosperm is complete around 8 dpa that starch accumulation really starts. The principal starch accumulation period continues until 25 dpa. As accumulation of starch and proteins in the starchy endosperm cells near completion they enter into PCD resulting in dismantling of all but the most essential cell functions. Despite being void of all organelles, the cell remains metabolically active for 1-2 days, during which deposition continues (Lan *et al.,* 2004). The aleurone cells remain alive

until germination, fulfilling a vital role in mobilising the storage reserves in the starchy endosperm. A few days after germination the aleurone cells enter PCD (Fath *et al.*, 2000).

The making of starch

Introduction

The ability to acquire, convert, use and store energy is a fundamental aspect of all life. For us humans it means to eat food which is converted into chemical energy, which our bodies use to sustain our activities. Excess energy is converted into glycogen and fat for short and long term storage, respectively, for use at a later time. Plants are faced with the same challenge of how to solve the energy problem but the majority of plants have solved it in quite a different way. Plants acquire energy by absorbing sunlight which is converted into chemical energy through the process of photosynthesis. The captured energy is used to fuel a myriad of activities within the plant. The reliance on sunlight to obtain energy has limitations since sunlight is not always available, *e.g.* during the night. To overcome this, a number of ways to store energy have evolved in plants. The most important way is the production of polysaccharides, commonly referred to as starch. Starch consists of polysaccharide macromolecules of defined non-random structure, packed into granules, small spheres. Starch is deposited in different tissues and fulfils different functions depending on the tissue. In leaves, so called transient starch accumulate during the day and is broken down during periods of low photosynthetic activity**.** In seeds, roots, tubers, stems and corms storage starch is deposited. The storage starch acts as long-term store of energy that can be utilised by the plant itself when necessary. In contrast, storage starch deposited in developing seeds is exclusively used during germination and establishment of the plantlet. The synthesis of starch from photoassimilates to granules involves a number of enzymes and regulatory steps. The importance of starch to both plants and humans make it an essential object of study. In the following pages the major aspects of starch synthesis will be covered and discussed. The main focus will be on the synthesis of storage starch in cereal seeds.

Assembling the building block of starch

The synthesis of the starch itself, especially cereal grain starch, is merely the last steps in a long chain of events that start with photosynthesis in leaves. The steps leading to the capture and conversion of sunlight into sugars through photosynthesis are outlined in an excellent review which forms the bases of the following passage (Nevins, 1995). High energy light photons are absorbed by chlorophyll situated in huge molecular antenna complexes in the chloroplast membrane. The excited chlorophyll passes its energy towards the centre of the antenna where a reaction centre is situated. The reaction centre initiates an electron transport in the thylakoid membranes, which in turn establishes a potential gradient, H^+ gradient, across the membrane that is used to drive the formation of ATP and NADPH. This is the first step towards starch as solar energy have been captured and temporarily stored as chemical energy in ATP. The next step is the fixation of carbon which is done by assimilating atmospheric

carbon dioxide. Carbon dioxide dissolves in the moisture of the leaf and moves into the chloroplast where it reacts with the enzyme rubisco. Within the active site of rubisco the carbon dioxide reacts with ribulose bisphosphate forming two molecules of 3-phosphoglyceric acid. The 3-phosphoglyceric acid is then energized by ATP and NADPH to form glyceraldehydes-3-phosphate, a triose hydrocarbon. In the next step, the triose molecules are either exported out of the chloroplast or remain in the chloroplast. Some of the retained trioses are used to regenerate ribulose bisphosphate. Remaining trioses react pair-wise to form a phosphorylated fructose, a hexose. The fructose phosphate is isomerized in two steps within the chloroplast to glucose-1-phospate. Glucose-1-phospate can, with the help of AGP-glucose pyrophosphorylase (AGPase) and ATP, turn into ADP-glucose, the basic building block of starch, transient leaf starch in this case. The two triose molecules that were exported into the cytosol are also converted to hexoses and finally form UDP-glucose and fructose-6-phospahate, which through the action of sucrose phosphate synthase combine into sucrose-6-phospate. After dephosphorylation by sucrose-phosphatase, sucrose is formed as a rather stable temporary end product that can be exported to other parts of the plant via the phloem (Lunn & MacRae, 2003).

From its site of synthesis in the mesophyll cells sucrose is believed to move via plasmodesmata to the sites of phloem loading where it is released in the apoplasm surrounding the phloem through an unknown mechanism. The uptake into the phloem from the apoplasm is mediated by H^+-sucrose co-transporters. Once inside the phloem, sucrose is believed to move through an osmotically generated flow from source regions with high levels of sucrose to sink regions with lower levels (Lalonde, Wipf & Frommer, 2004). Sucrose is unloaded into the developing endosperm by H^+-sucrose co-transporters via the crease vain containing specialized transfer cells (Weschke *et al.*, 2000). The crease vain is a single vascular bundle extending across the length of the grain and is the only route of import to the developing grain. Movement from the transfer cells to the starchy endosperm cells is thought to be symplastic through plasmodesmata (Wang *et al.*, 1995). Within a starchy endosperm cell sucrose can either be broken down, within the cytosol, through the action of sucrose synthase to fructose and UDP-Glucose or to glucose and fructose by an invertase, either in the apoplastic space or in the cytosol (Figure 2). Through the further action of various phosphorylases, glucomutases and isomerases; glucose, fructose and UDP-glucose is converted to ADP-Glucose, the dedicated starch precursor (Vandeputte & Delcour, 2004).

The enzymes responsible for making starch

Considering that starch is a compound composed of just glucose moieties it is a remarkably complex molecule. This is true both in its primary structure and how it is packed into granules. Enormous diversity exists between starches from different plant species and plant organs and each are virtually unique. This diversity in starch structure originates from varying activity levels, of isoforms within species and homologues between species, of starch biosynthetic enzymes. Our understanding of how this diversity can be the result of a handful of classes of starch synthesis genes are beginning to emerge but much still remains unknown.

The basic enzymes needed in the starch synthesis, AGPase, starch synthases (SS), starch branching enzymes (SBE) and starch debranching enzymes (DBE) are conserved across a wide range of taxa. The number of isogenes in each class is greater for the starch storing plants, *e.g.* cereals and tuber crops, than non-starch storing plants. The following overview of starch synthesis will mainly focus on cereal seed storage starch and especially on what is known about barley seed storage starch. The understanding of starch synthesis in cereals is yet not fully understood. Wherever necessary appropriate parallels with leaf starch and tuber storage starch will be used to shed light on poorly understood aspects of seed storage starch synthesis. Cross tissue and species comparison should be used with caution since the processes are known to differ in some fundamental aspects.

Synthesis of ADP-glucose and its transportation into the amyloplast

The first committing step in the starch synthesis pathway is AGPase (Figure 2). AGPase catalyze the conversion of glucose-1-phosphate and ATP into ADP-glucose and pyrophosphate (PP_i). The active AGPase is a multimeric complex comprising two large subunits (LSU) and two small subunits (SSU). In all tissues, except endosperm of grasses, AGPase has an exclusive plastidial location. In the endosperm of grasses a second AGPase is located in the cytosol (Beckles, Smith & ap Rees, 2001). The cytosolic and plastidial forms of AGPase are expressed from two different sets of genes (Johnson *et al.*, 2003). In developing barley endosperm the cytosolic isoform is responsible for a majority of the ADP-glucose synthesis, roughly 85% (Thorbjornsen *et al.*, 1996) indicating a very important role in the synthesis of starch precursors.

Further support of AGPase as a rate-limiting step in starch synthesis comes from the molecular characterisation of various AGPase mutants. In barley, the low starch mutant *Risø16* was recently shown to carry a deletion in the gene encoding the SSU of cytosolic AGPase, which completely abolishes the cytosolic AGPase activity (Johnson *et al.*, 2003). In *Risø 16* the lack of cytosolic AGPase reduces starch content to 44% of normal without altering the timing of starch accumulation. This indicates that loss of cytosolic AGPase leads to a reduced capacity for starch synthesis in the endosperm (Johnson *et al.*, 2003). There are no other known AGPase mutants of barley, but the *Shrunken2* mutant in corn is affected in the cytosolic LSU gene which also leads to total loss of cytosolic AGPase activity and a low starch phenotype (Preiss *et al.*, 1990). Both *Risø 16* and *Shrunken2* have fully functional plastidial AGPase which results in normal levels of leaf starch. No mutants in either of the plastidial AGPase subunits have been molecularly characterized. Due to this it is difficult to predict the importance of the plastidic AGPase in endosperm starch synthesis.

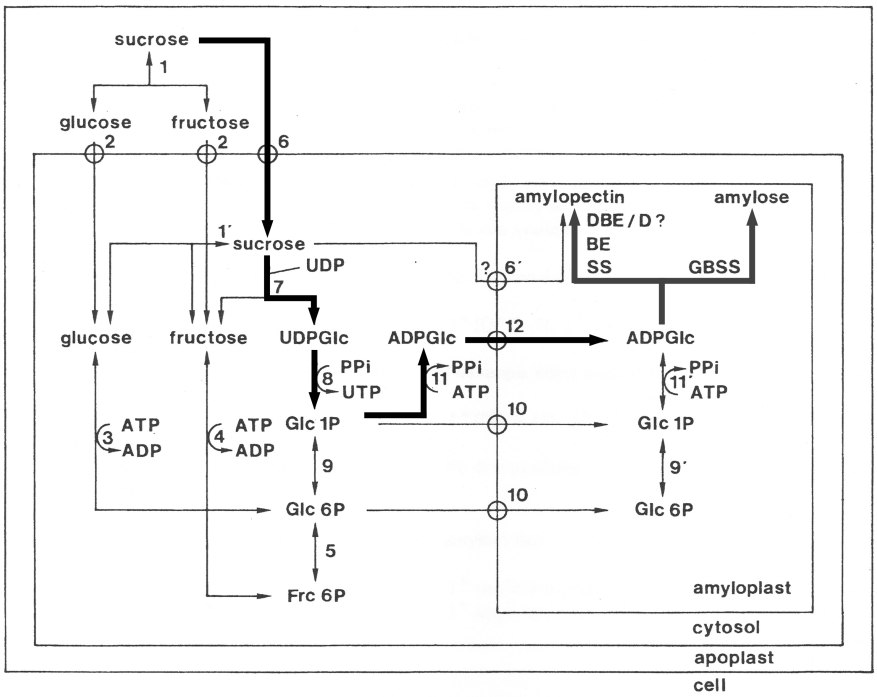

Figure 2 The starch biosynthetic pathway in non-photosynthetic cells.

Thick arrows indicate path with greatest metabolic flux
1 – invertase, 2 – hexose transporter, 3 – hexokinase, 4 – fructokinase, 5 – glucose phosphate isomerase, 6 – sucrose transporter, 7 – sucrose synthase, 8 – UDP glucose pyrophosphorylase, 9 – cytosolic phosphoglucomutase, 10 – phosphate translocator,
11 – ADP glucose pyrophosphorylase, 12 – ADP glucose translocator. Glc – glucose, ADP – adenosine diphosphate, UDP-uridine diphosphate. (After Vandeputte & Delcour (2004))

Synthesis of ADP-glucose in the cytosol requires transport of ADP-Glucose across the amyloplast membrane into the stroma and site of starch synthesis. This transport is accomplished by an adenylate translocator, the ADP-Glc transporter encoded in the *lys5* locus of barley (Doan, Rudi & Olsen, 1999). The *lys5* mutant in barley (Patron *et al.*, 2004) as well as the *Brittle-1* mutant of corn (Shannon *et al.*, 1998) lack functional plastidic ADP-Glc transporter and are therefore unable to transport cytosolic ADP-glucose into the amyloplast, resulting in high cytosolic levels of ADP-glucose as well as a low starch phenotype. The fact that mutants like *Risø16*, *Shrunken2* and *Lys5* accumulate endosperm storage starch indicates that alternative routs to AGP-glucose exist in amyloplasts. Although little molecular evidence exists, the most likely alternative is the import of glucose-1-phosphate and/or glucose-6-phosphate via hexose phosphate transporters. Once in the amyloplast the glucose-1-phosphate and glucose-6-phosphate are readily converted to AGP-glucose through phosphoglucomutase and plastidial AGPase (Kavakli *et al.*, 2000; Vandeputte & Delcour, 2004).

Being the first committed step in the starch synthesis pathway, AGPase activity is an obvious point of regulation. Interestingly, the two forms of AGPase, plastid and cytosolic, respond to regulatory signals in partly different ways. The plastid form are activated by 3-phosphoglycerate (3-PGA) and inhibited by PP_i whereas the cytosolic form is inhibited by PP_i, ADP and Fructose-1,6-bisphosphate (Ballicora, Iglesias & Preiss, 2004). The cytosolic form has no direct activator but the inhibitory effects can be lifted by 3-PGA and Fructose-6-phosphate. Although it has no classical activator its substrate affinity can be enhanced by 3-PGA at low ATP levels. This higher affinity can be reversed by PP_i (Ballicora, Iglesias & Preiss, 2004). AGPase from plant leaves and potato tubers have been shown to be under post-translational redox regulation (Hendriks *et al.*, 2003). An intermolecular disulfide bridge between Cys12 in the two SSU is present in the deactivated enzyme. The oxidative breakage of this linker results in enzyme activation (Tiessen *et al.*, 2002). The key amino acid, Cys12, for this type of regulation is conserved in AGPases from plant leaves and other tissues but not in the monocot cytosolic endosperm AGPases, which results in insensitivity to this particular type of regulation (Ballicora *et al.*, 1999).

Starch synthases

The role of SS in the synthesis of starch is the transfer of single glucose moieties from ADP-glucose to glucan polymers. This result in the sequential elongation and creation of a linear chain of glucose units connected with $\alpha(1 \rightarrow 4)$ linkage. All starch synthesizing organisms have multiple SS isoforms and specialized starch storing plants have multiple isogenes of each isoform. Studies of exon-intron structure and homologies in the C-terminal part of the various SS genes from such diverse organisms as *Ostreococcus tauri* and *Arabidopsis thaliana* indicate that emergence of multiple SS forms is a very ancient evolutionary event (Li *et al.*, 2003; Ral *et al.*, 2004). All SS have more or less conserved C-terminal domains containing two ADP-glucose binding domain and one glucosyl transferase domains (Denyer *et al.*, 2001). The N-terminal regions of various SS proteins are less conserved and vary considerably in length. The N-terminal region is not required for activity and it is believed to have a regulatory and/or substrate determining role (Tomlinson & Denyer, 2003). It has generally been accepted that SS adds glucose units one at a time to the non-reducing end of the growing chain. Recently an alternative mechanism have been proposed, according to which the enzyme remains bound to the growing chain and adds glucose residues to the reducing end (Mukerjea & Robyt, 2005). According to the reducing end theory the growing starch chain jumps between the two ADP-glucose binding sites and becomes elongated with one residue for each jump. In barley there are five classes of SS, four that are mainly located in the stroma around the granule and one class that are exclusively located inside the starch granule.

Soluble starch synthases

The soluble starch synthases (SSS) forms have been grouped into four different classes based on homology, named SSI to SSIV. In monocots, *e.g. Arabidopsis thaliana*, and alga, one member of each class exists whereas multiple members of

some groups exist in cereals (Li *et al.*, 2003; Hirose & Terao, 2004; Delvalle *et al.*, 2005). In barley, only two *sss* genes, *ssI* and *ssIIa*, have so far been reported but there is reason to suspect that several will be discovered since eight different SSS from all four classes have been found in rice (Hirose & Terao, 2004). Assemblies of available barley ESTs indicate the presence of *sss* genes of classes III and IV also in barley (personal observation). The soluble starch synthases play a crucial role in the synthesis of amylopectin. The different isoforms function cooperatively to assemble the complete amylopectin molecule. Since members of all classes have been conserved throughout evolution there is reason to believe that they have different, unique roles to fulfil in the synthesis of amylopectin (Delvalle *et al.*, 2005). Due to the abundance of isoforms the precise role of each class of SS is still not quite determined but mutant/transgenic studies have enabled a qualified guess based on changes in chain length pattern in the absence of single isoforms.

A mutant of SSI in rice (Nakamura, 2002) shows a shift in amylopectin chain length distribution with lower number of chains of 8-12 degrees of polymerisation (DP: number of glucose residues in the chain) and a higher number of chains of 6-7 DP. This would indicate a role in elongating chains of DP 6-7 to chains of DP 8-12. In rice *SSI* shows a steady level of expression during development through out the whole plant (Hirose & Terao, 2004). In leaves of *A. Thaliana*, SSI appears to have a similar role in elongating short chains of transient amylopectin starch (Delvalle *et al.*, 2005). In *A. thaliana* leaf SSI mRNA oscillates in a circadian rhythm with highest amount of transcripts at the end of the photoperiod and lowest just before the onset of illumination. SSI mRNA or protein levels are however not correlated to SSI activity indicating some form of post-translational regulation (Delvalle *et al.*, 2005).

Mutants from several species including barley (Morell *et al.*, 2003) have been found to be affected in the *ssIIa* gene. In barley, the *ssIIa* mutation, *sex6* locus, gives rise to striking phenotypical and biochemical changes. The amylopectin chain length distribution shifts towards more branches with DP 6-11 and less branches with DP 12-30 (Morell *et al.*, 2003), indicating a slightly longer chain preference compared to SSI. In barley the SSIIa mutation results in an increased level of amylose, above 60% compared with normal levels around 25% (Morell *et al.*, 2003). In corn, the corresponding null-mutation, *sugary2* (Zhang *et al.*, 2004) results in a more moderate increase from about 28% to 38% amylose. The difference may, in part, be explained due to experimental differences since it is known that the level of amylose in seeds of the barley SSIIa mutant is reduced during storage (personal observation). A pleiotropic effect of the SSIIa mutation is the loss of granule-bound SSI, SBEIIa and SBEIIb (Morell *et al.*, 2003). Levels and activities of SSI, SBEIIa and SBEIIb in the stroma are not altered, indicating either that the individual enzymes or a complex involving SSIIa can not bind to the granule (Morell *et al.*, 2003). Evidence for the later explanation was recently presented when it was shown that SBEI and SBEIIb form a complex *in vivo* after phosphorylation (Tetlow *et al.*, 2004). The rice homologue of SSIIa in barley, SSII-3, is mostly expressed in the grain filling stage, after 5 dpa, and exclusively in developing panicles (Hirose & Terao, 2004). SSIIb, although not detected in

barley, are expressed in leaves in rice (Hirose & Terao, 2004) and corn (Harn *et al.*, 1998).

Starch syntheses of class III have not been reported in barley but assembly of available ESTs shows that two transcripts, *ssIII-1* and *ssIII-2*, with high homology to SSIII from other cereals are present in barley (personal observation.). SSIII-1 is represented by 17 ESTs, all from green tissues, and shares highest homology with rice *ssIII-1*, which have been showed to be expressed in leaf tissue (Hirose & Terao, 2004). *ssIII-2*, represented by 5 ESTs from developing caryopsis, share greatest homology to *ssIII* from wheat, which shows endosperm specific expression during early, 4-15 dpa, endosperm development (Li *et al.*, 2000). The corn *ssIII* shows tissue specific expression confined to the endosperm and would thus appear to be a functional homolog of SSIII-2 in rice, wheat and barley. In corn, a mutation in the *ssIII* gene, encoded by the *dull1* locus, have been characterized (Gao *et al.*, 1998). The *dull1* mutant shows pleiotropic loss of SSII and SBEIIa in addition to SSIII loss (Gao *et al.*, 1998). The effect on amylopectin structure caused by loss of SSIII in corn is a decrease in chains with DP>17 (Tomlinson & Denyer, 2003). Due to pleiotropic effect it is not possible to exactly define the role of SSIII in starch biosynthesis.

Genes encoding starch synthesis proteins of class IV have so far only been cloned from a handful of species including rice (two isogenes) and wheat. The barley EST collection contains a number of ESTs with close homology to rice and wheat *ssIV* but the number of ESTs are not sufficient to assemble a full-length contig or to determine if the transcripts are from one or two *ssIV* genes (personal observation). In rice, both *ssIV* genes are expressed primarily in sink leaves and developing caryopsis at steady levels throughout development of the caryopsis but trace amounts are found in all tissues (Hirose & Terao, 2004). There is currently no information available concerning the role of SSIV in starch synthesis.

Granule bound synthases

One class of starch synthase proteins are exclusively localized within the starch granules and are therefore termed granule bound starch synthase (GBSS). In barley there are two types of GBSS, one sink-tissue specific, GBSSI, and one source-tissue specific, GBSSIb (Patron *et al.*, 2002) or GBSSII (Vrinten & Nakamura *et al*., 2000). GBSS is solely responsible for the synthesis of amylose (Smith, Zeeman & Denyer, 2001). This has been shown in so called *waxy* mutants that carry a mutation in the *waxy* locus, the gene encoding GBSSI, which produce no or little amylose in their starchy endosperm (Patron *et al.*, 2002). *Waxy* (high amylopectin) barley mutants are one of the oldest starch mutants to have been selected. Chinese archival records from the 16th century describe naked barley with *waxy* or glutinous endosperm. Then this mutant was grown especially for brewing purposes (Takahashi, 1955). From China the *waxy* barley is believed to have spread to Japan via Korea as early as the 17th century and from there to the rest of the world (Takahashi, 1955). The notion of a single mutational event is strongly supported by the finding that all natural *waxy* barley varieties carry the same deletion in the 5´-non-coding region of *gbssI* (Patron *et al.*, 2002).

It has been shown that GBSSI synthesize amylose in cavities within the intact amylopectin scaffold that make up the structural element of the starch granule (Tatge *et al.*, 1999). This is made possible by a network of pores permeating the granule (Fannon, Hauber & Bemiller, 1992). Amylose can be leached from intact granules by incubation in water at elevated temperatures, indicating that this network of pores have access to the granule surface (Banks, Greenwood & Thomson, 1959). Through these pores ADP-Glucose penetrates the granule and can reach the GBSSI enzyme bound to the starch chains in the interior of the granule. It has been shown that plants with reduced levels of GBSSI activity have granules where the amylose is confined to central regions of the granule (Blennow *et al.*, 2003). A mechanism for this phenomenon has been proposed based on relative substrate affinity of GBSSI compared to other SS (Clarke *et al.*, 1999). GBSSI has lower affinity for ADP-glucose and is thus out-competed by the other SS forms close to the granule surface. This leads to synthesis of amylopectin which forms a scaffold in which GBSSI is trapped. Once inside the granule it will have access to ADP-glucose, diffusing in through the granule pores, without having to compete against the other SS isoforms. This leads to a shift in starch synthesis towards more amylose farther from the granule surface (Clarke *et al.*, 1999). Apart from its role in amylose synthesis GBSSI is believed to be involved in the synthesis of extra long amylopectin branches (Fulton *et al.*, 2002).

In leaves, *gbssI* has been found to be expressed in a circadian controlled manner with high expression levels early in the light period (Merida *et al.*, 1999). It has been hypothesized that there is a need to replace GBSSI that have been degraded together with the granules during the night (Smith *et al.*, 2004). Since this relationship does not exist in the endosperm it is doubtful whether *gbssI* expression in the endosperm is subjected to similar regulation.

Starch branching enzymes

Within the linear glucose chain, where the glucose residues are coupled in an $\alpha(1 \rightarrow 4)$ manner, branches are introduced by coupling a chain of glucose residues in an $\alpha(1 \rightarrow 6)$ manner. This branching is done by starch branching enzymes. In barley, as in most cereals, several starch branching enzymes, SBE, belonging to two classes, SBEI and SBEII, have been characterized (Sun *et al.*, 1997, Peng *et al.*, 2000). Although the exact mode of action of SBEs have not been established, it is generally agreed that they act by first cutting a chain and attaching themselves to the severed chain. It then joins the attached chain to a neighbouring chain in an $\alpha(1 \rightarrow 6)$ manner (Tomlinson & Denyer, 2003).

The starch branching enzyme class I in barley consists, so far of SBEI and SBEIc. In barley and wheat it has been shown that *sbeI* is expressed during the later part of endosperm development (Morell *et al.*, 1997, Mutisya *et al*., 2003). SBEI preferentially transfers longer chains than SBEII and have a much higher branching activity towards amylose then amylopectin (Guan & Preiss, 1993). No SBEI mutant have been described in barely but a rice mutant affected in SBEI exists and have been shown to contain amylopectin with fewer long chains, DP>37, and fewer short chains, DP 12 to 21, with an increase of really short

chains, DP>10 (Satoh *et al.*, 2003). A SBEI mutant of corn has been shown to accumulate amylopectin with chain branch structure indistinguishable from wild type amylopectin (Blauth *et al.*, 2002). A double mutant in corn deficient in both SBEI and SBEIIb does, however, show deviant amylopectin structure, as compared to a SBEIIb single mutant, indicating a functional interaction between the branching enzymes, see below, (Yao, Thompson & Guiltinan, 2004). Additional insight into the role of SBEI have been gained by observing to what extent over-expressed corn *sbeI* could complement the absence of glycogen branching enzyme in *E. coli* (Seo *et al.*, 2002). Using that system it was shown that *sbeI*, when produced alone, could not induce branching and glycogen accumulation. However when produced together with SBEIIa and/or SBEIIb it could alter the branching structure. Very little is known about SBEIc other then that it appears to exist and be preferentially incorporated into large A-type starch granules in species with bimodal granule distribution, *e.g.* wheat, rye, barely and triticale (Peng *et al.*, 2000).

The second class of starch branching enzymes have two members in barley, SBEIIa and SBEIIb. The two genes are differentially expressed so that *sbeIIb* is only expressed in endosperm whereas *sbeIIa* is expressed in all starch synthesizing tissues (Sun *et al.*, 1998). A B-Box-like element in the 2nd intron of the *sbeIIb* is responsible for the difference in expression pattern (Ahlandsberg, Sun & Jansson, 2002). Both SBEII enzymes have similar affinity for amylose and generally tend to produce shorter branches than SBEI (Blauth *et al.*, 2001). Based on a sbeIIa mutant in corn (Blauth *et al.*, 2001), it was predicted that SBEIIa mainly affects amylopectin structure in leaves by reducing the amount of short chains. The *sbeIIa* mutation also caused an early senescence phenotype leading to low levels of photo-assimilation and very few seeds. Endosperm starch appears unaffected by the *sbeIIa* mutation and has an almost identical distribution of amylopectin chains (Blauth *et al.*, 2001). *sbeIIb* mutants, amylose extender (*ae*), on the other hand have been isolated from a range of species, *e.g.* corn (Kim *et al.*, 1998), rice (Mizuno *et al.*, 1993) and pea (Bhattacharyya *et al.*, 1990). Amylopectin from *ae* mutants have an increased amount of long chains, DP>38, and a marked decrease of short chains, DP<17 (Nishi *et al.*, 2001). Despite almost identical substrate specificities the *ae* phenotype of endosperm starch can not be compensated by SBEIIa activity although it is unaffected in the ae mutants (Nishi *et al.*, 2001). The *ae* mutation shows pleiotropic effect on SSI activity, which is lowered by almost 50% in rice endosperm (Nishi *et al.*, 2001). If this is due to shortage of SSI substrate, SSI favourably elongates short chains, or some unknown complex formation is not known.

It has recently been shown that all SBE enzymes in wheat depend on phosporylation to be activated. Upon phosporylation SBEI and SBEIIb forms a complex together with starch phosphorylase in order to attain *in vivo* activity (Tetlow *et al.*, 2004).

Starch debranching enzymes

The traditional role of DBE has been in starch degradation and not in starch synthesis. Although the role of DBE in starch synthesis is still unclear, it is obvious that they do have an important role in the synthesis of starch (Sun *et al.*, 1999; Burton *et al.*, 2002; Dinges *et al.*, 2003). Two classes of DBEs exists in barley, isoamylase represented by three genes (Hussain *et al.*, 2003) and pullulanase represented by one gene (Kristensen *et al.*, 1999). The two classes are separated based on substrate specificity with isoamylase debranching amylopectin and glycogen whereas pullulanase debranches amylopectin, pullulan and glycogen.

Two mutant lines in barley, *Risø17* and *Notch-2*, affected in the isoamylase 1 gene, *isa1*, have been characterized (Burton *et al.*, 2002). The mutants were found to have reduced starch content and altered amylopectin structure. It also accumulates phytoglycogen, a branched amylopectin-like glucose polymer that can not crystallize into granules. The most striking difference is however in the initiation and growth of starch granules. In *Risø17* there is only a single wave of granule initiation resulting in up to five times as many granules during early developmental stages but never the less an equal number at maturity compared to wild type. Several granules initiate within each plastid and as the granules grow, compound granules forms. This suggests that isoamylase plays a fundamental role in granule initiation (Burton *et al.*, 2002). The barley *isa1* mutant accumulates a mixture of granule forming starch and soluble phytoglycogen whereas the equivalent mutant in rice only accumulates phytoglycogen (Nakamura *et al.*, 1997).

The role of isoamylase in amylopectin branch length distribution, although obviously important, remains to be determined. Isoamylases are known to exist and function as part of large multimeric complexes (Dauvillee *et al.*, 2001). The total loss of all isoamylase activity in *isa1* mutants may indicate that *isa1* subunits are required to produce functional complexes (Tomlinson & Denyer, 2003). In corn a mutation in the isoamylase 1 gene results in sweet corn, the normal eating corn.

Whereas mutants of isoamylase genes have been characterized in a range of species, a pullulanase mutant have so far only been isolated in corn through a focused PCR based screening effort of induced mutation lines (Dinges *et al.*, 2003). As expected the mutant shows a so called starch excess phenotype with slightly elevated levels of leaf starch during periods of darkness. However, after extended periods of darkness, leaf starch levels returned to wild type levels, indicating a non-essential role in starch degradation for pullulanase. The absence of pullulanase shifted the amylopectin chain length distribution towards shorter chains in leaf starch whereas there was no shift in endosperm amylopectin. In endosperm the only difference between mutant and wild type was the appearance of short branched polysaccharides in the absence of pullulanase, indicating a role of debranching short polysaccharides for pullulanase (Dinges *et al.*, 2003). A barley transformation line with anti-sense towards a pullulanase inhibitor has been

isolated and characterized (Stahl *et al.*, 2004). Reduction in pullulanase inhibitor levels lead to increased levels of pullulanase. The mayor effect on starch was a reduction in the number of B-type starch granules and a shift in amylopectin chain length distribution towards shorter chains.

Other enzymes believed to be involved in starch synthesis

In addition to the enzymes described above, a number of proteins suspected of being involved in starch biosynthesis have been discovered. The putative roles in starch synthesis of these proteins remain highly hypothetical and in many cases the link to starch synthesis is the presence in starch granules or amyloplasts during periods of starch synthesis. Starch contains phosphate monoesters covalently linked to the glucose residues, tuber starch more so then cereal starch. The so called R1 protein is an α-glucan water dikinase believed to be involved in starch phosphorylation (Ritte *et al.*, 2002).

Starch phosphorylase, which catalyses the reversible addition of one glucose unit to a glucose chain from glucose-1-phosphate, is another protein potentially involved in starch synthesis (Yu *et al.*, 2001). The hypothesis that starch phosphorylase is involved in starch synthesis was considerably strengthened recently when it was showed that it forms a complex with the active forms of SBEI and SBEIIb (Tetlow *et al.*, 2004). Its role is however still unknown. A number of enzymes, *e.g.* amylase, disproportionating enzyme and pullulanase inhibitor, traditionally known to participate in starch degradation, might have a role in starch synthesis since they are present in starch-synthesizing amyloplasts (Smith, 1999).

Structure and synthesis of the granule

The non-random branch distribution of amylopectin enables it to form semi-crystalline, insoluble inclusions. These inclusions are generally referred to as starch granules. Shape, size and size distribution of granules varies between species and are typical of its botanical origin (Reichert, 1913). The ability to form granules results in a much higher packing density enabling more starch to be stored in a given volume. There are several layers of organization within the granule from the most basic level being the chain distribution of individual amylopectin molecules to the highest level, shape and size of the granule itself. Barley has a bimodal size distribution of its granules with the larger A-type granules ranging from 10-35 μm in diameter and the smaller B-type granules between 1 and 8 μm in diameter (Tang, Watanabe & Mitsunaga, 2002). The A-type granules are synthesized during the first part of the starch accumulation period whereas B-type granules are initiated and synthesized from 15 dpa and until maturity.

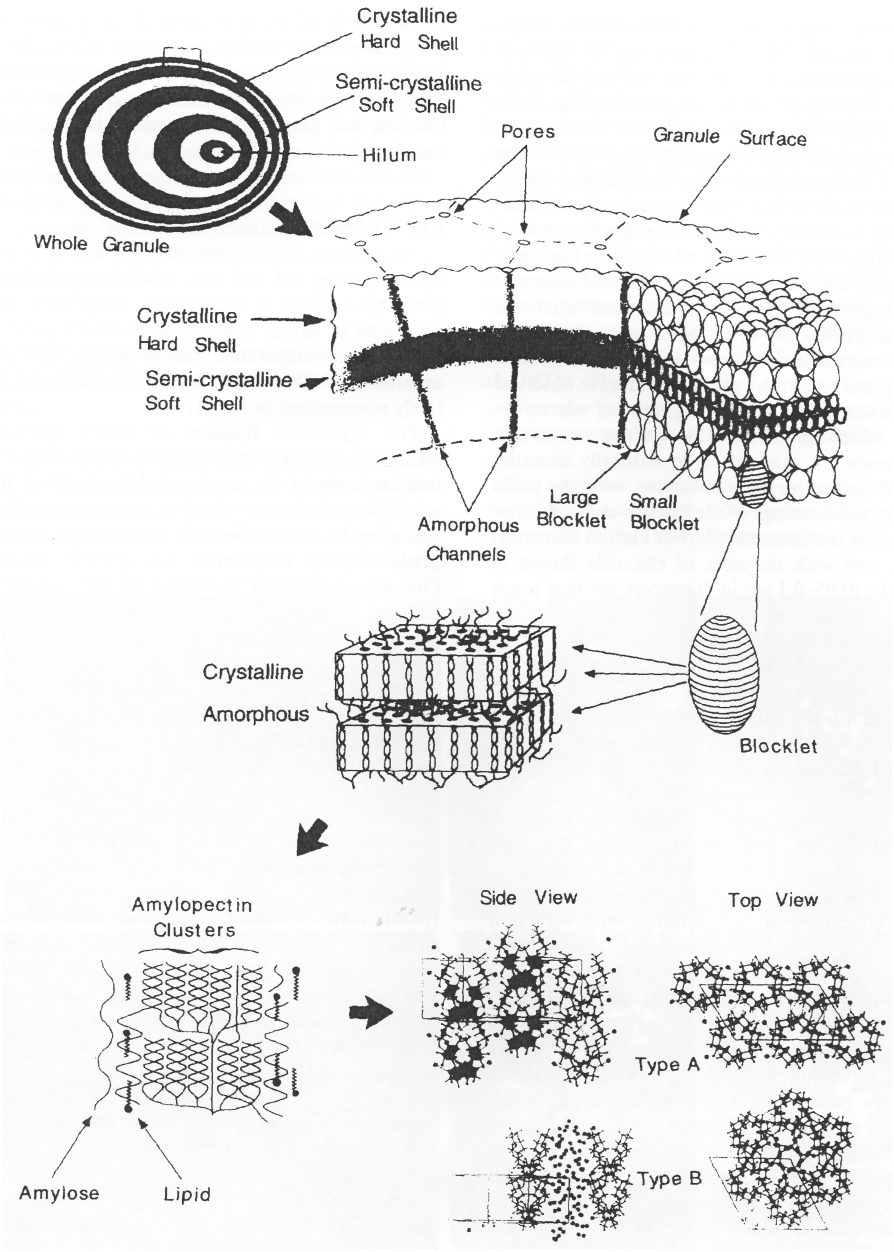

Figure 3 Overview of starch granule structure

From upper left, cross section of granule with growth rings, alternating shells of crystalline and semi-crystalline zones made up of large and small blocklets, respectively. The blocklets are made up of several alternating crystalline regions of parallel amylopectin chain-clusters and amorphous layers containing the branch points of the amylopectin molecule. In the crystalline regions parallel amylopectin chains form double helices that pack together in either of two ways, dense Type A order and loss Type B order. (After Gallant (1997))

Before discussing how the granules are organized we should consider how they are initiated. This most basic aspect of the granule is probably the least understood. Two main hypotheses have been proposed. The first proposes that the process is analogues to glycogen initiation which requires glycogenin, a self-glycosylating protein, to act as primer. It was recently shown that when levels of a protein homologous to glycogenin are reduced in *A. thaliana*, the amount of leaf starch decreases (Chatterjee *et al.*, 2005). The second hypothesis postulates that granules form spontaneously via a process called spherulitic crystallization due to properties of the amylopectin molecule (Ziegler, Creek & Runt, 2005). This process has been shown to occur *in vitro* resulting in sphericals with internal structures resembling the core of *in vivo* starch granules. Although the initiation and formation of granules is not fully understood, it is clear that it is not a random process since shape, size and size distribution are consistent within species but different between species.

Several barley mutants show differences in granule morphology, size, and initiation. The isoamylase mutant mentioned above is one such mutant (Burton *et al.*, 2002). Despite morphological differences between granules from various biological sources the internal structure is mostly conserved. A hilum forms the centre of the granule and consists of a hollow core surrounded by a region of amorphic starch molecules (Buleon *et al.*, 1997). Surrounding the hilum are several concentric shells of alternating zones of semi-crystalline and amorphous material known as growth rings (Pilling & Smith, 2003) (Figure 3). In cereals it is presumed that growth rings are the result of variations in sucrose supply between light and dark periods as cereal grown under constant light does not form rings (Buttrose, 1962). This is different from tuber starches which does have rings also when grown in continues light or dark (Pilling & Smith, 2003). Throughout the granule, extending inwards from the surface, there is a network of pores (Fannon, Hauber & Bemiller, 1992). The exact role of these pores are not clear but they have been hypothesized to be important during starch synthesis by allowing ADP-Glc to penetrate the granule, facilitating amylose synthesis, and during degradation of the granule by giving degrading enzymes access points towards the interior of the granule.

The next smaller level of granule organization is the blocklets that make up the growth rings (Gallant, Bouchet & Baldwin, 1997). Larger blocklets, Ø ≈ 80-120 nm, make up semi-crystalline growth rings whereas the amorphous zones are composed of much smaller blocklets, Ø ≈ 25 nm. The blocklets are made up of alternating layers of crystalline and amorphous zones, made up of parallel amylopectin branches in super-helical formation and branch point clusters, respectively (Vandeputte & Delcour, 2004). One repeat of a crystalline and an amorphous layer are roughly 9 nm thick. This distance is remarkably conserved independent of botanical source and presumably represents some innate property of the amylopectin molecule. The crystalline layers are roughly between 12 and 16 glucose residues thick and one repeat roughly 28. A single amylopectin molecule is believed to span between 4 and 10 repeats (Vandeputte & Delcour, 2004). Within the crystalline layers the amylopectin branches form pair-wise super-helixes. The superhelixes can arrange in two ways, either closely packed, side by

side in layers where each successive layer is shifted by half of a superhelix, or loosely in a hexagonal pattern with one superhelix at each corner. The close packing results in A-type crystal pattern found in cereal starch and the loose pattern gives B-type crystal pattern found in tuber starches. A mixture of the two types is mainly found in starch from legumes and is called C-type starch (Vandeputte & Delcour, 2004).

Amylose, structure and synthesis

Barley amylose consist of mostly linear, between 5 and 12 branches per molecule (Takeda *et al.*, 1999), glucose chains with chain lengths between 180 and 16300 DP, average DP 1120 (Schulman *et al.*, 1995). Despite its rather simple structure, issues regarding how the molecule is initiated and elongated still remain open to debate. Two main hypotheses have been proposed. They differ mainly regarding if a priming molecule is needed to initiate the amylose molecule or not. The first model propose that amylopectin branches are elongated while still attached to amylopectin and once of sufficient length released from amylopectin to form amylose. Once released, the chain elongation is terminated (van de Wal *et al.*, 1998). This model is supported by *in vitro* pulse-chase experiments on isolated starch granules from *Chlamydomonas reinhardtii* that show that [^{14}C]Glu is first incorporated into amylopectin before it emerges in the amylose fraction, with forms faster then the de-novo starch synthesis rate, indicating cleavage of elongated amylopectin branches (van de Wal *et al.*, 1998). This work has been repeated with *A. thaliana* leaf starch and no evidence of amylose synthesis from amylopectin primers could be detected (Zeeman, Smith & Smith, 2002). Whether this discrepancy is due to starch source or experimental conditions are currently unknown.

In the second model of amylose synthesis, amylose is primed by short malto-oligosaccharides (Zeeman, Smith & Smith, 2002). From these short glucan chains the amylose molecule is proposed to be *de-novo* synthesized by the successive addition of glucose residues through the action of GBSSI. Experiments have shown that [^{14}C]Glu will be added to malto-oligosaccharides when [^{14}C]ADP is incubated together with starch granules (Zeeman, Smith & Smith, 2002). It has however been suggested that malto-oligosaccharides act as acceptors rather then primers since the most likely addition is one glucose residue and not the initiation of a whole chain (Mukerjea & Robyt, 2005).

Amylopectin, structure and synthesis

Barley amylopectin consists of highly branched, between 320 and 400 branches per molecule, glucose chains with average DP 20 (Tang, Watanabe & Mitsunaga, 2002). Amylopectin is one of the largest and most complex molecules on earth and at least three models have been proposed for its synthesis.

The first model is the so called glucan trimming model (Myers *et al.*, 2000). The glucan trimming model is based on two different phases of growth, first one phase with branching followed by debranching and one where the newly formed

branches are elongated. Debranching enzymes have a pivotal role in reducing the number of new branches that are to be elongated so that a limited number of branches are formed which will allow crystallization.

The second model is called water soluble polysaccharide clearing model (Smith, Zeeman & Denyer, 2001) (Figure 4 A). In this model the debranching enzymes only have an indirect involvement in starch synthesis as scavenging enzymes by removing water soluble polysaccharides which, if left in the stroma, could be elongated and initiate granules and/or phytoglycogen. The resent finding of increased number of granules and phytoglycogen accumulation in isoamylase mutants give some support for this model (Burton *et al.*, 2002).

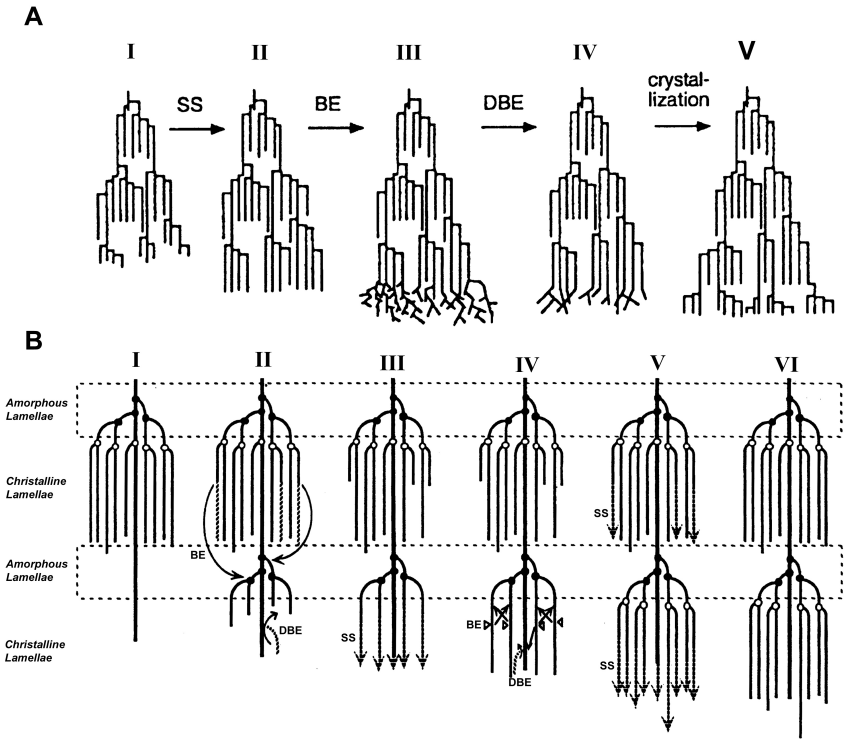

Figure 4 Models of amylopectin synthesis

A – Glucan trimming model: I→II Branch elogation, II→III New branches form, III→IV Excess branches are removed, IV→V After removal of excess branches newly formed parallel branches can crystallise. (After Ball et al. (1996))
B – two step branching and improper branch clearing model: II Branches are formed by moving strands from the previous layer. III Newly formed branches elongate IV Intra cluster branching to facilitate double helix formation V Elongation of all branches VI Double helix formation and crystallisation. (After Nakamura (2002))

The third model is called two-step branching and improper branch clearing model (Nakamura, 2002) (Figure 4 B). As the name implies it involves a two-step branching mechanism with a first round of branching carried out mainly by SBEI by transferring chains from the preceding cluster to a single longer chain extending from the previous cluster. This creates a branching zone which will turn into the amorphous lamellae. The newly formed branches elongate and once of a certain length, suitable for SBEIIb, branching will start by transferring chains between the elongating chains of the new crystalline lamellae. DBEs are involved in both branching steps by removing "incorrect" branches. In a final step all branch chains are elongated to proper length. With this model, two zones of branches are formed, one mayor in the amorphous lamellae and one minor within the crystalline lamellae. The occurrence of branch points within both lamellae has been suggested based on experimental evidence (Jane, Wong & McPherson, 1997).

Plant proteomics

A proteome is made up of the proteins expressed by a genome in a specific situation. The rational build up and use of knowledge of proteomes is called proteomics. In contrast to the genome, which is constant in all cells under all natural conditions, the proteome is constantly changing in response to various stimuli. Due to its ever changing composition, study of proteomes presents somewhat of a challenge, especially since the equivalent of PCR for protein amplification has yet to be developed. Understanding of the proteome and its changes and interactions of proteins are going to be an essential part in understanding the mechanistic working of life.

In order to acquire an understanding of an organism's total proteome, all proteins an organism is capable of expressing, data from a great many proteomic studies carried out under various conditions, tissues and developmental stages must be complied. Due to the dynamic nature of the proteome a proteomic study can be designed in many ways depending on what aspect of the proteome is being studied. The most basic type of studies asks what proteins are present at a particular instant. A protein reference map can then be constructed indicating what proteins are present and at what levels. By constructing such reference maps at different developmental time points as well as before and after various stimuli, so called comparative proteomics can be done to find out how the proteome changes. Detected changes in protein levels can then ideally be used to determine how the organism under study responds to stimuli. Unfortunately this is far from trivial but as molecular knowledge of cellular pathways increase, changes in protein levels will be more informative in the future. What is usually as interesting as changes in levels are changes in protein activity. Activity changes, at constant protein levels, are usually accomplished by post-translational modifications or complex formation. Activity regulating modifications are usually of a transient nature making isolating of an *in vivo* representative sample difficult. Furthermore correlating a change in modification status with a change in activity will require

much further study. Protein microarray chips, although relatively novel, shows great potential in helping to solve that problem. The use of native gels to investigate intact protein complexes also show great potential since it is becoming evident that protein complex formation is a very important biological process.

Proteomic characterization has been pioneered in human and microbial systems but due to the potential gains, proteomic studies will be a greater part of plant systems biology. Although a lot of notable proteomic work has been carried out on plants much remains to be done if we are to gain a fuller understanding of plants.

Aims of this study

This project has been a part of a larger effort with the long-term goal of investigating the development of barley caryopsis and especially how deposition of starch is accomplished and regulated. This project has mainly focused on using proteomic techniques to further that goal. The initial specific aims were:

- Establish a proteomic 2-D reference map of intrinsic barley starch granule proteins
- Make a comparative proteomic study on intrinsic starch granule proteins between *amo1* and Midas (wt) at a number of developmental stages.

- As the project progressed the aims were expanded to also include the investigation of PCD during caryopsis development and especially the occurrence of a VEIDase.

Results and discussion

Proteins integral to starch granules in barley

The proteome of an organism can usually be divided into sub-proteomes. In barley the starch endosperm proteome, a sub-proteome in its own right, can be divided into two main fractions or sub-proteomes, the soluble proteome and the granule-associated proteome. The soluble proteome, consisting of the soluble proteins can potentially be further subdivided depending on which solute is used. The granule-associated proteins can be divided into two sub-proteomes, the surface-localised proteins, proteins that can be removed from the granules by washing or through enzymatic cleavage, and the intrinsic proteins which are truly inside the starch granule.

The intrinsic starch granule proteins are believed to be trapped inside the granule during synthesis of the granule. It has been hypothesized that trapped proteins are involved one way or another in the synthesis or deposition of starch. As our main interest is starch, its synthesis and deposition, it was natural to start the proteomic characterisation of the *amo1* mutant by analyzing the intrinsic granule proteins. There were no previous reports about the extraction and separation of intrinsic starch granule proteins using two dimensional SDS-PAGE, so a methodology for that had to be developed. The main parameters that had to be optimized were: amount of washing before release of intrinsic proteins, removal of SDS and concentration of the sample after gelatinization and conditions for 2-D separation.

Since the 2D separation of intrinsic granule proteins had not been reported before our first objective was to produce a 2D reference map where the majority of the protein spots visualized had been identified (Figure 2, Paper I). This was done and out of 96 spots picked from the gel, 74 were identified. The most striking about the list of identified proteins were that 49 spots had been identified as GBSSI. The GBSSI spots are spread over a large area with variation both in molecular weight and pI. The tryptic peptide distribution on the full length protein clearly indicates that a majority of the GBSSI spots are truncated forms. Apart from GBSSI, three other proteins, SSI, SSII, and SBEIIb were identified as intrinsic granule proteins based on their increase in abundance after washing of the granules. All these proteins are involved in starch synthesis and have previously been shown to be localized within starch granules. Apart from these four intrinsic proteins five other proteins were identified in multiple spots. Those proteins were B and D hordein, serpin Z4, pyruvate orthophosphate dikinase (PPDK) and thiamine biosynthetic enzyme. They all showed decreasing abundance after granule washing and could be almost completely removed by enzymatically shaving the granules with thermolysin. Hordeins and serpin Z4 are very abundant proteins in the soluble fraction from endosperm and would be expected as major contaminations on the granule surface. PPDK and thiamine biosynthetic enzyme on the other hand have to my knowledge not been previously identified directly from 2D gels of barley soluble endosperm proteins. This indicates that they are not present in large amounts in the endosperm and that they may have some sort of affinity for starch.

In an effort to determine the cause of the massive GBSSI fragmentation a number of fragments were subjected to Edman degradation in order to determine N-terminal sequence and hopefully cleavage site. Seven fragments, five GBSSI and one each from SSI and SSII had N-terminals resulting from internal cleavage (Figure 2, Paper III). All fragments had been cleaved C-terminal of an aspartic acid (D). This indicates a specific mechanism, biological or chemical, with the ability to cleave next to an aspartic acid. A possible biological, enzymatic, explanation could be the involvement of a protease with caspase-like cleavage specificity. To test this hypothesis we assayed for the presence of caspase-like activity in developing endosperm. High proteolytic activities against several consensus caspase recognition sequences were found. These activities were further analysed as detailed in the next chapter.

Due to the apparent specificity in cleavage pattern we were initially inclined to expect a biological explanation. However, when it was brought to our attention that a similar phenomenon had been observed with corn starch granules and that the amount of fragmentation had been shown to depend on extraction conditions, we started to look for a non-biological explanation (He-Mu *et al.*, 1998). A search of the literature uncovered acid induced hydrolysis of aspartic acid bonds and especially aspartic-proline bonds as a plausible explanation. This mechanism had been well known and well documented in the older literature but was rarely mentioned in more resent papers. Further literature search revealed that fragmentation via this mechanism increased with increasing temperature and decreasing pH. A further complication turned out to be the use of a Tris-based

buffer as Tris buffers are known to decrease in pH during heating, so the initial pH of 6.8 turned into about pH 5.5 at 95 °C making the acid induced hydrolysis worse. By shifting to a phosphate-based buffer at pH 8.6 and doing the gelatinization at a lower temperature for a shorter time the amount of fragmentation could be substantially reduced (Figure 3, Paper III). It was even possible to extract granule bound proteins at sub-gelatinization temperatures through the leakage of amylose and protein from the intact granules. However the amount of amylose in the final protein pellet increased as the extraction temperature dropped. The increased amylose made isoelectric focusing considerably worse, especially for proteins with high molecular weight. This creates a dilemma where a choice between fragmentation and poor resolution had to be made. An acceptable compromise can be reached by reverting to 95 °C during gelatinization but keeping the high pH and short incubation time. With those conditions the granules are completely dispersed and released amylose is presumably "filtered" from the supernatant phase by the starch past during centrifugation. At these conditions, remaining fragments are no longer a major feature on the 2D gels and can easily be disregarded since their locations are known.

VEIDase, a caspase-like proteolytic activity in developing barley caryopsis

Caspases are a group of proteolytic enzymes that have well characterized roles in PCD in animals. In plants no true caspases have been found and the closest homologues, metacaspases, have been shown to have different cleavage preferences. A number of caspase-like proteolytic activities have been shown to exist in plants (Rotari, He & Gallois, 2005) and two candidate proteins have been identified (Coffeen & Wolpert, 2004; Hatsugai *et al.*, 2004). Caspase-like proteases in plants are believed to have a similar role in PCD as caspases have in mammals and have been shown to be active during response to pathogen attacks and during development.

As mentioned above, proteolytic activity, against a range of mammalian caspase substrates, was detected in developing barley caryopsis. Highest activity was found against the caspase-6 recognition sequence, Valine-Glutamic acid-Isoleucine-Aspartic acid (VEID). As with true caspases, the VEID cleaving activity (VEIDase) in barley caryopsis could only be inhibited with caspase specific inhibitors based on preferred recognition motif (Figure 2, Paper II). General inhibitors against various protease groups were ineffective or only marginally inhibiting. EDTA showed some inhibitory effect indicating the need for a divalent cat-ion for proper enzymatic activity. The caspase-like activity was shown to be present both in developing embryo and endosperm throughout development but with markedly higher activity during early phases of development. The severe change in activity suggests a greater involvement during certain developmental periods and indicates some form of developmental regulation of activity. The period of greatest VEIDase activity does not correlate with the period of greatest PCD activity as indicated by classical PCD markers,

e.g. Evan's Blue staining and TUNEL staining. We confirmed this for barley using TUNEL staining and showed that positive TUNEL staining nuclei, indicative of cells undergoing PCD, appear between 13 and 19 dpa. Both Evan's Blue and TUNEL are indicators of late stage PCD during terminal phases of development. If the barley VEIDase does have a role in PCD, it appears more likely to be in developmental PCD as part of the process to ensuring correct development.

The sub-cellular localization of VEIDase in young barley endosperm was determined using confocal microscopy and a peptide substrate that becomes fluorescent upon cleavage. The VEIDase activity was found localized in discrete regions within the cell. Not all cells contained VEIDase activity but no apparent pattern could be discerned with regards to which cells did. In endosperm pre-treated with VEIDase inhibitor no or little activity could be seen, indicating that observed fluorescence was the result of VEIDase activity.

Characterization of *amo1*

The *amo1* barley mutant was isolated some 40 years ago because of its elevated amylose levels (Merritt, 1967). Several unsuccessful attempts have been made at isolating the causative mutation. It has however been possible to pinpoint its location to chromosome 5S (Schondelmaier *et al.*, 1992). The *sbeIIb* locus, which has been shown to be linked to similar high amylose phenotypes in other crops, has also been localized to chromosome 5 in barley (Sun *et al.*, 1998). To test the possibility that *sbeIIb* carried the causative mutation we started our characterization of *amo1* by sequencing the *sbeIIb* transcript. Using RT-PCR the transcript was amplified from both mutant and wild type indication expression in both varieties. Sequencing showed no difference between the two varieties from 300 bp upstream of the ATG down to the poly-A tail. Western blotting further showed that SBEIIb was present throughout development of the endosperm at similar levels in both mutant and wild type. Taken together this shows that SBEIIb are present at similar levels and that there are no differences in the primary sequence between mutant and wild type.

This does however not role out the possibility of a role for SBEIIb in creating the *amo1* phenotype through differences in activity and/or post-translational modifications. This was tested by assaying starch branching activity in endosperm at three different developmental time points (Figure 10, Paper IV). A clear trend away from SBEIIa/b activity towards SBEI activity during development can be seen both in the wild type and mutant sample. The levels of branching activity and rate of change is however different. The mutant shows much lower levels of SBEIIa/b activity during early development and appears to switch later compared with wild type. Also the SBEI activity emerging during later stages of development appears to be less. Having in mind that there are no differences in the primary sequence for SBEIIb between mutant and wild type the difference in activity would indicate a difference in post-translational regulation. It was recently shown that SBEs in wheat needs to be phosphorylated in order to be activated (Tetlow *et al.*, 2004). A difference in phosphorylation state leading to a difference

in activity between mutant and wild type SBEs would be a plausible explanation for the observed discrepancy in SBE activity, especially since the SBEIIb level appears similar. In the same article it was also shown that SBEI and SBEIIb form a complex with phosphorylase in the active state. An inability to form an active complex due to lack of post-translational modification or proper ligand molecules could be another explanation for the observed difference in SBE activity.

In an effort to find genetic markers closely linked to the *amo1* mutation, for eventual positional cloning of the causative mutation, an AFLP (Amplified Fragment Length Polymorphism) based, bulked segregant analysis (Michelmore, Paran & Kesseli, 1991) procedure was initiated. Crosses were made and the F2 population screened for phenotype. Pools of F2 plants exhibiting similar phenotypes were compared using AFLP fragment patterns. Unfortunately no consistent differences in AFLP patterns between mutant and wild type pools could be found. At the time the reason for this was unknown but it was later, unfortunately too late, shown that F3 seeds derived from F2 seeds with mutant phenotypes did not always show mutant phenotype, indicating that a few heterozygous F2 seeds had been present in the original pools. This was unexpected since each F2 seed exhibiting *amo1* phenotype had been screen by two independent methods, amylose measurement and granule phenotype by microscopy, to confirm correct phenotype.

Amo1 was further characterized by comparing the proteins integral to starch granules isolated from endosperm at three different developmental stages. 2D protein patterns were compared and found to differ in a number of places (Figure 11, Paper IV). Protein spots with different abundance were identified (Table 1, Paper IV). The proteins identified showed no obvious correlation with high-amylose phenotype and were mostly predicted as non-plastid proteins. That, taken together with the fact that all spots showing a difference in abundance were more abundant in *amo1,* suggests that observed differences relate to some general differences between mutant and wild type granules. The most striking difference between *amo1* and wild type granules is the deep fissures found in *amo1* granules. A possible explanation would be that proteins get imbedded in these fissures where they remain protected from the washes that remove ordinary surface proteins. This notion is further strengthened by the fact that several proteins that are known to be surface-localized, *e.g.* hordein and serpin Z4, are found among the proteins showing differential abundance. The possibility of differential retention of starch granule surface proteins between varieties with morphologically different granules makes comparative proteomic studies difficult to carry out. However there is a poor correlation between relative protein abundance in the cytosol, as determined by relative spot intensity in 2D separated cytosolic proteins (Ostergaard *et al.*, 2004), and relative protein abundance of the granule proteins showing differential abundance. This may indicate that cytosolic proteins isolated from starch granules and detected as showing differential abundance may have some kind of starch affinity.

Future perspectives

Further characterization of *amo1*

The potential of *amo1* as an industrial source of high amylose starch has been overshadowed by corn and potato varieties with much higher amylose levels. However its potential as a means of understanding the process of starch synthesis remains high. In fact, in light of discovering that SBEIIb activity is altered while the primary sequence remains unaffected, underscores the mutants potential as a source of knowledge especially in understanding the regulation of starch branching. Towards this end a number of potential investigations could be undertaken:

• Preliminary investigations have indicated that transcript and protein levels are roughly equal in both mutant and wild type. It would be useful to investigate this in more detail to determine to what extent differences in activity is due to differences in enzyme concentrations.

• A possible explanation for the observed difference in SBE activity could be differences in phosphorylation status of SBEs. It would be of interest to investigate both the position of native phosphorylation and the difference in phosphorylation status of SBEs between *amo1* and wild type.

• *Amo1* appears to be affected in some regulatory aspect rather then in a key synthetic enzyme. Regulatory proteins usually have a low copy number and it would therefore be difficult to detect changes with current proteomic or other analytical methods. So, in order to ultimately pin point the causative mutation, a direct genomic approach will probably have to be pursued. Since the reason for initial failure to use a BSA-AFLP strategy has been discovered it should now be possible to resume work along those lines after having checked the F3-seeds from mutant F2 plants to ensure homozygosity.

Further exploration of the VEIDase activity

The discovery of a caspase-like activity, normally associated with cell death, during early stages of barley caryopsis development suggests the presence of previously uncharacterized mechanisms during caryopsis development. Only two plant proteins, both found in leaf tissue, representing two different families have so far been characterized as having caspase-like activity. The barley VEIDase is different both in its developmental regulation and tissue specificity. These differences make it probable that barley VEIDase represent a previously unknown caspase-like protein involved in poorly characterized cellular events. This makes its isolation and characterization interesting ventures that could be pursued along several lines:

• Initial investigations into affinity purification of VEIDase have shown that the biotinylated peptide probe, Biotin-VAD-FMK, which has been used successfully to purify mammalian caspases, will bind a number of barley endosperm proteins but apparently not covalently as intended. The addition of a 6-carbon spacer, X, between the biotin and VAD does help but not towards forming a covalent bond. It is possible that a similar probe but with the correct recognition sequence, Biotin-X-VEID-FMK, could allow formation of a covalent bond and facilitate isolation.

• An alternative way of identifying the VEIDase would be to clone full-length cDNAs into an expression library. Expression from these clones would then be induced and a flow cytometer based cell sorter would be used together with a fluorescent substrate to select VEIDase expressing clones. Positive clones can then be sequenced to hopefully reveal VEIDase and other enzymes upstream of the VEIDase.

• Traditional chromatographic techniques, used successfully to isolate saspases, caspase-like proteins from oat (Coffeen & Wolpert, 2004), could also be a potential way of isolating the barley VEIDase. However, VEIDase have been shown to have a rather short half life, which may prove to make chromatographic separation of the active enzyme impossible.

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