Plant UDP-glucose Pyrophosphorylase: Function and Regulation

Meng Meng

Doctoral dissertation
To be defended on Friday 12th September 2008, 10:00 at the lecture hall
KB3B1, KBC, Umeå University

Faculty opponent: Prof. Javier Pozueta-Romero, Spanish Research Council, Spain

Department of Plant Physiology
Umeå Plant Science Center
Umeå University, Sweden
Plant UDP-glucose Pyrophosphorylase: Function and Regulation
Meng Meng, Department of Plant Physiology, Umeå University, 90 187 Umeå, Sweden

Abstract

UDP-glucose pyrophosphorylase (UGPase) is an important enzyme of carbohydrate metabolism in all living organisms. The main aim of this thesis was to investigate the function and regulation of plant UGP genes as well as the UGPase proteins. Both in vivo and in vitro approaches were used, including the use of transgenic plants deficient in UGPase activity, and using purified proteins and their mutants to elucidate the structure/ function properties of UGPase.

In both Arabidopsis and aspen, there are two highly similar UGP genes being actively transcribed, but not to the same extent. For both species, the UGP genes could be classified into two categories: a “house-keeping” gene and a subsidiary gene, with the former functioning universally in all the tissues to support the normal growth, whereas the latter usually expressed at a lower level in most of the organs/tissues tested. Besides, the two UGP genes were also found being differentially regulated under abiotic stress conditions, e.g. low temperature. By investigating the Arabidopsis T-DNA insertion mutants, which respectively have one or both of the UGP genes knocked out, we noticed that as little as 10% of the remaining UGPase activity could still support normal growth and development under controlled conditions, with little or no changes in carbohydrate contents, including soluble sugars (e.g. sucrose), starch and cell wall polysaccharides. Those plants, however, had a significantly decreased fitness under field conditions, i.e. the plants most deficient in UGPase activity produced up to 50% less seeds than in wt. Therefore, we concluded that UGPase is not a rate-limiting enzyme in carbohydrate synthesis pathways, but still is essential in viability of Arabidopsis plants.

In order to characterize two Arabidopsis UGPase isozymes, both proteins were heterologously overexpressed in prokaryotic cells and purified by affinity chromatography. The two isozymes showed little differences in physical and biochemical properties, including substrate specificity, $K_m$ values with substrates in both directions of the reaction, molecular masses, isoelectric point (pI), and equilibrium constant. On the other hand, possibilities of distinct post-translational regulatory mechanisms were indicated, based on amino acid (aa) motif analyses, and on 3D analyses of derived crystal structures of the two proteins.

We used the heterologous bacterial system also to overexpress barley UGPase and several of its mutants, both single mutants and those with whole domains/ exons deleted. As a result, we have identified several aa residues/ protein domains that may be essential for structural integrity and catalytic/ substrate-binding properties of the protein. For instance, we found that the last exon of UGPase (8 aa at the end of C-terminus) was important for the protein ability to oligomerize and that Lys-260 and the second-to-last exon were essential for pyrophosphate (but not UDP-glucose) binding. The data emphasized the critical role of central part of the active site (so called NB-loop) in catalysis, but also pointed out to the role of N-terminus in catalysis and oligomerization, but not substrate binding, and that of C-terminus in both catalysis/substrate binding and oligomerization.

Key words: UDP-glucose pyrophosphorylase, carbohydrate metabolism, in vivo regulation, T-DNA knockout, heterologous expression, isozyme, mutagenesis, kinetic property, oligomerization
Plant UDP-glucose Pyrophosphorylase: Function and Regulation

Meng Meng
孟蒙

Doctoral dissertation

Department of Plant Physiology
Umeå Plant Science Center
Umeå University, Sweden
Dedicated to

My beloved father and mother

献给

亲爱的爸爸妈妈
# Table of contents

List of papers

Abbreviations

Introduction............................................................................................................. 1
  UGPase and its reaction.................................................................................. 2
  Role of UGPase in plants............................................................................ 3
  Isozymes of UGPase................................................................................... 4
  Evolution of UGPases and related proteins............................................... 6
  Kinetic properties....................................................................................... 7
  *In vivo* regulation...................................................................................... 8
  Tissue and subcellular localization............................................................ 11
  Three-dimensional structure..................................................................... 12

Materials and methods...................................................................................... 16
  Plant materials............................................................................................. 16
  Obtaining genetically modified plants....................................................... 17
  Heterologous overexpression and purification of UGPase proteins........ 18
  UGPase assay.............................................................................................. 19

Results and discussion...................................................................................... 20
  Two *UGP* genes in Arabidopsis and aspen............................................... 20
  Can one kinetically distinguish between UGPase isozymes in crude
  plant extracts? .......................................................................................... 23
  UGPase does not catalyze rate-limiting step, but plays essential role
  in *Arabidopsis*......................................................................................... 25
  Biochemical properties of *Arabidopsis* UGPase isoforms.................... 26
  Structure/ function studies on barley UGPase........................................... 27

Conclusions......................................................................................................... 29

Acknowledgment............................................................................................... 30

References.......................................................................................................... 31
List of papers

This thesis is based on the following papers, which will be referred to in the text by their roman numbers.


Papers I and III are reprinted with the kind permission of Elsevier Ltd.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>ABA</td>
<td>Abscisic acid</td>
</tr>
<tr>
<td>AGPase</td>
<td>ADP-glucose pyrophosphorylase</td>
</tr>
<tr>
<td>AGX</td>
<td>UDP-(N)-acetylglucosamine pyrophosphorylase</td>
</tr>
<tr>
<td>DK</td>
<td>(ugp1/ugp2) double-knockout mutant</td>
</tr>
<tr>
<td>HXK</td>
<td>Hexokinase</td>
</tr>
<tr>
<td>I-loop</td>
<td>Insertion loop</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NB-loop</td>
<td>Nucleotide binding loop</td>
</tr>
<tr>
<td>OKA</td>
<td>Okadaic acid</td>
</tr>
<tr>
<td>Pi</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>PPi</td>
<td>Pyrophosphate</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative real-time PCR</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>SPS</td>
<td>Sucrose-6-phosphate synthase</td>
</tr>
<tr>
<td>SuSy</td>
<td>Sucrose synthase</td>
</tr>
<tr>
<td>UGPase</td>
<td>UDP-glucose pyrophosphorylase</td>
</tr>
<tr>
<td>USPase</td>
<td>UDP-sugar pyrophosphorylase</td>
</tr>
<tr>
<td>wt</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
Introduction

Sunlight is the source of all biological energy on our planet. From fossil fuels to everyday foods and clothes, human beings are enjoying the energy captured and fixed through photosynthesis, which is primarily run by green plants. By making carbohydrates from “raw materials”, CO$_2$ and H$_2$O, plants convert light energy to chemical energy and store it in the bonds of sugar. A huge number of different forms of sugars, including monosaccharides (e.g. glucose, fructose, galactose, xylose, etc.), disaccharides (e.g. sucrose) and polysaccharides (e.g. starch and cellulose), are synthesized by plants during either photosynthesis or other anabolic pathways using the intermediate and subsequent products of photosynthesis.

Nucleotide sugars are monosaccharides that have a nucleotide bound to the carbohydrate chain through an energy rich linkage. Among them, uridine diphosphate glucose (UDP-glucose) is one of the most important (Feingold and Barber 1990, Kleczkowski 1994, Gibeaux 2004). It is the major glucosyl donor for carbohydrates in higher plants, serving as direct precursor for the synthesis of sucrose, which is a major product of photosynthesis in green leaves, accounting for much of CO$_2$ fixed during photosynthesis. Sucrose also serves as the principal (if not the only) long-distance transport compound in most plants and as a storage compound. Furthermore, UDP-glucose is a direct precursor for cellulose and callose synthesis occurring at the plasmalemma (Amor et al. 1995, Schlümpmann et al. 1994). Cellulose is the principal scaffolding component of all plant cell walls, a polymer derived from β-1, 4 linked glucose units. Callose differs from cellulose in consisting of β-1, 3-D-glucan chains. It is produced in response to wounding or infection by pathogens, and also in a few cell types it is made at specific stages of cell wall development, such as in growing pollen tubes and in the cell plates of dividing cells.

Plants also contain non-cellulosic polysaccharides, e.g. hemicellulose and pectin. These polymers consist of hexoses, pentoses, and uronic acids. The precursor of all these polymers (except starch) is, although indirectly, also UDP-glucose (Feingold and Barber 1990, Johansson et al. 2002, Gibeaut 2004, Seifert 2004). In addition, UDP-glucose is involved as well in the synthesis of carbohydrate moiety of glycolipids, glycoproteins and
proteoglycans, among other functions (Feingold and Barber 1990, Mikami et al. 2001, Bishop et al. 2002).

UDP-glucose can be formed from glucose-1-phosphate (glucose-1-P) and UTP by UDP-glucose pyrophosphorylase (UGPase), the enzyme that will be studied in detail in this thesis, or from sucrose by sucrose synthase (SuSy). In photosynthetic cells, where sucrose is synthesized, the pool of UDP-glucose is mainly derived from glucose-1-P by UGPase.

UGPase and its reaction

UGPase (EC 2.7.7.9) is one of the key enzymes of the carbohydrate metabolic pathway widely found in plants, animals and microorganisms. It catalyzes the reversible transfer of a uridylyl group from UDP-glucose to pyrophosphate (PPi), producing glucose-1-P and UTP. The catalytic activity of UGPase appears to be initiated by binding of UTP or UDP-glucose prior to the binding of glucose-1-P or PPi (Tsuboi et al. 1969). UGPase reaction may be channeled toward the forming of glucose-1-P or UGP-glucose because of metabolic coupling with other pathways of sugar metabolism, involved either in synthesis or degradation of sucrose (Figure 1). In source tissues, e.g. photosynthetic leaves, where sucrose is being produced and stored in vacuole or sent to sink tissues, UGPase is thought to be the primary source of UDP-glucose for sucrose synthesis by being coupled to the reaction catalyzed by sucrose phosphate synthase (SPS). On the other hand, in sink tissues, e.g.
roots and developing tubers, where sucrose is hydrolyzed, UGPase is considered to work in the metabolism of UDP-glucose formed by SuSy from sucrose breakdown. The glucose-1-P produced from UGPase reaction can either be catabolized through glycolysis pathway or converted into ADP-glucose by ADP-glucose pyrophosphorylase (AGPase) and finally stored as starch (Kleczkowski 1994).

Role of UGPase in plants

Despite the core location of UGPase and the importance of its product UDP-glucose in carbohydrate metabolism, relatively little is known about its role in plants. Also, it is still unknown whether UGPase reaction is a rate-limiting step, i.e. whether its regulation has any effects on overall carbohydrate metabolism in plants.

Since 1990, UGP cDNAs have been cloned and sequenced from various plants (Abe et al. 2002, Pua et al. 2000, Lluisma and Ragan 1999, Eimert et al. 1996, Spychalla et al. 1994, Katsube et al. 1990, Wu et al. 2002). Subsequently, antisense RNA and/or RNA interference (RNAi) technologies were employed to reduce the expression level of UGP genes in transgenic plants, in order to investigate the role of UGPase in plant metabolism. The most extensive UGPase studies with antisense RNA technique were done with potato. It was demonstrated that only 4% of UGPase activity was sufficient to maintain normal growth and development of potato tubers and no change in soluble sugar content was observed (Zrenner et al. 1993). This may indicate that UGPase activity is far in excess in vivo and the reaction it catalyzes is not a rate-limiting step at all. On the other hand, Spychalla et al. (1994) Borovkov et al. (1996) found a significant decrease of sucrose content associated with 30-50% reduction of UGPase activity in transgenic potato tubers. These results differ greatly from those of Zrenner et al. (1993) One possible explanation is that the researchers of those two studies happened to inhibit two different UGP genes that play different roles in potato. Furthermore, the potato tubers used in these two kinds of studies were of different developing stages, so that UGPase was involved in different directions of sucrose metabolism.
In *Dictyostelium discoideum*, a soil-growing amoeba, which contains two UGP genes, mutation in one of those genes led to aberrant cell differentiation and development, ending with small, gnarled fruit bodies (Bishop et al. 2002). Furthermore, in studies of Chen et al. (2007) with rice (*Oryza sativa* L.), as UGP1 gene was silenced by RNAi or cosuppression, male sterility was observed in transgenic plants, where normal callose deposition was disrupted in pollen mother cells during meiosis. On the other hand, a recent study on transgenic tobacco overexpressing a bacterial UGP gene revealed that the transgenic plants had increased height growth and, subsequently, increased biomass (Coleman et al. 2006). In a separate study, Coleman et al. (2007) reported that the overexpression of bacterial UGP in hybrid poplar led to increased contents of soluble sugars, starch and cellulose, but the plants had impaired growth rates, reduced stem height and smaller leaves. Thus, the elevated UGPase activity/content did affect the carbon allocation, although the outcomes obviously differed for both tobacco and hybrid poplar. The above-mentioned studies suggest that UGPase may play a critical role in plant growth and development, at least in some species and/or during some specific development stages.

In *Arabidopsis*, a model system for plant biologists, antisense inhibition of UGP gene caused ca. 20% decrease in UGPase activity and no obvious morphological phenotype, but there was a significant decline of carbohydrate levels in leaves (Johansson 2003). These results resembled those from the studies of Spychalla et al. (1994) and Borovkov et al. (1996) on potato tubers, suggesting the rate-limiting role of UGPase in carbohydrate metabolism of another model species. In this thesis, function and regulation of *Arabidopsis* UGP genes will be further studied using T-DNA insertion mutant lines.

**Isozymes of UGPase**

Isozymes are enzymes that differ in amino acid (aa) sequence but catalyze the same chemical reaction, and are usually coded for by homologous genes that have diverged over time or by different alleles of the same gene. In some cases, two related proteins are products of the same gene, arising by the process of differential splicing. In this case such
two proteins are called isoforms. Both isozymes and isoforms may display different kinetic parameters and/or regulatory properties. The existence of isozymes/ isoforms permits the fine-tuning of metabolism to meet the particular needs of a given tissue or developmental stage.

The UGPase, although known as an enzyme required in all tissues throughout the life of a plant, used to be assumed as being represented by a single gene in plants. For instance, all Ugp cDNAs from different tissues of barley (Hordeum vulgare) are identical (Eimert et al. 1996), suggesting all of them are transcribed from one gene on the chromosome. Nevertheless, subsequent studies based mostly on analyses of genomic and EST databases, revealed at least three plant species that had two UGP genes. Those plants include Arabidopsis, rice and aspen. There have been a few papers on the presence of different isozymes/ isoforms of UGPase, but whether those proteins are coded by separate genes is unknown. For instance, in rice (Oryza sativa), direct evidence has been provided by Chen et al. (2007b) that multiple UGPase isoforms, which differ in pi and N-terminal acetylation status, are present in vivo. In potato tubers, several highly conserved UGP cDNAs were cloned, and at least two isoforms/ isozymes were purified and characterized present in cold-sweetening resistant and cold sensitive potatoes (Sowokinos 2001, Sowokinos et al. 2004). The genes for those isoforms/ isozymes or the proteins themselves are possibly responding to distinct developmental regulation factors.

In the genomic and EST databases of Arabidopsis, a model species for plant biology, there are two highly homologous UGP genes. Although both genes have already been cloned, the differences in their respective expression patterns, metabolic roles and regulatory mechanisms in vivo are still unclear, not to mention the enzymatic properties of the proteins encoded by those genes. In another important model plant, aspen (Populus tremula x tremuloides), there are also two UGP genes. Unlike Arabidopsis, which is herbaceous, an aspen is a tree. Aspen makes and utilizes carbohydrate reserves during the annual cycle of activity and dormancy and has highly developed ability to synthesize secondary walls. In all those processes, the formation and utilization of UDP-glucose is central to overall carbon fluxes. Therefore, it would be interesting to
investigate how the roles and regulation of $UGP$ genes and UGPase protein/activity in a woody perennial species differs from that of herbaceous plants, e.g. $Arabidopsis$.

**Evolution of UGPases and related proteins**

As shown in Figure 2, all plant UGPases form a single monophyletic group, suggesting a single ancestral gene. Plant UGPases also have relatively high identity (39%-51%) with UGPases from slime mold (Ragheb and Dottin 1987, Bishop et al. 2002), animals (Konishi et al. 1993, Chang et al. 1996) and yeast (Daran et al. 1995). However, eukaryotic UGPases are significantly divergent from those of bacterial origin, with very little or no identity at aa sequence level (Hossain et al. 1994, Kleczkowski et al. 2004). This may indicate that genes for eukaryotic UGPase branched off at the very early stage of evolution, or that they have evolved independently. There is also 10%-16% identity and 20%-30% similarity between plant UGPase and SPS (Eimert et al. 1996, Geisler et al. 2004), suggesting a common early ancestor. On the other hand, bacterial UGPase appears to be more related to SuSy than to eukaryotic UGPase or plant SPS.

Another type of pyrophosphorylase, UDP-sugar pyrophosphorylase (USPase), can catalyze the same reaction as UGPase, and has also been purified and characterized from higher plants (i.e. pea and $Arabidopsis$) (Kotake et al. 2004, Litterer et al. 2006, Kotake et al. 2007). USPase has broad substrates specificity. Besides UDP-glucose, it also catalyzes the reversible formation of UDP-galactose, UDP-glucuronic acid, UDP-I-
arabinose and UDP-xylose from respective monosaccharide-1-phosphates in the presence of UTP as a co-substrate. However, although sharing the same catalytic function, plant USPases and UGPases have little or no identity in protein sequence. Phylogenetic analyses revealed that USPase and UGPase proteins should be categorized into distinct groups (Geisler et al. 2004, Litterer et al. 2006).

**Kinetic properties**

The reaction catalyzed by UGPase is freely reversible, and slightly favors glucose-1-P formation. The in vitro activity and kinetic properties had been studied for this enzyme from many plants species and different tissues. Reported $K_m$s of plant UGPases are usually similar for all substrates in both directions of the reaction, and most of the $K_m$s vary around 0.1 – 0.4 mM (Kleczkowski et al. 1994). However, some relatively high or low $K_m$s were also detected. For instance, UGPase of etiolated sorghum seedling was reported to have $K_m$s of 0.06, 0.05, 0.03 and 0.05 mM, respectively, for UDP-glucose, PPI, UTP and glucose-1-P (Gustafson and Gander 1972).

Several aa in some conserved motifs of UGPase have been reported to be involved in substrate binding and catalysis (Geisler et al. 2004). For example, Cys-99 residue (based on barley UGPase sequence) is conserved in all eukaryotic UGPases. A Cys-99 to Ser mutation of barley UGPase caused 50% reduction in activity and 12-fold higher $K_m$ for PPI, compared to the wild-type (wt) protein (Martz et al. 2002). This suggests that Cys-99 is located at or near PPI-binding site, or at least near a site that affects PPI binding. This aa residue was also demonstrated as important for substrate binding in the UGPase from human liver (Chang et al. 1996). In addition, LIV to NIN and KK to LL mutations of barley UGPase led to partially insoluble proteins, lowered the $V_{max}$ by 12- and 2-fold, respectively, and displayed about 50% lower $K_m$ values with both UDP-glucose and PPI, compared to wt protein (Martz et al. 2002). Thus, these mutations slightly improved the accessibility of substrates to their respective binding sits on the enzyme, at the cost of lower activity. In earlier studies, five Lys residues were identified essential for catalytic activity or substrate binding in potato UGPase, particularly Lys-367 (Kazuta et al. 1991,
Katsube et al. 1991, Fukui et al. 1993). Considering that all the substrates of UGPase contain phosphate group(s) with negative charges and may react with the positively charged Lys residues, it may suggest that the UDP-glucose binding site contains a net positive charge due to the presence of lysines. Likewise, at least three Lys residues were implicated in substrate binding of the UGPase from bovine liver (Konishi et al. 1993).

**In vivo regulation**

There are several levels of regulations that can be envisaged for UGPase. They involve regulation at the gene expression level (eventually having an effect on UGPase activity/protein content), but also post-translational regulation (e.g. protein phosphorylation), protein interaction effects (perhaps resulting in metabolite channeling), and direct inhibitory/activatory effects of metabolites at the active site of UGPase. Concerning gene regulation, in *Arabidopsis* leaves, it had been demonstrated that the expression of *UGP* mRNA was strongly up-regulated by sucrose, and to some extent by glucose or osmoticum (Ciereszko et al., 2001). In the same study it was found that light exposure to dark-adapted *Arabidopsis* leaves could mimic the effect of external sucrose feeding, possibly because of the sucrose formation during photosynthesis. Sucrose stimulation of *UGP* expression was also observed for potato tubers (Spychalla et al. 1994). Several studies have implied a possible role for hexokinase (HXK) in sugar signaling, with HXK being proposed as a sugar sensor (Jang et al. 1997, Jang et al. 1997b, Pego et al. 1999). However, sucrose specific signaling may have different components from HXK mediated glucose (hexose) signaling or osmoticum signal pathways (Rook et al. 1998, Loreti et al. 2001). Indeed, in *Arabidopsis* the sucrose effect on *UGP* was independent of HXK status, and was completely blocked by okadaic acid (OKA) (Ciereszko et al. 2001b), a potent inhibitor of protein phosphatases 1 and 2A (Bialojan and Takai 1998). The inhibitory effect of OKA on *UGP* expression indicates the involvement of one or both of the phosphatases in sugar mediated regulation of the gene.

Abiotic stresses are also important factors that affect *UGP* gene expression in plants. Reported by Ciereszko et al. (2001), *UGP* from *Arabidopsis* was highly up-regulated by
cold treatment at both mRNA and protein level. As short as 1 day of exposure at low
temperature was sufficient for a significant increase in the transcription level, as well as
higher UGPase activity and protein content. Low temperature effects on UGP expression/
UGPase activity were also implicated in the phenomenon of “cold sweetening” of cold-
stored potato tubers (Spychalla et al. 1994, Borovkov et al. 1996). It has been known that
cold treatment leads to a rapid and sustained accumulation of soluble carbohydrates
(hexoses and sucrose) in plant cytosol (Strand et al. 1997, ap Rees et al. 1988, Dejardin et
al. 1999), but it is still unclear if the up-regulation of UGP by cold is via the same pathway
(or part of it) as the sucrose mediated regulation. The effect of low temperature on UGP
expression was apparently transmitted independently of abscisic acid (ABA), as found by
using ABA-deficient mutants (Ciereszko et al. 2001).

In other study which involved Arabidopsis plants, a crucial role of Pi deprivation in up-
regulating UGP gene expression was demonstrated (Ciereszko et al. 2001b). The study
involved either wt plants grown on liquid media or soil-grown wt and mutants (pho1 and
pho2) impaired in inorganic phosphate (Pi) content. The mRNA level of Arabidopsis UGP
gene(s) showed dependence on internal Pi status as did the protein content and enzymatic
activity, although different organs (leaves, stems/flowers and roots) had different
sensitivity to Pi-starvation. Since sucrose synthesis/ hydrolysis is closely linked to Pi
availability, the up-regulation of UGP by Pi starvation may be explained as a mechanism
to readjust plant nutritional status. The sucrose and Pi-deficiency up-regulation
mechanisms of UGP, although independent from each other, might share similar
components (Ciereszko et al. 2005).

In the cases described above, the up-regulation of UGP mRNA level was subsequently
accompanied by increased UGPase activity and protein content, suggesting that under
these conditions UGP was regulated at the transcriptional and/or post-transcriptional level.
A similar mechanism may also be responsible for increase in UGPase protein/ activity in
plants under other stress conditions, e.g. those exposed to cadmium poisoning (Repetto et
al. 2003), salt stress (Yan et al. 2005) or to exogenously applied ethylene (Pua et al., 2000).
Besides the regulation at transcriptional and post-transcriptional level, a few types of post-translational modifications may also be involved in the regulation of UGPase, e.g. phosphorylation, O-glycosylation, binding to 14-3-3 proteins, etc. In yeast, localization and function of UGPase was found to be affected by PAS kinase-dependent serine phosphorylation in the N-terminus domain (Smith and Rutter 2007). Whereas there is no published evidence for phosphorylation of plant UGPase, two other enzymes of the sucrose pathway, SPS and SuSy (both are are functionally “coupled” to UGPase reaction), have already been demonstrated to be under the control of phosphorylation/ dephosphorylation (Winter and Huber 2002, Winter et al. 1997). The in vivo O-glycosylation was reported for a mammalian UGPase (Wells et al. 2003). Interestingly, immunoblot analyses of UGPases (following SDS-PAGE) in different tissues of *Arabidopsis* (Ciereszko et al. 2001) revealed slight differences (1-2 kD) of protein size for UGPases from various tissues, i.e. the band corresponding to UGPase(s) from root fraction was slightly larger than that from other tissues, suggesting a possible phosphorylation and/or O-glycosylation for the root protein(s).

In addition to the regulatory factors discussed above, investigations with barley UGPase had established that this protein readily oligomerizes, forming a mixture of monomers, dimers and higher-order oligomers for both purified recombinant protein and UGPase in crude leaf extract, with only the monomers as enzymatically active (Martz et al. 2002). Likewise, for human UDP-N-acetylglucosamine pyrophosphorylase (AGX), which is functionally related and has approximately 40% protein sequence similarity to UGPases, monomer is also by far the most active form. Dimers of AGX were proposed to dissociate to monomers under assay conditions (Peneff et al. 2001). Also another pyrophosphorylase, ADP-glucose pyrophosphorylase (AGPase), was reported to have oligomerization as an important regulatory mechanism in several species (Hendriks et al. 2003). Furthermore, a recent study had demonstrated that the oligomerization status affected the cellular localization of SuSy (Duncan and Huber 2007), whose in vivo function in non-photosynthetic tissues/ organs is tightly coupled with UGPase.
Based on further studies on mutant proteins of UGPase from barley, the deoligomerization step (formation of monomers) was found as rate-limiting. For instance, the LIV to NIN mutant of barley UGPase resulted in strongly impaired ability of deoligomerization, and consequently, enzymatic activity of the mutant was over 90% reduced, compared to wt protein (Martz et al. 2002).

There is little evidence for metabolic regulation of UGPase activity. The enzyme was shown to be inhibited by products of its reaction, as reviewed by Kleczkowski (1994), but the inhibition constants were relatively high and, thus, product inhibition as a regulatory mechanism for UGPase is rather unlikely under in vivo conditions. This does not preclude the possibility that other metabolites, not yet tested against UGPase, may have regulatory role.

**Tissue and subcellular localization**

UGPase is required for all the tissues of all living organisms. High level of enzymatic activity, protein content and/or mRNA of *UGP* had been detected in both source and sink tissues of many plant species, e.g. leaves, seed embryo and seed endosperm of barley (Eimert et al. 1996), banana fruit (Pua et al. 2000), rice endosperm (Abe et al. 2002), *Arabidopsis* mature and young leaves, stem/flowers and roots (Ciereszko et al. 2001), various tissues of potato except roots (Katsube et al. 1990), etc.

UGPase was also reported to be differentially expressed during various growth and/or developmental stage of plants. In banana (*Musa acuminata*), for instance, *UGP* transcript accumulated more abundantly in reproductive tissues than in other tissues, and was up-regulated along with the increase of ethylene, degradation of starch and synthesis of sucrose in banana pulp during fruit ripening (Pua et al. 2000). Furthermore, in sapwood of *Juglas* species, catalytic activity of UGPase showed elevation from late summer till early winter, and then declined until the next cycle. It was also observed that UGPase activity gradually decreased with age of the sapwood towards the transition zone and was absent in the heartwood proper (Magel et al. 2001). Interestingly, in the same study, similar
expression patterns were shown by SPS and Susy, supporting their involvement, along with UGPase, in sucrose metabolism of trees.

As the supplier of UDP-glucose, a key metabolite for carbohydrate metabolism in both photosynthetic and nonphotosynthetic tissues, UGPase is considered to be mainly localized in cytosol. Immunogold labeling of rice cells had revealed that most of the UGPase was located in the cytosol, but also to some extent in the amyloplast and Golgi (Kimura et al. 1992). Fractionation of rice and tobacco cells yielded some UGPase activity in the microsomes (Golgi bodies) (Mikami et al. 2001), whereas in barley relatively high UGPase activities were found in a membrane fraction (Becker et al. 1995). Considering that UDP-glucose is used not only in sucrose pathways, but also in the biosynthesis of cell wall, the membrane association of UGPase may be for the sake of providing UDP-glucose efficiently to cellulose synthase and/or callose synthase. In addition, UDP-glucose is also a direct or indirect precursor for all other noncellulose polysaccharides in the cell wall, e.g. hemicelluloses and pectins, as well as the carbohydrate chains of glycolipids and glycoproteins. Unlike cellulose and callose, hemicelluloses and pectins are made in Golgi bodies (Gibeaut 2000), so does the glycosylation of lipids and proteins. UDP-glucose used in these processes must be produced in Golgi or transported from cytosol through the Golgi membrane by transporters. Were fraction of UGPase activity associated with Golgi bodies, the enzyme would have a direct role in the supply of UDP-glucose for those biosynthetic steps.

Three-dimensional structure

In studies by Geisler et al. (2004), the 3-D structure of barley UGPases was derived from the crystal structure of human AGX, which has 40% protein sequence similarity and is functionally related to UGPases. According to this 3-D structure, the UGPase monomer, a bowl-shaped protein, can be divided into three large domains corresponding to N-terminal domain at one end, a centrally located catalytic domain including nucleotide binding loop (NB-loop), and a C-terminal domain that includes the insertion loop (I-loop), possibly involved in dimer formation and stabilization (Figure 3A). The N-terminal part
**Fig. 3 Predicted 3-D structure of barley UGPase**

**A.** Human AGX1-based barley UGPase structure. The N-terminal domain is shown in blue, the central domain is in green, with the NB-loop in yellow, and the C-terminal domain is in red with the insertion I-loop in dark green. (modified from Geisler et al., 2004)

**B.** Arabidopsis UGPase-based barley UGPase structure. The NB-loop is shown in yellow, and the region used to be considered as “I-loop” is shown in green.

of UGPase is tightly packed and includes a loop corresponding to aa 335-356 from middle part of the aa sequence. The central domain is built of a dominant nine-stranded β-sheet which is located at the core of the domain and surrounded by helices and loops. This domain contains the active site of the enzyme. These structural constrains form/surround the active center which is in a form of cavity, with the NB-loop at the entrance. The aa in the active pocket and NB-loop, including several important aa for substrate binding and catalysis (Kleczkowski et al. 2004, Geisler et al. 2004), are conserved in all UGPases. Based on AGX-derived structure, the central domain is linked to the C-terminus through a single helix, and the C-terminal domain is built from five β-sheets connected by loops, with the I-loop being the longest loop.

In a recent research, McCoy et al. (2007) successfully determined the crystal structure of *Arabidopsis* UGPase1 (At3g03250). This first ever crystal structure of plant UGPase showed high similarity to the homology model of barley UGPase (based on AGX structure), but only for the first 360 residues, including the NB-loop that is of key importance to the catalytic activity. Substantial differences between this and the AGX-based model concerned the whole C-terminal domain. In *Arabidopsis* UGPase 3D
structure, the C-terminus domain consists of six antiparallel β-sheets, instead of five β-sheets connected by loops as in the AGX-based model. Since barley UGPase protein shares 92% similarity and 82% identity with Arabidopsis UGPase1, it should be much more reliable to base the barley UGPase 3-D structure on that of the crystallized Arabidopsis UGPase rather than that of human AGX (Figure 3B).

The X-ray-resolved structure of Arabidopsis UGPase revealed the presence of both monomers and dimers of UGPase. For human AGX, which also has the ability to oligomerize and form dimers, an extended loop (I-loop) at C-terminal makes extensive contacts with the active site of its dimeric partner, whereas this loop is not present in Arabidopsis UGPase. According to McCoy et al. (2007), during oligomerization of Arabidopsis UGPase, the N-terminal domain of each monomer is positioned against the C-terminus β-helix of the other monomer and directly across its active site. Thus the oligomerization process would not only restrict the entry of substrate into the active site, but would also restrain the movement of the β-helix that may be necessary for catalysis. This would also provide a probable explanation for why the dimers and higher order oligomers showed little or no enzymatic activity.

In addition to the Arabidopsis protein, UGPases had also been successfully crystallized from two other eukaryotic species: yeast (Saccharomyces cerevisiae) (Roeben et al. 2006) and protozoan parasite Leishmania major (Steiner et al. 2007). Similar to Arabidopsis UGPase, the 3-D structure of L. major UGPase contains an N-terminal domain, a central catalytic domain and a C-terminal domain, with the highly conserved NB-loop located on the active site. This protein has also been demonstrated to exist exclusively as monomer under native conditions in vitro (Lamerz et al. 2006). On the other hand, in contrast to the monomeric L. major and plant UGPases (Martz et al. 2002), the animal and yeast UGPases are oligomeric (Levine et al. 1969, Roeben et al. 2006). The yeast UGPase (Ugp1p) forms homooctamers, which represent the enzymatically active form of this protein. The Ugp1p harbors a C-terminal domain with a left-handed β-helix that mediates the association in the homooctomer. The residues that participate in the formation of the Ugp1p octameric complex are highly conserved in animal and fungal
UGPase sequences. However, while plant UGPase aa sequences are generally similar to yeast Ugp1p, conspicuous differences are apparent in the C-terminal oligomerization domain. From the sequence alignments, it could be observed that plant UGPases are consistently missing hydrophobic residues in the C-terminus extension to the β-helix. This suggested the importance of C-terminal domain, especially the elongated β-strand 18 of Ugp1p, on the protein oligomerization process. Although it had been proved that plant UGPase was capable to oligomerize, the key aa and protein domains behind formation of dimers and higher order oligomers must be different from those found in Ugp1p octamers.
Materials and Methods

Plant materials

In this thesis, both Arabidopsis thaliana and aspen (Populus tremula x tremuloides) were used as model organisms to investigate the in vivo role and regulation of UGPase in plants.

Arabidopsis thaliana is widely used in many aspects of plant biology. It plays a similar role for plant sciences as mice and fruit flies play in human biology. Although Arabidopsis has little significance for agriculture, several advantages including small genome, small size, rapid growing cycle and easy transformation protocol made it the most famous model organism of plant sciences. In 2000, A. thaliana became the first plant to have its entire genome sequenced. Its genome sequence, along with a wide range of information concerning Arabidopsis, is maintained by the TAIR database. Furthermore, a large number of Arabidopsis mutant lines and genomic resources have already been available from stock centers. All these advantages have made Arabidopsis a widely used model organism for studies of the cellular and molecular biology of flowering plants.

Over 750 natural accessions of Arabidopsis have been collected from around the world. The most commonly used background lines include Columbia, Landsberg and Wassilewskija. In this thesis, all the Arabidopsis plant strains used are of ecotype Columbia.

Concerning aspen, the other species used in my studies, it is used as a model in forest genetics and woody plant studies. Unlike Arabidopsis, which is herbaceous and annual, aspen is a perennial arbor. Compared to other trees, aspen has a small genome size, grows rapidly, and is easily transformed. The genome sequence of Populus trichocarpa is already publicly available, as well as the EST libraries of 18 different tissues of hybrid aspen Populus tremula x tremuloides (Sterky et al. 2004). Moreover, a Populus microarray database that contains hybridization results for a wide variety of different aspen tissues and experimental conditions has been established and developed by the
Umeå Plant Science Center. This proved to be convenient for my studies on aspen UGPases.

**Obtaining genetically modified plants**

Transgenic *Arabidopsis* plants containing GUS reporter gene, driven by either *UGP1* or *UGP2* promoters, were constructed by Agrobacterium-mediated gene transformation. T-DNA insertion mutants *ugp1* and *ugp2* were both made by Salk Institute, and ordered from *Arabidopsis* Biological Resource Center (ABRC) of the Ohio State University. The homozygous lines were screened for by RT-PCR on total RNA using gene specific primers that locate at different sides of the T-DNA insertion site (Figure 4).

Double-knockout mutant (DK) was obtained by crossing *ugp1* and *ugp2*, and then the double-homozygotes were screened by three-primer-PCR that contains two gene specific primers and one primer corresponding to the sequence of T-DNA left border, using the genomic DNA as template (Figure 5).

---

**Fig. 4** RT-PCR used to screen for homozygotes of T-DNA insertion mutants

A. Sketch of RT-PCR using primers that locate at different sides of insertion sites

B. Agarose gel electrophoresis showing the RT-PCR results. Lines that failed to have target region amplified (*ugp1*1, *ugp1*-8, *ugp2*-3 and *ugp2*-5) were selected as homozygous.
Heterologous overexpression and purification of UGPase proteins

The wt and mutagenized barley Ugp cDNAs were respectively cloned into the prokaryotic expression vector pET23d. T7 promoter, which is highly induced by IPTG, as well as a short DNA sequence that codes for a polyhistidine peptide, are included on this plasmid. The vectors containing cDNAs of recombinant proteins were respectively transformed into BL21 (DE3), the E. coli strain optimized to overexpress heterologous proteins in a T7 promoter based system. After IPTG induction (final concentration 1 mM) and lysis processes, the raw lysate of BL21 (DE3) contained the recombinant protein among many other proteins derived from the bacteria. Then the bacteria lysate was loaded onto the column of Talon resin, an affinity medium that contains bound metal ions (nickel) to which the polyhistidine-tag binds with micromolar affinity. After washing with buffer to remove the unspecific proteins, the protein of interest could be eluted from the resin by adding higher concentration of imidazole (200 mM) to the elution buffer. While an excess of imidazole is passed through the chromatography column, it displaces the His-tag from nickel co-ordination, and as a result the His-tagged proteins are eluted from the column.

Fig. 5 Three-primer-PCR used to screen for homozygous lines of T-DNA insertion mutant
A. Sketch of three-primer-PCR protocol. Arrows indicate primers. LBb1 is the primer that recognizes the left border of T-DNA. Other arrows indicate gene specific primers.
B. Sketch of the agarose electrophoresis result after three-primer-PCR. WT: wild type; HT: heterozygote; HM: homozygote.
UGPase assay

In this thesis, UGPase activity assays (in pyrophosphorolysis direction) and UGPase equilibrium constant, but also assays of contents of soluble sugars and starch (in hexose equivalents), were all carried out by NADP-associated spectrophotometric quantification method. Although UGPase reaction itself does not include NADP/ NADPH as substrate or product, coupling enzymes added in the system could use glucose-1-P, which (together with UTP) is the product of UGPase pyrophosphorolysis reaction, to subsequently synthesize NADPH, so that the “real-time” reaction rate could be monitored. Similar approach was used to quantify UTP (in equilibrium constant measurement system).

In UGPase assays that were carried out in the direction of UDP-glucose and PPI formation (synthesis direction), PPI was hydrolyzed into two phosphate molecules by pyrophosphatase, and then quantitatively determined by heteropoly-molybdenum blue reaction. The blue color arises because the near colorless phosphomolybdate anion can be reduced to form an intensely colored mixed valence complex.
Results and Discussion

Two UGP genes in *Arabidopsis* and aspen (*Papers # I-III*)

In both *Arabidopsis* and aspen, there are two UGP genes. For *Arabidopsis*, the genes localize respectively at chromosome 3 and 5, and contain high number of introns (18 for both UGP1 and UGP2 genes, respectively). For aspen, on the other hand, each of the two UGP genes contain 21 exons and 20 introns, with 18 corresponding exons having the same length (except exons 9, 10 and 18), and with all corresponding exons having conserved boundaries (Figure 6). In both species, the two UGP genes are actively being transcribed, although not at the same level.

For both *Arabidopsis* and aspen, quantitative real-time PCR (qPCR) was done with cDNA from different tissues to estimate the respective expression of UGP genes there. For all the tissues studied in *Arabidopsis*, UGP1 showed constitutive expression at relatively high level. On the other hand, the expression of UGP2 was obviously lower than UGP1, except in the reproductive organs, i.e. flower and silique, where the two genes were expressed at approximately equal level. Similarly, analyses by qPCR, but also EST and microarray data, on aspen UGP genes demonstrated that UGP1 and UGP2 were differentially expressed in almost all the tissues examined. In no tissue was the expression of UGP2 more prevalent than that of UGP1, with the two genes being about equally expressed only in female flower and stem xylem. Based on these results, we proposed that, in both *Arabidopsis* and aspen, UGP1 has a role as a “house-keeping” gene, whereas UGP2 serves a subsidiary function. The “house-keeping” gene (UGP1 in both *Arabidopsis* and aspen) functions universally in all the tissues to support the normal growth, whereas the subsidiary gene (UGP2) is enhanced probably only when the expression of UGP1 is not sufficient to fulfill the requirement for UGPase.

In addition to qPCR analysis, *Arabidopsis* UGP1 and UGP2 promoters were respectively used to drive the expression of GUS reporter gene in transgenic plants, in order to study the expression pattern of both genes. The results of GUS staining confirmed the qPCR data. The UGP2: GUS had high staining intensity only in mature flowers, whereas UGP1:
Fig. 6 Intron/ exon diagram for aspen UGP genes

The $UGP_1$ clone was mapped to the gene model eugene3. 00440223, whereas the intron/ exon structure of $UGP_1$ was assembled from partial sequences of genome fragments LG_IV (nt 7840332- 7859932) and Scaffold_5051 (198-3165) in $P.\ trichocarpa$ genomic database (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html)
GUS showed a more constitutive staining pattern in other tissues also. It is interesting that in both Arabidopsis and aspen the “subsidiary” UGP2 gene was expressed at roughly equal level to that of UGP1 in reproductive organs, especially pollinated flowers and/or developing seeds (flower and silique of Arabidopsis; female flower of aspen). This could be linked to an intensive transport of sucrose during development of reproductive organs, and to an intensive starch synthesis during early stages of seed filling. In the seeds, sucrose is hydrolyzed by both SuSy and invertase, and UGPase may have a key role in converting UDP-glucose (from the breakdown of sucrose by SuSy) to glucose-1-P, the precursor of starch synthesis (Martin and Smith 1995). It has been reported that the activity of UGPase in pod tissues of mung bean remained higher than the activity of AGPase during seed development, showing UGPase to be an important enzyme controlling carbon flux (Chopra et al. 2005). Thus, it is plausible that a relatively high expression of the UGP2 gene in reproductive organs serves as a back-up to UGP1 expression, in order to ensure the successful reproduction of a plant.

The regulation of two UGP genes under inductive and stress conditions may give a clue as to their roles in plants. From the Arabidopsis GUS staining results, for example, UGP2 had a much lower basic expression but responded more dramatically than UGP1 to sucrose induction. Similar pattern was also seen from the qPCR results. This further confirmed that in Arabidopsis UGP2 is playing a “helping” role to UGP1, at least under some growth conditions. In aspen, however, UGP2 mRNA level showed no obvious increase after external sucrose feeding, whereas under cold treatment it increased steadily in stem and temporarily in leaf, but not in root at all. These results possibly suggest that the “trigger” to mobilize high UGP2 transcription is stricter in aspen than in Arabidopsis.

By studying the Arabidopsis T-DNA insertion mutants, ugp1 and ugp2, we could estimate the respective contribution of the two UGP genes to UGPase activity/content in wt plants. When UGP1 expression was impaired in ugp1, the UGPase activity decreased to 30%, compared to wt. This was the case both for leaves and roots of the mutant. Since the ugp1 mutant is a “leaky” one, it is likely that the UGPase1 contribution to overall UGPase activity in wt may exceed 70%. On the other hand, the ugp2 mutant had
about 80% UGPase activity, when compared to wt, suggesting that it contributes ca. 20% to an overall UGPase activity in the wt plants. The differences in UGPase activity between wt and ugp1 and ugp2 mutants were correlated with UGPase protein content, in both shoot and root, as determined by SDS-PAGE and western blots.

It is not unusual that in a given species there are more than one genes coding for homologous enzymes that catalyze the same reaction. Besides UGPase, other enzymes that “couple” with UGPase reaction at upstream and downstream steps of carbohydrate metabolism, i.e. SPS and SuSy, have several isozymes, each coded by a separate gene (Hirose et al. 2008, Bieniawska et al. 2007, Lutfiyya et al. 2007). In many cases, isozymes display different kinetic parameters and/or different regulatory properties. The existence of isozymes permits the fine-tuning of metabolism to meet the particular needs of a given tissue, growth condition or developmental stage.

**Can one kinetically distinguish between UGPase isozymes in crude plant extracts?**

Regulation and expression patterns of the two UGP genes had been revealed by qPCR and EST/ microarray approaches in both *Arabidopsis* and aspen. However, at the UGPase activity/protein level, we could not distinguish between UGPase1 and UGPase2 contributions in plant tissues/organs. On the other hand, at least theoretically, there should still be a way to distinguish between these isozymes via kinetic approaches, provided the two UGPases have distinct kinetic properties.

To address the contribution of both UGPases in plants, we determined UGPase $K_m$ values with both UDP-glucose and PPi in the crude extract from *Arabidopsis* leaves and roots (Figure 7A, B). Similar analyses were done with desalted crude extracts from mature leaf, root xylem and stem xylem of aspen (Figure 7C, D). In all cases, we observed a typical hyperbolic response, reflected by straight lines on reciprocal plots. All the $K_m$ values were relatively low and essentially identical for different tissues of the same species. This may suggest that either the same isozyme is prevalent in all the tissues examined or,
Fig. 7  Kinetics of Arabidopsis and aspen UGPase from different tissues
Assays of UGPase were carried out for Arabidopsis leaves and roots (A, B), as well as aspen mature leaves (ml), stem xylem (sxy) and root xylem (rxy) (C, D), using varying [PPi] (A, C) or varying [UDPG] (B, D), and maintaining the other substrate at 0.85 mM (UDPG) and 0.5 mM (PPi), respectively.

more likely, that both UGPase isozymes have similar or identical kinetic properties for each species. The latter explanation is especially evident in aspen stem xylem, where two types of Ugp cDNAs were expressed almost at the equal level and any considerable difference in $K_m$ values between two UGPase isozymes from this tissue would have resulted in a nonlinear reciprocal plot. Interestingly, when we overexpressed and purified both of the Arabidopsis UGPase isoforms, the $K_m$ values for each of the purified recombinant proteins were very similar to those determined for an overall UGPase activity in crude Arabidopsis extracts (see Paper #3 of this thesis). This indicates that crude extracts do not contain activities/metabolites that may seriously interfere with the accuracy of the $K_m$ determinations for UGPase. Also, the data suggest that the presence of the poly-histidine tag on the recombinant proteins is not interfering with their substrate kinetics.
UGPase does not catalyze rate-limiting step, but plays essential role in *Arabidopsis* (*Paper #II*)

*Arabidopsis* mutant lines *ugp1* and *ugp2*, which respectively had UGP1 or UGP2 knocked out by T-DNA insertion, showed no morphological phenotype compared to wt. Even though *ugp1* had only 30% of wt UGPase activity left, the remaining activity was apparently sufficient to support normal growth and development under standard conditions when the plants were cultivated in growth chambers.

In order to further decrease the UGPase activity, the two mutant lines *ugp1* and *ugp2* were crossed to obtain the double knockout mutant (DK), which showed only 10% of wt UGPase activity. When grown under normal light and temperature, there were still no morphological differences observed between DK and WT, and this was the case throughout the entire life cycle - from germinating till flowering and seeding stage. The only exception was the length of hypocotyls and roots of very young agar-grown plantlets which were longer than in wt plants. Both soluble sugar and starch contents decreased very little, if at all, in the DK mutant and cell wall content/composition analyses also yielded little or no differences. This strongly suggested that either as little as 10% of wt UGPase activity is sufficient to support basic requirements of normal growth and development or that UGPase activity is not important in those processes.

In apparent contrast to the above-described results, earlier studies, done in the same laboratory, on the role of UGPase in *Arabidopsis* revealed that “antisense” inhibition of UGP expression resulted in plants with only ca. 20% lower UGPase activity than in wt, but the transgenic plants had significantly lower contents of soluble sugars and starch (Johansson 2003). Apart from the fact that the “antisense” approach is not as straightforward as the use of knockouts, those results were obtained using our old growth chamber facilities where especially the light conditions/light quality were not as well defined as in new facilities. Those plants frequently had problems with light intensities as low as 120 μm⁻²s⁻¹ and appeared stressed (purple rosettes). Thus, it is still possible that growth conditions may hold the key to the rate-limiting role of UGPase, and it would certainly be of interest to test several growth conditions for the *Arabidopsis ugp*
knockouts prepared in the present study, especially with respect to light quality/quantity during the entire plant growth.

When all the four genotypes, i.e. wt, ugp1, ugp2 and DK, were grown in the field, under natural conditions, very significant differences were found with respect to the number of seeds (field-fitness test) produced by the wt vs. mutants, with the DK producing 50% less seeds than wt plants. The ugp1 and ugp2 mutants also produced less seeds than wt. This low seed yield suggests that, in the field, ugp knockouts would be outcompeted by wt in few generations. Similar approaches were earlier used in studies on photosynthetic mutants/knockouts which showed little or no phenotypic changes when grown under controlled conditions (Külheim et al. 2002, Frenkel et al. 2007). Interestingly, the DK knockout had an even larger reduction in field fitness than Arabidopsis mutants lacking PsbS and violaxanthin de-epoxidase which are considered as essential for photosynthetic de-excitation under fluctuating light conditions (Külheim et al. 2002).

**Biochemical properties of Arabidopsis UGPase isozymes (Paper #III)**

The cDNAs of Arabidopsis Ugp1 and Ugp2 were overexpressed in E. coli and the recombinant proteins were respectively purified by affinity chromatography. Both proteins shared very similar molecular masses (53 kD) and pI values (ca. 6.12), and had similar equilibrium constant of 0.3, suggesting slight preference for the pyrophosphorolytic direction of the reaction (glucose-1-P formation). Under standard assay conditions (saturated concentration for both substrates), UGPase2 showed higher activity than UGPase1 in both synthesis and pyrophosphorolysis directions of the reaction. On the other hand, the $K_m$s with all four substrates for UGPase2 were at least twice higher than for UGPase1. The $K_m$ values were generally low and ranged from 0.03 mM for PPi (UGPase1) to 0.36 mM for glucose-1-P (UGPase2). It is feasible to assume, based on estimates of metabolite content in plant cytosol (Winter et al. 1994, Geigenberger et al. 1998), that in vivo both isozymes would be essentially saturated with their substrates. Both UGPases were “true” UGPases, and did not react with ADP-glucose or galactose-1-P.
Even though the crystal structure of *Arabidopsis* UGPase1 contained both monomers and dimers of the protein (McCoy et al. 2007), we could detect only monomer when the oligomerization status of the purified proteins was analyzed by native PAGE. From earlier work on barley UGPase (Martz et al. 1992) it is known that only monomeric form is active, and that the oligomerization status of UGPase can be easily affected in vitro by subtle changes in hydrophobicity and protein crowding conditions. Thus, it is possible that conditions to induce oligomerization for barley UGPase differ from those for *Arabidopsis* UGPase.

**Structure/ function studies on barley UGPase (Paper #IV)**

Purified barley UGPase was used as a model to study structure/ function properties of the UGPase protein. From earlier studies with site-directed mutants for UGPase of both plant and animal origin (Katsube et al. 1991, Geisler et al. 2004, Martz et al., 2004), there has been evidence for a number of aa that are potentially important/essential for catalysis, substrate-binding and/or oligomerization properties of the UGPase protein. In this work we have designed several mutants, either site-directed or domain-based deletions of whole exons. The targets for mutagenesis were selected by taking into account the UGPase crystal structure by McCoy et al. (2007) and exon/ intron structure of *Arabidopsis UGP* genes. Both the wt and mutants were respectively overexpressed in a heterologous system and purified on affinity columns, and then the proteins were analyzed with respect both to their kinetic properties and their oligomerization status. As a result we have identified several aa residues and domains that may be essential for structural identity and catalytic/ substrate binding properties of the protein.

For instance, we found that the central part of the active site (so called NB loop) was essential for activity, whereas Lys-260 and both N- and C-terminal domains were important, but not essential, for catalysis. Both K260A and mutants lacking parts of C-terminal domain (except Ccut-8 mutant lacking last exon), but not N-terminus deletion mutant, had their $K_m$ values with substrates largely increased, especially with respect to PPi. Whereas high activity Ccut-8 mutant existed almost solely as a monomer, all the
other deletion mutants were more or less oligomerized and had very low activity. The data pointed out to the role of N-terminus in catalysis and oligomerization, but not substrate binding, and that of C-terminus in both catalysis/substrate binding and oligomerization.
Conclusions

- There are two UGP genes in both *Arabidopsis* and aspen. In both species, the two genes have different expression pattern in various tissues and respond differentially to inductive and stress condition treatments.
- The two UGPase isozymes of *Arabidopsis* have very similar physical and enzymatic properties.
- UGPase exists in far excess in *Arabidopsis*. It is not rate-limiting, but still essential for *Arabidopsis*.
- Lys-260, NB-loop, N-terminal domain and C-terminal domain of barley UGPase are all essential, or near-essential, for the enzymatic activity, whereas N-terminal domain is not important for substrate binding.
- Both N-terminal and C-terminal domains of barley UGPase are important for the protein oligomerization process.
Acknowledgements

I would like to thank everybody working now and who had worked in Umeå Plant Science Center during the years of my study, for creating such a friendly atmosphere. I really enjoy the time spent in UPSC.

Many thanks for my reference group, Stefan, Per and Ewa, for the valuable discussions about my project.

I also wish to thank everyone that worked in the same group with me during these years, Henrik, Iwona, Estelle, Erik, Sylwia, Marilo and Elizabeth. Your advice and encouragement helped me very much.

Special thanks:
My nice supervisor, Leszek, I just hope all my bosses in the future will be as kind as you are. 😊

My angels Matt and Jane, thank you for everything.

Malgorzata, the first person who taught me how to run proteins on gels, thanks for your kind help with native-PAGE, especially when I almost felt hopeless with it.

Bo, thanks for being a good friend, and for all the brilliant ideas.

All my lovely office-mates and lab-mates, Maria, Catherine, Peter, Marie-Noéelle, Paulina, Bas, Tuula, Oliver and Gunilla, I can’t help smiling when typing all these names. Thanks for sharing all the happy and unhappy moments with me during these years of study. I am so lucky to have all of you around.

Junko, thanks for helping me deal with the tree samples, especially grind the stem xylem (the hardest thing I had ever ground). And I spent very happy time with you in Denmark.

Many thanks for all the Chinese friends in UPSC. I really enjoy our dumpling parties and everyday “lunch symposiums”.

Tianyan, thanks for taking care of a sick girl after she just experienced a bloody surger.

And finally, Mo, thanks for your patient waiting on the other side of Atlantic Ocean. I am coming…….
References


**Chen RZ, Zhao X, Shao Z, Wei Z, Wang YY, Zhu LL, Zhao J, Sun MX, He RF, He GC (2007a)** Rice UDP-glucose pyrophosphorylase1 is essential for pollen callose
deposition and its cosuppression results in a new type of thermosensitive genic male sterility. Plant Cell 19: 847-861


Gibeaut DM, Carpita NC (1994) Biosynthesis of cell wall polysaccharides. FASEB Journal 8: 904–915


