Plant Sugar Signaling: Regulation of Starch and Fructan Metabolism

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Abstract

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In plants, sugars are not merely a carbohydrate metabolite or a photoassimilate of photosynthesis. They also play an important role in the intricate machinery of signal transduction. Sugar signaling is part of an ancient system for cellular adjustment to shifting environments and has been found to be crucial in responses to various stimuli, most importantly to the carbohydrate status of the plant. Gene responses to a changing carbohydrate status can vary greatly between plants and plant tissues. In general, carbohydrate depletion upregulates genes for photosynthesis, reserve mobilization and export processes, while high carbohydrate levels induce genes involved in storage or growth. Starch is the major storage compound in many plants and, hence, regulation of starch synthesis is to a large extent mediated via sugar signaling. Our aim in this project was to investigate regulators of carbohydrate metabolism in barley, sorghum and the model plant *Arabidopsis thaliana*. In barley, two highly similar and novel transcription factors, SUSIBA1 and SUSIBA2, were isolated and studied for their involvement in the regulation of two fructan synthesis genes, *6-SFT* and *1-SST*, and two starch synthesis genes *ISO1* and *SBEIIB*. SUSIBA2 was found to bind as an activator to the promoter of the *ISO1* and *SBEIIB* genes. Regulation of of *SBEIIB* also depended on binding of an unknown transcription factor to an element in the second intron. SUSIBA1 serves as a repressor and binds to the promoter of the *6-SFT* and *1-SST* genes. By the use of T-DNA insertion mutants we found two new sugar-inducible genes, *AtWRKY4* and *AtWRKY34*, involved in the regulation of three different *isoamylase* genes in *Arabidopsis*. The *AtWRKY4* and *AtWRKY34* genes were also involved in the regulation of a nucleoside diphosphate kinase, *NDPK3a*. Both the two SUSIBAs, and AtWRKY4 and AtWRKY34, belong to group I of the WRKY family of transcription factors. These transcription factors display sequence similarities and bind to the same promoter element, the *W-box*. However, despite sequence similarities between the SUSIBAs, AtWRKY4 and AtWRKY34, they show diversity in function, which illustrates the complexity of sugar signaling in plants.

Keywords: *Arabidopsis thaliana*, Fructan, *Hordeum vulgare*, ODN *Sorghum bicolor*, starch, WRKY,

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“You sort of start thinking anything is possible if you’ve got enough nerve”
J.K.Rowling

Contents

Abbreviations

List of selected abbreviations used in the text:

ABA	Abscisic acid
ADPglc	ADP glucose
1-SST	sucrose: sucrose 1-fructosyl transferase
6-SFT	sucrose: fructan 6-fructosyl transferase
AtISA	*Arabidopsis* isoamylase
EMSA	Electrophoretic mobility shift assay
GFP	Green fluorescent protein
HXK	hexokinase
NAG	N-acetyl-glucosamine
SPF1	sweet potato factor 1
STK	storekeeper protein
SURE	sugar responsive element
SUSIBA1	sugar signaling in barley 1
SUSIBA2	sugar signaling in barley 2
qPCR	quantitative real-time PCR

Appendix

Papers I-V

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

I. Sun, C., Palmqvist, S., Olsson, H., Borén, M., Ahlandsberg, S. & Jansson, C. A Novel WRKY Transcription Factor, SUSIBA2, Participates in Sugar Signaling in Barley by Binding to the Sugar-Responsive Elements of the iso1 Promoter. 2003. *Plant Cell*, 15, 2076-2092

II. Rosenquist, S., Sun, C., Olsson, H., Persson. C., Höglund, A-S. & Jansson, C. SUSIBA1; A transcription factor involved in regulation of fructan synthesis in barley leaves. (Submitted)

III. Rosenquist, S., Hammargren, J., Knorpp, C. & Jansson, C. *Arabidopsis* WRKY Mutants Impaired in Sugar Signaling. (Submitted)

IV. Hammargren, J., Rosenquist, S., Jansson, C., & Knorpp, C. A novel connection between nucleotide and carbohydrate metabolism in mitochondria: The *Arabidopsis* Nucleoside Diphosphate Kinase 3a gene is regulated by sugars. (Submitted)

V. Mutisya, J., Sun, C. X., Palmqvist, S., Baguma, Y., Odhiambo, B. & Jansson, C. Transcriptional regulation of the sbellb genes in sorghum (Sorghum bicolor) and barley (Hordeum vulgare): Importance of the barley sbellb second intron. 2006. *Journal of Plant Physiology*, 163, 770-780

Additional publications

1. Edqvist, J., Rönnberg, E., Rosenquist, S., Blomqvist, K., Viitanen, L., Salminen, T.A., Nylund, M., Tuuf, J., & Mattjus, P. Plants Express a Lipid Transfer Protein with High Similarity to Mammalian Sterol Carrier Protein-2. 2004. *Journal of Bioogical Chemistry*, 279(51):53544-53

2. Mutisya, J., Satish, P., Sun. C., Andersson. L., Ahlandsberg. S., Baguma. Y., Palmqvist. S., Odhiambo. B., Åman. P. & Jansson. C. Starch branching enzymes in sorghum (*Sorghum bicolor*) and barley (*Hordeum vulgare*): comparative analyses of enzyme structure and gene expression. 2003. *Journal of Plant Physiology*, 160(8):921-30

1 Introduction

1.1 Plants and carbohydrate metabolism

Carbohydrate metabolism, particularly the production and breakdown of starch, cellulose and sucrose, is fundamental to plant growth and development. Understanding the mechanisms behind the flux of carbon in the plant is of central interest in both plant science and life in general. Sucrose is a key molecule in carbohydrate metabolism in plants, and serves as the major transport compound in most of our crop plants. Starch is the second most abundant carbohydrate in the world and is a part of everyday life through the food that we eat or in industrial applications. In plants, starch serves as an important carbohydrate storage molecule. The breakdown of starch ensures retaining essential life processes in plants during periods of non-photosynthetic activity, especially in darkness or during seed germination.

Plants are extremely sensitive and responsive to their surroundings because of their immobility and needs to adjust to altered carbohydrate availability for their survival. The responsiveness to carbohydrate levels occurs within a complex structure. Sugar concentrations vary over a wide range in plant tissues, which provides plants with a broad range of signals (Koch 1996).

1.1.1 Photosynthesis and source activities

Plants capture light and use its energy to fix and reduce carbon dioxide from the surrounding air. The resulting triose phosphates produced can supply carbon to leaf cells or be converted to sucrose for export to other parts of the plant. Photosynthesis also supplies many biosynthetic pathways with reducing equivalents and ATP. The processes associated with light absorption are diurnal, and cause plants to cope with variations in the supply of nutrients during the light and dark periods. This creates the need for flexibility in metabolism. For example, during the night period in leaves or in resting organs such as potato tubers, starch is broken down to supply the plant with energy. Plant organs can be divided into source and sink tissues, depending on their function in carbohydrate metabolism (Buchanan *et al.*). Cells in plants engaged in solute accumulation act as sources and cells engaged in solute utilization act as sinks. The source and sink may be reversed depending on the season, or the plant's needs. Sugar stored in roots may be mobilized to become a source of energy in the early spring when buds in the trees develop.

1.1.2 The importance of starch

Starch is a renewable carbohydrate that can be produced cost-effectively in enormous quantities with modern agronomic methods. The challenge today lies within the ability to design the molecular structure of starch produced by cereal plants. This is one of the most important objectives of plant biotechnology. Designed starches can be used for bulk or value-added food and feed and in non-food applications as in chemicals or as bioenergy (Morell & Myers 2005).

1.1.3 Starch synthesis

Starch accumulates both in leaf cell chloroplasts as transitory starch, and in the amyloplast of plant storage tissue as storage starch. Starch is packed in the plastids in a complex granular structure of α-glucans, linked together by α-1,4 linkages and α-1,6 branches. Starch is usually divided in two distinct classes, amylose and amylopectin. Amylopectin is the most abundant form and is a highly branched molecule with a molecular weight of 10^7- 10^9 Da. Amylose is a smaller and linear molecule with a molecular weight of 10^5- 10^6 Da. Amylopectin is crucial in the production of granules and can by itself generate full size granules, which has been studied both in wild-type starch and mutant plants lacking amylose (Ball *et al.* 1996). The structure of the amorphous and crystalline segments within the amylopectin molecules in a starch granule is displayed in Figure 1.

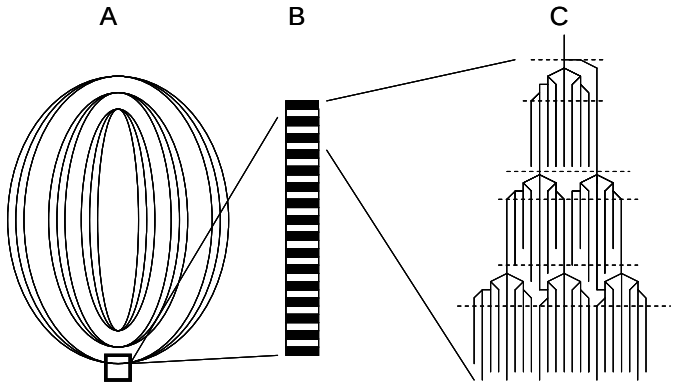


Figure 1. The structure of starch granules and the amylopectin molecule.

A; The native form of starch with amylose and amylopectin molecules are organized in granules as semi-crystalline and amorphous layers that form growth rings. B; A semi-crystalline layer consists of ordered regions composed of crystalline lamella (white box) and amorphous lamella (black box). C; Amylopectin clusters with crystalline lamella, is formed by α-1,4 linked glucans in double helices, of the short amylopectin branches and the amorphous regions consist of the α-1,6 branch points of the amylopectin cluster (Modified from Ball *et al*. 1996).

Starch synthesis includes four different steps, initiation, elongation, branching and granule formation. There are at least three classes of enzymes involved in the synthesis of starch (for review see (Ball *et al.* 1996; Kossmann & Lloyd 2000; Martin & Smith 1995; Ohdan *et al.* 2005; Tetlow *et al.* 2004a). The enzymes are ADP glucose pyrophosphorylase (AGPase; EC 2.7.7.23), starch synthase (SS; EC 2.4.1.21), and starch branching enzyme (SBE; EC 2.4.1.28). Granule-bound isoforms of SS are referred to as GBSS, leaving the abbreviation SS to usually mean the soluble forms of starch synthases. Also enzymes that traditionally have been associated with starch degradation are involved in starch synthesis, such as

isoamylases, also termed debranching enzymes (DBEs, EC 3.2.1.41, and EC 3.2.1.68). In short, amylose is synthesized by AGPase and GBSS while amylopectin is synthesized by the co-ordinated actions of AGPase, SS, SBE and DBE.

AGPase is the enzyme responsible for the production of ADPGlc, the soluble precursor of starch synthases. Elongation of the glucan chain by SS is achieved by catalyzing the transfer of the glycosyl moiety of ADPGlc to the reducing end of a pre-existing α-1,4-linked glucan primer. Amylose is elongated by GBSS, which includes GBSSI and GBSSII. Generally, GBSSI appears to be most common in storage organs, while GBSSII is responsible for amylose synthesis in leaves (Fujita & Taira 1998; Nakamura *et al.* 1998; Vrinten & Nakamura 2000). Elongation of amylopectin is made by the second group of SSs, namely SSI, SSII, SSIII and SSIV, and their distribution between plastids and amyloplasts differ between species, tissue and developmental stage of the plant. SSI have been reported to primarily be responsible for the formation of the shortest glucan chains, with a degree of polymerization (DP) of 10 glycosyl units or less (Commuri & Keeling 2001). The SSII and SSIII isoforms are suggested to act on progressively longer glucan chains. SSIV appears in a wide range of higher plants according to EST databases, but to this date a specific role for this isoform has not yet been clarified (Tetlow *et al.* 2004b).

SBE hydrolyzes *α*-1,4 linkages in the glucan chains and catalyzes the formation of *α*-1,6 linkages in the amylopectin molecule. The reaction results in the formation of a branched chain and an additional non-reducing glucan end which can be further elongated by SSs (Martin & Smith 1995). There are two major classes of SBEs, namely SBEI (also known as SBE B) and SBEII (SBE A). These two classes differ both in terms of amino acid sequence, glucan chains transferred and substrate specificities. The SBEII enzyme transfers shorter chains and has a higher affinity towards amylopectin. SBEI shows higher rates of branching with amylose (Takeda *et al.* 1993). In monocots, the SBEII class is divided into SBEIIa and SBEIIb (Rahman *et al.* 2001). Gene expression of *SBEIIB* was found to be endosperm-specific in barley while the *SBEIIA* gene was expressed in both endosperm and leaves (Sun *et al.* 1998). SBEIIa has primarily been suggested to be involved in the formation of transitory starch in leaves (Blauth *et al.* 2001).

Two groups of DBEs exist in plants; the isoamylase-type and the pullulanase-type (also known as limit-dextrinases), which efficiently hydrolyze (debranch) α-1,6-linkages in amylopectin and pullulan (a fungal polymer of maltotriose residues), respectively, and are part of the alfa-amylase ‘super-family’ of enzymes (Tetlow *et al.* 2004b). The decrease or loss of specific isoamylase isoforms is thought to be responsible for the accumulation of phytoglycogen rather than starch, in mutants of potato (Bustos *et al.* 2004), maize (James *et al.* 1995) and algae (Mouille *et al.* 1996). The precise role for the isoamylase-type and pullalanase-type DBEs in starch synthesis is not yet fully understood.

One model, the glucan-trimming model, proposes that glucan trimming is required for amylopectin to be packed properly into the insoluble granule structure (Ball *et al.* 1996; Myers *et al.* 2000). The DBEs are responsible for the removal of branches positioned inaptly at the surface of the growing granules. An alternative model to the glucan-trimming model is that the DBEs function in starch synthesis indirectly in a cleaning role. The DBEs removes soluble glucans from the plastid

stroma that are not attached to the granule surface. By this removal of substrates, the DBEs prevent synthesis of random glucan polymers by SSs and SBEs and the formation of phytoglycogen. With this model, phytoglycogen is not an intermediate product in the amylopectin synthesis (Zeeman *et al.* 1998). A third model proposes that the DBE activity is required for proper starch granule initiation (Burton *et al.* 2002). The importance of DBEs in starch synthesis is illustrated in the sugary mutants (*Su1)* of sweet corn where deficiency in one DBE isoform causes inhibition of starch synthesis, which, in turn, results in accumulation of sucrose (James *et al.* 1995).

Other enzymes that have been proposed to be involved in starch synthesis are the disproportioning enzyme (DPE) and the phosphorylase enzyme (PHO). The precise mechanisms, however, are not yet clear (Ohdan *et al.* 2005; Tetlow *et al.* 2004a).

Synthesis and accumulation of transitory starch typically occur during the light period and degradation during the night. In seeds and other starch storage tissues, starch is deposited during a specific developmental stage of the organ. Therefore, it seems likely that the regulation of activity of the starch biosynthetic enzymes is different in amyloplasts and chloroplasts. Many factors, such as light, sink and source strength, temperature, are involved. A more precise control of starch synthesis relies on the regulation of participating enzymes.

1.1.4 Starch degradation

Starch degradation has been extensively studied in tissues such as endosperm of germinating cereals but the regulation of the process is not yet fully uncovered.

Studies have revealed a large variation in starch degradation between tissues. The pathway of starch degradation in germinating cereal endosperm is different from the degradation in, for instance, *Arabidopsis* leaves at night. There is good reason to think that the process in endosperms differs from that in other organs because the mature endosperm is not a living tissue, whereas starch degradation in all other plant organs occurs within living cells (Smith *et al.* 2005). Multiple forms of different starch degrading enzymes have been found in almost all organs studied. These enzymes include endo- and exoamylases (α- and β-amylases, respectively), glucosidases, debranching enzymes, starch phosphorylases, and disproportionating enzymes (Smith *et al.* 2003).

In short, the major pathway of degradation in *Arabidopsis* leaves starts in the chloroplast where isoamylases and β-amylases act on the granular starch and the resulting maltose is exported to the cytosol for further metabolism to sucrose. In germinating legume cotyledons starch degradation is initiated by the disintegration of the amyloplast membrane. Starch degradation is then catalyzed by cytosolic enzymes such as limit dextrinases and α-amylases to linear glucans, which subsequently are degraded by glucan phosphorylases. The sucrose synthesized from starch is exported to growing roots and shoots. In germinating cereal endosperm starch degradation involves breakdown of both the amyloplast membrane and the plasma membrane and the degradation takes place in a nonliving tissue. The granule is attacked by *α*-amylases and limit dextrinases to linear glucans. Formation of maltose is then catalyzed by β-amylases. Glucose and

maltose produced in the endosperm are exported to the scutellum and then converted to sucrose for the growing embryo (Smith *et al.* 2005).

One group of newly discovered enzymes involved in the initiation of starch degradation is the glucan water dikinase (GWD) and phosphoglucan, water dikinase (PWD). Studies on mutants lacking GWD accumulate excess amounts of starch (Caspar *et al.* 1991; Yu *et al.* 2001b; Zeeman & Ap Rees 1999). Studies on the GWD enzyme from potato show that it transfers the *β*-phosphate of ATP at either the 6- or the 3-position of glucosyl residues within amylopectin and is important in initiating the starch degradation (Mikkelsen *et al.* 2004).

1.1.5 Fructan synthesis

Most plants store starch or sucrose as carbohydrate reserves, but approximately 15% of all flowering plants store fructans, among them many of great economic importance, such as the cereals (Vijn & Smeekens 1999). Fructans are linear or branched polymers of fructose units and are stored in the vacuole and may serve functions other than carbon storage. For example, fructans have been implicated in protecting plants from drought and cold stress, or as osmotic regulators (Bieleski 1993; Kawakami & Yoshida 2002; Pilonsmits *et al.* 1995; Vijn & Smeekens 1999). The substrate in fructan synthesis is sucrose and by the consecutive action of three enzymes the fructose unit is transferred from sucrose to the fructan chain. Fructan biosynthesis enzymes are related to sucrose-hydrolyzing enzymes. One enzyme, sucrose: sucrose 1-fructosyltransferase (1-SST), catalyzes the conversion of two sucrose molecules to a trisaccharide, 1-kestose, and a leaving a glucose molecule. The fructan chain is the elongated by fructose: fructose 1-fructosyltransferase (1-FFT). Branched fructans are created by the action of sucrose: fructose 6-fructosyltransferase (6-SFT). The 6-SFT enzyme converts either 1-kestose and sucrose to bifurcose and glucose, or two sucrose molecules to 6-kestose and glucose.

1.2 Sugar signaling in plants

For all living organisms on this planet the survival is dependent on photosynthesis, i.e. fixation of carbon and light energy from the sun. As the sugar molecule is the prime carbon and energy source, sugars have acquired important regulatory functions in different organisms early on in evolution. Plants are photosynthetic, sugar-producing and sessile, where maintaining homeostasis requires a complex and flexible regulatory machinery. Hence, sugar signaling plays an important role in controlling metabolism, stress resistance, growth and development (Rolland *et al.* 2006). The regulation is controlled at numerous levels in plants, for example by allosteric regulation of metabolic enzymes, and tissue-specific or temporal-specific expression of genes (Rook & Bevan 2003).

It has been reported previously that expression of genes involved in starch and fructan metabolism, among other pathways, are regulated by sugars (Ishiguro & Nakamura 1994; Khoshnoodi *et al.* 1998; Rook *et al.* 2001; Sun *et al.* 1999; Vijn & Smeekens 1999). In contrast to the situation in bacteria, yeast and mammals, where sugar signaling cascades are extensively studied, the sugar signaling transduction pathways in plants are poorly understood. Generally, in higher plants,

high sugar levels stimulate expression of genes involved in sink function, such as growth, storage of proteins and biosynthesis of starch and other carbohydrates, whereas low sugar levels promote photosynthesis and mobilization of energy reserves, such as breakdown of storage starch or lipids. Sugar signaling can be dissected into three steps, sugar sensing, signal transduction and target gene expression. Further complicating matter is the dual function of sugars as nutrients and signaling molecules and also the interaction between sugar signaling and hormonal networks. Hexoses, sucrose and trehalose might serve as elicitors of plant sugar signaling (Goddijn & Smeekens 1998; Jansson 2005; Muller *et al.* 2001). Sucrose serves as one of the most important molecules in sugar signaling. However, monitoring the sucrose specific signaling is more complicated since sucrose can be hydrolyzed to glucose and fructose and in addition, these hexoses can be recombined to sucrose. The use of sucrose analogs that cannot be metabolized assists in addressing this problem (Jansson 2005).

1.2.1 Sucrose and other disaccharides

Sucrose is well known to cause a wide range of transcriptional signaling, by repression of photosynthesis genes and induction of starch synthesis genes (Jang & Sheen 1994; Rolland *et al.* 2002a, 2002b; Smeekens 2000; Sun *et al.* 2003). Sucrose is the major transport form of photassimilate from source to sink organs and sucrose signaling provides information about the energy status of the plant (Jansson 2005). Sucrose repression has been shown to be important during germination and in postgerminative processes such as seedling development, where *α-Amy* gene expression is repressed by sucrose or in repression of glyoxylate cycle genes, whose gene products act in the mobilization of storage lipids, in early germination (Graham *et al.* 1994b; Loreti *et al.* 2000). The reason for sucrose repression might be that high sucrose levels reflect suboptimal growth conditions and the plant is protected by restraining the developmental programs (Lopez-Molina *et al.* 2001; Rolland *et al.* 2002a). Many events activated by sucrose might also be activated by glucose or fructose following sucrose hydrolysis. It has been suggested that in the developing seed, sucrose controls processes involved in differentiation and storage, and fructose or glucose regulate growth and metabolism (Jansson 2005; Weber *et al.* 1997; Wobus & Weber 1999). Sucrose-specific signaling has been demonstrated in for instance regulation of the patatin gene in potato (Jefferson *et al.* 1990), the *rolC* promoter of *Agrobacterium rhizogenes* Ri plasmid in transgenic tobacco plants (Yokoyama *et al.* 1994), and the *ATB2* bZIP transcription factor gene in *Arabidopsis* (Rook *et al.* 1998). Several sucrose analogs such as lactulose (β-galactose-1,4-fructose), palatinose (glucose-1,6-fructose) and turanose (glucose-1,3-fructose), are not metabolic sugars but were found to repress α-amylase activity. All three have a fructose moiety and further analysis of these analogs revealed that the intact fructosyl region was required for the repression (Loreti *et al.* 2000). Another analog, melibiose (galactose-1,6-glucose), which is also not metabolized, had very low effect on α-*Amy* (Loreti *et al.* 2000). Trehalose (glucose-1,1-glucose) is metabolized by plants and was found to be able to induce the *APL3* gene in *Arabidopsis*, which encoding an AGPase subunit (Wingler *et al.* 2000). Trehalose is hydrolyzed by trehalase and inhibition of trehalase lead to accumulation of a

compound similar to trehalose and a strong reduction of starch and sucrose content (Muller *et al.* 2001). This implies that trehalose might be involved in the regulation of carbohydrate metabolism. Recent studies also suggest that sucrose along with trehalose regulate specific responses that are not affected by hexoses (Eastmond *et al.* 2002; Rolland *et al.* 2006).

1.2.2 Glucose and other hexoses

Glucose is likely the most dominating hexose signaling molecule for gene regulation in plants, as well as in other organisms. As seen with sucrose, glucose is able to repress photosynthesis and germination in different plants (Rolland *et al.* 2002a; Smeekens 2000). Glucose repression of photosynthetic genes provides a strong metabolic signal that overrides light activation (Sheen 1990). Experiments have shown that hexose transport as such is not sufficient for gene repression, the sensor is intracellular and hexose phosphorylation is required (Loreti *et al.* 2001; Smeekens 2000). Glucose phosphorylation is either "unspecific" by hexokinases (HXKs) or specific by glucokinases. Furthermore, it has been shown that the phosphorylation event as such, and not the accumulation of hexose phosphates, is important for repression of sugar-regulated genes (Loreti *et al.* 2001; Rolland *et al.* 2002a). The alternative explanation that P_i and ATP is the reason for reduced gene activity can be discounted since addition of P_i failed to "induce" repression (Graham *et al.* 1994a; Jang & Sheen 1994).

Other hexoses, such as fructose and galactose, are also phosphorylated by HXKs or specific fructokinases and galactokinases. It is likely that they mediate signals by the same pathway as glucose as they have also been shown to be involved in repression of photosynthesis or glyoxylate cycle genes (Graham *et al.* 1994b; Jang & Sheen 1994).

1.2.3 Hexokinases

In yeast, it has been known since the 1970:s that HXK2 is involved in glucose repression (Rolland *et al.* 2002c). In a large number of mutant yeast strains a good correlation between glucose repression and phosphorylating capacity of mutated HXK was observed. Moreover, the same effects were shown with *HXK1/HXK2* hybrid constructs and, interestingly, no further metabolization beyond sugar phosphorylation was needed to generate the glucose repression response (Rose *et al.* 1991). The glucose analog, 2-deoxyglucose, which is transported and phosphorylated by HXK but not further metabolized, could also trigger glucose repression (Rolland *et al.* 2002c). Recent isolation and characterization of the *Arabidopsis glucose insensitive2* mutants (*gin2*) have established that AtHXK1 is crucial in plant sugar signaling (Moore *et al.* 2003). Along with studies on different sugars, sugar analogs, metabolic intermediates, and phenotypic analyses of transgenic *Arabidopsis,* further evidence for the role of HXK, was found (Jang *et al.* 1997). The participation of HXK as a sugar sensor in plants has been supported by three different approaches. First, the requirement for phosphorylation in glucose repression indicated that HXK is involved (Loreti *et al.* 2001; Smeekens 2000). Second, glucose repression was reversed by antisense inhibition of HXK activity in transgenic *Arabidopsis* plants, and overexpression of HXK activity in tomato resulted in a glucose hyper-sensitive phenotype (Dai *et al.* 1999;

Jang *et al.* 1997). When overexpressing the heterologous yeast HXK (YHXK) in *Arabidopsis*, the hyper-sensitive effect was not observed and it was concluded that YHXK supplies transgenic plants with phosphorylation capacity but the signaling effect of HXK was lost (Jang *et al.* 1997; Smeekens 2000). Thus the sugar-sensing capacity of HXK in plants is separate from its metabolic function. Third, HXK inhibitors such as glucosamine and mannoheptulose were found to inhibit glucose repression of gene activity (Jang & Sheen 1994; Pego *et al.* 1999; Umemura *et al.* 1998).

The intracellular localization of the HXKs is expected to play an important role in their functions. Although HXK is generally considered to be a soluble enzyme involved in glycolysis in the cytoplasm (Moore *et al.* 2003), two *Arabidopsis* HXKs, AtHXK2 and AtHXK3, were found to be associated with the mitochondrial membrane (Giege *et al.* 2003). This might enable coordination of both optimal cellular energy demand and substrate availability. HXK activities have also been shown to be associated with plastids (Wiese *et al.* 1999). The AtHXK1 was found to be able to translocate to the nucleus (Yanagisawa *et al.* 2003). Apart from the different *HXKs* and *HXK-like* (*HKL*) genes found in plants, there are also fructokinase and fructokinase-like genes, and genes for galactokinase and arabinose kinase (Kaplan *et al.* 1997; Pego & Smeekens 2000; Sherson *et al.* 1999). Whether fructokinases, galactokinases and arabinokinases participate in sugar signaling is not yet known. However, there are indications of fructokinases being involved in some cases (Jansson 2005; Pego & Smeekens 2000).

Three different pathways for glucose signal transduction in plants have been suggested (Xiao *et al.* 2000). In the HXK-dependent pathway, gene expression is mediated through the signaling function of HXK. It is not the phosphorylating activity of glucose that is important in this pathway but the signaling function that HXK provides. This was demonstrated by overexpressing the heterologous *YHXK2* in *Arabidopsis*, which caused no or little effect on the expression pattern of several photosynthesis genes, e.g. *CAB1, PC, PLD* and *rbcS*, despite several-fold increase in HXK catalytic activity after glucose treatment. The second pathway is dependent on the catalytic activity of HXK and is referred to as the glycolysis-dependent pathway. The pathogenesis-related genes *PR1* and *PR5* were both induced by glucose and the expression was enhanced in plants overexpressing AtHXK1 and lost in plants with antiAtHXK. The glucose induction was also seen in plants overexpressing the yeast *HXK2*. The expression of *PR* genes may depend on the levels of an unknown metabolite downstream of HXK in the glycolytic pathway. There is also evidence for a HXK-independent pathway and it has earlier been reported that two non-HXK substrates, 6-deoxyglucose and 3-O-methylglycose, have the ability to activate certain genes (Ehness & Roitsch 1997; Hilgarth *et al.* 1991; Martin *et al.* 1997; Roitsch *et al.* 1995).

1.2.4 Sugar sensing

Another important issue is whether sensing is intracellular or extracellular. These questions have been addressed by the use of sugar analogs (Jansson 2005). The interconvertibility between sucrose and hexoses makes it difficult to study direct

sensing of sucrose as a specific signaling agent. One way to attack this problem is to investigate the genes affected exclusively by sucrose, for example the repression of the gene encoding a proton-sucrose symporter (Chiou & Bush 1998; Loreti *et al.* 2001). It has also been proposed that sucrose transporters (SUTs) may have a sensing function in plants, as sucrose is the predominant form of photoassimilate that is imported to heterotrophic organs, such as seeds or tubers (Barker *et al.* 2000; Lalonde *et al.* 1999). The SUTs might also serve as sensors but this is still an open question (Barth *et al.* 2003; Eckardt 2003). Another suggestion is that sucrose sensors have evolved from SUTs but lost their translocation activity. One SUT found in tomato and *Arabidopsis* SUT2, which lacks transport activity, was suggested to be a sucrose sensor in sieve elements, as opposed to the high-affinity translocator, SUT1 (Barker *et al.* 2000).

1.2.5 Sugar and hormone crosstalk

There is now sufficient evidence of cross talk between sugar signaling pathways and hormonal networks (Arenas-Huertero *et al.* 2000; Arroyo *et al.* 2003; Gibson *et al.* 2001; Huijser *et al.* 2000; Laby *et al.* 2000; Leon & Sheen 2003; Price *et al.* 2004; Yanagisawa *et al.* 2003). Through studies on sugar signaling mutants the extensive interactions between sugar and hormonal signaling was revealed, particularly for ABA and ethylene (Leon & Sheen 2003). Glucose activates ABA synthesis and signaling and both ABA signaling and glucose signaling is antagonistic to ethylene signaling (Leon & Sheen 2003; Rolland *et al.* 2006). There have also been some indications of an auxin and a cytokinin connection to sugar signaling (Rolland *et al.* 2002a). Apart from the hormonal pathways, stress-related genes, such as the *PR* genes, can be triggered by elevated sugar levels (Roitsch 1999). Both biotic and abiotic stresses, such as cold and drought stress can cause major alterations in carbohydrate metabolism (Jansson 2005; Thomashow 1999; Wanner & Junttila 1999).

1.2.6 Transcriptional regulation

A great number of genes have been found to be transcriptionally regulated by sugars. Feedback-regulation of genes coding for metabolic proteins involved in sugar signaling by their own products is a common phenomenon. The challenge in investigating transcriptional regulation of genes lies in finding DNA elements in promoters of the regulated genes and proteins mediating the regulation. The large expression datasets generated by microarray experiments provide a platform and opportunity to study regulatory *cis*-elements involved in sugar signaling. Today, most information on sugar signaling *cis*-elements comes from genes in sweet potato tubers, cereal seeds, and genes coding for proteins in maize photosynthesis. Some of the first described sugar-induced *cis*-element were the sugar responsive-(*SURE)* element (Grierson *et al.* 1994), *SP8* (Nakamura & Yuki 1992), TGGACGG (Maeo *et al.* 2001), *G-box* (Giuliano *et al.* 1988) and *B-box* (Grierson *et al.* 1994; Zourelidou *et al.* 2002). A link between nutrient stress and other environmental stress responses was found with the identification of *G-box* elements and the closely associated *S-box* elements in light regulated promoters (Acevedo-Hernandez *et al.* 2005; Rolland *et al.* 2006). Other *cis*-elements were found in the sugar/ABA-induced *sporamin A* promoter in transgenic tobacco, the

minimal sporamin promoter *Spo*^{min}, which contains negatively acting regions and two carbohydrate metabolite signal responsive (*CMSRE*) elements in addition to the previously described *SP8a* element (Masaki *et al.* 2005; Morikami *et al.* 2005). Investigations of promoter sequences of maize photosynthesis genes propose the involvement of several regulatory elements in sugar repression (Sheen 1990). In the rice *α-amylase* gene, three elements with sugar responsive sequence (SRS) were identified, including the *GC-box*, *G-box* and *TATCCA*-element.

1.2.7 Post-transcriptional regulation

Transcriptional regulation is not the only way for sugars to control gene expression. Post-transcriptional regulation is also very important. One example of this mechanism is sugar repression of the expression of the rice *αAMY3* gene, which is regulated both at the level of transcription and mRNA stability (Chan & Yu 1998). It appears that the sequence of the 3'-untranslated regions (UTR) of several gene transcripts can control sugar dependent mRNA stability (Chan & Yu 1998; Cheng *et al.* 1999; Ho *et al.* 2001). Another level of regulation of expression, besides mRNA stability and transcriptional regulation, is by selective mRNA translation during stress or nutrient deficiency. There have been a few reports of sucrose-specific regulation of translation of the *Arabidopsis* transcription factor ATB2/bZIP11, which is stimulated by light and moderate concentration of sugars but repressed by higher levels of sucrose. The specific sucrose-induced repression of translation (SIRT) is dependent on the unusually long 5'UTR of the *ATB2/bZIP11* gene. This regulatory mechanism involves short open reading frames in the 5'UTR region of the transcript (Rook *et al.* 1998; Wiese *et al.* 2005). However, the exact molecular mechanism behind this type of control is not yet resolved (Rolland *et al.* 2006). Control of protein stability is another example of sugar-dependent regulation. Many proteins are subjected to ubiquitin- and 26S proteasome-dependent degradation. One example is the degradation of the EIN3 protein in *Arabidopsis* mediated by glucose, which antagonizes the ethylene signaling pathway and promotes proteasome-dependent degradation of the EIN3 transcription factor (Leon & Sheen 2003; Yanagisawa *et al.* 2003). Phosphorylation of proteins has also been suggested to be important in targeting proteins for degradation (Planchais *et al.* 2004; Rolland *et al.* 2006).

1.2.8 The WRKY family of transcription factors

In plants, the WRKY transcription factors constitute a large family. The first described WRKY proteins were SPF1 from sweet potato, and ABF1 and ABF2 from wild oats (Ishiguro & Nakamura 1994; Rushton *et al.* 1995). There are now over 70 *WRKY* genes found in *Arabidopsis* (Dong *et al.* 2003; Eulgem *et al.* 2000) and over 80 in rice (Goff *et al.* 2002; Zhang *et al.* 2004). The family is defined by a domain of 60 amino acids, which contains the amino acid sequence WRKY at its N-terminal end and a putative zinc finger motif at its C-terminal end. Some of the WRKY proteins contain two WRKY domains (group 1), while others have only one (group 2 and 3). Most of the published WRKY proteins bind to the cognate cis-acting element, the *W-box*, with a sequence of (C/T)TGAC(T/C), in the promoter (Eulgem *et al.* 2000) or the 5′ -UTR of target genes (Yu *et al.* 2001a). For the WRKY proteins containing two WRKY domains, such as SUSIBA2 (Sun

et al. 2003), SPF1 (Ishiguro & Nakamura 1994), and AtZAP1 (dePater *et al.* 1996) the C-terminal domain has the major DNA-binding activities.
WRKY proteins function as transcriptional activators or repressors. For example, *Arabidopsis* ZAP1 binds to and activates a synthetic promoter containing the *W-box* in yeast and *Catharanthus roseus* suspension cells (dePater *et al.* 1996). Some WRKY proteins can function both as repressor or activator. For instance, *AtWRKY6*, suppresses its own promoter as well as the promoter of a closely related WRKY family member, whereas it activates the promoters of a receptor-like protein kinase (*SIRK*) and the senescence- and pathogen defense-associated *PR1* genes (Robatzek & Somssich 2001).

WRKY proteins are involved in various functions in the plant cell, such as pathogen defense (Chen & Chen 2000; Eulgem 2006; Eulgem *et al.* 1999; Ryu *et al.* 2006; Shen *et al.* 2007; Turck *et al.* 2004), trichome development (Johnson *et al.* 2002), seed development (Luo *et al.* 2005), and leaf senescence (Hinderhofer & Zentgraf 2001; Miao *et al.* 2004). In addition, WRKY proteins are involved in plant responses to wounding (Hara *et al.* 2000), freezing (Huang & Duman 2002), drought, salinity, cold, heat (Pnueli *et al.* 2002; Rizhsky *et al.* 2002; Seki *et al.* 2002). One WRKY protein has also been found to mediate both salisylic acid and jasmonic acid responses and another was found to mediate responses to ABA in rice (Li *et al.* 2004; Xie *et al.* 2005). We reported on *WRKY* genes involved in regulating starch synthesis (Sun *et al.* 2003).

The WRKY family seems to originate from early eukaryotes and expanded greatly in plants. It also appears as if the group I WRKY proteins are the more ancestral WRKY form. This is based on findings of a single copy *WRKY* gene with two WRKY domains in the primitive eukaryote, *Giardia lamblia*, the slime mold, *Dictostelium discoideum*, and the green algae, *Chlamydomonas reinhardtii* (Zhang & Wang 2005).

1.3 Mono- and dicotyledonous plants, similarities and differences

1.3.1 *Arabidopsis thaliana* as model plant

There are many reasons why *Arabidopsis* is currently the most popular model plant in plant molecular sciences. To mention a few, *Arabidopsis* is a small-sized plant with a short generation time, its genome is fully sequenced, and many phenotypic and biochemical mutants have been mapped.

1.3.2 Barley (*Hordeum vulgare*)

Barley has played an important role in agriculture and was among the first crops to be domesticated. Barley is an annual cereal grain and a member of the grass family Poaceae. Barley serves as a major animal feed crop, with smaller amounts used for malting and in health food. In 2005, barley was ranked the fourth largest cereal crop, in quantity produced and in area of cultivation of cereal crops, in the world (FAO 2005). Today, the top-three barley producing countries are Russia, Canada, and Germany (FAO 2005). The barley genome is not yet sequenced, but because of its importance large efforts have been made in collecting germplasm from the whole world. There are currently almost 400 000 barley accessions in gene banks over the world (van Hintum & Menting 2003). As research is now focused more

on the molecular characterization of genes and proteins, valuable tools for exploring barley genes have been established. For example, the HarvEST barley database was developed as a useful tool for molecular biologists. HarvEST is principally an EST database-viewing software that emphasizes gene function, and is oriented toward comparative genomics and the design of oligonucleotides, in support of activities such as microarray content design, function and annotation, physical and genetic mapping (http://harvest.ucr.edu/).

Genetic improvements of crops have traditionally been performed by crossing and selection. Through the introduction of new tools in biotechnology crossing barriers between unrelated species are now overcome, and new genes can be introduced asexually into plants. Cereal crops were initially difficult to transform both with biolistic transformation and *Agrobacterium*-mediated transformation. The first transgenic barley line was not established until 1994 (Wan & Lemaux 1994). There are now systems developed for *Agrobacterium*-mediated barley transformation (Fang *et al.* 2002; Tingay *et al.* 1997).

1.3.3 Sorghum (*Sorghum bicolor* L. Moench)

Sorghum bicolor is usually an annual or short-term perennial cereal grain and one of the world's most important cereals after rice, wheat, maize and barley. In the Sub-Saharan Africa it ranks the second most important cereal after maize. The crop is a C4 grass and widely adapted to temperate and marginal lands. Sorghum is a staple food for over 750 million people in the developing world. The value of sorghum is accentuated by its ability to grow in marginal areas lacking sufficient moisture for production of maize, wheat or rice. In addition, the photosynthetic efficiency of sorghum surpasses that of most cultivated crops. Taken together, these aspects strongly argue for an increased utilization of sorghum as a starch crop for food and non-food products.

2 Aims of this study

The long-term objective with this research project has been to obtain information regarding the relationship between plant sugar signaling and carbohydrate metabolism, specifically starch synthesis. The understanding of mechanisms behind regulation of carbohydrate metabolism will provide tools and techniques for altering starch composition in economically important crops like barley and sorghum. By the use of *Arabidopsis thaliana* as a model plant we studied sugar signaling pathways regulating carbohydrate metabolism. A reverse genetic approach was used; investigating insertion mutants of *Arabidopsis* where the impact of specific genes involved in sugar signaling was studied. By the use of biolistic transformation techniques transient expression of specific genes were monitored in barley and sorghum.

The project goals included:

1. To clarify the roles of the SUSIBA transcription factors in barley starch synthesis.

2. As the SUSIBA project progressed, the aim was extended to include investigation of the role of the SUSIBAs in fructan synthesis.

3. To study WRKY transcription factors in *Arabidopsis thaliana* and their involvement in sugar signaling and regulation of genes involved in starch metabolism. To elucidate the regulatory effects of sugars on starch synthesis and develop a model for sugar signaling in plants and study the relationship between starch metabolism in grasses and the model plant *Arabidopsis*.

4. As the WRKY project developed the aim was extended to include the study of nucleotide metabolism and sugar signaling.

5. To investigate the transcriptional regulation of *SBEIIB* in sorghum and the involvement of sugar regulating elements within the *SBEIIB* promoter.

3 Results and Discussion

3.1 Sugar-regulated cis and trans factors in barley

By the time this project started, only a few putative transcription factors with relevance to plant sugar signaling had been isolated. The sweet potato factor 1, (SPF1) binds to the *SP8* sequence motif in sweet potato, a repressor of the *sporamin* gene (Ishiguro & Nakamura 1992). The storekeeper protein (STK) binds to the *B-box* element and induces expression of the patatin gene in potato (Zourelidou *et al.* 2002). By studies of expression in transgenic lines of *Arabidopsis*, it was found that several sugar regulated genes, including those for β-amylase (*βAMY*) and the AGPase large subunit (*APL3*) were activated and two transcription factors, WRI1 and ASML2, were found to play an important role in directing the carbon flow to storage when sugar concentrations are high (Masaki *et al.* 2005).

In **paper I**, we report the finding of a novel transcription factor, SUgar SIgnaling in Barley 2, (SUSIBA2). We isolated cDNA from barley and purified the corresponding protein. SUSIBA2 was shown to bind to *SURE* and *W-box* elements but not to the *SP8a* element in the *ISO1* promoter. We could also demonstrate the nuclear localization of SUSIBA2 in a transient assay system with a SUSIBA2:GFP fusion protein. By the use of an oligodeoxynucleotide decoy strategy with transformed barley endosperm we could provide experimental

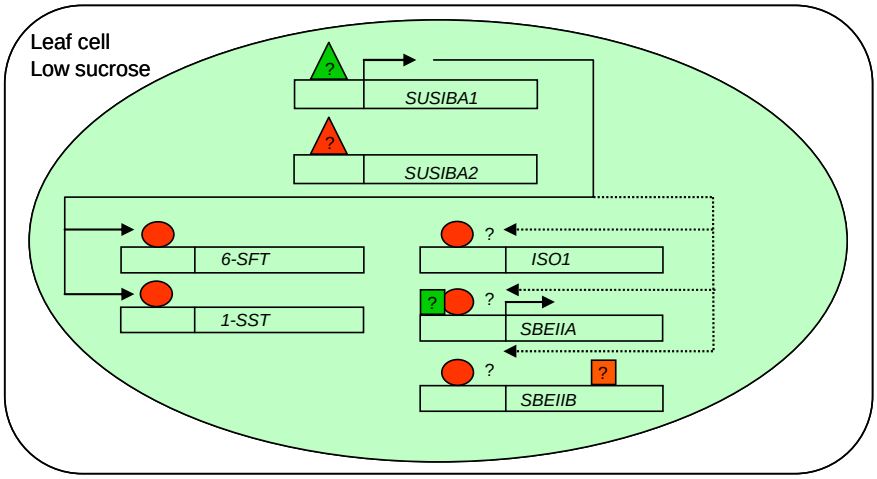
evidence for the importance of the SURE elements in *ISO1* transcription. We also found that *SUSIBA2* is expressed in endosperm but not in leaves and that transcription of *SUSIBA2* was sugar inducible. Ectopic *SUSIBA2* expression was obtained in sugar-treated leaves. Likewise, binding to *SURE* elements was observed for nuclear extracts from sugar-treated but not from control barley leaves. The temporal expression of *SUSIBA2* in barley endosperm followed that of *ISO1* and the endogenous sucrose levels, with a peak at 12 days after pollination. Our data indicate that SUSIBA2 binds to the *SURE* elements in the barley *ISO1* promoter as an activator. Along with the previously described SPF1 transcription factor from sweet potato, SUSIBA2 belongs to group 1 of the WRKY family of plant transcription factors (Eulgem *et al.* 2000).

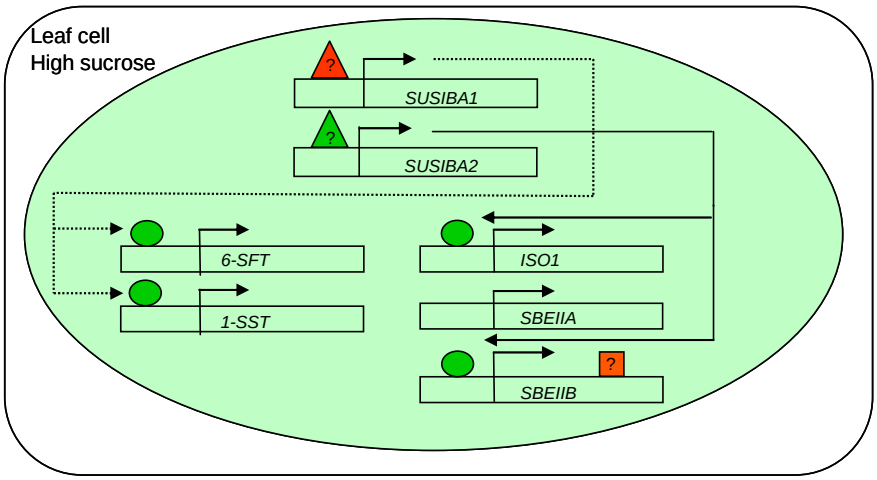
3.2 Transcriptional regulation of 6-SFT gene expression

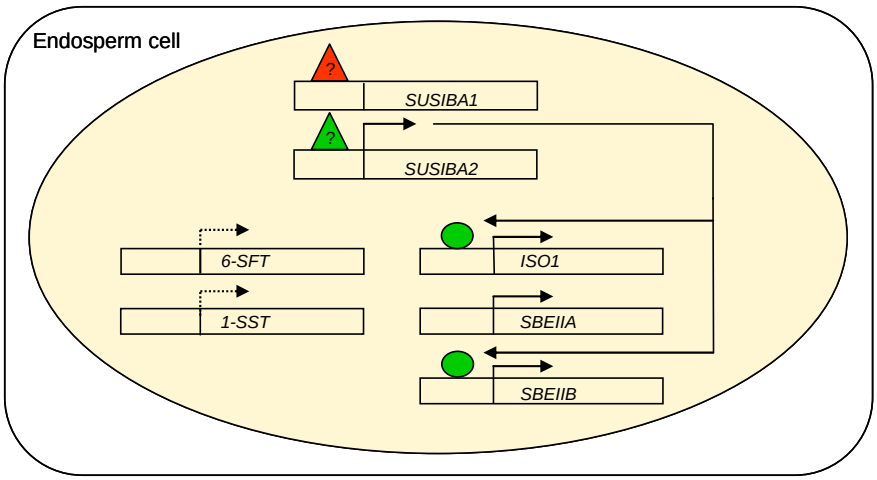
In **paper II** we report that another WRKY transcription factor, SUSIBA1, is involved in regulation of fructan synthesis. Here, the function of SUSIBA1 and its relation to SUSIBA2 were investigated. The barley *SUSIBA1* gene was cloned and overexpressed in *E. coli* and functional studies of purified SUSIBA1 were performed. Gene expression of *SUSIBA1* was studied in leaves and endosperm. By the use of ODN antisense strategy towards the *SUSIBA1* transcript we discovered that *6-SFT* and *1-SST* gene expression was repressed by SUSIBA1. Interestingly, *SUSIBA2* expression was induced by sucrose treatment of barley leaves, while *SUSIBA1* expression was down-regulated (Fig. 2).
Several questions still remain on how or if SUSIBA1 is involved in the regulation of starch synthesis. Preliminary qPCR experiments show that ODN antisense treatment of *SUSIBA1* in barley leaves induces expression of *SBEIIa*, which might indicate that SUSIBA1 is a repressor of both starch synthesis and fructan synthesis in barley. The SUSIBA proteins are, though sharing a high degree of sequence identity, functionally different and expressed in different tissues. The evolutionary relationship between SUSIBA1 and SUSIBA2 remains to be solved.

Figure 2. Model of SUSIBA regulation of transcription in barley

In barley leaves *SUSIBA1* gene is activated by an unknown factor (triangle) under low-sucrose conditions and the SUSIBA1 protein represses expression of the *6-SFT* and *1-SST* genes. SUSIBA1 might also be involved in repression of *ISO1*, *SBEIIA* and *SBEIIB* (dotted line). However, the *SUSIBA2* expression is repressed by an unknown factor (triangle). An additional unknown factor (square) suppresses expression of *SBEIIB* by binding to the *Bbl*-element in the second intron. In barley leaves at high sucrose levels, *SUSIBA2* is activated and the protein induces expression of *ISO1* and *SBEIIB*. *SUSIBA1* is suppressed. The *6-SFT* and *1-SST*, genes are expressed In barley endosperm *SUSIBA2* is expressed and the SUSIBA2 protein induces expression of *ISO1* and *SBEIIB*.


Leaf cell
Low sucrose
?
SUSIBA1
?
SUSIBA2
6-SFT
1-SST
?
ISO1
?
?
SBEIIA
?
?
SBEIIB


Leaf cell
High sucrose
?
SUSIBA1
?
SUSIBA2
6-SFT
1-SST
ISO1
SBEIIA
?
SBEIIB


Endosperm cell
?
SUSIBA1
?
SUSIBA2
6-SFT
1-SST
ISO1
SBEIIA
SBEIIB

3.3 Sugar-mediated transcriptional regulation of two WRKY genes in Arabidopsis

In **paper III** we found two new WRKY transcription factors in *Arabidopsis*, AtWRKY4 and AtWRKY34, involved in sugar-mediated regulation of three *isoamylase* genes.
Although WRKY transcription factors are typically involved in pathogen- and stress-related responses in higher plants, SUSIBA2 from barley and SPF1 from sweet potato, both belonging to group I, participate in sugar signaling regulation of starch metabolism (Fig. 3).

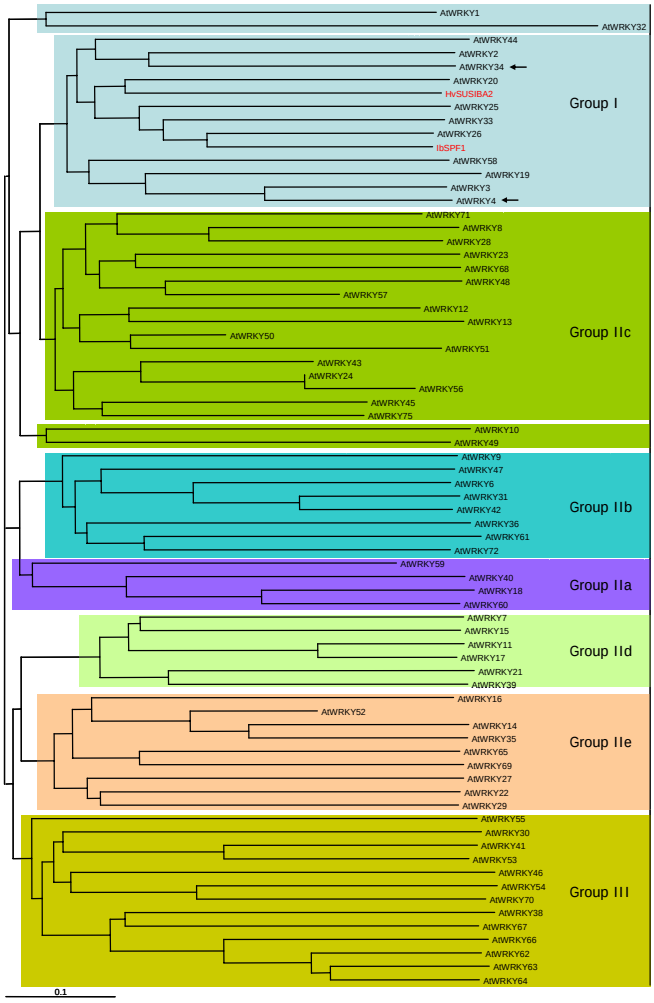


Figure 3. Phylogenetic tree of 71 WRKY proteins from *Arabidopsis* together with SUSIBA2 and SPF1

Figure 3. 72 ORFs encoding WRKY proteins were found through database searches. AtWRKY5 was excluded and the 71 remaining *Arabidopsis* WRKY proteins were aligned with SPF1 from Sweet potato (IbSPF1) and SUSIBA2 from barley (HvSUSIBA2). The classification of the groups was based on the number of WRKY domains and the features of the zinc-finger motifs as described by Eulgem et al. 2000 and is displayed by different colors. Sequences were aligned using ClustalW v1.83, with default settings. The neighbor-joining tree from full-length proteins was made from the aligned sequences using the TREEVIEW software. The IbSPF1 and HvSUSIBA2 proteins are indicated in red.

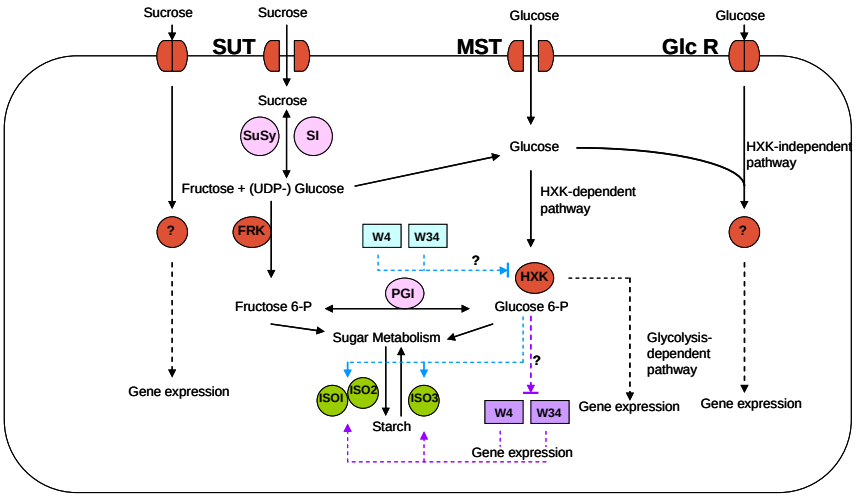


Figure 4. Model of sugar signaling in plants.

Sucrose and glucose are either sensed at the plasma membrane by specific sensors or receptors (Suc R and Glc R). Sucrose and glucose molecules are transported into cells via sucrose/monosaccharide transporters (SUT and MST), which also might confer signaling transduction. Glucose is sensed by HXK through two possible routes, either distinct from glycolysis or through a glycolysis-dependent pathway. Glucose might also be sensed by a HXK-independent pathway. Fructose is sensed and phosphorylated by FRK. Sucrose invertase (SI) and sucrose synthase (SuSy) are involved in the degradation of sucrose to glucose/UDP-glucose and fructose. The phosphoglucoisomerase enzyme (PGI) is involved in converting the reversible reaction of fructose 6-P to Glucose 6-P from the hexose phosphate pool. Hexose phosphates can then further be used in sugar metabolism, glycolysis, TCA cylcle and respiration, or for synthesis of starch.
In *Arabidopsis,* expression of three isoamylase genes *AtISA1*, *AtISA2* and *AtISA3* (ISO1, ISO2 and ISO3) are induced by sucrose and glucose. Two transcription factors, AtWRKY4 and AtWRKY34 (W4 and W34) are mediating the signaling transduction through two possible routes, either downstream of HXK or upstream of HXK as repressors.

We investigated the potential role of the two sugar-inducible group I WRKY genes in Arabidopsis, AtWRKY4 and AtWRKY34, in regulation of starch metabolism. By investigating T-DNA knock-out mutants for the AtWRKY4 and AtWRKY34 genes we studied sugar-dependent regulation of three genes central to starch synthesis and degradation, *AtISA1*, *AtISA2*, and *AtISA3*, encoding, isoamylase1, 2 and 3, respectively. We observed that sucrose and glucose, but not the non-metabolizable sucrose analogs turanose and palatinose, could induce

expression of all three genes in both mutants and wild-type Arabidopsis leaves. In the presence of the HXK inhibitor N-acetyl-glucosamine (NAG), sucrose induction of the genes was up-regulated in wild-type plants but not in the mutants. We suggest after these observations that sucrose induction of the *AtISA1*, *AtISA2*, and *AtISA3* genes proceeds via HXK-dependent repression, and that the WRKY4 and WRKY34 transcription factors are involved in sucrose-mediated regulation of starch metabolism in Arabidopsis (Fig. 4).

3.4 Sugar-mediated regulation of NDPK3a gene expression in Arabidopsis

Further studies on the two sugar-inducible genes, *AtWRKY4* and *AtWRKY34*, in connection with a sugar-inducible nucleoside diphosphate kinase (*NDPK3a*) gene was investigated in **paper IV**. As sugar metabolism is associated with mitochondria through the conversion of sugars to ATP, and through the production of carboskeletons, investigations of the NDPK gene family provide useful information about the link between nucleotide and carbohydrate metabolism. The basic metabolic function of NDPK enzymes (NDPK, EC 2.7.4.6) is to catalyze the transfer of a γ-phosphate group from a nucleoside triphosphate (NTP) to a nucleoside diphosphate (NDP), thereby balancing the nucleotide pool. This results in, either production of (d)ATP, or (d)CTP, (d)GTP, (d)TTP and (d)UTP. There are three different groups of NDPKs in plants with distinct subcellular localization. NDPK1, which is cytocolic, NDPK2, which is located in the stroma of the chloroplast and NDPK3, located in the mitochondrial intermembrane space and lumen of the chloroplast (Hammargren *et al.* 2007; Spetea *et al.* 2004; Struglics & Hakansson 1999; Sweetlove *et al.* 2001; Tanaka *et al.* 1998; Yang & Lamppa 1996). In *Arabidopsis* two isoforms of NDPK3 have been found, NDPK3a and NDPK3b (Hammargren *et al.* 2007). We investigated the sucrose inducibility of the NDPK gene family in *Arabidopsis* using qPCR. We could show that only the *NDPK3a* gene, whose gene product is located predominantly in mitochondria, is subject to sucrose and glucose induction; no other *Arabidopsis NDPK* gene was sucrose inducible. The induction reached a half-maximum after about 6 hours of incubation, and was stable for at least 48 hours. The sucrose and glucose inductions were shown to be HXK-independent by experiments in the presence of NAG. Furthermore, turanose, a sucrose analog that is not metabolized in plant cells, did not induce the *NDPK3a* gene expression. An analysis of the *NDPK3a* gene revealed two *W-boxes* in the promoter region, suggesting that the *NDPK3a* gene expression is regulated by WRKY transcription factors. Studies of the two T-DNA insertion mutants, *Atwrky4* and *Atwrky34*, indicated altered gene expression of *NDPK3a* after sucrose and glucose induction. Interestingly, glucose induction of *NDPK3a* gene expression decreased in the *Atwrky34* mutant while expression in *Atwrky4* mutant was similar to that in wild-type leaves. The reverse effect was observed in **paper III**, where glucose induction of *AtISA2* gene expression was decreased in the *Atwrky4* mutant. Further, the induction of *isoamylase* genes followed an HXK-dependent pathway while that of *NDPK3a* was HXK-independent. This demonstrates the complexity of sugar signaling and that the signal transduction pathway of nucleotide conversion is different from that of starch metabolism.

3.5 Regulation of expression of SBEIIB in sorghum and barley

In **paper V**, the possible involvement of sugar-dependent regulation of *SBEIIB* gene expression was investigated. By deletion mutagenesis of the *SBEIIB* gene promoter of sorghum and a transient expression system with GFP, the minimal promoter required for expression was discovered. However, the expression of sorghum *SBEIIB* was not sugar inducible, in contrast to the expression of barley *SBEIIB*. Moreover, barley *SBEIIB* expression is limited to endosperm tissue while sorghum *SBEIIB* is expressed in both endosperm and embryo. It has previously been suggested that the second intron of the barley *SBEIIB* gene is conferring the endosperm specificity (Ahlandsberg *et al.* 2002). The sorghum and barley genes are very similar with the notable exception that the barley *SBEIIB* second intron is approximately one order of magnitude longer compared to the second intron in sorghum *SBEIIB*. Experiments with the barley *SBEIIB* second intron fused together with the sorghum promoter showed no expression of the reporter gene in embryonic tissue, lending support to the hypothesis that the barley *SBEIIB* second intron is important in regulation of gene activity (Fig. 2).

4 Future perspectives and concluding remarks

4.1 Further exploration of the SUSIBAs in barley

There are still many questions to address when it comes to elucidating the function of the SUSIBAs in barley. A major difference between SUSIBA1 and SUSIBA2 is the number of WRKY domains, SUSIBA1 has only one WRKY domain, while SUSIBA2 has two. Apart from lacking one WRKY domain, the amino acid sequence of SUSIBA1 is identical to that of SUSIBA2. The C-terminal WRKY domain from group I WRKY proteins is proposed to be involved in the sequence-specific binding to cognate DNA elements while the N-terminal WRKY domain is involved in facilitating the DNA binding or involved in protein-protein interactions. This implies that SUSIBA1, which only has the "C-terminal" WRKY domain, has the same sequence-specific DNA binding properties as SUSIBA2 but lacks the N-terminal WRKY domain which might be crucial for activation of gene expression. One suggestion of how this mechanism functions would be that SUSIBA2 is recruiting other proteins and that the protein complex is enabling activation of gene expression. SUSIBA1, on the other hand, is merely binding to several of the sugar-inducible elements and blocks activation and thereby acts as a repressor. There are several ways to investigate this further, for example competitive EMSAs with SUSIBA1 and SUSIBA2 proteins or site-directed mutagenesis of the SUSIBAs in combination with binding assays. Another intriguing question is if the *SUSIBA1* and *SUSIBA2* transcripts originate from two different genes, or if they are splice variants from the same gene. It would be useful to retrieve the genomic sequences and investigate the promoters of the barley *SUSIBAs*. By studying the rice genome we found clues about elements within the SUSIBA rice ortholog. Inspections of promoter sequence of the rice SUSIBA2 ortholog also known as *OsWRKY78*, revealed both *SP8b* and *W-box* elements. However, there is no known ortholog to SUSIBA1 in rice. The finding

of *W-boxes* in *SUSIBA2* suggests that it can be autoregulated by binding to the *W-box* element. It has previously been suggested that the *W-boxes* in promoters of defense-related *WRKY* genes are subject to autoregulation or controlled by other members of the WRKY family (Dong *et al.* 2003).

The idea of SUSIBA1 acting as a more general repressor might be supported by the finding that it represses not only *6-SFT* and *1-SST*, but also *SBEIIA* expression in barley leaves. This remains to be investigated further. On the other hand, there is evidence for SUSIBA2 acting as a specific activator of starch synthesis as experiments with antisense ODN treatment of barley leaves against *SUSIBA2* suggests that *6-SFT* is neither induced nor repressed.

4.2 Finding other WRKY proteins involved in sugar signaling

In *Arabidopsis* there are at least 13 known group I WRKY proteins, of which WRKY4 and WRKY34 are two. The obvious question is if there are other putative candidates involved in sugar signaling. The most interesting group I protein for us to investigate further is the SUSIBA2 ortholog AtWRKY20. Unfortunately, until now, retrieving homozygous knock-out mutants from stock centers have been impossible. Another interesting WRKY protein to investigate further is the SPF1 protein and its putative orthologs in *Arabidopsis,* AtWRKY26 and AtWRKY25, and their role in sugar signaling. There might also be other WRKY proteins outside group I that is of interest in sugar regulation, and investigations of cross-talk between different signaling pathways.

We identified two transcription factors involved in the sugar-inducible regulation of three *isoamylase* genes in *Arabidopsis* and we suggest that there are two possible ways for AtWRKY4 and AtWRKY34 to mediate this signaling (Fig. 4). We can not exclude that the first potential pathway of AtWRKY4 and AtWRKY34 regulation is upstream of HXK as repressors of HXK expression. By investigating the *AtHXK1* promoter we found 6 *W-boxes* which might suggest that WRKY proteins can attach to the *HXK* gene promoter and regulate the expression. The other possibility is that AtWRKY4 and AtWRKY34 are operating downstream of HXK, either by HXK itself acting as repressor of *AtWRKY4* and *AtWRKY34* genes, or some other unknown downstream factor. There are reports of HXK being found in the nucleus and there is a possibility that this is a key in the regulation (Moreno & Herrero 2002). The mechanisms behind sugar signaling, and the participation of other group I WRKY proteins, remain to be explored in future studies.

4.3 Other regulating elements in starch and fructan synthesis genes

In line with further investigations of the SUSIBA proteins, the promoter elements of other genes involved in starch and fructan synthesis is of high interest. In addition, the finding of the *Bbl*-element in the *SBEIIB* second intron suggests that it is not only the promoters of genes that confer specific gene expression. By retrieving genomic sequences of selected genes and further deletion mutagenesis of promoters and introns we hope to find other interesting elements involved in sugar signaling.

4.4 General conclusion

The great challenge in elucidating the complete sugar signaling cascades of plants is the complexity of multicellular, photosynthesizing organisms, with both source and sink tissues. However, the establishment of *Arabidopsis* and rice as model plants, with the complete genome sequences available, increasing number of knock-out lines, along with microarray technologies have improved the genetic research significantly. Microarray analyse provide a powerful tool to gain insight in global transcript dynamics. However, the molecular details of signal transduction and crosstalk can only be revealed by the use of a combination of "omics" techniques, merged with more biochemical approaches. The limitations today lies within the ability to visualize the location and quantify the exact concentration of sugar molecules and other metabolites in the cells. By the use of novel microscopic and fluorescence techniques this obstacle might be overcome in the future.

The identification of the regulatory mechanisms that control both starch and fructan biosynthesis will be of vital interest for many years to come, as they are the keys in finding ways to manipulate carbohydrates in important crop plants.

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and
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