Carbon Allocation in Underground Storage Organs

Studies on Accumulation of Starch, Sugars and Oil

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Cover: Starch granules in cells of fresh potato tuber visualised by iodine staining.

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Carbon Allocation in Underground Storage Organs. Studies on Accumulation of Starch, Sugars and Oil.

Abstract

By increasing knowledge of carbon allocation in underground storage organs and using the knowledge to improve such crops, the competitiveness of these types of storage organs can be strengthened. Starch is the most common storage compound in tubers and roots, but some crops accumulate compounds other than starch. This thesis examined representative underground storage organs accumulating starch, oil and sugars. These were: the oil-accumulating nutsedge (*Cyperus esculentus*), a half-grass which possesses the unusual ability to accumulate triacylglycerol in considerable levels in small tubers physiologically resembling those of potato; the sucrose-storing taproot of sugar beet (*Beta vulgaris*); and the starch- and sugar-storing taproot of parsnip (*Pastinaca sativa*). The lack of starch formation in sugar beet was examined in relation to expression of starch biosynthesis-related genes and enzymes in the taproot.

In parallel studies on potato (*Solanum tuberosum*), a classical starch accumulator, two different transgenic approaches to alter the metabolism and starch biosynthesis of tubers were tested. A novel finding was that expression of the oil transcription factor WRINKLED1 in potato tubers resulted in tubers accumulating oil and negatively affected starch biosynthesis. The oil-accumulating potato tubers shared structural similarities with young nutsedge. Assays on the transcriptome of sugar beet and parsnip revealed that transcripts of two plastidial genes responsible for energy import and phosphate hydrolysis were lower in sugar beet than in parsnip, indicating potential importance for starch accumulation. In potato, the importance of these two genes was assayed by silencing the genes. The outcome was potato tubers with severely affected starch biosynthesis, granule morphology, tuber yield, tuberisation and starch quality, confirming that the silenced genes play an important role in starch biosynthesis in potato tubers.

Keywords: carbon allocation, starch, sucrose, hexose, oil, *Solanum tuberosum*, *Beta vulgaris*, *Cyperus esculentus*, *Pastinaca sativa*, GMO, transcription factor

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Dedication

To my family

The more I learn, the more I realize how much I don't know.
Albert Einstein

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Turesson, H., Marttila, S., Gustavsson, KE., Hofvander, P., Olsson, M., Bülow, L., Stymne, S., and Carlsson, A. S. (2010). Characterization of oil and starch accumulation in tubers of *Cyperus esculentus* var. *sativus Cyperaceae*. *American Journal of Botany* 97(11), 1884-1893.

II Turesson, H., Andersson, M., Marttila, S., Thulin, I., and Hofvander, P. (2014). Starch biosynthetic genes and enzymes are expressed and active in sugar beet tap-root. *BMC Plant Biology* 14(104). DOI: 1471-2229/14/104

III Hofvander, P., Turesson, H., Carlsson, A. S., Andersson, M. (2014). Expression of Arabidopsis *WRINKLED1* in potato tubers lead to increased amounts of fatty acids in the form of triacylglycerols and membrane lipids while affecting tuber phenotype and starch composition. (Manuscript)

IV Andersson M., Turesson, H., Fält., A, and Hofvander, P. (2014). Tuber-specific inhibition of inorganic pyrophosphatase and ATP/ADP translocator leads to major alterations in starch accumulation and tuber formation. (Manuscript)

The contribution of Helle Turesson to the papers included in this thesis was as follows:

I Planned and performed the experimental work. Participated in evaluation of the data and writing of the manuscript together with the co-authors.

II Planned and performed most of the experimental work. Participated in evaluation of the data and writing of the manuscript together with the co-authors.

III Performed the southern experiment, prepared the samples for all structural studies and performed the light microscopy studies. Evaluated the results and participated in writing the manuscript together with the co-authors.

IV Planned and performed a large part of the experiments. Evaluated the data and wrote the manuscript together with the co-authors.

Abbreviations

G3P	Glycerol 3-phosphate
ACP	Acyl carrier protein
ADPgase	ADPglucose pyrophosphorylase
ATP	Adenosine triphosphate
BE	Branching enzyme
CoA	Coenzyme A
DAG	Diacylglycerol
DGDG	Digalactosyldiacylglycerol
ER	Endoplasmic reticulum
G1P	Glucose 1-phosphate
G6P	Glucose 6-phosphate
GBSS	Granule bound starch synthase
GPT	Glucose 6-phosphate transporter
LDA	Limit dextrinase
LEC	Leafy cotyledon
MGDG	Monogalactosyldiacylglycerol
MOS	Maltooligosaccharide
PGM	Phosphoglucomutase
Pi	Inorganic phosphate
PL	Phospholipid
PPi	Pyrophosphate
qPCR	Quantitative polymerase chain reaction
SS	Starch synthase
SUSIBA	Sugar signalling in barley
TAG	Triacylglycerol
Wri1	WRINKLED1

1 Introduction

Throughout the history of agriculture, the use of land and the yield of crops have both increased considerably (FAOSTAT, 2013) As a result, world production of plant commodities has continually increased. However, in order to provide for the growing global population and to move towards sustainable agriculture and a sustainable economy, systems for more efficient use of the world's natural assets must be implemented, in parallel with other actions such as general respect for natural resources. Possibilities for exploiting the use of land more efficiently and decreasing the pressures on the environment need to be explored, which is a challenge but not impossible. For example, crops modified by modern plant breeding can be used to achieve more efficient production of carbon compounds, both with regard to yield and to special qualities. Such plant breeding methods can also enable environmental stresses such as drought, pests and diseases to be overcome, leading to a decrease in harvest losses and lower use of input chemicals as beneficial outcomes.

Plants store carbon in different forms with the universal function of supplying the developing next generation with energy until it is self-sustainable through photosynthesis. Seeds, tubers and roots are the most common sites for this energy storage and the forms in which energy is stored are predominantly oil, starch and sugars. Underground storage organs mainly store starch and sugars, while seeds from different plant varieties accumulate high levels of starch and also oils and proteins. These energy-rich organs are used by humans for food and animal feed and also for biofuel and a broad range of industrial applications.

The average global yield of the main tuber and root crops grown in agriculture has increased by almost 50% during the last 50 years, to 11 tonnes per hectare (Figure 1). However, this is still a low figure in view of the fact that individual root and tuber crops can display much higher yields. For instance, the average global potato yield in 2011 was 19.5 metric tonnes per hectare

(t/ha) and that of sugar beet was almost 55 t/ha (FAOSTAT, 2013). However, potato is a global crop with many different applications (CIP, 2014), while sugar beet is grown mainly in Europe and North America and exclusively for sugar extraction (EastAgri, 2009). The global area used for growing tubers and roots has increased from approximately 54 million ha to almost 61 million ha, an increase of just over 10%. However, compared with the cultivated area of *e.g.* wheat (220 million ha), it is still a very low figure. If root and tuber crops were to possess additional beneficial traits, there is certainly room for expansion of these kinds of crops in terms of both areal yield and cultivated area.

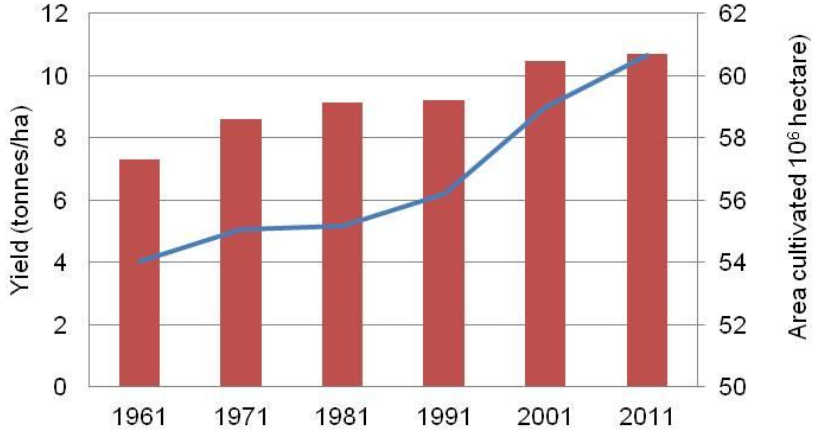

Figure 1. Global yield (tonnes/ha) and cultivated area (million ha) of tubers and roots (cassava, potatoes, sweet potatoes, sugar beet and yams), 1961-2011. Source: FAOSTAT, 2012.

In order to advance plant breeding and crop improvement, it is necessary to understand the mechanisms behind carbon allocation in crops. Increased knowledge of these mechanisms would generate opportunities to adapt the storage organs of plants to meet the global food challenges described above.

This thesis focuses on carbon allocation in underground storage organs. Starch is the most common storage compound in tubers and roots, but some crops accumulate compounds others than starch. Two plant species that store non-starch compounds, yellow nutsedge and sugar beet, were studied here. Nutsedge stores oil in considerable quantities and sugar beet uniquely stores sucrose.

In parallel studies, a classic starch accumulator, potato, was genetically engineered by introducing WRINKLED1, a known transcription factor triggering genes involved in oil synthesis, in an attempt to produce tubers that can accumulate oil. Another study on potato examined the effects of downregulation of two genes, plastidial ATP/ADP transporter (*NTT1)* earlier shown to play an important role in starch biosynthesis, which we wanted to assay further, and plastidial pyrophosphatase (*PPa6)* which when expressed transiently in photosynthetic tissue displayed alterations in starch biosynthesis (George *et al.*, 2010; Tjaden *et al.*, 1998). The overall aim of the work described in this thesis was to contribute new information on the complex issue of carbon allocation and identify new directions for future research.

2 Background

2.1 Underground storage organs

Plants store energy in different forms and in different organs of the plant. Seeds, roots and tubers are the most common sinks and the most occurring form of energy stored in underground storage organ is mostly starch and sugars.

There are some characteristics that distinguish underground storage organs from organs located above ground. One obvious difference is the location of the storage organ, *e.g*. roots and tubers are beneath the ground, seeds and stems above. This means that the harvesting procedure for underground organs differs from that used for seed. Another feature of roots and tubers is that in northern Europe, seeds can in many cases be sown as a winter crop, while roots and tubers can seldom be sown before winter unless intended for seed production (biennials).

Underground storage organs often have a higher water content compared with seeds. For instance, cassava contains about 60% water (OECD, 2009) and potato and sugar beet 65-75% (EastAgri, 2009; Hofvander *et al.*, 2004; OECD, 2002), while cereal seeds have a water content typically below 20% (Herrera-Saldana *et al.*, 1990). However, the net dry weight yield per unit land area of tubers and roots is generally higher than that of cereals. A simple calculation example based on winter wheat and starch potato harvests in Sweden 2013 results in a twice as high potato yield (10.5 t/ha) compared to wheat (5.4 t/ha) (Swedish Board of Agriculture, 2014). Some drawbacks of high water content in a crop, in view of their application areas as a raw material, are the added weight during transportation and the additional effort required removing the water during processing of the crop. Tubers and roots are also sensitive to drought, while seeds are not as sensitive. Furthermore, seeds from cereals

contain both the embryo and storage tissues, while tubers and roots act only as a storage organ and the embryo is located elsewhere in the plant.

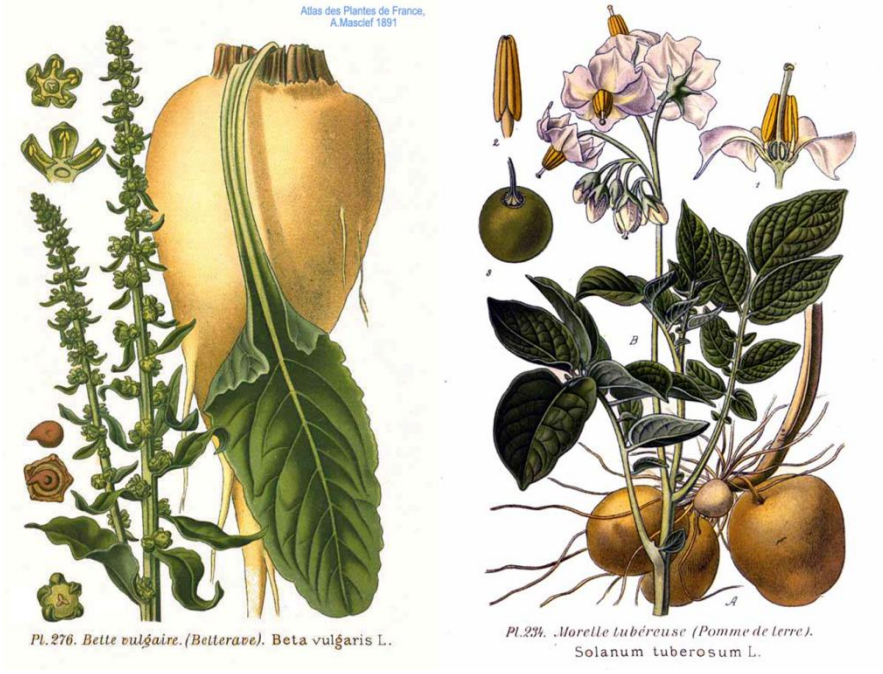

Figure 2. Illustration of (left) a typical taproot, that of sugar beet (*Beta vulgaris*) and (right) a typical tuber, that of potato (*Solanum tuberosum*). Source: The public domain. Wikimedia commons, Atlas des plantes de France by A. Masclef.

2.1.1 Taproots

The characteristic feature of a taproot is an enlarged, swollen primary root (Figure 2, left). The energy in the taproot is used for bolting, flowering and setting of seeds, providing for the coming generation. Mining of energy from a biennial taproot usually starts after a change in day length or exposure to a cold period, *i.e*. after winter dormancy (Sung & Amasino, 2004). Annual plant species with taproots also exist. In that case, the plant starts to produce seeds at an earlier stage than in biennial plants, which means that the carbon compounds accumulated in the taproot of annuals start to be reallocated before the taproot has time to grow very large.

2.1.2 Tubers

A storage organ referred to as a tuber can grow on the plant in different ways. The growth habits of tubers, sometimes called root tubers (*e.g.* sweet potato

(*Ipomea batata*)), and tuberous roots, (*e.g*. cassava (*Manihot esculenta*)) differ from stem tubers, they develop from the base of the plant while stem tubers develop from underground stems, stolons. A difference between a taproot and tuber plant is that tuber plants generate several storage organs on one plant, opposed to the taproot which has only one storage organ per plant. A well-known stem tuber crop is potato (*Solanum tuberosum*) (Figure 2, right), Potatoes are usually grown by planting seed tubers, and not true potato seeds. True potato seeds are not very viable and degeneration rates are high (Pallais, 1987). Consequently, seed tubers are used for planting and need to be produced on a yearly basis.

After establishment of the plant, stolons evolve at the base of the plant. In physiological terms, these are designated underground stems. The tip of the stolon swells and tubers begin to develop and these in turn can provide energy for coming generations (Figure 3). Potato plants are sensitive to frost, resulting in non-persistence of generations over seasons in northern Europe, but in theory tubers remaining in the soil can give rise to the next generation. Another tuber crop, yellow nutsedge (*Cyperus esculentus*), displays similar behaviour, although it is not grown in northern Europe.

Figure 3. Demonstration of a stem tuber evolving by swelling of the tip of a stolon on yellow nutsedge (*Cyperus esculentus*) . Scale bar = 5 mm. Photo H. Turesson.

2.1.3 Storage compounds in different underground organs

The predominant storage compound in underground storage organs is starch, which constitutes a large proportion of the dry weight in potato and parsnip (*Pastinaca sativa*) and is also found present at considerable levels in *e.g*. horse radish (*Armoracia rusticana*) and carrot (*Daucus carota*) (Figure 4). Plant species that do not store starch also exist, for example sugar beet (*Beta vulgaris*), in which the main storage component is sucrose, and the fructan-storing species onion (*Allium cepa*), which accumulates inulin, and Jerusalem artichoke (*Helianthus tuberosus*), which accumulates polyfructan (Ritsema & Smeekens, 2003; Frehner *et al.*, 1984). To the best of my knowledge, the only crop plant to store oil in significant quantities in its underground organs is yellow nutsedge, hereafter referred to as nutsedge. The tubers of this sedge species contain high levels of both oil and starch.

The occurrence of hexoses in an underground organ can mostly be detected when the tissue is very young and in a build-up phase (Ap Rees, 1974), or as a response to stress or wounding (Rolland *et al.*, 2006; Benhamou *et al.*, 1991). Propagation of a new generation of a plant also causes rearrangements in carbon composition, with conversion of the accumulated carbon compounds to hexoses. However, hexoses seldom occur as a storage compound.

In general, one compound is the dominant storage form in the plant, but storage of two or more is not unusual. A combination of accumulates can be the result of leakage of metabolites between different biosynthetic systems, or of parallel processes during storage. For example, transition of accumulated starch in fresh, mature tubers and roots to sugars during storage or cold treatment is common. Examples of this are the active sweetening of parsnip through cold treatment (Wismer *et al.*, 1995) or the generally considered non-starchy carrot, which in fact contains starch when harvested (Figure 4). In light of this, the definition of what a mature storage organ comprises has to be revised. In many cases, harvesting of a crop does not take place when photosynthesis is still active or has just ceased. Instead, the storage organ is often left on the plant after cessation of photosynthesis, allowing the organ to rearrange the accumulated carbon, *e.g*. dehaulming of potatoes and maturation of cereals.

Figure 4. Demonstration of presence of starch in various tubers and roots. Slices of root and tuber tissue stained with iodine, which stains starch purple/brown. Photo: H. Turesson

2.2 Accumulation process – from photosynthate to sink filling

2.2.1 Photosynthate entering the sink

There is generally good knowledge of the biosynthetic pathways for storage compounds, but factors determining the channelling of the photosynthate produced into oil, sugar or starch in different plant species are not equally well known. The cells of storage organs of plants, such as seeds, roots and tubers, have in common the import of sucrose, generated from photosynthesis in the green part of the plants (Fu *et al.*, 2011). Sugars in plants generally have several functions, *e.g*. as signalling substances and as nutrients, which means that the pathways involved can be quite complex.

Sucrose is the main photosynthate transported through the phloem between source and sink under concentration and pressure gradients generated by invertases hydrolysing the sucrose to hexoses during the transport through the plant (Ayre, 2011). When delivered to the cells, sucrose is subcellularly compartmentalised or converted to hexoses to maintain a sucrose gradient along the phloem (Rolland *et al.*, 2006). In the initial development of sink cells, sucrose is converted by invertases to hexoses, which are channelled to construction of cell structures and to provision of energy for respiration (Sturm & Tang, 1999). Examples of species with higher hexose levels in young storage organs are nutsedge and parsnip (Paper I and II). On a switch by the plant to storage accumulation, sucrose is either transported directly into the vacuole or converted to uridine diphosphate glucose (UDP-glucose) and fructose by sucrose synthase. The UDP-glucose is subsequently transformed to hexose phosphates. The hexose compounds generated, *i.e*. hexoses and hexose phosphates, are further partitioned to different subcellular compartments destined for various end products or directed further into the plant as signalling substances (Rolland *et al.*, 2006).

2.2.2 Transcription factors

A biological process in a plant is rarely operated by a single enzyme and instead a whole set of enzymes interact. The transcription of genes leading to the translation to enzymes/proteins is often organised by a transcription factor. A transcription factor is a protein modulating processes in plants by activation or repression of mRNA transcription of groups of genes involved in a specific process. The genes to be affected often have binding sites which are recognised by the transcription factor.

Examples of transcription factors involved in carbon allocation:

- LEAFYCOTELYDON1 and 2 (LEC1&2) are transcription factors involved in embryo maturation, seed maturation and seed oil storage (Lotan *et al.*, 1998; West *et al.*, 1994). Their involvement in fatty acid biosynthesis has been proven by over-expression of LEC1 in *Arabidopsis thaliana* resulting in upregulation of most of the genes involved in *de novo* fatty acid synthesis, a mechanism confirmed in Arabidopsis seedlings (Mu *et al.*, 2008). LEC2 has also been shown to upregulate genes involved in fatty acid biosynthesis and induction of LEC2 occurs at the onset of triacylglycerol (TAG) accumulation in mature Arabidopsis embryos (Santos-Mendoza *et al.*, 2008).

- WRKY is a large family of transcription factors participating in several processes, for example plant immune responses (Rushton *et al.*, 2010). One example of WRKY transcription factors related to carbon allocation are the SUSIBA transcription factors (Olsson *et al.*, 2003; Sun *et al.*, 2003). SUSIBA1 (repressor) and SUSIBA2 (activator) have been shown to act in barley (*Hordeum vulgare*) endosperm as a carbon source-sink communicator. SUSIBA2 expression levels mirror endogenous sugar concentrations, which results in altered starch accumulation.

- WRINKLED1 (Wri1) is considered to be a transcription factor upholding a key regulatory function of carbon allocation towards fatty acids. Wri1 was first identified in an *Arabidopsis thaliana* mutant producing wrinkled seeds with clearly affected seed oil accumulation, leading to the conclusion that wri1 is involved in carbon flux during the development of the seeds (Cernac & Benning, 2004; Focks & Benning, 1998). Wri1 mutants display lower oil content and higher content of sugars than the wild type. Furthermore, overexpression of endogenous wri1 in maize (*Zea mays*) resulted in 30% higher seed oil content, although at the expense of starch accumulation (Shen *et al.*, 2010).

- In rice, Rice Starch Regulator 1 (RSR1) has been proposed to be a transcription factor having a negative effect on starch biosynthesis. Mutants of RSR1 display altered starch granule morphology and starch properties due to higher amylose concentration and altered amylopectin structure (Fu & Xue, 2010).

2.2.3 Starch accumulation

In underground plant organs, starch is synthesised and accumulated in a specialised plastid located in non-photosynthetic tissue, the amyloplast (Figure 5). Glucose-6 phosphate (Gluc-6-P), derived from the photosynthetic sucrose, is transported into the amyloplast by a glucose 6-phosphate transporter (GPT) and in four dedicated steps generates two different kinds of glucose polymers, amylopectin and amylose. These polymers are turned into inert sophisticated starch granules that do not require energy to maintain their structure.

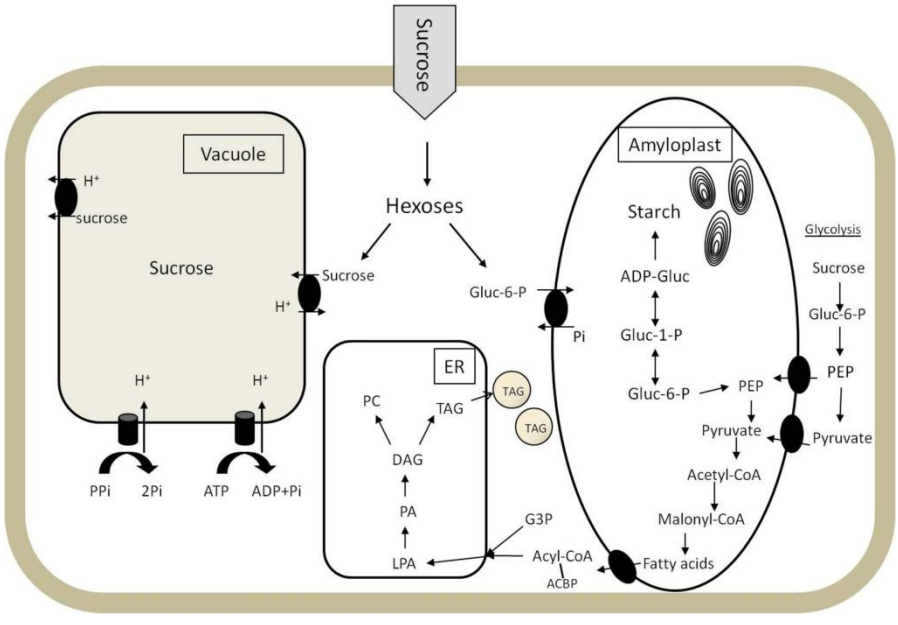

Figure 5. Simplified schematic overview of the accumulation process of sucrose, starch and oil in a storage cell of a tuber or a root. Illustration: H. Turesson & P. Hofvander.

Gluc-6-P is converted to glucose-1 phosphate (Gluc-1-P) catalyzed by phosphoglucomutase and further irreversibly converted to adenosine diphosphoglucose (ADP-glucose) by ADP-glucose pyrophosphorylase (ADPgase) (Stark *et al.*, 1992). Starch synthases and branching enzymes catalyse the formation of polymeric glucose chains linked together by α-1,4 and α-1,6 linkages. One granular bound starch synthase (GBSS) and four soluble starch synthases (SSI-IV) have been identified. The significance of these starch synthases is well described in numerous publications (*e.g.* Roldán

et al., 2007; Edwards *et al.*, 1999b; Abel *et al.*, 1996; Edwards *et al.*, 1995; Kuipers *et al.*, 1994).

Several enzymes participate in building starch granules

With availability of ADP-glucose, the building of starch polymers can commence. It has been shown that starch synthases are dependent on the type of substrate available, such as longer or shorter molecules, and thus inhibition of one starch synthase isoform alters the substrate composition and availability for the remaining starch synthase isoforms to act on, consequently leading to altered quality and quantity of the starch produced. Five isoforms of starch synthases are known to date (GBSS and SSI-IV). All use the same substrate, ADP-glucose, but each isoform is specialised, resulting in different unique insoluble glucans in plants. GBSS, is responsible for synthesis of amylose and displays activity in the physical centre of the granule, where amylose also is found (Zeeman *et al.*, 2010). The amylose is not branched, possibly because branching enzyme (BE) is not available in the matrix of the granule where the GBSS is situated (Kuipers *et al.*, 1994). SSI is reported to be most important in photosynthetic tissues and genetic inhibition of the isoform does not affect tuber starch (Kossmann *et al.*, 1999). SSII is responsible for 10-15% of the total starch synthase activity, but a reduction in its activity has little effect on starch biosynthesis when studied in potato tuber (Edwards *et al.*, 1999a). A reduction in SSIII, on the other hand, has a major effect on the starch synthesised, which results in cracks in granules. SSIII accounts for approximately 80% of the total starch synthase activity and the isoform is also expressed in the green part of plants (Abel *et al.*, 1996). Starch synthases I-III also are believed to elongate different lengths of glucans (Zeeman *et al.*, 2010). SSIV is thought to be involved in granule initiation, since in an Arabidopsis mutant lacking SSIV activity only one large starch granule is initiated, compared with several starch granules per chloroplast in the wild type (Szydlowski *et al.*, 2009; Roldán *et al.*, 2007). Branching enzyme (BE) does not produce net starch, but instead the existing starch is rearranged by BE hydrolysing α-1,4 bonds and reattaching the glucan with a α-1,6 bond, creating a novel branch.

Starch phosphorylase is reversible, but due to substrate availability releasing Gluc-1-P from the non-reducing ends of α-1,4 glucans, it is believed mainly to have a starch hydrolysis function (Yu *et al.*, 2001). However, it has been postulated that phosphorylase can act to prolong shorter glucans, so that SSI-IV can act upon the glucans and amylopectin can be synthesised, thus giving phosphorylase a synthetic function (Satoh *et al.*, 2008).

Debranching enzymes, such as pullullanases or limit dextrinases (LDAs) and isoamylases, are considered to perform the function of trimming glucans to transform the structure of the branched glucan into a self-organising structure. The consequences of these debranching activities are starch hydrolysis and promotion of amylopectin condensation. Isoamylase is believed to hydrolyse α-1,6 linkages, restricting the glucans in unstructured growth, with different isoforms having different affinities to glucan chain lengths (Hussain *et al.*, 2003). Studies on mutants in various crops (maize, barley, Arabidopsis) which display a decrease in amylopectin accompanied by an increase in soluble phytoglycogen, an evenly branched glucan, have concluded that isoamylase works on soluble glucans (Burton *et al.*, 2002; Zeeman *et al.*, 1998). Thus, by debranching soluble glucans, the formation of the large, insoluble molecule amylopectin can be promoted.

The initiation of starch molecules is dependent on an initiation/starting point. Small glucans of about six glucose units are believed to be required for starch synthases to be active, although it has been reported that SSIII has the ability to polymerize glucans in absence of primers (Szydlowski *et al.*, 2009). In yeast and animals, glycogen production is initiated by the glycogenin protein creating short chains of glucose on which glycogen synthases can operate in building the polymer (Cheng *et al.*, 1995). No strong evidence of an equivalent function has been demonstrated in plants, although attempts have been made with plant-derived genes sharing sequence homology with the yeast isoform (Chatterjee *et al.*, 2005; Hofvander, 2004). There are different theories as to what constitutes the starting point of amylose synthesis. One suggestion is that maltooligosaccharide (MOS), a soluble 4-10 monosaccharide glucan, acts as a primer which can diffuse into the centre of the granule and there be utilised by GBSS (Denyer *et al.*, 2001). Another theory of starch initiation is from amylopectin, on which presumably debranching activity works, providing primers (van de Wal *et al.*, 1998). GBSS then attaches ADP-glucose on the primers, building the fairly unbranched amylose molecule.

Amylopectin is the backbone of the starch granule, forming different regions with its highly branched structure. A starch granule consists of alternate amorphous and crystalline layers due to the density of branching points, formed as growth rings into a stable granule. The crystalline layer consists of tighter clusters of amylopectin chains, while the amorphous layer contains the part of the amylopectin molecules that are less dense in glucans.

Starch granules are stable when isolated, but in their natural environment in the plant, small pores in the granule surface are believed to allow starch-degrading enzymes to hydrolyse the starch by undermining the structure of the

granule. This releases glucose, which can be used as energy for the coming generation (Sarikaya *et al.*, 2000).

2.2.4 Sucrose accumulation

Sucrose is a disaccharide composed of fructosyl and glucosyl units connected by the reducing ends of the hexoses with a glycosidic bond. The non-reducing ends of the molecule are less reactive and therefore the molecule is suitable for transportation in plants. As in other accumulation processes, sucrose generated from photosynthesis is transported through the phloem and is believed to symplastically enter the cytosol of the storage cell, where invertases initially convert the sucrose to the hexoses fructose and glucose (Koch, 2004). On switching to the accumulative phase of plant development, invertase activity decreases, while sucrose synthase activity increases (Sturm & Tang, 1999). Due to the availability of substrates favouring the breakdown of sucrose, sucrose synthase generally works in the opposite direction to synthesis of sucrose by catalysing sucrose + UDP to UDP-glucose and fructose. These products generate fewer hexoses, which presumably leads to lower hexose signalling and in turn allows accumulation of storage carbohydrates to proceed undisturbed. The UDP-glucose can only be utilised for re-synthesis of sucrose in the cytosol by sucrose phosphate synthase (SPS) (UDP-glucose + fructose-6P → sucrose-P + UDP). In a later step, sucrose phosphate combines with UDP-glucose to form sucrose + inorganic phosphate (Pi), a reaction catalysed by sucrose phosphate phosphatase (SPP) (Wind *et al.*, 2010).

The vacuole is the subcellular compartment in a sink cell where sucrose is stored. Accumulation of sink sucrose in the vacuole starts with active transport into the vacuole by sucrose transporters, sucrose proton antiport transporter and tonoplast-bound H^+/sucrose symporter (Koch, 2004). The difference in sucrose concentration, which can be up to seven-fold higher in the vacuole compared with the cytoplasm (Saftner *et al.*, 1983), is actively maintained by proton pumps (v-PPase and v-ATPase) (Krebs *et al.*, 2010; Hedrich & Schroeder, 1989) As a consequence, there is a continuous inflow and outflow of protons through the tonoplast membrane surrounding the vacuole. The vacuole also harbours invertase activity, which is triggered when the accumulated sucrose needs to be utilised (Roitsch & González, 2004). To the best of my knowledge, import of hexoses to the vacuole has not been demonstrated and vacuolar SPS and SPP have not been found. Thus re-synthesis of sucrose in the vacuole is unlikely, with the consequence that hexoses generated in the vacuole are transported to the cytoplasm and re-synthesised there if destined for this (Winter & Huber, 2000).

2.2.5 Oil accumulation

Oil is accumulated as a storage compound in plants predominantly in the form of triacylglycerol (TAG), a molecule consisting of a glycerol backbone with three fatty acids esterified to it. The path from photosynthate to an oil body full of TAG in a plant cell involves a multitude of enzymatic steps in different subcellular compartments (Figure 5).

Initially, Gluc-6-P is converted to phosphoenolpyruvate (PEP) and further to pyruvate. These reactions can take place in both the plastid and the cytosol and the intermediates can be transported into the plastids from the cytosol. The subsequent steps, including *de novo* fatty acid synthesis, are plastidial (Figure 5) (Ohlrogge and Jaworski, 1997). The pyruvate is transformed to acetyl-CoA, which is used as a building block for fatty acid synthesis after its conversion to malonyl-CoA catalysed by acetyl-CoA carboxylase (ACCase) (Rawsthorne, 2002). Activity of plastidial ACCase is suggested to be a possible regulator of carbon into oil accumulation and conversion of acetyl-CoA is considered to be the first step in fatty acid synthesis (Sasaki & Nagano, 2004). Acetyl-CoA works as a starting unit of the fatty acid and malonyl-ACP, with the malonyl group transferred from CoA to an acyl carrier protein (ACP), supplying two carbons at each cycle of fatty acid synthesis, catalysed by fatty acid synthases. The cycle of building fatty acids continues by adding two carbons until a 16C-ACP or 18C-ACP is obtained. A desaturase can work on the 18:0-ACP and convert the fatty acid to a desaturated 18:1-ACP. These fatty acids are transported through the double plastidic membrane and the ACP is hydrolysed and activated to CoA. The fatty acid-CoAs are exported through the cytosol to the endoplasmic reticulum (ER). Three fatty acids are esterified one-by-one to a glycerol molecule, glycerol-3-phosphate (G3P), in a process known as the Kennedy pathway (Kennedy & Weiss, 1956). Modifications such as acetylation, epoxidation and hydroxylation can be made to the fatty acids when attached to the glycerol backbone. TAG is budded off from the endoplasmic reticulum as oil droplets or oil bodies (Frandsen *et al.*, 2001). When present in high amounts oil droplets can fuse into large oil drops (Leonova *et al.*, 2010; Heneen *et al.*, 2008). Oil bodies are small, simple structures consisting of TAG molecules covered with a phospholipid and oleosin layer, which appears to have a shielding function to protect the TAG from cytosolic components and thus for instance withstand the strains of dehydration and rehydration in seeds (Siloto *et al.*, 2006; Jacks *et al.*, 1990; Yatsu & Jacks, 1972). The amount of oleosins also determines the size of the formed oil bodies (Siloto *et al.*, 2006).

2.3 Gene expression analysis

Gene expression analysis reveals which genes are being transcribed at the time of sampling, thus indicating which proteins or enzymes are being translated at a given time-point. The benefits of this kind of analysis are many. The information on gene expression itself, or expression of genes in a treated sample compared with a non-treated sample or one part of a plant compared with another, can be very useful, alone or in combination with information obtained in complementary analyses on *e.g*. cell biochemistry or metabolites. However, there are drawbacks in this kind of analysis, *e.g*. a transcript is not always the sole factor responsible for translation of a protein. Transcript levels may correlate poorly with the active protein (Schwanhausser *et al.*, 2011; Maier *et al.*, 2009; Gygi *et al.*, 1999). Additionally, the activity or function of a protein can be affected by factors such as post-translational functions, which are not always influenced by the transcript level of the corresponding gene. Three different types of gene expression analysis representing different approaches are described below.

2.3.1 qPCR

Real-time quantitative polymerase chain reaction, or qPCR, is a relatively narrow approach, mostly used when studying one or a few genes in a sample. The relative expression of the gene under study or the copy number of genes inserted is determined, which can be of interest for example when analysing a transgenic line. However, there can be difficulties and obstacles to making this method a standard procedure. For example, analysing the quantitative expression requires a stable reference gene, which can be a challenge in itself. Many reference genes considered to be stable have proven not to be so, and therefore in every individual case the genes chosen have to be thoroughly validated (Pfaffl *et al.*, 2004; Vandesompele *et al.*, 2002). In addition, the chemistry of the amplification must be assayed to allow conclusions to be drawn from the measurements made. As an example, reference genes used in potato were studied by Nicot *et al*. (Nicot *et al.*, 2005) by exposing potato to different treatments/stresses and assaying seven commonly used reference genes. The results showed large variations in expression levels of the different genes, depending on the treatment applied. An obvious conclusion from the study is the importance of evaluating the stability of the housekeeping gene in the treatments applied before using it as a comparison.

2.3.2 Microarray

A broader kind of study is the microarray. When microarray is chosen as the analysis method, the aim is generally to determine and compare gene

expression of large groups of genes in different treatments (Schena *et al.*, 1995). Several thousand probes of choice are attached to a microchip that is then exposed to the complementary DNA (cDNA), which hybridises to the chip. The amount of DNA attached to the chip is detected by luminescence and the level of emitted signal is measured. Microarray is an efficient way to compare different treatments by identifying changes in expression levels and patterns in a large number of genes.

2.3.3 Massive parallel sequencing

The demand for more reliable, cheaper and faster high-throughput sequencing methods in order to analyse expression of whole genomes has led to the application of available DNA sequencing methods such as Illumina and 454-pyrosequencing to cDNA. These methods determine sequences of DNA, which can be assembled by sophisticated software algorithms to full-size genes, de-novo transcriptome assembly. The obtained sequences can also be mapped against a reference genome, where the number of mapped reads describes the expression level of the gene in question in comparison to for instance another treatment or plant species. If the genome in question has not been analysed previously, the usual procedure is to annotate against an already mapped genome, in a plant context very often Arabidopsis. In the past decade this form of analysis has gone from being very expensive and laborious to being a basic tool that most researchers can afford and use (Wolf, 2013). Nowadays many high-throughput sequencing analyses are conducted to investigate a specific and limited research question, *i.e*. utilising only a small part of the information obtained. However, it is of course possible to return to the data obtained and these are often made available in public databases, enabling other researchers to make use of them.

3 Aim and objectives

The overall aim of this thesis was to advance existing knowledge on carbon partitioning to underground storage organs. Carbon allocation in underground storage organs mostly revolves around starch accumulation. However, two plant species diverging from the typical starch accumulation pattern, nutsedge and sugar beet were studied here. In addition, potato was in two different approaches genetically engineered in order to study the effect of modifications potentially redirecting the tuber metabolism.

- Nutsedge (*Cyperus esculentus*) was biochemically and structurally characterised from tuber initiation to 42-day-old tubers, with emphasis on starch and oil accumulation (Paper I).

- A biochemical, structural and transcriptomic comparison of sugar beet (*Beta vulgaris*) and parsnip (*Pastinaca sativa*) was made, from the perspective of absence of starch accumulation in sugar beet (Paper II).

- The transcription factor WRINKLED1 was introduced into potato tuber (*Solanum tuberosum*) in order to study whether the tuber was then able to redirect photosynthates to accumulation of oil (Paper III).

- Two genes in potato, *NTT1* and *PPa6*, were downregulated separately and in combination, in order to study the importance of their encoded functions for starch accumulation (Paper IV).

4 Carbon allocation in underground storage organs

4.1 Characterisation of tuberous nutsedge (*Cyperus esculentus*) *(*Paper I)

4.1.1 The nutsedge tuber, an organ with special traits

The variation in the quantities or special qualities of oils accumulated in seeds or fruit mesocarp in plants has been well studied and possible novel application areas in several plant species have been identified (Dyer *et al.*, 2008). *Arabidopsis thaliana* often serves as a model crop when studying the accumulation process and genes of interest connected to seed oil accumulation (Jako *et al.*, 2001; Focks & Benning, 1998). Paper I describes a unique underground storage organ which accumulates oil in small tubers. Nutsedge is a sedge, a half-grass, characterised by stem tubers containing high levels of starch, sugars and oil (Linssen *et al.*, 1989). The specific properties of these tubers make them a suitable non-seed storage tissue to study. The nutsedge tuber has features that resemble the behaviour of a seed and which cannot be assigned as classical storage accumulation of tubers or roots. One example is the high dry weight of the tubers. As presented in Paper I, at 42 days after tuber initiation (DAI) the nutsedge tubers had a dry weight of 58%, compared with 20-25% in sugar beet and potato. Mature nutsedge tubers are also resistant to drought; they maintain viability even after exceeding 95% dry weight, a property which seeds also possess (Steiner & Ruckenbauer, 1995). In Paper I, the nutsedge tubers were grown in an aeroponic system, which allowed them to be inspected and sampled on a frequent basis and thereby monitored from initiation to an age of 42 DAI.

4.1.2 Identifying time points crucial for switches in development

Important key time points regarding carbon allocation can be identified by studying the developmental phases of plant storage organs. Nutsedge tuber development displayed a conventional pattern, with an initial high sugar content used for cell building and respiration. A clear switch was noted at 7-9 DAI, when the sugar content in tubers dramatically decreased and starch accumulation commenced and increased to comprise over 30% of dry weight after 13 DAI. In parallel, the dry mass of the tubers also strongly increased, to level out at almost 60%. Linssen *et al*. (1989) reported sugar levels in yellow nutsedge of up to 20% of dry matter, but those measurements were made on dry tubers, most likely stored for a period of time. In Paper I the sugar levels in tubers harvested at 42 DAI were below 2%. A portion of the tubers harvested at 42 DAI were stored one month and then sugar contents were measured and it was found that they indeed displayed an increase (to about 7%), confirming reallocation of accumulates during storage. Assaying the lipid distribution of the tubers confirmed the switch from establishment of the cells to commencement of storage accumulation at between 5 and 9 DAI. Polar lipids were the major lipid class at 5 DAI, representing membrane structures, while at 9 DAI 86% of the lipids were TAG. The TAG content further increased in the 22 DAI sample to 95%. These results were also supported by structural analysis of the material. Light micrographs of the youngest sample examined, 7 DAI, displayed storage cells to a large extent consisting of a vacuole and very small starch granules and oil bodies, while older samples had denser starch granules and oil bodies and the vacuoles were reduced in size. These findings reveal a storage organ with clear switches in the accumulation process, enabling identification of time points to further study carbon allocation, including enzyme activities, metabolic profiles and transcriptome analysis. Similar studies in other plant species, for example *Arabidopsis thaliana*, have been conducted (Baud *et al.*, 2002; Ruuska *et al.*, 2002). Also oats (*Avena sativa*), a cereal where some cultivars are rich in oil compared others, has been studied in this regard (Leonova *et al.*, 2010; Ekman *et al.*, 2008).

4.2 Absence of starch accumulation in sugar beet taproot (Paper II)

4.2.1 Sugar beet sink content – why sucrose?

The main feature of the sugar beet taproot is that the sole storage component is sucrose. Very low levels of hexoses are found in the taproot, even in early

development, when higher levels of hexoses are common in other plant species (*e.g.* the nutsedge and parsnip studied in Papers I and II). One exception is when the sugar beet plant is exposed to stress, i.e. there is an increase in hexoses following wounding of the taproot (Rosenkranz *et al.*, 2001).

The vacuole is central in sucrose compartmentalisation. A key feature of the vacuole in sugar beet cells is the continuous need to import protons to support several tonoplast-located pumps, which are dependent on protons (H^+) as antiporters or symporters (see section 2.2.4). An example of a proton pump specific for a halophyte (salt-tolerant plant species) is the tonoplast-located Na^+/H^+ antiporter activity in root and leaves, which prevents Na^+ from accumulating in the cytosol in saline growing conditions (Blumwald & Poole, 1987; Blumwald & Poole, 1985). The sugar beet plant can be categorised as a marginal halophyte, since it can tolerate moderate levels of sodium chloride (5 g NaCl/L, 85 mM) (Glenn *et al.*, 1999). This indicates that the sugar beet vacuole maintains a well-developed transport system for protons in and out across the tonoplast. Furthermore, it can be hypothesised that the high sucrose content in the sugar beet taproot is inherited from its ancestor, sea beet (*Beta vulgaris* spp. *maritima*). Sucrose is a metabolite contributing to osmolytic adjustment and it has been shown that sucrose concentration in cells increases with increased ambient saline levels (Koyro *et al.*, 2006; Hasegawa *et al.*, 2000).

4.2.2 Sugar beet sink content – why not starch?

Another intriguing fact regarding the sugar beet taproot is that the storage tissue does not accumulate starch. The absence of starch accumulation in sugar beet is a rare feature among agricultural crops. Sugar cane (*Saccharum officinarum*), another sucrose-storing crop and the largest sucrose provider in the world, actually accumulates low levels of starch (Wang *et al.*, 2013; Figueira *et al.*, 2011; Ferreira *et al.*, 2008).

Paper II examined the absence of starch formation in sugar beet taproot by structural, biochemical and transcriptomic analyses, in an attempt to identify whether factors crucial for starch accumulation are present or lacking. A comparison was made with the taproot of parsnip, which stores both sugars and starch. The structural analyses confirmed the known fact that sugar beet taproot storage cells do not contain granule-resembling structures. However, the biochemical analyses revealed activity of four enzymes central in building starch polymers: phosphoglucomutase, ADP-glucose pyrophosphorylase, starch synthase and branching enzyme. The activity level of these enzymes, reported per unit soluble total protein, was of the same order of magnitude as in parsnip. The transcriptomic analyses focused on starch biosynthesis and related

processes. Examining the results generated for sugar beet taproot, gene expression related to mechanisms involved in starch biosynthesis was shown to be present, although transcript levels were generally lower than in parsnip. Although exceptions of genes not being transcribed were identified, *e.g.* putative glucose phosphate transporters (GPT1, GPTlike), the plurality of the transcripts believed to participate in starch formation were present, which contradicts the absence of starch accumulation in sugar beet taproot.

4.3 Changes in metabolism of potato tubers caused by introduction of WRINKLED1 (Paper III)

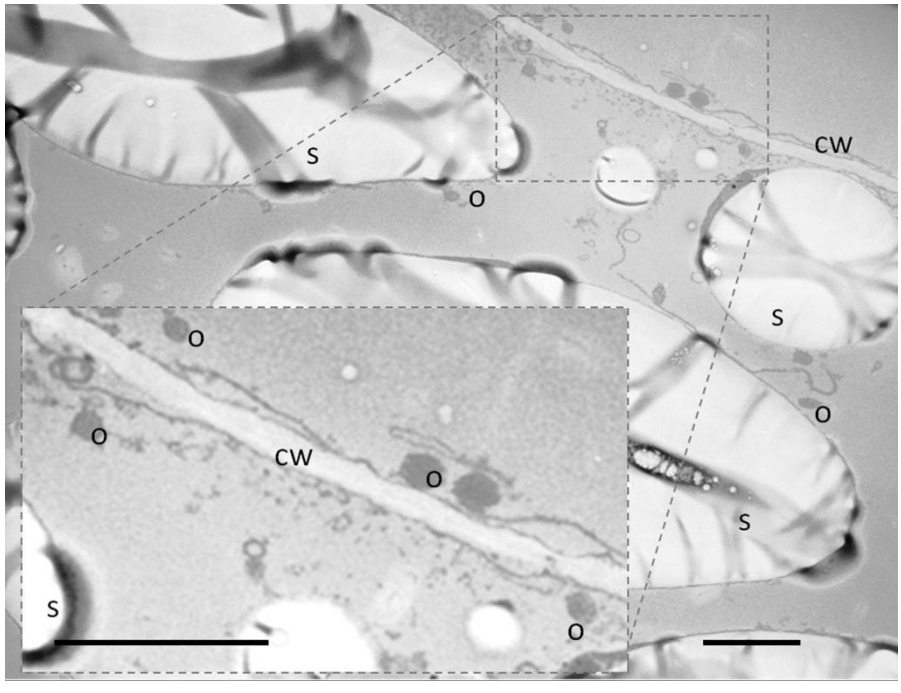

Figure 6. Transmission electron micrograph of transgenic potato 8016 (wri1) demonstrating presence of oildroplets adjacent to the cell wall. S – starch granule, cw – cell wall, o - oil droplet. Scale bars=1 μm. Photo S. Marttila

Oil accumulation in plants mainly occurs in seeds, fruit mesocarp and kernels. The most common form in which the oil is stored in cells is as TAG in oil bodies, droplets or fused into a large drop. Accumulation of oil in underground storage organs is unusual, with only one known crop storing appreciable

amounts of oil in underground parts, the nutsedge tuber (Paper I). The potato tuber is considered to accumulate a fairly pure starch compared with other starches, for instance in maize starch the level of lipids is higher (Vasanthan & Hoover, 1992). Besides starch, the potato tuber accumulates storage proteins, predominantly the glycoprotein patatin (Höfgen & Willmitzer, 1990). By introducing the transcription factor WRINKLED1 (wri1) into potato, the metabolism of the tuber was challenged and the function of the transcription factor in a tuber environment was then assayed. Wri1 has been found to positively regulate genes involved in late glycolysis and fatty acid synthesis and has been associated with an increase in TAG when overexpressed (Ma *et al.*, 2013; Ruuska *et al.*, 2002). In Paper III, *Atwri1* was introduced behind the tuber-specific GBSS promoter in the potato variety Kuras, which is grown for starch production (Hofvander *et al.*, 1992).

4.3.1 Expression of *Atwri1* in tubers leads to accumulation of polar lipids and triacylglycerol

Up to 30-fold increase in triacylglycerol in transgenic tubers

The initial experiments assaying lipid classes in transgenic *in vitro* microtubers revealed accumulation of TAG up to 2% of dry weight (dw), compared with 0.1% of dw in the wild type. Furthermore, the TAG accumulated in transgenic tubers grown in the greenhouse displayed a 30-fold increase. Analysis of the TAG profile revealed a fatty acid composition in tuber which was similar to that in true potato seed, with the exception of higher palmitic acid (16:0) and lower oleic acid (18:1) levels. Comparing the fatty acid composition of the transgenic tubers with wild-type tubers revealed that the wild type contained more linolenic acid (18:3) and very low levels of palmitic acid. The higher the TAG content was in the transgenic tubers, the more closely the TAG profile resembled that of potato seed. Whether this is due to increased fatty acid synthesis or other factors that determine composition of fatty acids in TAG could not be determined. Transcriptome analysis of *AtWRi1* transgenic lines revealed an upregulation of transcripts for plastidial fatty acid synthesis as well as genes involved in fatty acid modifications and processing. The lines also displayed increased diacylglycerol (DAG) levels, indicating higher availability of substrate for TAG synthesis.

Although a 30-fold increase of TAG was noted, the oil content of the potato tubers was generally low, about 1% of dw, and was also found to decrease during maturation of the tubers. The structural studies of the material revealed small oil bodies adjacent to the cell walls and around the starch granules (Figure 6), very similar to those in a developing nutsedge tuber (Figure 7). Oil droplets of TAG are usually shielded by a layer of proteins and phospholipids

to protect the accumulated oil from cytosolic enzyme activity. A possible explanation for the relatively low accumulation of TAG in combination with the increase in membrane-related lipids could be absence of oleosins in potato tuber TAG oil droplets. This has been shown in Arabidopsis seeds, where silencing of OLEO1 resulted in fewer and larger oil bodies and a significant decrease in total accumulated lipids (Siloto *et al.*, 2006). However, an increase in transcripts sharing homology with oleosins was noted in wri1 transgenic tubers compared with the wild type. This was confirmed by stained micrographs displaying oil droplets testing positive for protein (Figure 4 in paper III).

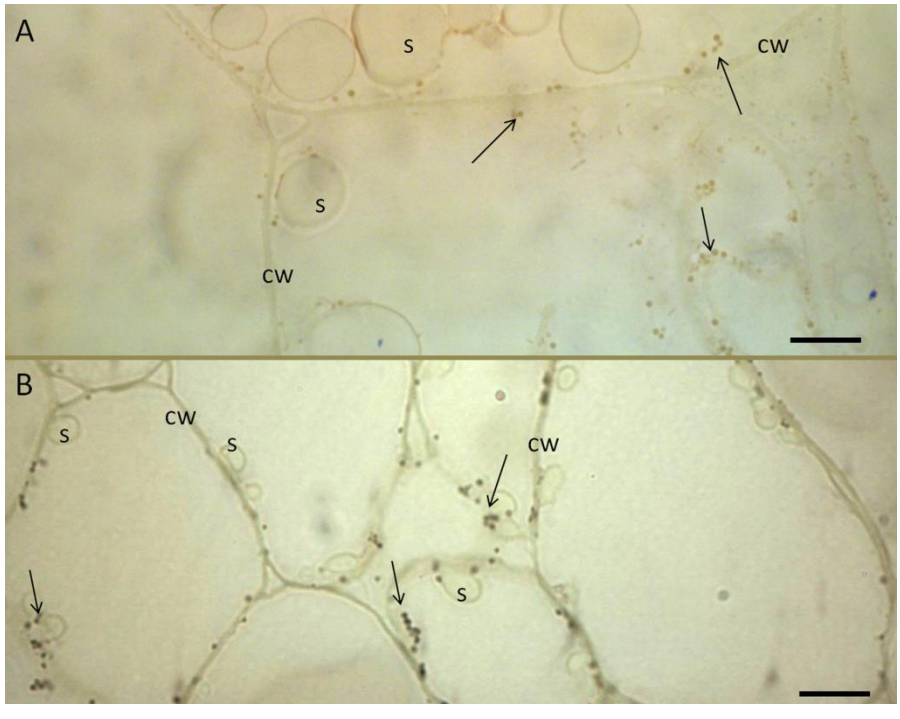

Figure 7. Micrographs stained with Sudan Black (oil-specific stain) of tubers from A) wri1 transgenic potato (*Solanum tuberosum*) and B) nutsedge (*Cyperus esculentus*) 7 days after tuber initiation. Oil droplets/bodies are indicated by arrows, S = starch, CW = cell wall. Scale bar = 10 µm. Photo H. Turesson

Polar lipids increase substantially in wri1 transgenic tubers

Due to the low TAG content in wild type potato, the dominating lipid class is the polar lipids. Further detailed analysis of mature tuber material grown in the greenhouse revealed that a substantial part of the accumulated lipids in transgenic potato could be attributed to polar lipids, which are usually associated with membrane structures (Simon, 1974). In the wild-type potato tuber the ratio between polar lipids and TAG was 24:1, while in the transgenic line it was 1:2. However, taking into consideration that the TAG increased 30-fold, the membrane-associated lipids subsequently also increased, about 2.5 times, in the transgenic line. This increase in polar lipids was confirmed by ultrastructural studies, which revealed frequent membrane invaginations in the transgenic line, but not the wild type (Figure 5 in Paper III). A possible theoretical explanation for the increased accumulation of polar lipids can be an increase in phosphate availability. Phosphate starvation is generally coupled with a decrease in phospholipids in plants in order to mobilise the phosphate needed. However the plastid galactolipids not containing phosphate (DGDG and MGDG) have been shown to be activated by phosphate deficiency (Jouhet *et al.*, 2003).

Starch-bound lipids

Lipids associated to starch can be found both at the surface of the starch granule and inside the granule (Morrison, 1981). It has been reported that lipids associated to starch are linked to the amylose molecule and that the amount of lipids decreases with decreased amylose content (Yasui *et al.*, 1996; Maniñgat & Juliano, 1980). Thus lipids located inside the starch granule might interact with amylose, which is reported to be more dense in the core of the starch granule (Fulton *et al.*, 2002; Kuipers *et al.*, 1994). Many plant-derived starches have lipids associated to the granule, for example maize, rice and wheat starch. However, starches from crops generally not accumulating lipids, such as tubers and roots of potato and cassava, also display lipids associated to the starch, but at lower levels (Vasanthan & Hoover, 1992). Levels of starch-bound lipids in the wri1 transgenic line studied in Paper III were found to have increased to comparable with those in maize starch reported by Vasanthan & Hoover, despite the amylose levels being slightly decreased compared with those in the wild-type tubers studied. The structural analyses of the transgenic tubers revealed oil droplets/bodies located in connection with the starch granules (Figures 6 and 7). The increase in starch-bound lipids in the wri1 transgenic lines suggests the possibility of altered starch properties, such as inhibition of swelling of the starch granules (Tester & Morrison, 1990).

4.3.2 Novel metabolic route affects starch biosynthesis

From a starch biosynthetic perspective, redirection of photosynthate by introduction of wri1 can in theory occur via one of two scenarios: Either the expression of wri1 inhibits starch biosynthesis by competition from the novel metabolic pathway, lowering the amount of metabolites available to be utilised for starch biosynthesis; or expression of wri1 indirectly triggers an increase in the sucrose gradient due to the increased demand for sucrose in order for both metabolic pathways to function. The starch content of transgenic wri1 tubers was in fact found to have decreased, but the underlying mechanism causing the decrease was not determined. Lower levels of starch in transgenic tubers are generally reported to be associated with higher levels of sucrose, glucose and fructose (Hofvander *et al.*, 2004; Geigenberger *et al.*, 2001; Tjaden *et al.*, 1998).

Starch granule morphology

The amylose level, or more specifically the molecular structure and level of amylopectin, is important for starch granule morphology. Amylopectin is considered to uphold the structure of the starch granule and in general high amylopectin mutants or transgenics do not display altered granule morphology (Andersson *et al.*, 2003; Buléon *et al.*, 1998). Comparisons of wild-type starch with high-amylose starch have revealed smaller and more irregularly shaped starch granules in the wild type (Hofvander *et al.*, 2004; Jane *et al.*, 1994). Thus a lower amylopectin/amylose ratio generates altered starch granule morphology. This theory is not applicable in Paper III, where the granules in wri1 transgenic potato tubers were smaller and the shape more irregular, but the amylose content was only slightly altered and in a negative direction. Instead, the altered metabolism, consisting of a combination of factors such as TAG accumulation, altered amylopectin/amylose ratio and higher levels of starch-bound lipids, most likely acted together to disturb granule formation, causing altered morphology.

4.4 Significance of energy supply and phosphate efflux in the amyloplast (Paper IV)

The amyloplast is the subcellular location of starch biosynthesis in non-photosynthetic tissue. As in most synthetic reactions, energy needs to be

provided and by-products from metabolic and enzymatic reactions need to be removed. The first committed step towards starch formation in the amyloplast is considered to be the ATP-dependent conversion of glucose-1 phosphate to ADP-glucose, which is catalysed by ADP-glucose pyrophosphorylase (ADPgase) and simultaneously generating pyrophosphate (PPi). PPi is further hydrolysed to inorganic phosphate (Pi), which inhibits ADP-glucose pyrophosphorylase by allosteric regulation (Sowokinos, 1981) and thus needs to be exported out of the amyloplast. The import of glucose-6-phosphate and ATP to the amyloplast is conducted at the plastid membrane in counterexchange with Pi (Flügge *et al.*, 2011), which indicates co-dependence of ATP import and PPi hydrolysis generating Pi in the amyloplast.

Paper IV examined whether the downregulation of two genes, responsible for plastidial ATP import (*StNTT1*) and PPi hydrolysis (*StPPa6*), both through single silencing and in combination, had an impact on starch biosynthesis and to what extent.

4.4.1 Restricting energy supply to the amyloplast causes substantial alterations in starch and tubers.

A plastidial ATP/ADP translocator was originally identified by expressing cDNA from Arabidopsis in spinach chloroplasts, which demonstrated counter-exchange of adenylates over a plastidial envelope (Neuhaus *et al.*, 1997). In Paper IV, the potato ATP/ADP translocator in tubers was downregulated by silencing the genes with RNAi technology and using a tuber-specific GBSS promoter (Hofvander *et al.*, 1992). This genetic modification resulted in tubers substantially affected as regards number, morphology, dry weight, starch content, amylose content and yield. Starch granule size and shape were also affected, appearing as smaller and more circular-shaped granules than the elongated type found in native potato starch. Assays on expression of the silenced gene displayed very low levels of transcript, which can be due to the combination of the silencing technology used and the strong tuber-specific promoter. Previous studies of this type in potato have used the *StNTT1* silenced by antisense and driven by the constitutive 35S promoter (Geigenberger *et al.*, 2001; Tjaden *et al.*, 1998). The results in Paper IV were similar, but the effects were more pronounced to those reported by Tjaden *et al*. (1998),

4.4.2 Plastidial pyrophosphatase is essential for starch synthesis and accumulation

Six isoforms of pyrophosphatases have been identified in Arabidopsis, one of which is located in the plastid (PPa6) (Schulze *et al.*, 2004). In Paper IV, the potato isoform of PPa6 was identified and the gene was downregulated using the same type of gene construct as was employed for downregulating *StNTT1*, *i.e.* with the *GBSS* promoter and RNAi technology. The transgenic plants displayed severe impacts on tuber morphology, yield and starch quality, more serious than caused by the *StNTT1* transgenes. Very low levels of amylose were recorded and starch granule size was reduced to about one-tenth of that in the wild type. The shape of the granules was also more circular than in the wild type. It has been postulated that PPi can be exported to the cytosol and thus starch synthesis not be affected by the down regulation of *StPPa6* (Lara-Nunez & Rodriguez-Sotres, 2004; Lunn & Douce, 1993). However the results in Paper IV contradict that theory, especially the severe effect of downregulation of *StPPa6*, which was most likely due to inhibition of ADPgase by the presence of PPi. Either the PPi inhibits the ADPgase reaction or the prevention of hydrolysis of PPi to Pi leads to decreased import of metabolites with which Pi counterexchanges. To our knowledge the function of this gene has not been assayed in a non-photosynthetic tissue such as the potato tuber previously, but a homologue has been transiently downregulated in *Nicotiana benthamiana* chloroplasts, resulting in decreased levels of starch (George *et al.*, 2010).

4.4.3 Effects of silencing *StNTT1* and *StPPa6* simultaneously

In Paper IV, the silencing of *StNTT1* and *StPPa6* in combination presumably caused both loss of energy import and an increased level of PPi in the amyloplast, thus leading to a lowered level of ADP-glucose. The two single gene approaches appeared to result in similar effects, with slightly varying impacts on starch biosynthesis leading to decreased dry weight, starch content, amylopectin/amylose ratio and subsequent characteristics of the tubers such as size and tuberisation. Stacking the genes in one construct did not add any additional effects regarding starch content and quality, but did alter the number of tubers obtained and tuber yield. The double construct average tuber weight ranged between 6-35% of the average tuber weight of the wild-type tubers and the number of tubers obtained was either similar to that in the wild type or 3- to 6-fold higher than in the wild type. Stacking the genes led to difficulties in regenerating viable plants, indicating that the functions of the genes are essential in regeneration of the plants. The expression levels of the down

regulated genes were in fact higher than those in the single gene approaches, confirming the necessity of functional genes in order to maintain the plants.

Other double-gene approaches have been performed with the aim of influencing starch formation by overexpression of metabolites feeding the synthesis of ADP-glucose (Jonik *et al.*, 2012; Zhang *et al.*, 2008). These studies reported an increase in ADP-glucose, which in turn generated an increase in starch and amylose content. Silencing two genes whose function is most likely contributing to the formation of ADP-glucose in Paper IV led to a decrease in starch and amylose content, confirming the key role of ADP-glucose in connection with starch biosynthesis.

5 Conclusions and future perspectives

5.1 Conclusions

Yellow nutsedge (Cyperus esculentus) was shown to be a very suitable candidate for further studies on carbon allocation in underground sink tissues in general and oil accumulation in particular.

The studies on developing nutsedge tubers raised several points to be taken into consideration when further exploring the issues of carbon allocation. Nutsedge utilises the cell space very efficiently, *i.e.* has a high dry weight and also accumulates considerable levels of oil in the form of TAG. In this regard, the behaviour of the tubers resembles that of seed, in other words they act as 'underground seed'.

The taproot of sugar beet (Beta vulgaris) does not store starch, but still expresses enzymes and corresponding transcripts central in starch biosynthesis in non-negligible amounts.

The unique characteristic of the sugar beet taproot in having sucrose as its single sink component sucrose was confirmed. Biochemical, structural and transcriptomic analysis of the taproot from a starch biosynthesis perspective showed that the sugar beet plant maintains transcriptional and enzymatic activities required for starch formation. Our results did not explain the total absence of starch accumulation, but showed that it is not due to loss of gene functions related to starch biosynthesis.

Expressing WRINKLED1 in potato (Solanum tuberosum) results in tubers with increased TAG and phospholipids content, as well as clearly affecting starch biosynthesis and tuber formation

Accumulation of TAG to this extent in potato tubers has not been achieved prior to the work in this thesis and this result can thus be considered quite important. In the transgenic plants the fatty acids contained in complex lipids increased considerably and starch biosynthesis was clearly negatively affected. The somewhat unexpected increase in phospholipids, a novel finding in relation to overexpression of wri1, indicates that there may be mechanisms present in these potato tubers directing fatty acids also to membrane lipids in addition to TAG storage.

Downregulating the plastidial genes StPPa6 and StNTT1 in potato results in tubers with severely affected starch content and quality, tuber morphology and yield.

The functions of *StPPa6* and *StNTT1* were shown to be closely linked to starch biosynthesis. The consequences noted by silencing of these genes, both singly and in combination, were shown to disturb starch biosynthesis significantly. The isoform of *PPa6* was identified in potato and assessed as being of major importance not only for starch biosynthesis but also the process of forming tubers which to our knowledge not has been observed earlier in studies aiming to affect starch biosynthesis.

5.2 Future perspectives

By studying different plant species with both diverging and common traits, research questions can be formulated and investigated. If a crop could be improved in terms of *e.g.* increased accumulation of storage compounds or improved or altered quality, the pressures on agriculture to fulfil an important role in an expanding bio-economy could be alleviated. Underground storage organs are very suitable candidates for such research, as the sink capacity of the crops is relatively large, providing the potential to accommodate large quantities of carbon compounds. Some practical obstacles exist, not least public opinion about modern breeding technologies. However, to achieve long-term supply to food, feed and industrial applications, these kinds of technologies appear to be necessary for me and barriers such as negative public opinion and difficult approval processes must be overcome.

The transcriptome analysis in paper II has only been explored in view of starch biosynthesis. Data is at hand to study other active or not active biosynthetic processes in sugar beet and parsnip. For instance sugar beet possesses, as previously stated, the rare ability to accumulate exclusively sucrose and by comparison of transcriptomes analysis it can be speculated in what genes are of importance leading to that behaviour.

Another example is the yellow nutsedge, which has much higher dry matter content combined with storage of two different compounds in considerate levels, deviating from other root or tuber crops. Within the nutsedge family there is a large variation of storage compounds, for example purple nutsedge accumulates only low levels of fatty acids (Stoller & Weber, 1975) Transcriptome analysis of genotypes and species of nutsedge would most likely provide many interesting suggestions on gene candidates to be assayed related to oil accumulation which could be assayed *e.g.* in potato and compared with the results obtained in Paper III, an inspiring challenge to improve.

We have potato lines accumulating very little starch, thus free sugars/carbon source should be available for channelling to either sugar or oil accumulation. It would be interesting to study how these lines behave and if higher levels of TAG can be accumulated if WRINKLED1 is introduced. Plastidial intermediates partaking in oil accumulation are imported directly into the plastid, but some of the enzymatic steps leading to formation of fatty acids are ATP-dependent, thus the effects of the silenced genes may counteract the effects of the introduction of WRINKLED1.

Another interesting continuation of the work presented here could be to introduce identified transcripts into a plant species that does not produce the compound of interest, but has advantages such as low susceptibility to diseases. The sugar beet is a possible crop to focus on due to its' high yield and qualities described above. Therefore by introducing a new trait into sugar beet and thus changing its characteristic sugar accumulation to *e.g.* starch or oil accumulation, new knowledge could be used in regarding carbon allocation in underground storage and potentially lead to novel crops on a commercial level.

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