Subcellular network of starch synthesis in maturing embryos of pea *Pisum sativum* L. (Fabaceae)

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Stichworte: *Pisum sativum*, Erbse, Samen, Embryo, Entwicklung, Stärke Synthese, ADP-Glucose, ADP-Glucose Pyrophosphorylase, Sucrose Transporter

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List of abbreviations

Enzymes and transporters in starch synthesis pathway:		**Metabolites involved in starch synthesis:**	
AGPase	ADP-glucose pyrophosphorylase	ADP	adenine diphosphate
BT1	Brittle1	ATP	adenine triphosphate
FBP	fructose-1,6-bisphosphatase	Fru	fructose
GBSS	granule bound starch synthase	Fru6P	fructose 6-phosphate
Glc6PDH	glucose 6-phosphate dehydrogenase	Glc	glucose
GPT	glucose 6-phosphate transporter	Glc1P	glucose 1-phosphate
GT	putative hexose transporter	Glc6P	glucose 6-phosphate
HK	hexose kinase	Pi	inorganic phosphate
HT	hexose transporter	PPi	inorganic pyrophosphate
Inv	alkaline invertase	Suc	sucrose
iPPase	alkaline inorganic pyrophosphorylase	UDP	uridine diphosphate
NTT1	nucleotide sugar transporter	UTP	uridine triphosphate
PFP	PPi-dependent phosphofructokinase		
PGI	phosphoglucoisomerase	**Other abbreviations:**	
PGM	phosphoglucomutase	DW	dry weight
SP	starch phosphorylase	FW	fresh weight
SPP	sucrose phosphate phosphatase	HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
SPS	sucrose phosphate synthase	HPLC	high pressure liquid chromatography
SS	starch synthase	KOH	potassium hydroxide
SuSy	sucrose synthase	NAF	non-aqueous fractionation
SUT	sucrose H^+ transporter	SE	standard error
TPT	triose phosphate transporter	TRIS	Tris(-hydroxymethyl)aminomethane
UGPase	UDP-glucose pyrophosphorylase	x *g*	gravitational acceleration

Chapter 1: Introduction

Starch is an important plant biomass product, relevant not only for nutrition but also as a valuable chemical compound for industrial applications. Starch accumulation takes place mainly in the reproductive organs (*e.g.* seeds) or in storage tissues (e.g. roots, tubers), which form the main usable part of the plant. Plant growth, reproduction and metabolic processes are controlled via multiple regulations which are affected by both external and internal factors. Thereby starch synthesis and turnover are recently emphasized as highly influential on plant biomass production (Sulpice *et al.* 2009; Vriet *et al.* 2010; Gibson *et al.* 2011; Stitt and Zeeman 2012). Thus the detailed knowledge of starch synthesis is of great interest for breeding in order to improve the outcome of yield in terms of both the percentage and composition of starch (Jobling 2004). Historically, pea (*Pisum sativum*) has been the model plant for studying starch synthesis due to its relatively short reproduction cycle and high starch accumulation rate in large maturing embryos (Smith and Denyer 1992). Thus in the present dissertation starch synthesis was studied in developing embryos of pea.

Starch synthesis

Starch is a macromolecule consisting of numerous glucose units (Figure 1). Starch is usually a mix of linear and highly branched glucose chains. Amylose is the linear starch molecule with *α*-1, 4-glycosidic bonds (Figure 1 A), and amylopectin is the highly branched version (Figure 1B). In amylopectin the glucose units are connected to each other by the 1st carbon in one glucose unit and by the 4th or 6th carbon on the next glucose unit. The amylopectin typically accounts for the largest percentage of starch (70-80%).

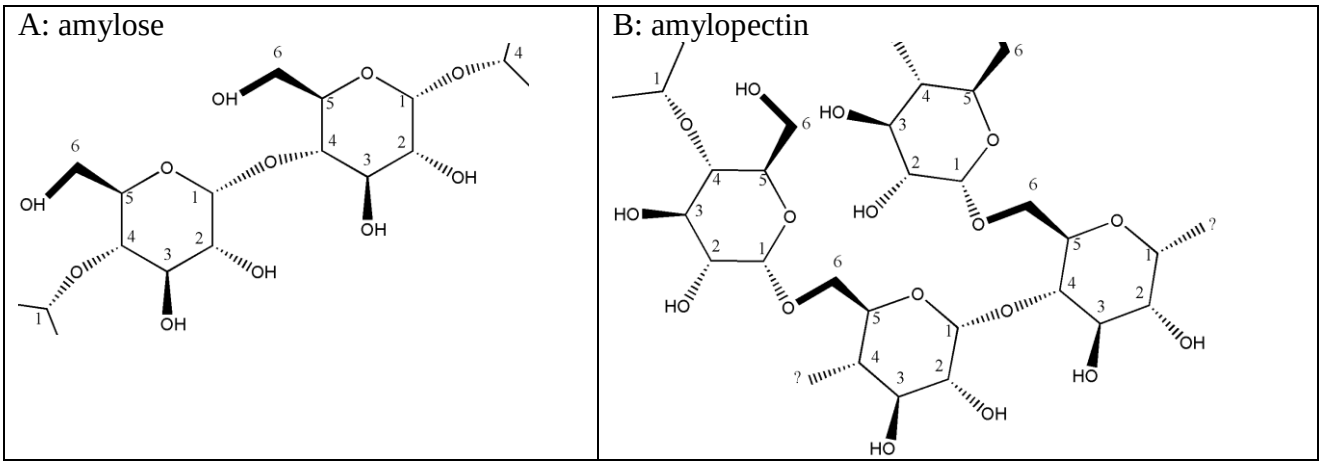


Figure 1. Amylose (A) and amylopectin (B) structure. Amylose is the linear starch molecule with α-1,4-glycosidic bonds. Amylopectin is highly branched and the glucose units can be connected to each other by the first carbon atom on one glucose unit and either the 4th or 6th carbon atom of the next glucose unit, this means by the *α*-1,4-glycosidic bond or the *α*-1,6-glycosidic bond.

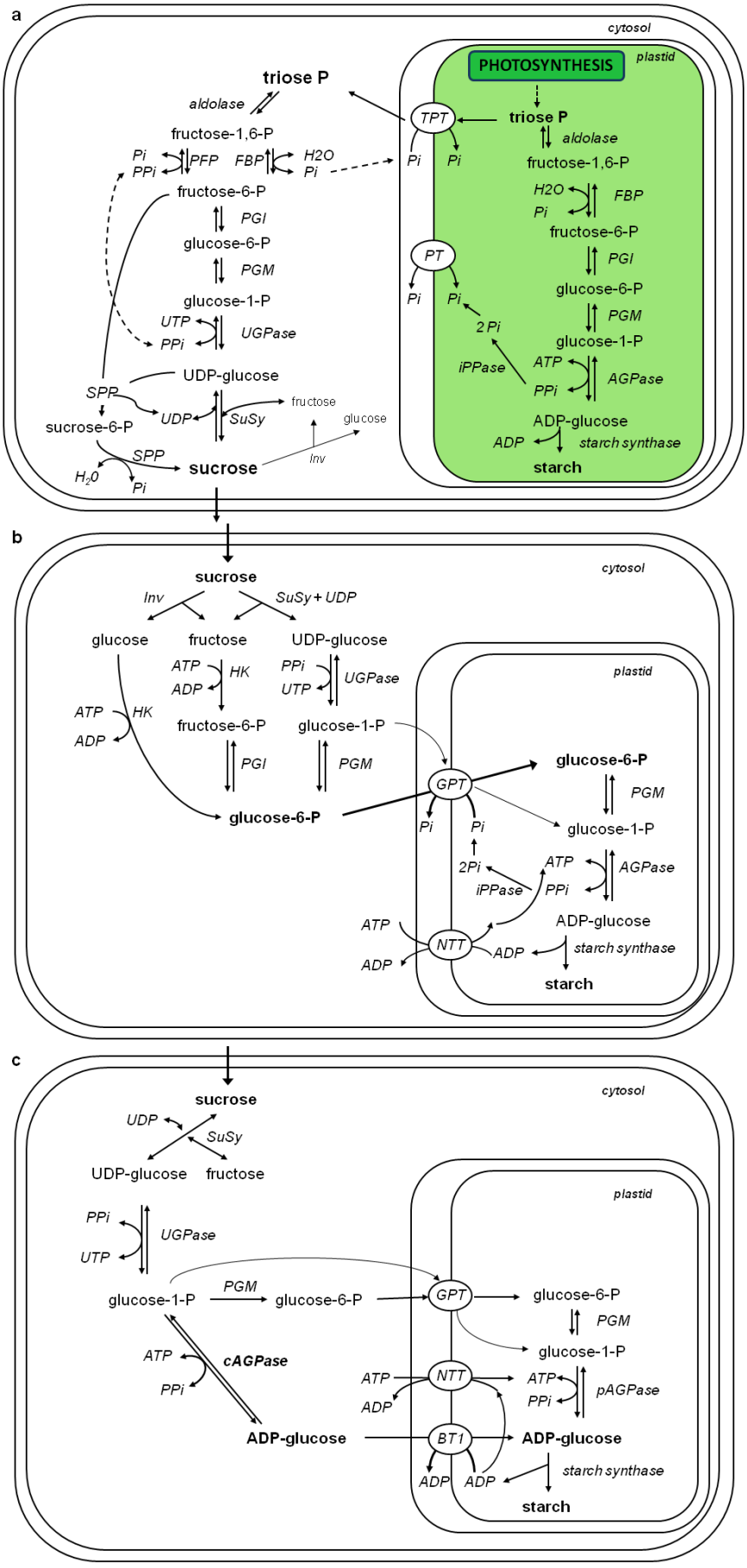
Starch synthesis can be divided into transitory and storage starch synthesis (Figure 2). Transitory starch synthesis takes place in chloroplasts of photosynthetic cells; whereby the substrates for starch

are derived from photosynthetic carbon fixation and starch synthesis competes with sucrose synthesis (Figure 2 a). Transitory starch has relevance as a carbon source at night, ensuring continuous growth, therefore the appropriate balance between sucrose and starch synthesis is needed (Stitt and Zeeman 2012). Thereby, efficient starch degradation and translocation of degradation products β-maltose and glucose are crucial at night. Transitory starch synthesis begins with triose phosphates (3-phosphoglycerate, glyceraldehyde 3-phosphate and dihydroxyacetone phosphate) derived from photosynthetic carbon fixation. The enzyme aldolase (EC 4.1.2.13) converts (reversibly) glyceraldehyde 3-phosphate and dihydroxyacetone phosphate into fructose-1,6-phosphate (Fru-1,6-P) which is converted in chloroplasts by fructose-1,6-bisphosphatase (FBP; EC 3.1.3.11) into fructose-6-phosphate (Fru6P). Fru6P is converted by phosphoglucoisomerase (PGI; EC 5.3.1.9) to glucose-6-phosphate (Glc6P), which is a substrate for phosphoglucomutase (PGM; EC 5.4.2.2). PGM produces glucose-1-phosphate (Glc1P), which is a substrate for ADP-glucose pyrophosphorylase (AGPase; EC 2.7.7.27). AGPase produces ADP-glucose (ADP-Glc), which is a substrate for starch synthases (soluble and granule bound isozymes, respectively EC 2.4.1.21 and EC 2.4.1.242), which add glucose units to the non-reducing end of the glucan chain to build up a linear starch molecule with α-1,4-glycosidic bonds (amylose) (see Figure 1). The starch branching enzyme (SBE; EC 2.4.1.18) contributes to amylopectin synthesis as it branches the linear starch chain in two steps: 1) it hydrolyses the α-1,4-glycosidic bond in one chain and 2) binds the hydrolyzed chain with another chain creating the α-1,6 bond (Myers *et al.* 2000; Hamada *et al.* 2007).

In contrast, the storage starch synthesis takes place in amyloplasts, which are usually localized in the heterotrophic (*e.g.* roots) or mixotrophic tissues (*e.g.* developing seeds), and depends on ATP produced by respiration and on imported sucrose synthesized in photosynthetic tissues (Figure 2 b, c). Thereby the two types of storage starch synthesis can be distinguished: the dicotyledon and the monocotyledon type (Figure 2 b, c, respectively). The difference lays in the existence of active cytosolic and plastidial isoforms of AGPase in the starch accumulating cells of monocot grasses (Beckles *et al.* 2001b; Tetlow *et al.* 2004). Dicotyledonous species (*e.g.* pea, potato) have only a plastidial AGPase and no cytosolic AGPase. Starch synthesis in storage tissues of dicots, *e.g.* pea embryos or potato tubers, depends directly on sucrose imported from the photosynthetically active maternal parts of the plant (Tegeder *et al.* 1999; Geigenberger 2003). The cleavage of sucrose in these cells is crucial for starch synthesis. Sucrose can be metabolized via invertase (Inv; EC 3.2.1.12) or sucrose synthase (SuSy; EC 2.4.1.13), whereby the main flux is via SuSy into starch, as SuSy has a much higher sucrose cleaving activity than Inv in starch storing organs (Edwards and ap Rees 1986; Morrell and ap Rees 1986; Doehlert *et al.* 1988; Wang *et al.* 1993). Several studies have

indicated the crucial role of SuSy on starch synthesis and development (Zrenner *et al.* 1995; Déjardin *et al.* 1997b; Li *et al.* 2013). As both SuSy and Inv have relatively low affinities for sucrose in the developing pea embryos (Peter 2011), it is most probable that the ratio of SuSy and Inv activities has a large influence on metabolism (Ruan 2012). Substrate and product concentrations of SuSy and Inv, especially those of sucrose and glucose, play a role in the regulation of development (Ruan 2012; Rolland *et al.* 2006). It has been shown that a high sucrose concentration acts as a signal for the developmental switch and for the start of storage product synthesis (Wobus and Weber 1999). Glc6P, synthesized from sucrose by the coupled reactions of SuSy, UDP-glucose pyrophosphorylase (UGPase; EC 2.7.7.9) and cytosolic PGM, is the preferred substrate transported into the plastids in most plants, as the import of other substrates involving Glc1P is considered much less active (Kim *et al.* 1989; Hill and Smith 1991; Kang and Rawsthorne 1994; Mohlmann *et al.* 1995). However, recent studies have indicated substantial uptake and corresponding relevance of Glc1P in potato tubers and also in leaves of *Arabidopsis thaliana* (Fettke *et al.* 2011, 2012). Glc6P is translocated into amyloplasts in exchange for an inorganic phosphate (Pi) and is then again within the plastids converted by PGM to Glc1P. Glc1P is the direct substrate for the most crucial step in starch synthesis – ADP-Glc synthesis via AGPase – as starch synthase has a very strong affinity towards ADP-Glc. Therefore ADP-Glc is the primary donor for starch (Ghosh and Preiss 1966; Denyer *et al.* 1996a).

In monocot grasses and cereals the storage starch synthesis differs from that in dicots in two ways. First, due to the existence of cytosolic AGPase (cAGPase), which was discovered in maize (Giroux and Hannah 1994; Shannon *et al.* 1996; Shannon *et al.* 1998) and can contribute up to 99% of starch synthesis (Thorbjørnsen *et al.* 1996; Denyer *et al.* 1996b; Comparot-Moss and Denyer 2009; Tiessen *et al.* 2012). The second difference lays in the existence of the ADP-Glc transporter brittle1 (BT1), as the ADP-Glc synthesized in the cytosol has to be transported into plastids. Thus the monocot pathway of starch synthesis is seen as more energy efficient; which can be calculated from the stoichiometry of the reactions (Comparot-Moss and Denyer 2009; Schuster and Junker 2011). The efficiency relies on the cytosolic ATP synthesis and cycling as well as on the cycling of used Pi back to PPi by the PPi dependent phosphofructokinases (PFP; localized only in the cytosol). The cycling of PPi is relevant for insuring Glc1P synthesis by UGPase (Figure 2 a).



Abbreviations:
ADP – adenine diphosphate,
AGPase – ADP-glucose pyrophosphorylase,
ATP – adenine triphosphate,
BT1 – Brittle1,
c – cytosolic,
FBP – fructose-1,6-bisphosphatase,
GTP – glucose phosphate transporter,
HK – hexose kinase, Inv – invertase,
iPPase – inorganic pyrophosphatase,
NTT –adenylate nucleotide transporter,
p – plastidial,
PFP – PPi-dependent phosphofructokinase,
PGI – phosphoglucoisomerase,
PGM – phosphoglucomutase,
Pi – inorganic phosphate,
PPi – inorganic pyrophosphate,
SPP – sucrose phosphate phosphatase,
SPS – sucrose phosphate synthase,
TPT – triose phosphate transporter,
UDP – uridine diphosphate,
UGPase – UDP-glucose pyrophosphorylase,
SuSy – sucrose synthase,
UTP – uridine triphosphate.

Figure 2. Overview of transitory starch synthesis (a), storage starch synthesis in dicots (b) and monocots (c).

Contradictions in starch synthesis

Starch synthesis is of continuous interest and may seem to have been extensively investigated, which is reflected by the numerous experimental and review papers published each year (*e.g.* Geigenberger 2011; Stitt and Zeeman 2012). However starch synthesis remains a controversial topic that has raised multiple scientific discussions lasting decades. One of the first discussions (1940s to late 1950s) dealt with the hypothesis of phosphorylase being the final step in starch synthesis using a Glc1P as a primer for starch (De Feteke *et al.* 1960). This hypothesis was soon rejected due to the localization of phosphorylase activity in the cytosol and unfavorable kinetic characteristics (Stocking *et al.* 1952; Ewart *et al.* 1954; De Feteke *et al.* 1960). The next hypothesis regarded UDP-glucose (UDP-Glc) as a primary donor for starch (De Feteke *et al.* 1960). This was soon disproved by what is now the accepted knowledge that ADP-Glc is the primary donor for starch, and that ADP-Glc is solely synthesized by AGPase (Ghosh and Preiss 1965, 1966).

There remain several open questions regarding the regulation of starch synthesis and its actual pathway. The fundamental reasons for these continuing debates are (1) specialization of starch synthesis in higher plants in one of two general categories of plastids: chloroplasts and amyloplasts and (2) diversification of plant species themselves; whereby the starch synthesis differs between monocotyledonous and dicotyledonous species. However, the starch synthesis pathway is widely conserved and similar in higher plants (Ball *et al.* 2011) and the questions raised regarding the regulation of starch metabolism and the effects of starch turnover on the whole plant metabolism and development are similar among different plant species *e.g. Lotus japonicus*, *Solanum tuberosum* and *Arabidopsis thaliana* (Vriet *et al.* 2010; Fernie *et al.* 2002; Sulpice *et al.* 2009; Stitt and Zeeman 2012).

One of the most actively discussed questions is the source of ADP-Glc and its transport into plastids. It is doubted that the localization of AGPase activity and the source of ADP-Glc is solely in the plastids in dicots. This issue has been under active discussion between several workgroups for more than two decades. This debate was initiated and still led by the workgroup of Pozueta-Romero in (Pozueta-Romero *et al.* 1991a, b; Baroja-Fernández *et al.* 2001, 2003, 2004, 2009; Munoz *et al.* 2005; Bahaji *et al.* 2011). Evidences provided by this workgroup have been discussed sceptically by several other workgroups (Neuhaus *et al.* 2005; Streb *et al.* 2009; Smith *et al.* 2012). Due to the lack of final evidence of cAGPase in dicots, it is widely accepted that in dicotyledonous species the primary donor for starch synthesis, ADP-Glc, is synthesized directly in the plastids by plastidial AGPase. There are only a few published papers claiming to have found cAGPase in dicots *e.g.* Chen *et al.* (1998), whose work was contradicted by Beckles *et al.* (2001a). However the cytosolic AGPase activity of the tomato fruit was very low in comparison to the plastidial activity (Chen *et*

al. 1998). Furthermore the preproteins of AGPase subunits are synthesized in the nucleus and have to be targeted to the plastid; thus explaining the cytosolic AGPase detected by the antibodies. In monocots ADP-Glc synthesized in the cytosol is transported into the plastids in an exchange of ADP via the adenylate translocator called Brittle1 (Shannon *et al.* 1998; Bowsher *et al.* 2007; Kirchberger *et al.* 2007). In contrast, dicots seem to have no *in vivo* ADP-Glc transport from the cytosol through the plastid envelope membranes (Kirchberger *et al.* 2008). Thereby SuSy could be a possible ADP-Glc source in the cytosol due to an affinity of SuSy for ADP in addition to UDP (Delmer *et al.* 1972, Pozueta-Romero *et al.* 1991b; Muñoz *et al.* 2005; Angeles-Núñez and Tiessen 2010). The alternative model of starch synthesis supposes that the cytosolic SuSy synthesizes the ADP-Glc which is then transported into plastids via the adenylate transporter (Pozueta-Romero *et al.* 1991 a, b). The existence and functionality of a plastidial ADP-Glc transporter in dicots is until now unproven. Another question is the additional use of UDP-Glc by starch synthases if ADP-Glc production is inhibited, as some starch synthase isoforms have low affinity also towards UDP-Glc (Frydman and Cardini 1967; Macdonald and Preiss 1985; Denyer *et al.* 1996a). Furthermore the subcellular localization and role of several enzymes and transporters in the sucrose to starch pathway remains unclear *e.g.* hexose kinases in stroma (Giese *et al.* 2005).

The complex interrelated regulation of sucrose and starch synthesis continues to provoke ongoing scientific discussions. In the following paragraph, as well as throughout the whole dissertation, I will focus only on the storage starch synthesis.

Role of AGPase in starch synthesis

The role of AGPase for both starch accumulation and biomass formation under normal day/night (light/darkness) cycle in higher plants is undisputedly crucial, even when there exists some additional source of ADP-Glc. The AGPase activity in leaves has been shown to correlate with sink strength and yield of biomass, regulating carbon partitioning and starch turnover (Obana *et al.* 2006; Gibson *et al*. 2011). Even in oil crops, which do not store starch in mature seeds, the AGPase activity regulates the carbon availability for storage products (Vigeolas *et al.* 2004; Ragel *et al.* 2013). The cytosolic AGPase controls the rate of starch synthesis in several monocot cereals, *e.g.* wheat, rice and maize, where the overexpression of cAGPase has resulted in higher biomass yield and increased starch content in seeds (Smidansky *et al.* 2002; Sakulsingharoj *et al.* 2004; Nagai *et al.* 2009; Li *et al.* 2011c). In contrast, effects of elevated AGPase expression or activity on starch and yield in dicots are controversial. For example the overexpression of AGPase in potato tubers did not led to a significant increase in starch content or yield (Sweetlove *et al.* 1996), although the AGPase in potato plants possess relatively high control over the flux into starch (Sweetlove *et al.* 1999). Futhermore, in legume seeds the AGPase and starch synthase have a low flux control

coefficient, which means that there could be other enzymes crucial for the rate of starch synthesis (Denyer *et al.* 1995; Rolletschek *et al.* 2002). The capacities of AGPase and starch synthases are much higher than the actual substrate availability allows for; thus the moderate reduction in starch accumulation is explainable in conditions of reduced activities of these enzymes (Hylton and Smith 1992; Denyer *et al.* 1995; Fulton *et al.* 2002). This can be connected also to the fact that the AGPase in pea and faba bean embryos is relatively insensitive to allosteric regulation by 3-PGA and Pi (Hylton and Smith 1992; Weber *et al.* 1995b).

The repression of AGPase in several crops has led to a corresponding reduction in starch content. In monocots, *e.g.* maize, the starch content in seeds was reduced nearly 75% having the mutation shrunken-2 and brittle-2 (Dickinson and Preiss 1969; Tsai and Nelson 1966; Creech 1965). The shrunken-2 (sh2) mutation affects the AGPase small subunit while the brittle-2 (bt2) mutation affects the large subunit of AGPase; these mutations cause elevated soluble sugar concentrations and increased hexose cycling (Spielbauer *et al.* 2006). Similar results have been shown for barley, in which the mutation of the cytosolic small subunit of AGPase led to a nearly 44% reduction in starch content in the endosperm (Johnson *et al.* 2003; Tiessen *et al.* 2012). In dicots the effects of the reduction of AGPase on starch content has been studied in potato plants and tubers, *A. thaliana*, pea and faba bean embryos (Lin *et al.* 1988; Smith et *al.* 1989; Müller-Röber *et al.* 1992; Hylton and Smith 1992; Weber *et al.* 2000). While the AGPase activity was drastically reduced in these mutants, the corresponding reductions in starch synthesis ranged from drastic to merely moderate. Hence, either there is an excess amount of AGPase activity for starch synthesis with parallel existing isozymes that can partially compensate the repression or there are other sources of substrates for starch.

A recent study on the effects of repressed AGPase in pea embryo starch synthesis using metabolite profiling and transcriptome analysis throughout the embryo development was performed by Weigelt *et al.* (2009). In their study, moderately reduced starch content and elevated concentrations of soluble sugars were similar to those detected in faba bean (Weber *et al.* 2000b). The transcriptome analysis of developing pea embryos showed that the RNA interference repressed AGPase caused changes in the expression of several starch synthesis related genes. Embryos at 30 days after pollination (DAP), having a repressed AGPase small subunit expression, had upregulated expression of granule bound starch synthase I, starch debranching enzyme, as well as plastidial PGM, cytosolic PGI, UGPase and plastidial Glc6P translocator. These results together with upregulated sucrose phosphate synthase (SPS) (at 20, 30, 35 DAP) and SuSy in the earlier mid seed filling phase (20 and 25 DAP) indicate an enhanced sucrose and hexose cycling (Weigelt *et al.* 2009).

Effects of other enzymes on starch accumulation

Enzymes directly bound to starch synthesis (soluble and granule bound starch synthases and starch branching enzyme, abbreviated as SS, GBSS, SBE, respectively) have strong effects on starch composition and accumulation. In addition, some enzymes related to sucrose cleavage greatly affect starch accumulation. This could be due to the competition between pathways for the same substrate, whose availability regulates the metabolism and development; and as well as due to the regulation of gene expression and enzyme activities by sugars (Koch 1996; Wobus and Weber 1999). Thus, carbon partitioning between different pathways at the start of the sucrose degradation pathway is crucial for the entire metabolism.

Starch synthesis in sink tissues depends on imported sugars, mainly in the form of sucrose. Therefore it is not surprising that SuSy has been shown to exhibit strong control over starch accumulation and carbon partitioning in storage tissues, *e.g.* potato tubers and maize kernels, probably due to its much higher activity in comparison to Inv and its energy (ATP) saving character (Morell and ap Rees; Zrenner *et al.* 1995; Junker *et al.* 2006; Baroja-Fernández *et al.* 2009). The sucrose cleavage via SuSy is also linked to the yield, determining the sink strength, which is shown by the increased yield in SuSy overexpressed potato tubers (Baroja-Fernández *et al.* 2009) and reduced yield in tubers with repressed SuSy expression (Zrenner et al 1995). The expression and activity of SuSy and AGPase as well as starch accumulation throughout the development of the legume embryo are strongly possitivly correlated (Heim *et al.* 1993; Weber *et al.* 1996; Chopra *et al.* 2005).

Sucrose cleavage by Inv is an irreversible reaction producing glucose and fructose, which need to be phosphorylized for further metabolic steps by hexose kinases using ATP. Isozymes of Inv are located in the apoplast, cell wall, cytosol, vacuole and plastids, and have different relevance in carbohydrate partitioning and development (Fotopoulos 2005; Junker *et al.* 2006; Vargas *et al.* 2008; Nägele *et al.* 2010; Tamoi *et al.* 2010). Glucose, produced from sucrose cleaved by Inv, is functioning also as molecular signal. A certain glucose concentration and its ratio to other sugars is needed for active cell division, whereas specific sucrose level is required for the induction of storage product synthesis and differentiation (Wobus and Weber 1999). Fructose on the other hand can control sucrose synthesis, as a high concentration of fructose inhibits SuSy activity. In legume seeds, Inv of the maternal part – the seed coat – controls the development of embryos, by regulating the mitotic activity and therefore the cell number of seeds and possible seed size (Weber *et al.* 1995a, 1996). Later embryo development and storage product synthesis is more influenced by the ratio of Inv and SuSy, whereby the SuSy activity rapidly increases with the beginning of storage phase (Weber *et al.* 1995a, 1996). In potato tubers the elevated apoplastic and cytosolic Inv activity

leads to a reduction in sucrose and starch content and an increased flux through glycolysis (Sonnewald *et al.* 1997; Trethewey *et al.* 1998). In leaves and young seedlings the elevated Inv activity (in plastids, vacuole and cell wall) causes a reduction in growth and inhibition of photosynthesis, which can be accompanied by increased starch accumulation due to reduced export of photosynthetic products and sink strength (von Schaewen *et al.* 1990; Tamoi *et al.* 2010; Nägele *et al.* 2010). In summary, the effect of Inv on metabolism is connected to hexose kinases and to the role of glucose in the regulation of gene expression.

Hexose kinases (hexokinases and fructokinases; HK) contribute to the next step in sucrose cleavage, using ATP to phosphorylate glucose and fructose to Glc6P and Fru6P, respectively, which are essential substrates for both the oxidative pentose phosphate pathway and glycolysis. There are many unresolved questions regarding their functions in signalling and the localization of these enzymes in specific tissues and organelles (Granot *et al.* 2013). In higher plants HKs localize in the cytosol, mitochondria and as well as in plastids (*i.e.* stroma and envelope) (Granot *et al.* 2013). Thereby, the glucose phosphorylating HK (HK glc) have a special role as a signal mediator *e.g.* in leaf photosynthesis (Moore *et al.* 2003). Elevated glucose concentrations and overexpression of HK repress growth and photosynthesis (Jang *et al.* 1997; Dai *et al.* 1999; Xiao *et al.* 2000). Also the repression of HK can lead to growth retardation, as shown by RNAi silencing of HXK1 in *Nicotiana tabacum* (Kim *et al.* 2013). *Nt*HXK1 plays a crucial role in starch degradation in leaves at night by phosphorylating the glucose transported from chloroplasts and/or phosphorylating the glucose derived from β-maltose (via disproportionating enzyme, DPE; EC 2.4.1.25) in the cytosol thereby providing a substrate for glycolysis as well as for sucrose synthesis. The reduction of the *Nt*HXK1 expression leads to retardation of growth and photosynthesis, due to the feedback reactions caused by the excess accumulation of β-maltose in chloroplasts, which induce the degradation of the chloroplasts (Kim *et al.* 2013; Stettler *et al.* 2009). Therefore, HK indirectly influence starch synthesis via the regulation of photosynthesis.

PGM has one of the highest activities of enzymes in the sucrose degradation pathway in seeds (Doehlert 1990; Harrison *et al.* 1998; Troncoso-Ponce *et al.* 2009), in storage tissues (Sweetlove *et al.* 1996; Steinhauser *et al.* 2010) and in leaves (Gibon *et al.* 2009). PGM catalyzes the reversible reaction between Glc6P and Glc1P. Both cytosolic and plastidial isoforms of PGM are relevant for the high rate of starch synthesis in pea embryos as the Glc6P synthesized by cytosolic PGM is the main substrate for starch transported into plastids in pea embryos (Hill and Smith 1991). The high dependence of starch synthesis on imported Glc6P and plastidial PGM has been shown in studies with reduced plastidial PGM expression and activity in pea and other species (Harrison *et al.* 1998, 2000; Caspar *et al.* 1985; Hanson and McHale 1988; Lin *et al.* 1988).

In plants there are several (acid) phosphatases and pyrophosphatases which are confined within different subcellular compartments, *e.g.* vacuoles, plastids, apoplast. The alkaline inorganic pyrophosphatase (iPPase) is confined to plastids in leaves as well as in storage tissues (tubers, seeds) (Gross and ap Rees 1986; Weiner *et al.* 1987), whereas acid phosphatase and pyrophosphatase are confined to the apoplasm and vacuoles (Duff *et al.* 1994; Ferjani *et al.* 2012). In developing seeds there is a remarkable amount of both the acid and alkaline iPPase activity. Thereby, the alkaline iPPase is crucial for starch synthesis, making the AGPase catalyzed reaction (converting Glc1P and ATP into ADP-Glc and pyrophosphate) practically irreversible due to the thermodynamic overhead of converting pyrophosphate (iPP) into phosphate (iP). Therefore the starch accumulation rate is dependent on iPPase activity as shown in potato tubers (Viola *et al.* 1991, Geigenberger *et al.* 1998; Schulze *et al.* 2004). Similarly, the iPPase activity in legume seeds is positively correlated with starch accumulation throughout seed development (Chopra *et al.* 2005). Although there are several sources of iPP in plastids, the concentration of iPP in plastids is low; probably due to the high iPPase activity. Also the iP concentration in plastids is low, which may be due to the role of iP in the exchange of hexose P and triose P between the cytosol and plastid. The low iP concentration is crucial, as the AGPase activity is inhibited by iP (Weber *et al.* 1995b; Preiss *et al.* 1991).

The examples presented above do not complete the full system of starch synthesis and several other enzymes could be discussed, *e.g.* UGPase and PGI, which have a high expression and activity in storage tissues (Zrenner *et al.* 1993; Sowokinos *et al.* 1993). In developing pea embryos, UGPase and PGI seem to be close to equilibrium *in vivo*, and therefore are neither rate limiting nor regulatory for starch synthesis (Forster and Smith 1993). However, the discussed enzymes illustrate the complex regulatory network between enzymes and metabolic pathways and their effect on plant growth and development.

Sucrose transport into pea embryo cells

The allocation of sugars from photosynthetic tissues into various plant organs is crucial for plant viability and reproduction, due to the heterotrophic nature of several organs, *e.g.* roots and seeds. Among several sugars and some amino acids, sucrose is the main transported carbohydrate in plants. Translocated sucrose is a limiting factor for storage product synthesis in sink tissues, *e.g.* for starch, oil and protein synthesis. The uptake of sugars into pea embryos is well described and similar to that of other legume seeds and the cereal endosperm (Tegeder *et al.* 1999; Rosche *et al.* 2002; Weschke *et al.* 2000).

The development of pea embryos depends directly on sugars and amino acids, which are uploaded from the seed coat either over the endosperm or by direct physical connection (Tegeder *et al.* 1999;

Rosche *et al.* 2002; Borisjuk *et al.* 2002). The growth of a legume embryo is strongly correlated to sugar transport activities in the seed coat and epidermal cells of the embryo. During the early developmental phase the embryo has an active uptake of hexoses due to the high activities of invertases in endosperm vacuoles and a high density of hexose transporters (STP1) in the epidermal cells of the embryo (Weber *et al.* 1997). During the seed filling phase, the expression and activity of sucrose H^+ symporters (SUT) increases and becomes dominant over the hexose transport system when the embryo reaches mid cotyledon characterized by the initiation of storage compound accumulation. Active sucrose transport correlates with the start of the starch accumulation phase in legume embryos and the formation of transfer cells (Weber *et al.* 1997). The embryo epidermal transfer cells are responsible for high sucrose influx due to the dense localization of sucrose transporters on their membranes. Further transport inside the pea embryo cells is symplasmic; that is, the sugars are distributed via plasmodesmatal connections from the epidermal transfer cells to storage parenchyma cells, which is similar to that found in potato tubers (Tegeder *et al.* 1999; Viola *et al.* 2001, Kühn *et al.* 2003).

Two types of sucrose transporters are known in pea embryos: the sucrose H^+ symporter (SUT) and the sucrose facilitator (SUF) (Zhou *et al.* 2007; Melkus *et al.* 2009). SUT can be divided into three main clades, which have diverse roles in various organs throughout plant development (Aoki *et al.* 2003). For example, in rice (*Oryza sativa*), all five identified SUT genes are temporally and differentially expressed in all tissues. One of these, the *Os*SUT1, was shown to be essential for reproduction (Aoki *et al.* 2003; Hirose *et al.* 2010). Interestingly, only one isoform of sucrose H^+ symporters, the *Ps*SUT1, seems to be expressed in pea seeds. *Ps*SUT1belongs to the clade I, and its activity is found to be most relevant for sucrose uptake in pea embryos (Borisjuk et al 2002; Zhou *et al.* 2007; Zhou *et al.* 2009). In legume seeds SUT1 is primarily localized to plasma membranes of transfer cells localized in the cotyledon and the seed coat epidermis, and in the developing endosperm (Weber *et al.* 1997; Heim *et al.* 2001; Melkus *et al.* 2009).

The role of SUT1 and other sucrose transporters on yield remains controversial. The overexpression of various SUT has led in some studies to increased yield, *e.g.* in potato tubers expressing the rice sucrose transporters (*Os*SUT2 and *Os*SUT5) had a higher biomass yield per plant but not an enhanced starch content (Sun *et al.* 2011). Contrary to the expectations, the limitation of sucrose transport by repressing the *St*SUT1 in potato tubers did not cause a reduction of tuber yield and starch content (Kühn *et al.* 2003). Overexpression of the barley *Hv*SUT1 in the endosperm of wheat grains also did not lead to an increase in starch content, instead it increased protein content; thereby slightly increasing the yield of wheat under field conditions (Weichert *et al.* 2010). The overexpression of potato *St*SUT1 in pea embryos has led to an enhanced growth rate but did not

increase the seed dry weight (Rosche *et al.* 2002). Thus there remain open questions about the regulation of sucrose uptake by SUT1 and its translocation in pea cotyledon cells and the effects of sucrose on starch synthesis or on the synthesis of other storage products.

Legume seed development

Legume seed development is characterized by temporal and spatial changes in seed anatomy and chemical composition. The differentiation of cotyledon cells proceeds in a wave-like manner from the inner core to the outer region; therefore the cotyledons contain cells in different physiological ages over their development (Borisjuk *et al.* 1995, 2003).

Legume seed development can be divided into four main stages: cell formation, cell expansion, synthesis of storage reserves, and the maturation-desiccation-dormancy stage (Bain and Mercer 1966). The separation into phases according to Bain and Mercer (1966) considers not only the changes in seed structure and changes in accumulation of starch, protein and fat, but also changes in the cellular structure. These four phases can be distinguished from each other by their metabolism and subcellular organization in the parenchyma cells. Embryos in the beginning of the seed filling phase (phase II: cell expansion according to Bain and Mercer) have differentiated structure, containing all organelles (including plastids, mitochondria and large vacuoles) and the cells start to expand. The next phase – cell expansion and rapid synthesis of starch and storage protein – is characterized by structural changes of the endoplasmatic reticulum, formation of protein bodies along the vacuole membrane and endoplasmatic reticulum, as well as the filling of plastids with starch granules which disrupt the internal structure of the plastids. The last phase – maturation and dormancy – is characterized by continuing but slowed storage accumulation, an increase of dry weight, a reduction of fresh weight and the enlarging of the protein bodies and starch granules.

There are more detailed divisions of development, *e.g.* the 25 stages of pea seed development described by Marinos (1970). This detailed separation has its advantages for early seed analysis. However, if the main interest lay in seed filling and storage product synthesis, other classifications can be more useful. One well structured system suitable for this is described by Borisjuk *et al.* (1995) for faba beans and can be readily applied to other legumes. Borisjuk *et al.* (1995, 2003) distinguished seven stages based on anatomical and chemical changes: the globular, the early heart, and the late heart stage and four stages of early to late cotyledon development. The four stages of early to late cotyledon development overlap with the four developmental stages described by Bain and Mercer (1966). The cotyledons in stage IV are mitotically active; stage V is characterized by cell expansion starting from the inner core of the cotyledons, whereas the outer region maintains the mitotic activity; stage VI is the active storage accumulation phase; and at stage VII the embryo has

reached maturity, which is characterized by the ceasing of cell expansion and further accumulation of storage compounds.

Experimental systems to study cell compartmentation

The plant cell has a very complex organization containing multiple compartments, for example the cytosol, nucleus, mitochondria and plastids, which are responsible for different processes. To understand these processes and their networks in a cell is an ongoing aim of biological research. Thereby the knowledge regarding subcellular compartmentation of metabolites and enzymes is helpful to explain the coordination of metabolic networks.

There are only a few methods to analyze the subcellular localization and concentration of metabolites and enzymes at the same time from the same sample. One of the most promising methods is nuclear magnetic resonance (NMR), which is a non-invasive technique. At the present time NMR has a relatively low resolution; it is very effective at the tissue level and has been applied to several plant species (e.g. pea seed by Melkus *et al.* 2009; barley endosperm by Rolletschek *et al.* 2011). On the subcellular level there are only a few studies using NMR, *e.g.* for water content (Musse *et al.* 2010).

In order to analyze the organelle biology it is usually necessary to separate the organelles from each other. There are different methods for fractionation of the cell into compartments. The most widely used methods are based on a density gradient centrifugation using aqueous solutions, *e.g.* sucrose or Percoll. However, the aqueous methods are usually destructive and the aqueous conditions can lead to a leakage of metabolites and enzymes from their original compartment. Water-free methods can overcome this problem. The non-aqueous fractionation (NAF) of plant cells using density gradient centrifugation, as described by Gerhard and Heldt (1984), enables cell compartment separation and maintains the enzyme and metabolite stability and localization. NAF was first developed by Behrens (1932) for the separation of the nuclei from calf heart muscles and in plants it was first used for the separation of chloroplasts by Stocking (1959). The method of Gerhard and Heldt (1984) enables the analysis of the metabolite distribution between three cell compartments – the cytosol, vacuoles and plastids. Plant cell organelles have changing densities during cell maturation and therefore NAF has been successfully applied only on mature tissues (Jagendorf 1955; Stocking 1959). This NAF method has been applied to mature leaves from different species, *e.g.* spinach, barley, beans (Gerhard and Heldt 1984; Winter *et al.* 1993; Weise *et al.* 2004), potato tubers (Farré *et al.* 2001; Junker *et al.* 2006; Farré *et al.* 2008) and recently to barley endosperm (Tiessen *et al.* 2012). There is also a differential centrifugation (sedimentation) method of NAF (Shannon *et al.* 1998) which has been used for maize endosperm. The sedimentation method has an advantage over the equilibrium density gradient NAF due to the easier handling and centrifugation. Cell

fragmentation into compartments is possible due to the different densities of organelles, which is the main precondition for a successful separation.

Aims of the study

In order to elucidate the role of the enzymes and metabolites in starch synthesis, the subcellular localization and concentrations of metabolites, and the activities of enzymes in the sucrose to starch pathway have to be investigated. At the subcellular level, the starch synthesis network has only been investigated in some storage tissues, *e.g.* potato tubers (Farré *et al.* 2001) and barley endosperm (Tiessen *et al.* 2012). Therefore the aim of this study is to improve the understanding of storage starch synthesis at the subcellular level by analyzing maturing pea embryos.

In order to analyze the starch synthesis on the subcellular level in pea embryos the method of non-aqueous fractionation (NAF) was chosen, which was subsequently optimized for developing pea embryos.

One of the main biological aims is to determine the localization of AGPase and ADP-Glc, due to the recent evidence for cytosolic ADP-Glc in dicots (Baroja-Fernández *et al.* 2012). To solve this approach the transgenic pea line iAGP-3, having the RNA interference repressed AGPase expression (Weigelt *et al.* 2009), and its wild type parent cultivar Eiffel, will be used. This study should also provide answers to the role of AGPase on starch synthesis in pea embryos, and how the RNAi inhibited AGPase transgenic pea embryos retain high starch content.

AGPase is the enzyme which provides the direct substrate for starch synthesis and is therefore located at the very end of the pathway. Thus using the pea line SUT7, which has over-expressed sucrose transporter (*Vf*SUT1), effects of the changes at the very beginning of the pathway on starch synthesis were investigated. Open questions to study are the effect of increased sucrose influx on enzyme activities in the first steps of sucrose degradation and the influence on starch synthesis.

Chapter 2: Materials and methods

Plant material

Seeds of the pea (*Pisum sativum*) cultivar Eiffel, its transgenic line iAGP-3 (expressing the reverse fragment of the pea AGP small subunit fused to the LeB4 promoter; Weigelt *et al.* 2009), wild type SUT and its transgenic line SUT7, overexpressing the sucrose transporter SUT1 from *Vicia faba* under USP promoter, were obtained from Ruslana Radschuk (workgroup of Seed Development in IPK Gatersleben, Germany). Pea plants were grown in 2 L pots in a growth chamber under a light/dark regime of 16h/8h (20°C/18°C), with a light intensity of 108 µmol m^{-2} s^{-1} and relative air humidity of 70%. The cultivation substrate was a one to one mixture of the local compost (IPK Gatersleben) and Substrate 2 (Klasmann-Deilmann GmbH, Geeste, Germany). Pods were marked according to number of days after pollination (DAP) and collected at the end of the day's light phase. Collected pods were cooled and the embryos were rapidly dissected from their seed coats on ice and snap frozen in liquid nitrogen. Embryos were then stored at -80˚C until used.

Enzyme assays

Enzyme extraction was performed as in Trethewey *et al.* (1998), except that the BSA was omitted from the extraction buffer. Ground whole embryos or aliquots of the dried fractions of NAF were re-suspended on ice in 1 ml of enzyme extraction buffer [50 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 1 mM Na-EDTA, 1 mM EGTA, 5 mM dithiothreitol, 2 mM benzamidine, 2 mM 6-aminocaproic acid, 0.5 mM phenylmethylsulfonylfluoride, 0.1% (v/v) Triton X100 and 10% glycerol (v/v)] and centrifuged for 10 minutes (min) at 4˚C, 20800 g (which corresponds to a maximal speed of 14000 rpm) (centrifuge 5417R, Eppendorf, Germany, Hamburg). The supernatant was desalinated with Illustra NAP^{TM} columns equilibrated with extraction buffer (GE Healthcare, United Kingdom, Buckinghamshire). The gained extracts were aliquoted, frozen with liquid nitrogen and stored at -80˚C. Enzyme assays were performed in 96 well plates (with 300 µl well volumes) at 25˚C using coupled reactions leading to NADH formation from NAD. The NADH absorption signal was measured for at least 25 min with 30 sec intervals at 340 nm with a spectrophotometer (EL808, BioTek Instruments, Winooski, USA). Concentrations in the following assays are given in terms of the final concentration.

The AGPase assay modified after Weber *et al.* (1995b) contained 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 0.4 mM NAD, 2 units ml^{-1} (U ml^{-1}) glucose 6-phosphate dehydrogenase (Glc6PDH; Roche, Switzerland, Basel) and 2 U ml^{-1} phosphoglucomutase (PGM; Sigma-Aldrich). After incubation for 10 minutes the reaction was initiated by 2 mM sodium pyrophosphate (NaPP) and ADP-Glc.

The UGPase assay modified after Kleczkowski *et al.* (2005) contained 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 0.5 mM NAD, 2 U ml^{-1} Glc6PDH and PGM. The reaction was initiated by 2mM UDP-Glc and 1.5 mM NaPP.

The SuSy assay (Barratt *et al.* 2001) contained 50 mM HEPES-KOH (pH 7.5), 5 mM $MgCl_2$, 1 U ml^{-1} UGPase, 2 U ml^{-1} PGM, 2 U ml^{-1} hexokinase (HK), 2 U ml^{-1} phosphoglucoisomerase (PGI) (Roche, Switzerland, Basel), 2 U ml^{-1} Glc6PDH, 0.5 mM NAD and 0.5 mM ATP. The reaction was initiated by 250 mM sucrose, 2 mM UDP and 2 mM NaPP.

The hexose kinase (HK) assay modified after Copeland *et al.* (1978) contained 50 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 1.5 mM ATP, 1 U ml^{-1} Glc6PDH and 0.3 mM NAD. The reaction was initiated either by 1 mM glucose or 0.5 mM fructose.

The alkaline invertase (Inv) assay modified after Zrenner *et al.* (1995) contained 40 mM HEPES (pH 7.5), 1.5 mM NAD, 1 mM ATP, 2 U ml^{-1} HK and 2 U ml^{-1} Glc6PDH. The reaction was initiated by 300 mM sucrose.

The PGM assay (Foster and Smith, 1993) contained 50 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 0.4 mM NAD und 2 U ml^{-1} Glc6PDH. The reaction was initiated by 6 mM glucose 1-phosphate (Glc1P).

The PGI assay (Forster and Smith, 1993; Schaffer and Petreikov, 1997) contained 100 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 0.33 mM NAD and 1 U ml^{-1} Glc6PDH. The reaction was initiated by 3 mM fructose 6-phosphate (Fru6P).

The phosphoenolpyruvate carboxylase (PEPCase) assay (Colombek *et al.* 1997) contained 25 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, 0.2 mM NADH and 6 U ml^{-1} malate dehydrogenase (Roche Switzerland, Basel). The reaction was initiated by 4 mM phosphoenolpyruvate and $KHCO_3$.

The glyceraldehyde 3-phosphate dehydrogenase (GAPDH; NADPH dependent) assay buffer modified after Baalmann *et al.* (1994) contained 100 mM Tris-HCl pH (7.8), 5 mM 3-PGA, 3 mM ATP, 1 mM EDTA, 10 U ml^{-1} PGKinase and 0.35 mM NADPH. The reaction was initiated after 15 minutes incubation at room temperature adding the enzyme extract.

The iPPase assay modified after Gross and ap Rees (1986) and Taussky and Shorr (1956) contained 50 mM Tris-HCl (pH 7.5) and 5 mM $MgCl_2$. The reaction was initiated by 1.5 mM NaPP. After 30 min the reaction was stopped with 12% (w/v) trichloroacetic acid/water and the assay plate was incubated on ice for 10 min. The plate was then centrifuged for 10 min at 4˚C and 3220g. Then 150 µl supernatant was transferred to a new well plate and 150 µl Taussky Shorr reagent (1% w/v ammonium molybdate, 5% w/v $FeSO_4$, 0.5 M H_2SO_4) was added. After 5 min incubation at room

temperature the adsorption was measured at 595 nm. The phosphorus standard from Sigma-Aldrich was used for calculation of the produced orthophosphates.

Analysis of metabolites

Soluble sugars were extracted using 80% aqueous ethanol for the analysis of sucrose, glucose and fructose, and trichloroacetic acid (TCA) (Trethewey *et al.* 1998) for the analysis of hexose phosphates, UDP-Glc, iP and iPP (Jelitto *et al*. 1992). Soluble sugars and hexose phosphates were measured via enzyme-coupled spectrophotometric assays as described in Stitt *et al.* (1989).

Pellets from ethanol or TCA extractions were used to hydrolyse starch to glucose. The pellets were washed three times with 80% ethanol. Then the pellets were heated at 95˚C in a 50 mM sodium acetate buffer pH 5.2 for 30 min to gelatinize the starch. The starch was then hydrolysed with amyloglucosidase and α-amylase overnight. Then the samples were heated at 95˚C for 15 min and centrifuged 5 min at 20800g (centrifuge 5417R, Eppendorf, Germany, Hamburg); the supernatant was directly used for analysis or stored at -20˚C.

Adenylates (ATP, ADP, AMP and ADP-Glc) were extracted as in Gullberg *et al.* (2004) with 1 ml methanol-chloroform-water (2.5:1:1 v:v) mixture vortexing the samples for 15 min at 4˚C, then 400 µl of distilled water was added to the samples on ice. Next, the samples were shortly vortexed before centrifugation at 4˚C, 20800g for 5 min. The resulting upper phase (methanol-water) was collected and dried for approximately 3h in a SpeedDry 2-33IR Rotational-Vacuum-Concentrator (Martin Christ, Osterode, Germany). Dried aliquots were stored at -80˚C. Adenylates were analyzed by HPLC separation (Waters 2795 Alliance HT) and with a Waters 2475 Multi λ Fluoresence Detector (Waters GmbH; 65760 Eschborn; Germany) as described in Haink and Deussen (2003).

Histological methods and determination of subcellular volumes

Histological work was performed as in Weigelt *et al.* (2009). Subcellular volumes were calculated as in Farré *et al.* (2001). The determination of subcellular volumes was performed using three embryos per pea line at 30 DAP. Selected embryos of Eiffel and iAGP-3 had a fresh weight of 500 ± 20 mg and embryos of SUT wt and SUT7 had a fresh weight of 400 ± 20 mg. At least two microscopy class slides, each with eight 0.2 µm thick cuttings on it, and representing different parts of the embryo, were analyzed per embryo. Photos were taken with 5x and 10x lens magnifications using a Carl Zeiss AG (Oberkochen, Germany) microscope Axioskope and the software AxioVision 4.7. The 5x lens magnification from the microscope gave images covering 1.79 x 1.34 mm of the tissue, which resulted in approximately 196 cells in an image. With the 10x magnification lens, the microscope images covered 0.89 x 0.67 mm of the tissue, which resulted in approximately 64 cells

per image. The relative organelle volumes were determined using the open source software ImageJ (http://rsbweb.nih.gov/ij/index.html).

Methods of non-aqueous fractionation (NAF)

Plant material preparation

Pea embryos were rapidly dissected on ice, weighed and then cooled in liquid nitrogen before storing at -80˚C. For the NAF the embryos were pooled forming four to six gram (fresh weight) patches. Embryos were pooled while frozen in liquid nitrogen and ground using a swing mixer mill (MM400, Retsch GmbH, Haan, Germany) at swing frequency 30 Hz. The steel beakers and balls were precooled in liquid nitrogen. Four small aliquots (about 40 mg each) were taken from the ground material. Frozen ground material was transferred to a 50 ml plastic beaker and dried for 100 ± 10 hours at 0.52 mPa (-27˚C) in a freeze dryer (Alpha 1- 2 LD Plus, Martin Christ, Osterode, Germany). After lyophilization the samples were weighed and four aliquots (about 20 mg) from dry material were taken. The beakers were quickly closed and placed in plastic bags containing silica gel. These plastic bags were then placed into boxes containing silica gel, and were used immediately or stored at -80˚C.

The efficiency of grinding was controlled by analyzing the dried material through the microscope; the aim was to get mainly small particles on average 2 to 3 µm in diameter as described in Farré et al. (2001).

Linear density gradient NAF

The NAF method used here was modified after Farré *et al.* (2001) and Weise *et al.* (2005), and personal communication with Annika Nerlich (2009). The laboratory protocol can be found in Appendix 2.

Preparations and forming the linear density gradient

Preparations and forming of the linear density gradient were done at room temperature as in Farré *et al.* (2001). The dry powder of embryos was suspended in a 15 ml tetrachlorethylene/heptane mixture (66/34% [v/v], density 1.3 g ml^{-1}). The suspension was homogenized for 30 sec with 67% power and 8 sec pulses using an ultrasonic homogenizer (Sonoplus HD 200, MS 73/D, Berlin, Germany) while keeping the beaker in a vessel filled with cooled (4˚C) heptane in order to avoid heating. Four 100 µl aliquots were taken from this homogenate. The homogenate was then filtered through a polyester net (20 µm); the net was rinsed once with a 10 ml tetrachlorethylene/heptane mixture (66/34% [v/v]) and the gained filtered homogenate was then centrifuged for 10 min at room temperature at 3200 g. The supernatant was discarded and the pellet was resuspended in a 3 ml

tetrachlorethylene/heptane mixture (60/40% [v/v]). The suspension was mixed well and four 100 µl aliquots were taken.

The linear density gradient (30 ml, ρ = 1.3-1.62 g cm^{-3}) was formed using an Econo System Controller with a peristaltic pump (model EP-1 Econo Pump, Bio-Rad Laboratories Inc., Hercules, USA). The prepared suspension was carefully loaded on top of the gradient. The gradient was centrifuged for 60 min at 5000 g and 4°C in a swing out rotor (Sorvall DuPond HB-4) using a Sorvall RC5C Plus centrifuge (Thermo Fisher Scientific Inc., Waltham, USA). The gradient was then divided into ten fractions starting from the top and transferred with a Pasteur pipette into 15 ml tubes. 10 ml heptane was added to the fractions which were then centrifuged at 3200 g for 5 min and the supernatant was discarded. The remaining pellets were resuspended in 2 ml heptane and divided into four equal aliquots. Aliquots were again centrifuged at 3200 g for 5 min, the supernatant was discarded and the remaining pellet was dried in a SpeedDry 2-33IR Rotational-Vacuum-Concentrator evaporator (Martin Christ, Osterode, Germany) for 15 min at 25˚C using 4.3 mbar vacuum. The dried aliquots were stored at -80˚C until required for further analysis.

Differential sedimentation NAF

The differential centrifugation NAF was applied to pea embryos in different developmental stages. The method is basically similar to the method described above, however it relies on differential sedimentation of cell particles, not on the isopycnic positioning in the density gradient.

Method of four sedimentation-dilution steps

Plant material was prepared as previously described for the linear density gradient. The method described by Shannon *et al.* (1998) was tested. It involves four sedimentation steps, lowering the density of suspension after each centrifugation. In this study the solvent density steps were 1.44, 1.42, 1.37 and 1 g cm^{-3}, which correspond to the respective percentage of tetrachlorethylene in the solvent mixture of: 85, 83, 75, 50% (v/v).

First the lyophilized plant material was resuspended in a 12 ml solvent mix of 85:15 (v/v) tetrachlorethylene : heptane, having density of 1.44 g cm^{-3}. This suspension was homogenized by sonication for 30 sec. Four 100 µl aliquots were taken and the homogenate was filtered through a nylon net (20 µm pore size) into 15 ml tube. The volume of filtered homogenate was adjusted to 12 ml in order to allow the calculation of the right dilution for the next density value. Centrifugation was done at room temperature in 5 min at 3200 g. The gained pellet was retained and the supernatant was transferred to a new empty tube. Supernatant was then diluted with heptane to lower the density to 1.42 g cm^{-3} (corresponding to the solvent ratio 83:17 [v/v]). Centrifugation was then repeated to yield the new pellet fraction and the supernatant was again transferred to a new

tube. The supernatant was again diluted with heptane to the next density of 1.37 cm^{-3} and centrifuged as before to yield the next pellet. Before the last centrifugation the supernatant was diluted to at least a ratio of 50:50 (v/v) and centrifuged as before to yield the last pellet fraction. The remaining supernatant was discarded. The pellets were then shortly dried (10 min) at 25°C using a 4.3 mbar vacuum in the rotary vacuum evaporator (SpeedDry 2-33IR Rotational-Vacuum-Concentrator, Martin Christ, Osterode, Germany). The dried pellets were then resolved in 2 ml pure heptane and divided into four equal aliquots. Aliquots were dried for 30 min at 25°C using a 4.3 mbar vacuum in the rotary vacuum evaporator. The dried pellets were stored at -80°C until the extraction of metabolites and enzymes.

Final method of NAF via differential centrifugation

The non-aqueous fractionation method was modified after Shannon *et al.* (1998) and Farré *et al.* (2001). For the fractionation approximately 5 g total fresh weight (FW) pea embryos were used. Embryos, collected at 30 DAP and having a fresh weight of 500 ± 20 mg, were pooled. Embryos were pooled while frozen in liquid nitrogen and ground (2*3 minutes, 30 Hz) using a mixer mill MM400 (Retsch GmbH, Haan, Germany), which was precooled in liquid nitrogen. Four small aliquots (about 40 mg each) were taken from the ground material. Frozen ground material was transferred to a 50 ml plastic beaker and dried for about 100 hours at 0.52 mPa (-27˚C) in a Alpha 1- 2 LD plus freeze dryer (Martin Christ, Osterode, Germany). After freeze drying the samples were weighed and four aliquots (about 20 mg) were taken. The beakers were quickly closed and placed in plastic bags containing silica gel. These plastic bags were then placed into boxes containing silica gel, and stored at -80˚C (or were used immediately). The dry powder was suspended in 15 ml of tetrachlorethylene/heptane mixture (83/17% [v/v], density 1.43 g ml^{-1}). The suspension was sonicated for 30 seconds with 67 % power with 8 second pulses (Sonoplus HD 200, MS 73/D, Berlin, Germany). Four aliquots of 250 µl were taken from this suspension. The suspension was then filtered through a nylon sieve with a pore size of 20 µm (VWR, Radnor, USA) into a 15 ml reaction tube. Four aliquots of 250 µl were taken from the filtered suspension. The rest of filtered suspension was centrifuged at room temperature for 5 minutes at 3220g, after which the supernatant was transferred to the next reaction tube and diluted to the next density step with heptane and centrifuged as before. This procedure was repeated to gain a total of 6 different fractions (pellets). The solution mixtures for the fractions were: 83%, 80%, 77%, 74%, 71% and 50% (tetrachlorethylene/heptane v/v); these correspond to solution densities: 1.43, 1.41, 1.39, 1.36, 1.32 and 1 g ml^{-1}. The pellets gained were re-suspended in heptane and divided into four aliquots, which were then dried using a SpeedDry 2-33IR Rotational-Vacuum-Concentrator (Martin Christ, Osterode, Germany). Dried aliquots were stored at -80˚C.

Mathematical and statistical analysis

The distribution (confinement) of the metabolites and enzymes in cellular compartments was calculated by a quadratic best fit method (method of linear least squares values) using the command line tool BestFit (Klie *et al.* 2011, Krueger *et al.* 2011; http://csbdb.mpimp-golm.mpg.de/csbdb/bestfit/bestfit.html). This analysis is based on the distribution of organelle marker enzymes. In this study iPPase, GAPDH, PEPCase, UGPase and α-mannosidase were used as markers for plastids, cytosol and vacuole, as described in Farré *et al.* (2001).

A statistical analysis was performed using Fisher's variance analysis (ANOVA) and Student's *t*-test (two-tailed, unequal variation, α=0.05).

Western blot

Protein separation was done using SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and electrophoretic blotting using BioRad equipment (Mini-PROTEAN® II Electrophoresis Cell) following BioRad protocols. Separating gel contained 11.25% acrylamid. The separation conditions: constant voltage 150V for 30 minutes following 50 minutes 75V. Electrophoretic transfer of proteins on a PVDF membrane ((Immobilon-P; pore size 0.45 mm; Millipore, Eschborn, Germany) was done using semi-dry blotting procedure; blotting was performed 90 minutes at constant current 0.8 mA cm^{-1}. Primary antibodies (all raised in rabbit) used were for UGPase (product nr AS05 086) and Rubisco large subunit (product nr AS03 037) (both from Agrisera, Vännas, Sweden), and for AGPase (*Hordeum vulgare* small subunit AGPase antibody, which was kindly provided from workgroup of H.P. Mock). Secondary antibody was IRDye 800CW (coat anti-rabbit) (LI-COR, Lincoln, Nebraska, USA). Blots were analyzed and documented using LI-COR Odyssey software. The extended protocol and composition of buffers are located in Appendix 1.

Energy state

The energy change and the direction of starch synthesis related reactions were evaluated using the standard free energy change constants ($\Delta G`°$, from http://equilibrator.weizmann.ac.il) and calculated mass action ratios (Q) of the reactions.

Chapter 3: The establishment of non-aqueous fractionation for pea embryo cells

Subcellular analysis of enzymes and metabolites in developing pea embryos using non-aqueous fractionation (NAF) involved the establishment of (1) an optimal density gradient, (2) assays for marker enzymes, and (3) assays for metabolites and enzymes of interest. Of specific interest was the analysis of starch metabolism during the seed filling phase of pea embryos. The focus on the seed filling phase was based on its high rate of storage product synthesis and its near linear biomass gain during this stage, which enables the best insight into starch metabolism.

Linear density gradient NAF

Linear density gradient NAF is, by its nature, based on equilibrium density-gradient centrifugation, which separates cellular components according to their density. This method allows each particle to migrate to an equilibrium position in a gradient where the density of the surrounding liquid is equal to the density of the particle. The NAF method used here was modified after Farré *et al.* (2001) and Weise *et al.* (2005), and personal communication with Annika Nerlich (2009).

Crucial to the success of NAF are the homogenous and small particles gained by grinding. The aim was to get mainly small particles on average 2 to 3 µm in diameter as described in Farré *et al.* (2001). Therefore the efficiency of grinding was controlled by analyzing the dried material with a light microscope. The duration of grinding was a limiting factor, not only for the resulting particle size but also due to the possibility of thawing. The grinding efficiency was tested using intervals between one and four minutes, with a single repetition after cooling in liquid nitrogen. The optimum was the grinding twice, for three minutes, at 30 Hz (Figure 3). Increase of grinding duration mainly resulted in thawed samples or technical problems developed with the steel beaker due to ice condensation following the freezing

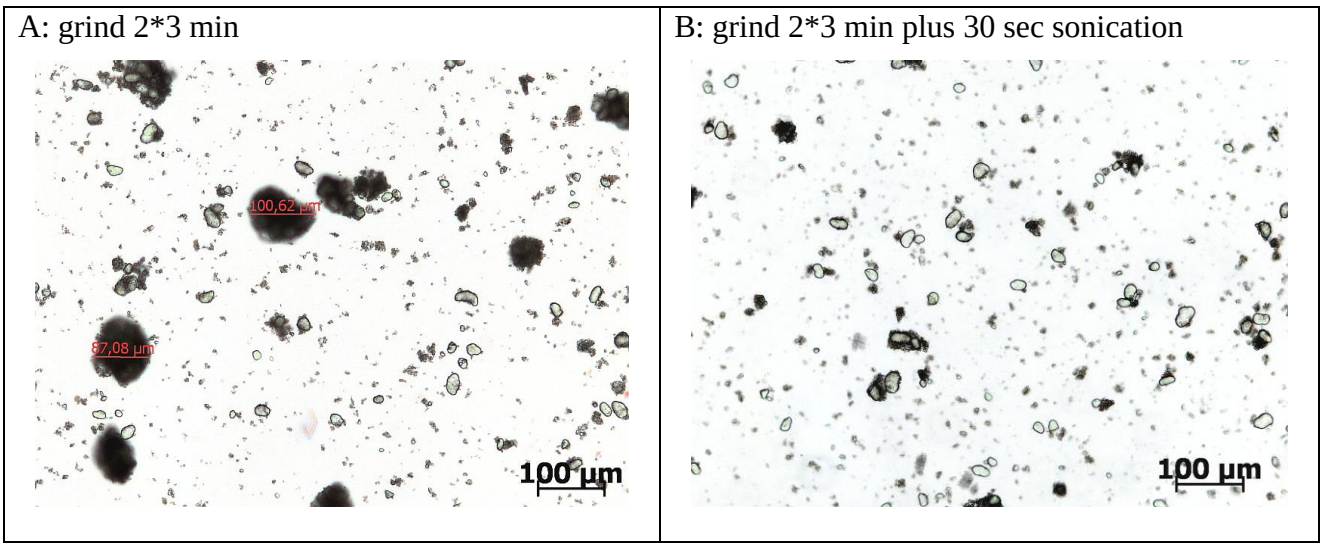


Figure 3. Optimized preparation of pea embryos for non-aqueous fractionation: grinding twice for three minutes using the swing mixer mill (A) and sonication for 30 seconds (B), which separated the aggregated particles.

Failed establishment of linear density gradient NAF

The establishment of the linear density gradient NAF encountered several technical and biological problems, which led to a repeated failure of fractionation. Technical problems involved inadequate plant material preparation and storage, which may have led to water contamination or insufficient grinding (many large particles). The efficiency of grinding was tested by measuring the particle size after lyophilization. This led to an optimization of the grinding and treatment with ultrasound vibrations (grinding twice for three minutes at a frequency of 30 Hz, and sonication for 30 seconds). The long storage of freeze dried material led to water contamination and affected enzyme activities. Therefore, it was decided that freeze dried powder was to be used immediately or to be stored at -80˚C just for a few days. The second main problem was creating the gradient with a peristaltic pump, which failed several times due to air bubbles in the tubing during the pumping of the gradient. Other sources for failure during pumping included unsuitable tubing materials for the used organic solvents as well as the fragile injection valve of the pump. The third source for failure was the gradient sensitivity to shaking due to the mixable nature of the solvents used. Centrifuged gradients were photographed for a quick visual comparison. Almost all formed gradients were different; Figure 4 illustrates only a small part of this work.

The biological problem was the relatively narrow compartment density range indicated by formed gradients. Density of subcellular compartments can change marginally during the development of pea embryo cells (Bain and Mercer 1966; Hoh *el al.* 1995). Furthermore the separation of the chloroplast by non-aqueous methods has been successful only with mature tissues of leaves, which may be explained by changing of plastid density over the development (Jagendorf 1955; Stocking 1959).

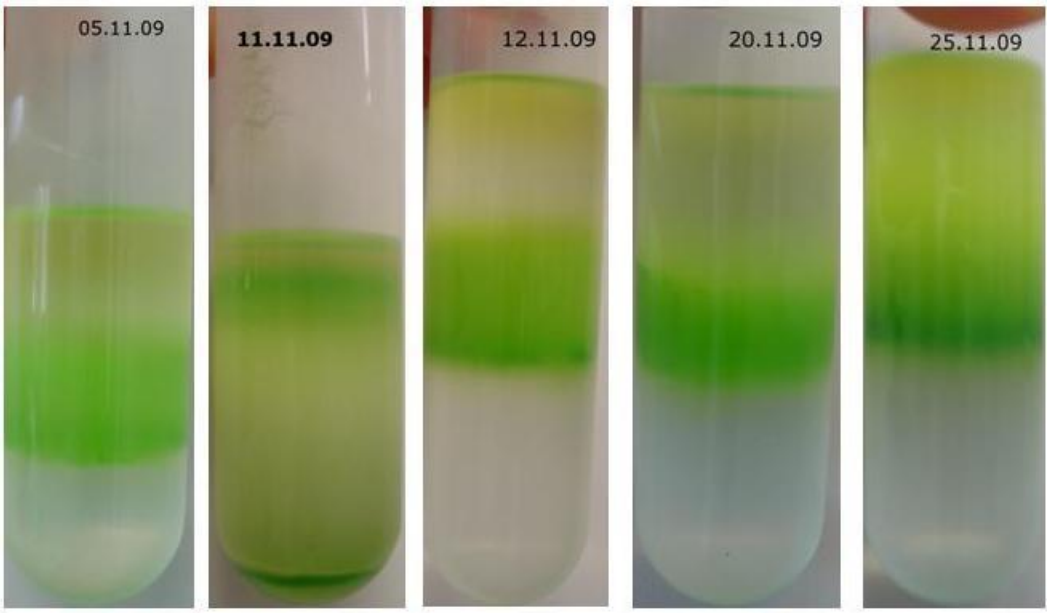


Figure 4. Example of linear non-aqueous gradients performed in November 2009. The photos illustrate the unreliability of the peristaltic pump, as a different gradient was formed with each repetition.

Differential sedimentation NAF

After unsuccessful tests with linear gradient, the differential centrifugation NAF (Shannon *et al.* 1998) was tested to determine whether NAF is applicable to developing pea embryos. The differential centrifugation NAF was applied to pea embryos in different developmental stages. The method is basically similar to the linear gradient method, although it relies on the differential sedimentation of cell particles, not on the isopycnic positioning in the density gradient.

Testing the NAF according to Shannon *et al.* 1998

Plant material was prepared as previously described for the linear density gradient and the method described by Shannon *et al.* (1998) was tested. This involves four sedimentation steps, lowering the density of suspension after each centrifugation. In this study the solvent density steps were 1.44, 1.42, 1.37 and 1 g cm^{-3}, which correspond to the respective percentage of tetrachlorethylene in the solvent mixture of: 85, 83, 75, 50% (v:v).

Results led to the conclusion that possibly only maturing embryos can be effectively analyzed with this method (Figure 5). The separation of plastids failed in early developmental stages (Table 1 and Figure 5); which was indicated by the confinement of starch to the cytosol according to compartment localization calculation by the command line tool BestFit. The reason for failure during early stages was most probably due to the similarity of organelle densities during the early phases of tissue development. Therefore, the following tests were applied to embryos in the mid to late developmental stage.

Table 1. Subcellular relative confinement into three cellular compartments – plastids, cytosol, and vacuole – calculated with the BestFit software tool

Four step differential centrifugation NAF was performed with pea embryos in three different development stages (110 mg FW, 190 mg FW and 520 mg FW corresponding to 16, 18 and 30 DAF).

	110 mg FW embryos			190 mg FW embryos			520 mg FW embryos		
	plastid	**cytosol**	**vacuole**	**plastid**	**cytosol**	**vacuole**	**plastid**	**cytosol**	**vacuole**
starch	4	**96**	0	100	0	0	100	0	0
glucose	31	42	27	**49**	**51**	0	18	82	0
fructose	0	0	100	0	12	88	0	100	0
sucrose	0	0	100	0	21	79	0	26	74

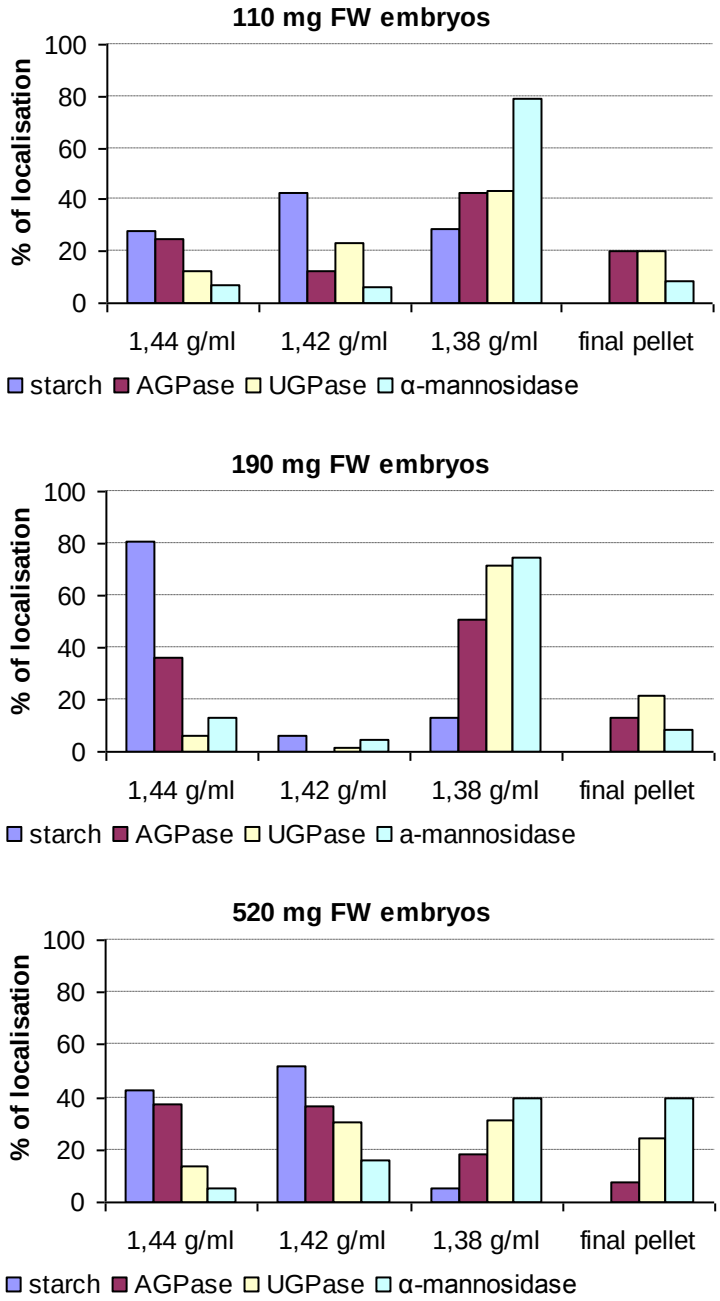


Figure 5. Distribution of marker enzymes and starch in centrifugation pellets from differential centrifugation NAF using four centrifugation steps applied for pea cv. Eiffel embryos at different developmental time points.

Optimized differential centrifugation NAF

The density range of the solvent mixture as well as the number of density steps were optimized for better separation of compartments and for mathematical resolution. The density range for organelles looked very narrow in the previous tests and the main plant material sedimented between 1.44 and 1.3. This led to the use of six solution density steps: 1.43, 1.41, 1.39, 1.36, 1.32, and about 1 g cm^{-3}, which corresponds respectively to the relative ratios of tetrachlorethylene and heptane: 83:17, 80:20, 77:33, 74:71, 50:50 (v:v). Analyses were performed using embryos of the pea cv. Eiffel having 60, 100, 200 and 300 mg fresh weight (FW). Similar to four sedimentation steps, the subcellular fractionation using six sedimentation steps was not successful with younger embryos as the starch was largely confined to the cytosol (Figure 6). Together with the results from the four sedimentation steps NAF using 520 mg FW embryos, led to the conclusion that for efficient fractionation and analyses the pea embryos from the maturing phase can be used. This was confirmed by successful tests with three separate fractionations of the cultivar Eiffel embryos having a fresh weight 500 ± 50 mg (Figure 7; Figure 8). The subcellular confinement of metabolites into compartments was in accordance with the expectations. But the marker enzymes for cytosol (UGPase) and vacuole (α-mannosidase) had very similar distributions (pairwise Student's t-test $\alpha = 0.05$). Also the resulting standard error was very large in the three compartment analyses. Therefore the NAF allows only two compartment analysis for the metabolite and enzyme distribution between plastids and the "*cytosolic compartment*". The "*cytosolic compartment*", later referred to mainly as the cytosol, is used here to represent a non-plastid space of tissue or in other words the whole cell with a cell wall and intercellular space but excluding the plastids and therefore also contains other organelles, *e.g.* mitochondria.

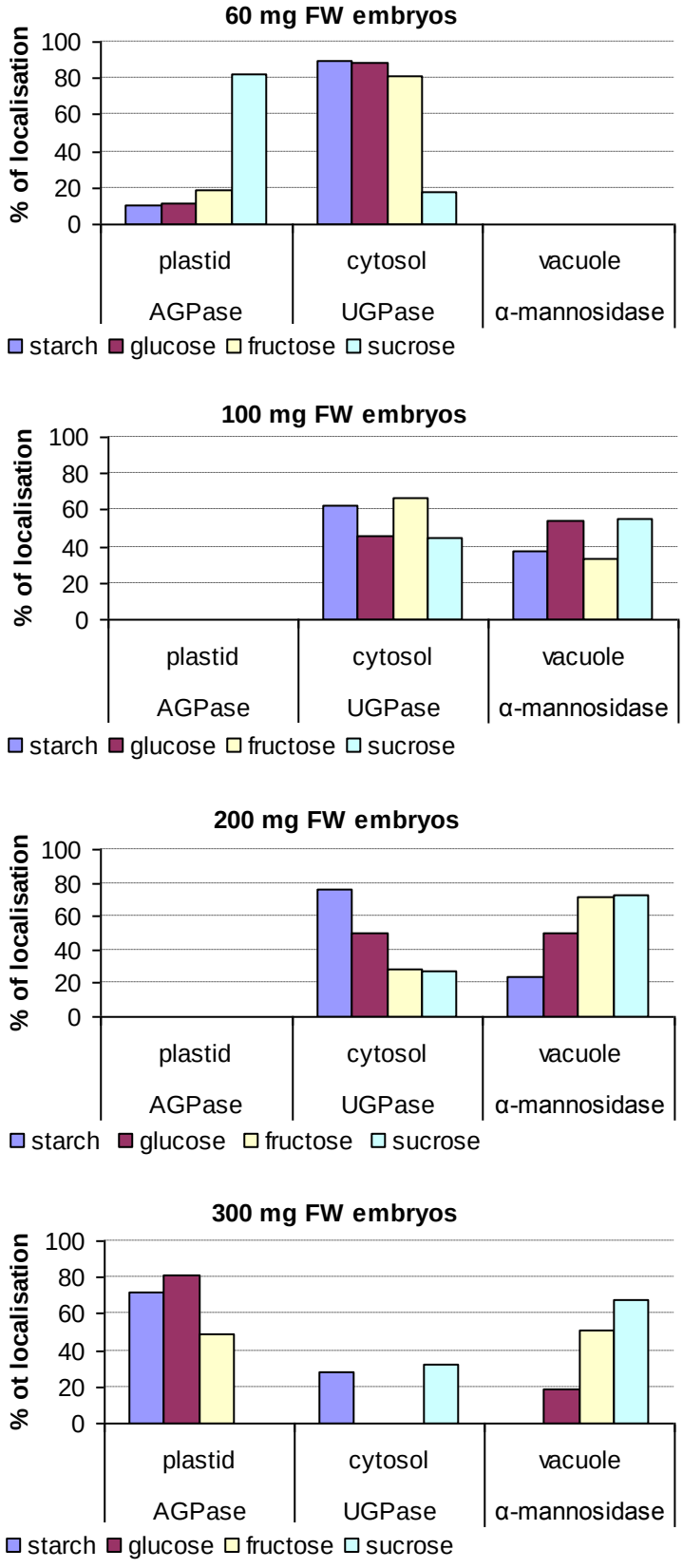


Figure 6. Test results for the six sedimentation steps of differential centrifugation using the pea cultivar Eiffel embryos in different developmental stages.

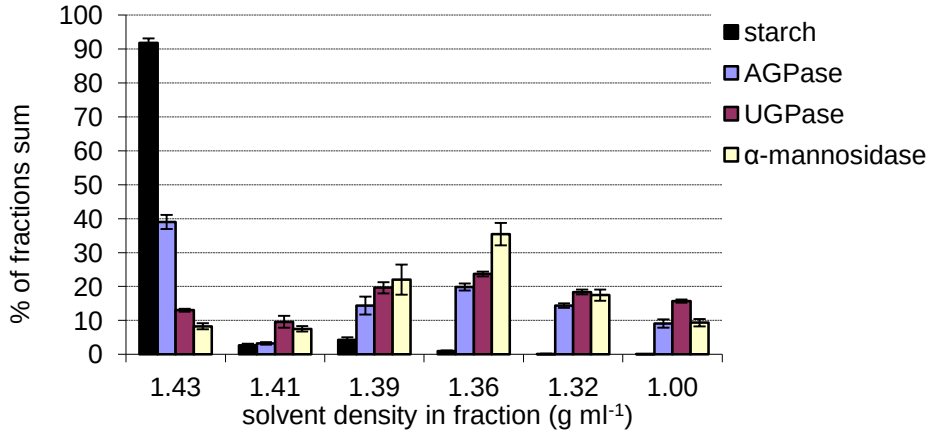


Figure 7. Marker enzymes and starch distribution over six fractions. Data represents the average ± SE of three separate NAFs performed with 500 ± 50 mg FW embryos.

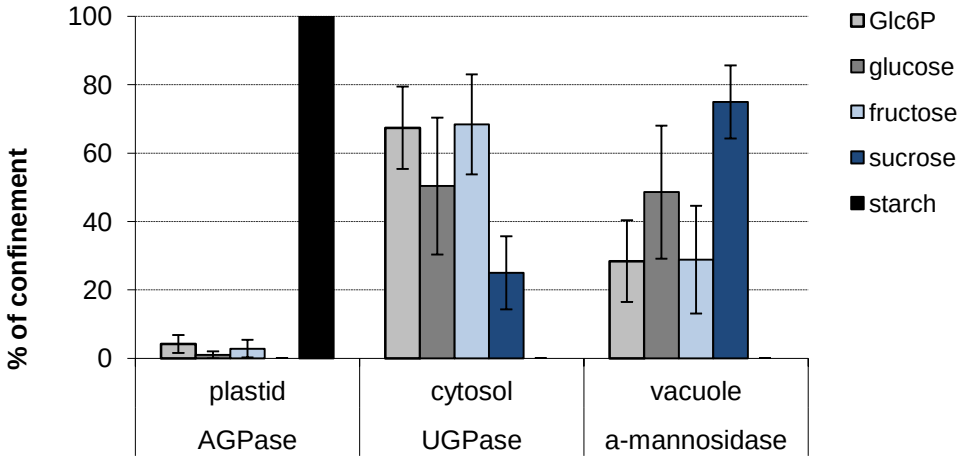


Figure 8. Subcellular metabolite confinement into three cell compartments (plastid, cytosol, and vacuole) in maturing cultivar Eiffel (500 mg FW) embryo cells according to the BestFit software tool. Data represents the average ± SE of three separate NAFs with 500 ± 50 mg FW embryos.

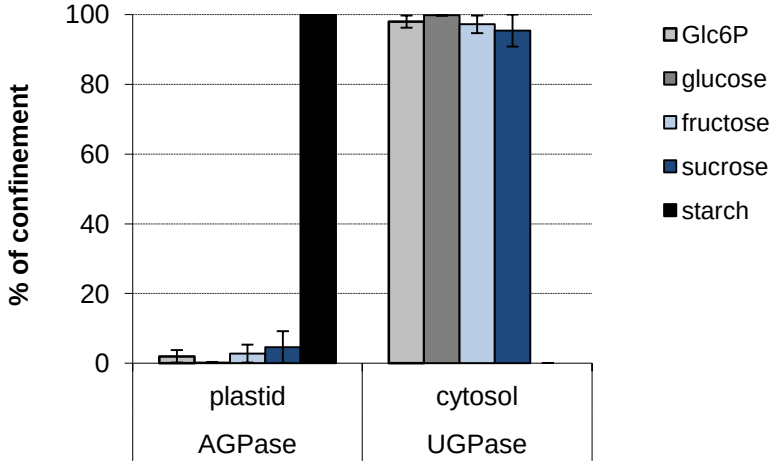


Figure 9. Subcellular metabolite confinement into two compartments (plastid and cytosol) in maturing cultivar Eiffel (500 mg FW) embryo cells according to the BestFit software tool. Data represents the average ± SE of three separate NAFs using 500 ± 50 mg FW embryos.

Discussion

NAF was expected to enable the analysis of starch metabolism during the seed filling phase in pea embryos. Efficient separation of subcellular compartement using NAF was limited to the maturing phase in developing pea embryos; this is probably due to the more homogenous and differentiated cells in this stage. The cells of pea as well as of other legume embryos differentiate over their development and their subcellular structure changes remarkably (Bain and Mercer 1966; Briarty 1980; Hoh *el al.* 1995). During the seed filling phase the legume embryo undergoes the differentiation from adaxial to abaxial surface of the cotyledon; therefore in the early and mid cotyledon phase the cells are more divergent (Borisjuk *et al.* 1995). Cells in differentiating tissue undergo several structural changes, which can be accompanied by changing densities of the membranes as well as that of the cytosol and organelle matrix.

Appling NAF for maturing pea cotyledon cells enabled to analyze the plastidial and cytosolic carbohydrate metabolism. Hence, only two subcellular compartments were distinguished in the pea embryo cells: the plastidial and the *cytosolic compartment*. This was probably due to the absence of large vacuoles in maturing cotyledon cells of wild type Eiffel, as well as due to the similar densities of the mitochondria, protein bodies, ER, and the cytosol. A similar cellular composition was also found in developing barley endosperm, where only the plastidial and cytosolic compartments were separated by NAF (Tiessen *et al.* 2012).The lack of large central vacuoles and the formation of dense protein bodies in maturing pea embryos explain the failure of the separation of plant material into three compartments using NAF, as described for leaves (Gerhard and Heldt 1984; Stitt *et al.* 1989). Fractionation of embryos in very early development (*e.g.* 12-14 DAP) could have been potentially successful as the cells are quite homogenous at this stage, but the too equal densities of organelles and membranes would have hindered this approach.

In hindsight, AGPase may have been the wrong plastidial marker for analyzing young embryos, due to the very low activity of AGPase in young pea embryos (Weber *et al.* 1995b; Weigelt *et al.* 2009). Due to this, and being one of the enzymes of interest, the use of AGPase as an organelle marker was omitted in the following studies.

Conclusions for NAF establishment

The specific interest in the establishment of NAF was to analyze the starch metabolism. Efficient separation in developing pea embryos was limited to the maturing phase, in which the embryos contain more homogenous cells. The lack of large central vacuoles and the formation of dense protein bodies in maturing pea embryos may explain the failure of three compartments analyses via NAF, as described for leaves (Gerhard and Heldt 1984; Stitt *et al.* 1989). Nonetheless, NAF enabled to analyze plastidial and cytosolic carbohydrate metabolism of maturing pea cotyledon cells.

Chapter 4: Subcellular effects of AGPase repression in starch synthesis

Results

Metabolites and enzyme activities in maturing pea embryos

The transgenic pea line iAGP-3 and its parent cultivar Eiffel have been described and several metabolites and the transcriptome covering embryo development were analyzed in Weigelt *et al.* (2009). Still, additional measurements were needed for a complete starch synthesis pathway analysis. In this study the embryos selected for the analyses of starch synthesis connected metabolite concentrations and enzyme activities had, at thirty days after pollination (DAP), an average fresh weight (FW) of 500 ± 20 mg.

The results of the present study were in accordance with Weigelt *et al.* (2009), although minor differences have occurred due to subtle differences in growth conditions, marking the point of pollination or measurement assays. As in Weigelt *et al.* (2009) the water content of embryos was much higher (approximately 25 %) in the transgenic line iAGP-3. The starch content of maturing embryos at 30 DAP added up to 20.5 % and 9.1 % of FW in Eiffel and iAGP-3, respectively (Table 2). In dry weight (DW) the starch content was 50.4 % and 30.3 % respectively. The iAGP-3 embryos contained two to three folds higher concentrations of soluble sugars (sucrose, glucose, fructose, Glc6P, Glc1P and Fru6P). Contrary to the increased soluble sugars the ADP-Glc concentration was significantly reduced in iAGP-3 embryos (27.8 ± 2.9 and 18.1 ± 1.8 nmol g^{-1} FW, *t*-test *p*-value 0.02; in Eiffel and iAGP-3, respectively).

As already shown in Weigelt *et al.* (2009) the AGPase activity in embryos of the iAGP-3 was drastically reduced (could be considered under detection limits) over the whole embryo development. The only significant difference found besides the AGPase activity in iAGP-3 embryos was the slightly increased (15 %) PGM activity (p=0.038, *t*-test). There was a slight tendency for enhanced iPPase activity (p=0.068, *t*-test). In the study of Weigelt *et al.* (2009) the activity of SuSy was increased in iAGP-3 embryos at 30 DAP. In contrast, in the present study the activities of SuSy were not significantly enhanced in iAGP-3 compared to Eiffel; not only at 30 DAP but at any investigated time point, although there was a slight tendency to increased SuSy activity from 16 DAF (Appendix 5).

Table 2. Content of dry matter (%), starch (% of FW), contents of metabolites (nmol g^{-1} FW) and enzyme activities (nmol min^{-1} g^{-1} FW) in maturing embryos (500 mg FW, 30 DAP) of wt Eiffel and its transgenic line iAGP-3

Results are given as mean ± SE of eight replicates. The significant differences were tested using ANOVA and the Student's *t*-test α=0.05. Measurement results below the limit of detection are marked by (<LOD).

	Eiffel	iAGP-3	t-test p-value	
dry matter	**40.7 ± 0.4 % FW**	**30.0 ± 1.4 % FW**	**0.000**	(n=6)
starch	**20.5 ± 1.2 % FW**	**9.1 ± 0.9 % FW**	**0.001**	
	nmol min^{-1} g^{-1} FW	**nmol min^{-1} g^{-1} FW**		
iPPase	673.9 ± 21.5	832.1 ± 68.0	0.068	
UGPase	4101.7 ± 128.6	4027.4 ± 297.2		
AGPase	**317.6 ± 31.0**	**4.6 ± 2.3 (<LOD)**	**0.000**	
SUSY	1036.6 ± 31.0	1093.7 ± 64.6		
PGM	**17504.5 ± 1085.1**	**20615.7 ± 718.2**	**0.038**	
PGI	13390.8 ± 621.2	12668.2 ± 557.4		
HK (glc)	125.3 ± 5.0	123.7 ± 6.9		
HK (fru)	88.7 ± 6.9	77.5 ± 2.5		
invertase	79.2 ± 3.3	109.9 ± 16.3		
	nmol g^{-1} FW	**nmol g^{-1} FW**		
sucrose	**58255.5 ± 6599.1**	**116723.8 ± 11265.8**	**0.001**	
starch	**923707.0 ± 61844.9**	**569116.8 ± 33386.9**	**0.001**	
ADP-glucose	**27.8 ± 2.9**	**18.1 ± 1.8**	**0.021**	
AMP	8.6 ± 1.5	9.1 ± 1.4		
ADP	16.8 ± 1.3	21.1 ± 1.4	0.051	
ATP	85.3 ± 7.7	98.8 ± 10.3		
glucose	**386.1 ± 48.3**	**1420.1 ± 78.8**	**0.000**	
fructose	**468.7 ± 47.4**	**1589.9 ± 84.5**	**0.000**	
Glc6P	**278.2 ± 11.0**	**857.2 ± 63.2**	**0.000**	
Fru6P	**56.1 ± 2.2**	**161.5 ± 14.7**	**0.000**	
Glc1P	**-0.8 ± 2.3 (<LOD)**	**21.3 ± 4.1**	**0.001**	
UDP-glucose	179.6 ± 10.0	169.3 ± 9.6		

Histology and subcellular volumes

In order to calculate subcellular concentrations, the subcellular volumes were determined by histological methods. Thereby the relative volumes of plastids, *cytosolic compartment*, cell wall and apoplast were measured. Similar to Weigelt *et al*. (2009), AGPase deficient pea embryos showed altered cell morphology compared to its parental line Eiffel. The storage parenchyma cells of embryos from Eiffel contained several large starch granules and that of iAGP-3 contained many smaller starch granules at 30 DAP (Figure 10). Plastids in both lines were nearly 100% filled with starch; therefore the relative area of starch granules was set equal to the relative plastid volume. The volume, or more precisely volume density, of organelles was calculated as the relative area of the embryo tissue at the sectional plane. The compartmental volume was calculated from micrographs using the ImageJ software and the principle of Delesse: "the areal density of profiles on sections is an unbiased estimate of the volume density of structures", as in (Winter *et al.* 1993). Starch granules in wild type embryo cotyledons added up to 42.7 ± 3.9 % and in iAGP-3 to 12.9 ± 2.7 % of the

embryo tissue (Figure 10 C, D, Table 3). Maturing pea embryo cells contained many small protein bodies and no distinguishable vacuole, as the large central vacuole present during early development turns into small vacuoles, which mainly contain storage proteins (Figure 10 A, B) (Boyer 1981; Craig *et al.* 1979; Hoh *et al.* 1995; Hillmer *et al.* 2001). The intercellular space of a cotyledon tissue was decreased in iAGP-3 embryos; the intercellular space amounted to 6.9 ± 0.2 and 5.4 ± 0.3 % (n=100; $p<0.001$) in wild type and iAGP-3, respectively. The volume of cell wall added up to 12.1 ± 0.1 % and 12.3 ± 0.1 % in Eiffel and iAGP-3, respectively (n=25; $p=0.74$). The density of maturing pea embryos did not differ significantly and was about 1 g ml^{-1} (1.08 ± 0.01 g ml^{-1} in Eiffel 1.01 ± 0.05 g ml^{-1} in iAGP-3; note that the density was only measured twice).

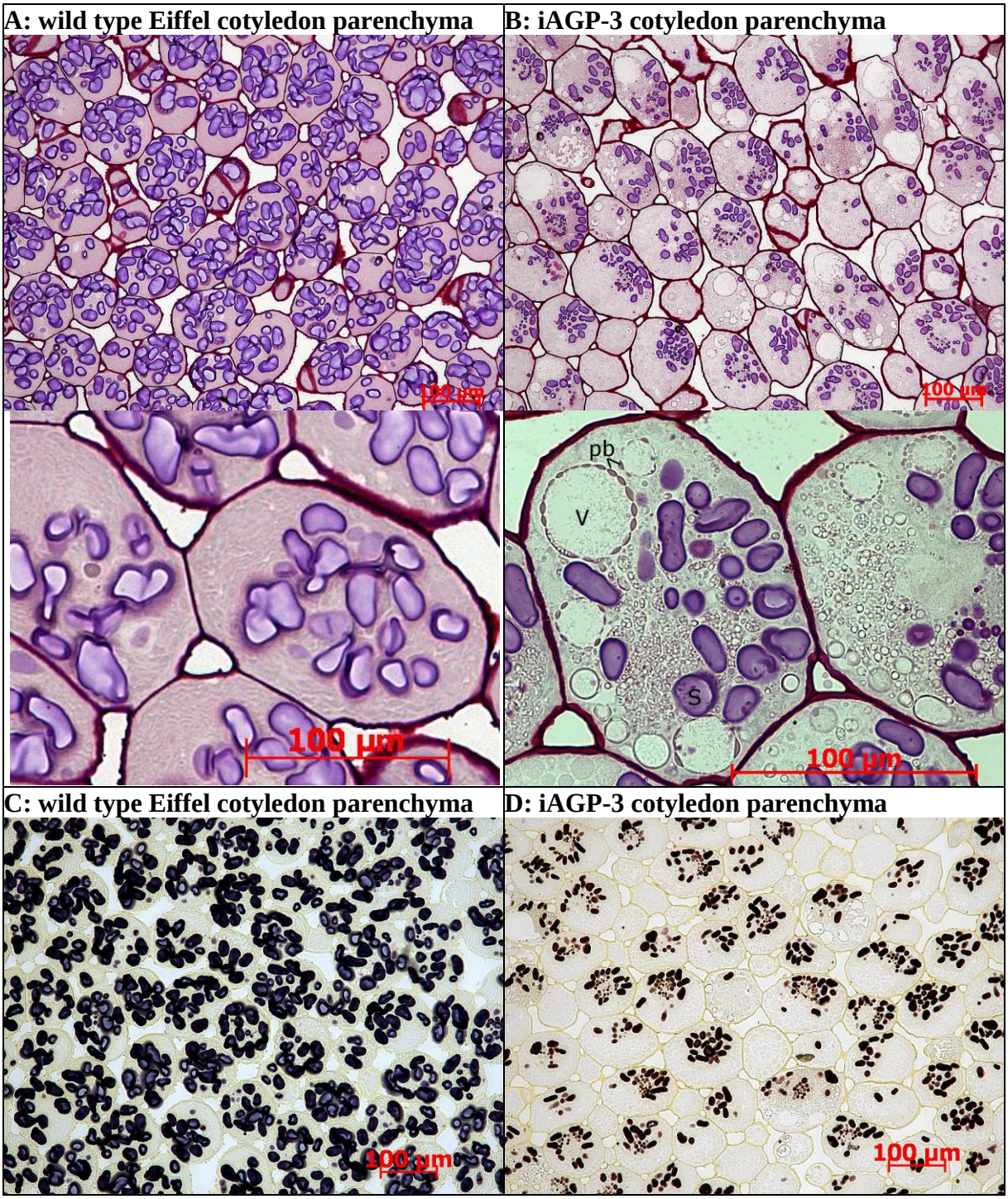


Figure 10. Cotyledon parenchyma of wild type Eiffel (A, C) and iAGP-3 (B, D). Slides (2.5 µm thin) photographed in A and B were stained with basic fuchsin followed by brief counterstaining with crystal violet. Slides (2.5 µm thin) photographed in C and D were stained with Lugol's solution. In A and B, the lower pictures represent a small section of the upper picture. The lower picture of B illustrates the big vacuoles (V) and protein bodies (pb) along the vacuole membrane in iAGP-3 embryos as well as starch granules (S).

Subcellular compartmental volumes and calculation of subcellular concentrations

The interest of the histological study was to define the reaction space volumes. Special interest was given to the plastidial and cytoplasmic reaction spaces. The conducted histological analysis did not allow a precise estimation of the volume of the cytosolic compartment; as the cytoplasm is a

complex compartment containing several organelles *e.g.* mitochondria, endoplasmatic reticulum (ER) and protein bodies. Therefore "*cytosolic compartment*" represents here the non-plastid liquid fraction, which contains the cytosol, apoplast, vacuoles and other minor liquid spaces. Due to this, the subcellular reaction spaces were evaluated, see Table 3, both for plastids and the non-plastid liquid fraction (later also referred to as the cytosol or *cytosolic compartment*). The non-plastid liquid fraction in Eiffel amounted to 8.6 % and 39.41 % in iAGP-3. The total volume of the plastids was approximately equal to the starch granule volume, which was directly measured. Still, the volume of the stromal and envelope space, which is the *plastid mobile phase*, had to be estimated. The volume of the *plastids mobile phase* is very small and the probable volume of the envelope space and stroma was estimated to be about 10 % according to the description for potato tuber amyloplasts (Kosegarten and Mengel 1994). Therefore the mobile phase volume of plastids was estimated to be 4.3 % and 1.3 % of total tissue volume and 5.3% and 1.5% of total cell volume for Eiffel and iAGP-3, respectively.

The calculations of subcellular relative and absolute concentrations were performed as follows:

$$Conc.\,in\,compartment = tissue\,content * \frac{confinement\ to\ compartment\ (\%)}{organelle\,volume\ \ \%}.$$

The values of metabolite concentrations and enzyme activities are given in Table 2 and compartmental confinement values (calculated using BestFit tool) are given in Appendix 3.

Table 3. Subcellular compartment volumes in maturing pea *cv. Eiffel and iAGP3) embryo cells

	Eiffel	**iAGP3**	
Data from this study	**% of tissue**		
starch granule	42.7±0.5	12.6±0.2	*p*=0.000
cell wall	12.1±0.1	12.3±0.1	*p*=0.745
apoplasm	6.9±0.2	5.4±0.3	*p*=0.000
cytosol + apoplasm	**8.59**	**39.41**	represents the non-plastidial compartment
Data from other publications			
nucleus	3.5	3.5	Briarty *et al.* 1980, *Phaseolus vulgaris* cotyledon
ER	22	22	Briarty *et al.* 1980, *Phaseolus vulgaris* cotyledon
mitochondria	0.5	0.5	Farre *et al.* 2001, potato tuber
protein bodies	10.57	9.70	Weigelt *et al.* 2009, Craig *et al.* 1979, pea Colombek *et al.* 2001, faba bean Weichert *et al.* 2010, wheat

Non-aqueous fractionation of pea embryo cells

Six separate fractionations of both pea lines – Eiffel and iAGP-3 – were performed. In this study GAPDH, iPPase and starch were used as organelle markers for plastids, and PEPCase and α-mannosidase as cytosolic and vacuolar markers, respectively (Figure 11). Pea belongs to the dicotyledonous species and therefore the localization of AGPase has been described to be only in

plastids. Previous studies utilizing NAF have used AGPase as a plastidial marker *e.g.* potato tuber (Farré *et al.* 2001) and spinach leaves (Gerhardt and Heldt 1984). AGPase was not used as a marker in this study, as the iAGP-3 embryos have an inhibited AGPase activity and because the localization of AGPase was subject of this study. The use of UGPase as a cytosolic marker was omitted due to its possible additional localization in plastids (Kimura *et al.* 1992; Okazaki *et al.* 2009). In embryos of cv. Eiffel the GAPDH and iPPase activity distribution over fractions correlated strongly with the starch and AGPase activity distributions (Table 4). The AGPase distribution in fractions of iAGP-3 embryos was very similar to the cytosolic as well as the vacuolar marker (Figure 11 b; Appendix 4). Furthermore the iPPase and GAPDH activity distribution is similar in both lines confirming them as suitable plastidial markers. No significant difference (*t*-test) was found between the cytosolic and vacuolar marker distribution (PEPCase and α-mannosidase, respectively) in either line. Therefore only two subcellular compartments were distinguished in the pea embryo cells: the plastidial and the *cytosolic compartment*.

The accuracy of NAF was tested using Western blot (protein immunoblot) with specific organelle antibodies (Table 5). Antibodies against the AGPase small subunit and the Rubisco large subunit (RbLs) were used to test the distribution of plastids in the NAF fractions and UGPase antibodies were used to test the distribution of the cytosolic compartment. Although UGPase is potentially also localized in plastids at a low rate, it was still used as an indicator for the cytosol due to the lack of alternative antibodies against cytosolic enzymes and as it had very high correlation with other cytosolic markers (PEPCase and SuSy; Table 4). Proteins were extracted from the six pellets (fractions) achieved by differential centrifugation NAF; see Chapter 2 for the detailed method. AGPase from the extracts of Eiffel was strongly confined to the first pellet fractions of the NAF, whereas the AGPase antibody reaction of iAGP-3 had in all fractions a very week signal. AGPase signal in iAGP-3 fractions correlated to the whole protein amount and also to the UGPase antibody signal. The RbLs antibody signal resembled more the wild type AGPase antibody signal distribution in the fractions than that of iAGP-3. The antibody blots showed some contamination interfering the fractionation efficiency.

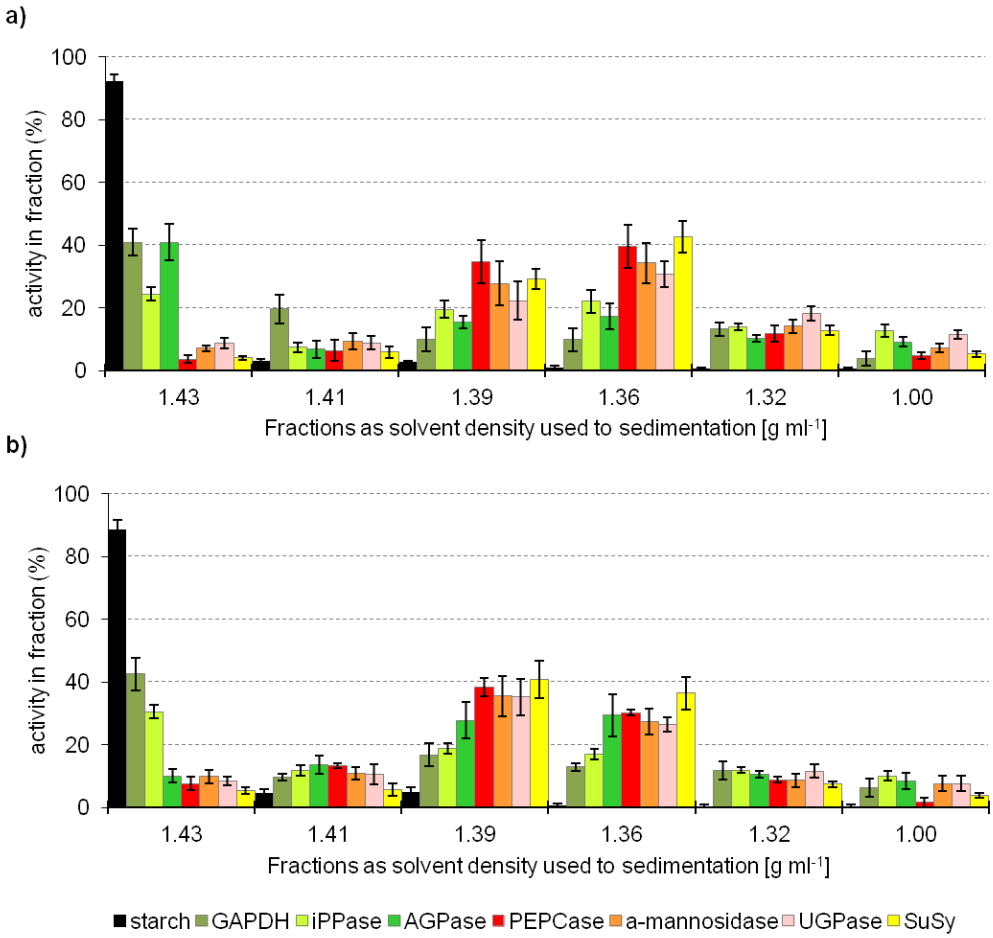


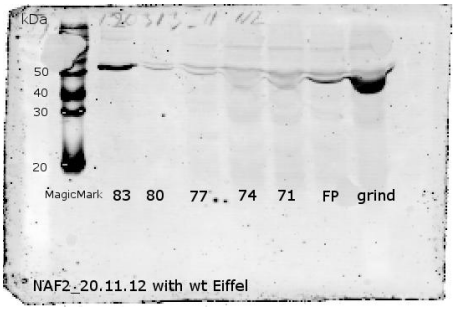
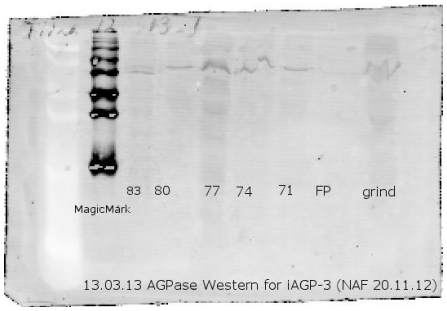
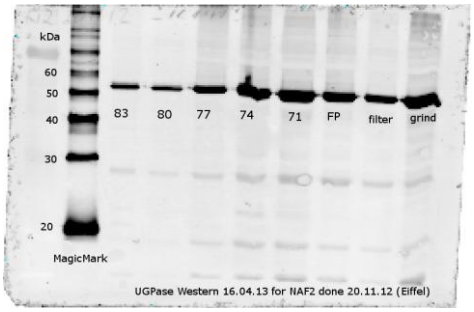
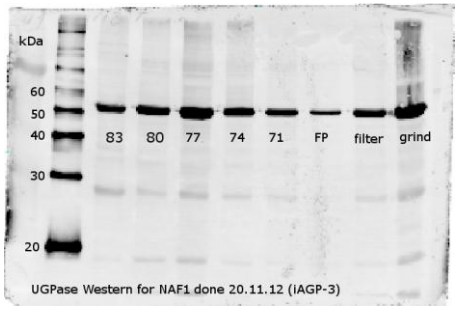
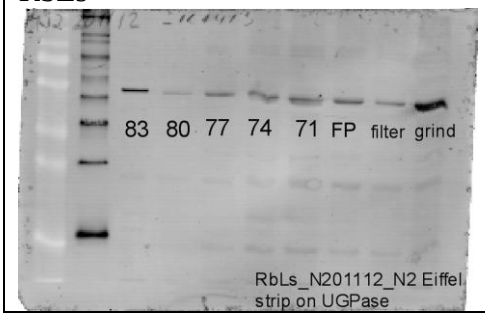
Figure 11. Subcellular distribution of organelle markers over sedimentation fractions a) of wt Eiffel embryos and b) its transgenic line iAGP3. Data points are the mean of six replicates of fractionation ± SE.

Table 4. Correlation of subcellular markers in NAF performed with maturing embryos of cv. Eiffel

Pearson's correlations coefficient *r* and its *p*-value at α=0.05 were calculated using relative activities in pellet fractions.

Pearson's *r*	**iPPase**	**AGPase**	**UGPase**	**α-mannosidase**	**SuSy**	**starch**	**GAPDH**
iPPase							
AGPase	0.65						
UGPase	0.38	-0.10					
α-mannosidase	0.50	0.12	0.78				
SuSy	0.54	0.11	0.86	0.97			
starch	0.42	0.72	-0.36	-0.32	-0.32		
GAPDH	0.26	0.52	-0.33	-0.27	-0.26	0.43	
PEPCase	0.14	-0.01	0.46	0.56	0.46	-0.32	-0.11
p-value	**iPPase**	**AGPase**	**UGPase**	**α-mannosidase**	**SuSy**	**starch**	**GAPDH**
iPPase							
AGPase	0.000						
UGPase	0.013	0.525					
α-mannosidase	0.001	0.451	0.000				
SuSy	0.000	0.504	0.000	0.000			
starch	0.006	0.000	0.020	0.040	0.041		
GAPDH	0.103	0.000	0.031	0.082	0.102	0.004	
PEPCase	0.379	0.955	0.002	0.000	0.002	0.039	0.508

Table 5. Localization of subcellular marker enzymes in six pellet fractions of NAF (83. 80,… 71) and in grind plant material determined by specific antibodies against AGPase, UGPase and RbLs using Westen blot.

Western blots for NAF done with Eiffel	Western blots for NAF done with iAGP-3
AGPase 	AGPase 
UGPase 	UGPase 
RbLs 	RbLs 

Subcellular confinement of enzymes and metabolites related to starch synthesis

Subcellular confinement of enzymes and metabolites into plastids and *cytosolic compartement* revealed only some differences between cv. Eiffel and the transgenic line iAGP-3 (Figure 12 a, b). Generally, both metabolites and enzymes were confined more strongly to the cytosol than to plastids with the exception of starch, which was 100% confined to plastids (Figure 12 c, d). Aside from AGPase itself, significant differences between Eiffel and iAGP-3 were found in the confinement of PGM and UDP-Glc. In iAGP-3 embryos the relative confinement of PGM to plastids was increased and the confinement of UDP-Glc to plastids was decreased.

The plastidial marker enzyme GAPDH had average plastidial confinements of 97.3 ± 0.2 % and 94.0 ± 1.7 % in Eiffel and iAGP-3, respectively. The cytosolic marker enzyme PEPCase had average cytosolic confinement of 98.3 ± 0.9 % and 95.3 ± 2.0 %. Subcellular markers – GAPDH, iPPase, PEPCase, α-mannosidase – had on average 6.2 % deviation from 100%, which indicates the reliability range of markers.

Some enzymes and metabolites were almost equally confined to plastids and cytosol. Fru6P localized nearly equally in plastids and cytosol in Eiffel and iAGP-3. Othen than Fru6P, in iAGP-3 PGM, HK (glc and fru), ADPglc, and ADP had relatively equal distribution between plastids and cytosol. Also AMP and Glc1P were equally confined to plastids and cytosol, but the results of AMP and Glc1P should be considered with caution due to the very low concentrations measured, which can lead to false interpretation (Tiessen *et al.* 2002).

In order to further test the significance of difference or similarity, the Fischer's LSD test was applied. According to Fisher's LSD test for Eiffel, similarly to starch, both GAPDH and AGPase were confined 100% to plastids. In contrast PEPCase, PGI and SuSy were confined 100% to the cytosol. In iAGP-3 the Fisher's LSD test showed that GAPDH and iPPase are similar to starch confined in plastids, in parallel the PEPCase, SuSy, PGI, α-mannosidase and UGPase were similarly confined to the cytosol. The shift of the UGPase confinement to the cytosol was similar to that of UDP-Glc in iAGP-3 embryos. Fisher's LSD for the metabolites showed that, sucrose, ADP and ATP were significantly cytosolic in Eiffel. In iAGP-3 the sucrose, ATP, glucose, fructose, Glc6P and UDP-Glc were predominantly cytosolic.

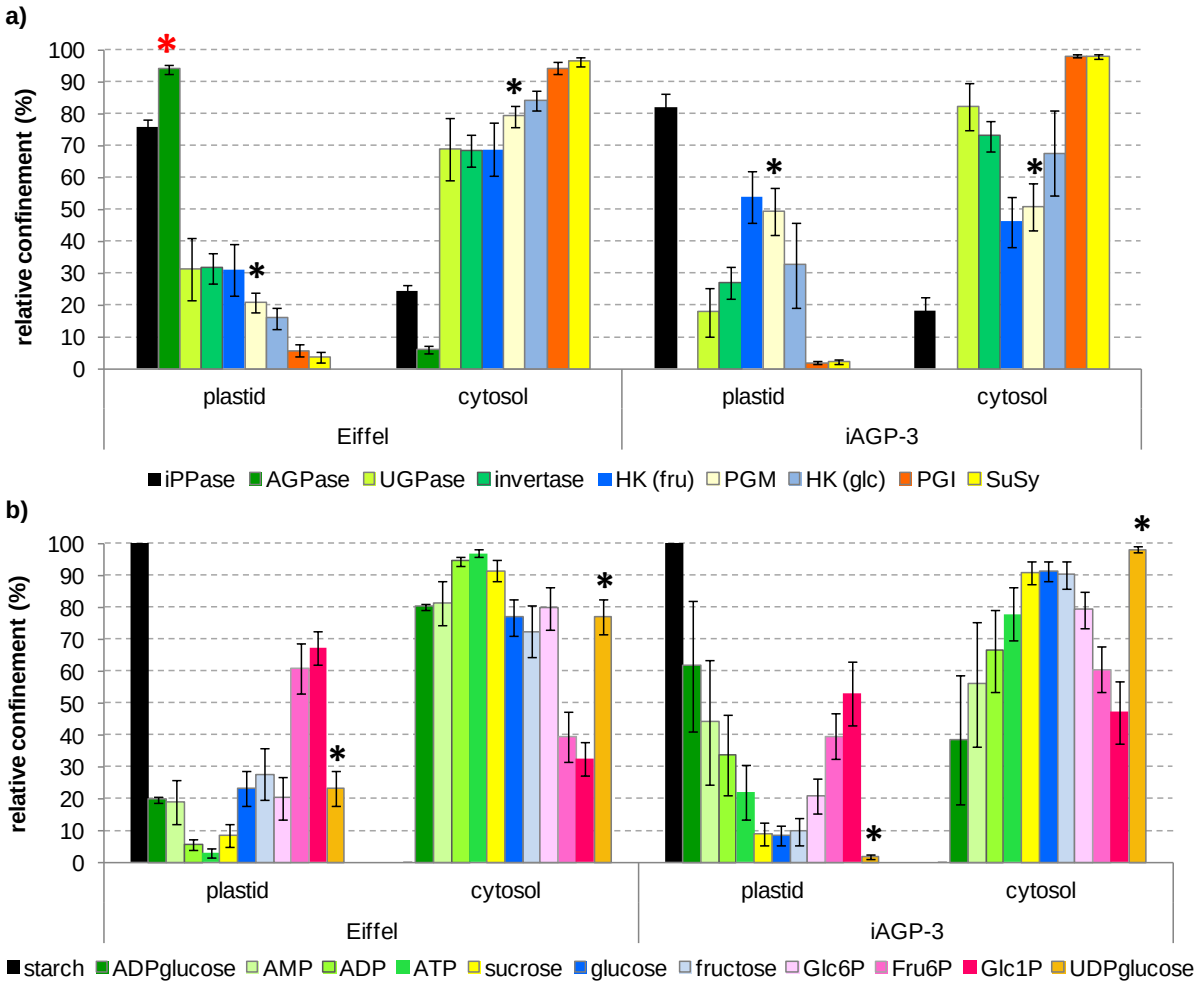


Figure 12. Relative cellular confinement of enzyme activities (a) and metabolites (b) in pea cv. Eiffel and its transgenic line iAGP3 embryos in two cell compartments – plastid and cytosol – according to BestFit analysis. Data points are means from three replicates of fractionation ± SE. The asterisks (*) indicates a significant difference at a 0.05% level between the wild type and Eiffel.

Subcellular concentrations of metabolites and enzymes related to starch synthesis

Several significant differences were detected in the absolute subcellular concentrations of metabolites and enzyme activities in pea embryos of cv.Eiffel and iAGP3 (Table 6).

Changes in absolute concentrations and enzyme activities were predominantly found in the *cytosolic compartment*. The *cytosolic compartment* of iAGP-3 maturing embryos had a much lower activities of starch related enzymes and concentrations of sucrose, adenylate nucleotides, and that of Glc6P, ADP-Glc and UDP-Glc. In the plastids of iAGP-3 the concentrations of Glc6P, Fru6P and Glc1P were significantly enhanced. Adenylate concentrations showed a trend towards elevated levels in the plastids of iAGP-3 embryos. This corresponds with the repressed AGPase activity as its substrates, ATP and Glc1P, cannot be used for starch synthesis. Contrary to the expected reduction in ADP-Glc concentration in iAGP-3 plastids, the analysis revealed similarly high concentrations of ADP-Glc as in the

plastids of Eiffel, even slightly higher. Simultaneously, the ADP-Glc concentration was reduced in the iAGP-3 cytosol. Significant change was also detected in UDP-Glc concentrations; thereby both the plastidial and cytosolic UDP-Glc concentrations were much lower in iAGP-3 than in Eiffel. The plastidial absolute activities of iPPase, PGM, HK (fru) and Inv were significantly increased in iAGP-3 embryos.

The absolute starch concentration in iAGP-3 plastids, based on the measured starch granule volume, was twice as high as that of the Eiffel plastids. This was in contraticton to the expectation that both lines should have approximately the same starch concentration in plastids, as both lines have fully developed amyloplasts consisting mainly of starch granules at the studied development stage. In iAGP-3 the plastidial volume was expected to be nearly 50% of the Eiffel plastids based on the 50% reduction of starch concentration in whole embryos, but the histological work showed about 70% reduction of starch granules in iAGP-3. It is conceivable, that the volume of plastids in iAGP-3 embryos was underestimated by the histological method employed. It might be that the plastids/starch granules of iAGP-3 are denser as the amylose rich starch has higher density (Yoon and Lin 2003). When the subcellular concentrations for iAGP-3 were calculated using the expected relative volume of plastids in a cotyledon tissue (25%), the results did not differ largely from values calculated with 12.6 % plastid volume (Table 7). Moreover, similar significant differences between Eiffel and iAGP-3 were observed. These results emphasize the strong effect of the repressed AGPase on the relative subcellular distribution of the enzymes PGM and HK (glc and fru), even though the subcellular volume of plastids in iAGP-3 may be underestimated.

Table 6. Subcellular enzyme activities (mM min^{-1} FW) and metabolite concentrations (mM)

Data is the mean of six fractionations performed both with embryos of Eiffel and iAGP-3 ± SE. The statistically significant (p<0.05) differences between Eiffel and iAGP-3 are marked by bold font; thereby the lower values are colored blue and higher values green. The values under detection limit are signed with *.

	subcellular concentrations (mM)		relative subcellular concentrations (%)	
Eiffel	**plastid**	**cytosol**	**plastid**	**cytosol**
iPPase	**11.97 ± 0.37**	**1.89 ± 0.18**	**86.3 ± 1.5**	**13.7 ± 1.5**
UGPase	29.97 ± 9.29	**32.84 ± 4.62**	43.4 ± 11.7	56.6 ± 11.7
AGPase	**6.99 ± 0.09**	**0.22 ± 0.05**	96.9 ± 0.7	3.1 ± 0.7
SUSY	0.88 ± 0.36	**11.63 ± 0.18**	**6.9 ± 2.7**	**93.1 ± 2.7**
PGM	**85.24 ± 13.08**	**161.38 ± 6.51**	**34.0 ± 4.4**	**66.0 ± 4.4**
PGI	17.85 ± 5.68	**147.01 ± 2.82**	**10.6 ± 3.1**	**89.4 ± 3.1**
HK (glc)	0.33 ± 0.07	**0.87 ± 0.03**	**26.7 ± 4.7**	**73.3 ± 4.7**
HK (fru)	**0.91 ± 0.24**	**1.01 ± 0.12**	**44.6 ± 9.5**	**55.4 ± 9.5**
invertase	**0.59 ± 0.09**	**0.63 ± 0.04**	**47.1 ± 5.5**	**52.9 ± 5.5**
sucrose	115.90 ± 47.59	**620.54 ± 23.67**	**14.8 ± 5.7**	**85.2 ± 5.7**
starch	**2161.99 ± 0.00**	0.00 ± 0.00	100.0 ± 0.0	0.0 ± 0.0
ADPGlc	0.13 ± 0.01	**0.26 ± 0.00**	**33.0 ± 1.3**	**67.0 ± 1.3**
AMP	0.04 ± 0.01	**0.08 ± 0.01**	**30.1 ± 9.8**	**69.9 ± 9.8**
ADP	0.02 ± 0.01	**0.18 ± 0.00**	**10.4 ± 2.7**	**89.6 ± 2.7**
ATP	0.06 ± 0.03	**0.96 ± 0.01**	5.7 ± 2.4	94.3 ± 2.4
Glc	2.09 ± 0.51	3.46 ± 0.25	35.9 ± 7.7	64.1 ± 7.7
Fru	3.03 ± 0.89	3.95 ± 0.44	40.4 ± 9.3	59.6 ± 9.3
Glc6P	**1.32 ± 0.43**	**2.58 ± 0.22**	**31.4 ± 8.9**	**68.6 ± 8.9**
Fru6P	**0.80 ± 0.10**	0.26 ± 0.05	**74.2 ± 6.2**	**25.8 ± 6.2**
Glc1P *	**0.02 ± 0.00**	**0.01 ± 0.00**	**80.1 ± 3.7**	**19.9 ± 3.7**
UDPGlc	**0.97 ± 0.23**	**1.61 ± 0.11**	36.1 ± 7.3	63.9 ± 7.3
iAGP-3	**plastid**	**cytosol**	**plastid**	**cytosol**
iPPase	**53.87 ± 2.89**	**0.38 ± 0.09**	**99.2 ± 0.2**	**0.8 ± 0.2**
UGPase	56.40 ± 23.91	**8.41 ± 0.77**	75.0 ± 7.7	25.0 ± 7.7
***AGPase* ***	**0.10 ± 0.05**	**0.01 ± 0.00**	64.9 ± 15.0	35.1 ± 15.0
SUSY	1.90 ± 0.60	**2.71 ± 0.02**	**35.6 ± 9.1**	**64.4 ± 9.1**
PGM	**804.01 ± 121.13**	**26.53 ± 3.88**	**96.2 ± 0.9**	**3.8 ± 0.9**
PGI	18.66 ± 4.45	**31.54 ± 0.14**	**34.3 ± 6.7**	**65.7 ± 6.7**
HK (glc)	1.99 ± 0.81	**0.13 ± 0.03**	**85.9 ± 5.3**	**14.1 ± 5.3**
HK (fru)	**5.27 ± 0.77**	**0.14 ± 0.02**	**96.6 ± 1.1**	**3.4 ± 1.1**
invertase	**2.34 ± 0.42**	**0.20 ± 0.01**	**89.7 ± 3.0**	**10.3 ± 3.0**
sucrose	841.67 ± 329.61	**269.18 ± 10.57**	**61.2 ± 11.2**	**38.8 ± 11.2**
starch	**4504.14 ± 0.00**	0.00 ± 0.00	100.0 ± 0.0	0.0 ± 0.0
ADPGlc	0.88 ± 0.29	**0.02 ± 0.01**	**93.8 ± 5.0**	**6.2 ± 5.0**
AMP	0.32 ± 0.14	**0.01 ± 0.00**	**82.3 ± 14.5**	**17.7 ± 14.5**
ADP	0.56 ± 0.21	**0.04 ± 0.01**	**84.4 ± 10.6**	**15.6 ± 10.6**
ATP	1.72 ± 0.66	**0.20 ± 0.02**	69.0 ± 23.1	31.0 ± 23.1
Glc	9.58 ± 3.43	3.30 ± 0.11	58.8 ± 13.7	41.2 ± 13.7
Fru	12.23 ± 5.27	3.64 ± 0.17	60.8 ± 13.5	39.2 ± 13.5
Glc6P	**14.08 ± 3.79**	**1.72 ± 0.12**	**78.6 ± 9.6**	**21.4 ± 9.6**
Fru6P	**5.05 ± 0.92**	0.25 ± 0.03	**92.9 ± 3.1**	**7.1 ± 3.1**
Glc1P	**0.89 ± 0.17**	**0.03 ± 0.01**	**95.2 ± 2.5**	**4.8 ± 2.5**
UDPGlc	**0.24 ± 0.11**	**0.42 ± 0.00**	26.9 ± 11.5	73.1 ± 11.5

Table 7. Subcellular concentration distribution and absolute concentrations calculated from expected plastid volume (25% of cotyledon tissue) for iAGP-3 pea embryos

Data represents six separately done NAFs of pea iAGP-3 maturing embryos (30 DAP, 500 ± 20 mg FW) ± SE. Data in bold marks the significant differences between wild type embryos and iAGP-3 embryos; green is for higher concentration and blue for lower compared to wild type. Significant differences between the iAGP-3 measured plastid volume and expected volume (26.3%) are marked with *.

iAGP-3	plastid	cytosol		plastid	cytosol
iPPase	**25.86 ± 1.39**	**0.59 ± 0.14**		**97.6 ± 0.7**	**2.4 ± 0.7**
UGPase	27.07 ± 11.48	**12.89 ± 1.17**		54.4 ± 11.1	45.6 ± 11.1
AGPase *	***0.05 ± 0.02***	***0.01 ± 0.00***		***52.0 ± 16.8***	***48.0 ± 16.8***
SUSY	0.91 ± 0.29	**4.16 ± 0.03**		16.8 ± 4.8	83.2 ± 4.8
PGM	**385.92 ± 58.14**	**40.65 ± 5.95**		**89.0 ± 2.4**	**11.0 ± 2.4**
PGI	8.96 ± 2.14	**48.33 ± 0.22**		15.1 ± 3.4	84.9 ± 3.4
HK (glc)	0.96 ± 0.39	**0.20 ± 0.04**		**69.7 ± 9.4**	**30.3 ± 9.4**
HK (fru)	**2.53 ± 0.37**	**0.22 ± 0.04**		**90.3 ± 2.9**	**9.7 ± 2.9**
invertase	**1.12 ± 0.20**	**0.31 ± 0.02**		**74.7 ± 5.7**	**25.3 ± 5.7**
sucrose	404.00 ± 158.21	**412.41 ± 16.19**		40.2 ± 11.0	**59.8 ± 11.0**
starch	2161.99 ± 0.00	0.00 ± 0.00		100.0 ± 0.0	0.0 ± 0.0
ADPGlc	0.42 ± 0.14	**0.03 ± 0.01**		**85.6 ± 10.7**	**14.4 ± 10.7**
AMP	0.15 ± 0.07	**0.02 ± 0.01**		72.1 ± 18.8	27.9 ± 18.8
ADP	0.27 ± 0.10	**0.05 ± 0.01**		**71.0 ± 15.6**	**29.0 ± 15.6**
ATP	0.83 ± 0.32	**0.30 ± 0.03**		59.1 ± 20.0	40.9 ± 20.0
Glc	4.60 ± 1.65	**5.05 ± 0.17**		39.4 ± 11.1	60.6 ± 11.1
Fru	5.87 ± 2.53	**5.58 ± 0.26**		41.1 ± 11.2	58.9 ± 11.2
Glc6P	**6.76 ± 1.82**	2.64 ± 0.19		61.8 ± 11.8	38.2 ± 11.8
Fru6P	**2.42 ± 0.44**	0.38 ± 0.05		82.2 ± 6.3	17.8 ± 6.3
Glc1P	**0.43 ± 0.08**	**0.04 ± 0.01**		87.6 ± 5.7	12.4 ± 5.7
UDPGlc	**0.11 ± 0.05**	**0.65 ± 0.01**		**13.2 ± 5.9**	**86.8 ± 5.9**

Bioenergetics of starch synthesis in pea embryos

The energy change and the direction of starch synthesis related reactions were evaluated using the standard free energy change constants (ΔG`°, from http://equilibrator.weizmann.ac.il) and mass action ratios (Q) of the reactions (Table 8).

The concentrations of UDP and UTP were taken from the studies of developing potato tuber (Farre *et al.* 2001 and 2006). The concentration of PPi was below the detection limit; therefore the value of 9.4 nmol g^{-1} was taken from Edwards *et al.* (1984). The Pi concentrations were 16164 ± 790 nmol g^{-1} and 10313 ± 1012 nmol g^{-1} in embryos of Eiffel and in iAGP-3, respectively (*t*-test p>0.001; n=6).

At whole embryo level the Q showed trends to be far from equilibrium (K'_{eq}), in particular the Q of Inv, HK (glc and fru) as well as iPPase and starch synthase (SS) (Table 8 A). Significant differences in mass action ratios between Eiffel and iAGP-3were found for the reactions of SuSy, Inv, UGPase and pPGM (*t*-test; α=0.05; n=6). In general, the calculated free energy change (ΔG) favored the reactions towards starch synthesis, although the cytosolic PGM reaction was directed towards Glc1P synthesis instead of Glc6P and the PGI reaction favored the Fru6P synthesis, which supports the flux towards glycolysis. Significant differences in ΔG between the Eiffel and iAGP-3 embryos were found for the reactions of SuSy, Inv and AGPase. The AGPase reaction estimated at the whole cell level in iAGP-3 seemed to be further from equilibrium and more strongly shifted towards starch synthesis.

At the subcellular level the biochemical energy parameters were quite similar to that at the whole tissue level (Table 8 B). The Q differed between Eiffel and iAGP-3 in cytosolic SuSy, Inv, HK (glc), PGI, UGPase and AGPase. In plastidial compartment only the Q of SS was significantly different between Eiffel and iAGP-3; thereby the starch synthesis reaction was slightly closer to equilibrium in iAGP-3. The ΔG in plastids was significantly different for AGPase, UGPase and the SS reaction. In the *cytosolic compartment* the ΔG was significantly different for SuSy, Inv, HK (glc), AGPase and UGPase. In Eiffel the ADP-Glc synthesis was further from equilibrium in plastids compared to iAGP-3 and directed rather towards Glc1P synthesis than towards ADP-Glc synthesis. This kind of tendency could be explained by very low ATP and Glc1P concentrations in Eiffel due to efficient starch synthesis and irreversibility of iPPase reaction *in vivo*. Higher concentration of Glc1P in iAGP-3 was also reflected by the plastidial UGPase reaction, which was out of equilibrium and energetically directed towards UDP-Glc synthesis. In the *cytosolic compartment* of both pea lines the UGPase reaction was directed towards the Glc1P synthesis.

Table 8. Bioenergetics of starch synthesis in maturing pea embryos of cv. Eiffel and iAGP-3: Gibbs free-energy change (ΔG), equilibrium constant ($K`_{eq}$) and mass action ratio (Q)

Values for the standard free energy change for the biochemical standard state (ΔG`°) was taken from http://equilibrator.weizmann.ac.il , except the starch synthase (SS), which was taken from the MetaCyc database.

A)Biochemical energy parameters at whole embryo level for pea cv. Eiffel and iAGP-3

The mass action ratio (Q) and free energy change (ΔG) were calculated using the equilibrium constant ($K´_{eq}$) and standard free energy change (ΔG´°) (constants for the biochemical standard state pH 7.0 and T=25°C).

Enzyme	Eiffel		iAGP_3		ΔG`° [kJ/mol]	$K`_{eq}$	reaction
	Q	ΔG	Q	ΔG			
SuSy	***0.19 ± 0.02***	***-2.74 ± 0.23***	***0.30 ± 0.03***	***-1.61 ± 0.26***	1.5	0.55	(UDPglc*fru)/(suc*UDP)
Inv	***3.90 ± 0.39***	***-22.63 ± 0.24***	***21.81 ± 2.45***	***-18.42 ± 0.33***	-25.9	34322	(glc*fru)/suc
HK (glc)	0.13 ± 0.01	-30.00 ± 0.26	0.14 ± 0.01	-29.77 ± 0.14	-24.9	22933	(Glc6P*ADP)/(glu*ATP)
HK (fru)	0.02 ± 0.00	-31.50 ± 0.17	0.02 ± 0.00	-31.30 ± 0.13	-22	7122	(Fru6P*ADP)/(fru*ATP)
PGI	0.21 ± 0.01	-4.13 ± 0.10	0.19 ± 0.00	-4.34 ± 0.06	-0.2	1.08	Fru6P/Glc6P
UGPase	***-0.07 ± 0.09***	-2.47 ± 0.95	***0.69 ± 0.07***	0.58 ± 0.25	1.6	0.52	(Glc1P*UTP)/(UDPglc*PPi)
PGM cyt	37.46 ± 29.41	1.76 ± 0.70	41.69 ± 3.58	1.36 ± 0.23	-7.8	23.22	Glc6P/Glc1P
PGM pla	***-0.01 ± 0.01***	-1.76 ± 0.70	***0.03 ± 0.00***	-1.36 ± 0.23	7.8	0.04	Glc1P/Glc6P
AGPase	0.47 ± 0.37	***-0.64 ± 0.43***	0.08 ± 0.01	***-8.02 ± 0.32***	-1.7	1.98	(ADPglc*PPi)/(ATP*Glc1P)
iPPase	27794920 ± 0.00	17.57 ± 0.00	11313971 ± 0.00	15.34 ± 0.00	-24.9	22933	(Pi*Pi)/PPi
SS	579946 ± 52597	-20.36 ± 0.24	687010 ± 58921	-19.92 ± 0.21	-53.15	2028658740	(starch*ADP)/ADPglc

B)The biochemical energy parameters calculated for the plastidial and *cytosolic compartment* of maturing pea cotyledon cells of cv. Eiffel and iAGP-3

The mass action ratio (Q) and free energy change (ΔG) were calculated using constants at the biochemical standard state (pH 7.0 and T=25°C).

Enzyme	Eiffel				iAGP-3			
	Q		ΔG		Q		ΔG	
	plastid	cytosol	plastid	cytosol	plastid	cytosol	plastid	cytosol
SuSy	0.88 ± 0.20	***0.17 ± 0.01***	-1.51 ± 0.91	***-3.12 ± 0.21***	0.00 ± 0.00	***0.42 ± 0.03***	-6.09 ± 1.20	***-0.67 ± 0.07***
Inv	0.13 ± 0.03	***0.02 ± 0.00***	-27.32 ± 2.33	***-35.48 ± 0.21***	0.13 ± 0.07	***0.04 ± 0.00***	-20.83 ± 2.73	***-33.62 ± 0.07***
HK (glc)	0.31 ± 0.09	***0.14 ± 0.00***	-28.81 ± 0.59	***-29.72 ± 0.07***	0.70 ± 0.27	***0.06 ± 0.01***	-19.63 ± 3.29	***-30.89 ± 0.15***
HK (fru)	0.28 ± 0.09	0.01 ± 0.00	-26.54 ± 0.71	-33.27 ± 0.30	0.14 ± 0.05	0.01 ± 0.00	-19.64 ± 3.27	-33.35 ± 0.20
PGI	1.09 ± 0.14	***0.09 ± 0.01***	-0.62 ± 0.32	-6.27 ± 0.21	0.77 ± 0.44	***0.14 ± 0.01***	-2.08 ± 0.38	-5.06 ± 0.08
PGM	0.03 ± 0.01	85.79 ± 11.54	-2.27 ± 0.45	2.27 ± 0.45	0.22 ± 0.13	27.92 ± 14.77	1.63 ± 0.66	-1.63 ± 0.66
AGPase	7.78 ± 1.75	***8.58 ± 0.96***	***2.73 ± 0.50***	***3.43 ± 0.29***	0.09 ± 0.05	***0.05 ± 0.03***	***-5.48 ± 0.99***	***-10.32 ± 0.78***
iPPase	71859 ± 0	289156 ± 0	2.81 ± 0.00	6.25 ± 0.00	98906 ± 0	25653 ± 0	3.60 ± 0.00	0.25 ± 0.00
UGPase	0.18 ± 0.03	***0.01 ± 0.00***	***-3.45 ± 0.35***	***-9.58 ± 0.22***	39.36 ± 33.02	***0.30 ± 0.06***	***5.97 ± 0.94***	***-1.73 ± 0.25***
SS	**376.6** ± 60.0	0.00 ± 0.00	***-38.80 ± 0.37***	0.00 ± 0.00	**2567.9** ± 316.4	0.00 ± 0.00	***-33.8 ± 0.17***	0.00 ± 0.00

Discussion

This study focussed on the starch synthesis network in pea embryos. The analyzed starch synthesis pathway starts with the import of sucrose into the cytosol and ends with starch synthesis in plastids. The emphasis here was on the subcellular network, described by enzyme activities (as maximal velocities) and concentrations of related substrates and products. Thereby the main aim was to investigate the metabolism of ADP-Glc, as the additional source of ADP-Glc was hypothesized to be in the cytosol. Therefore starch synthesis was studied by analyzing the transgenic pea line having repressed AGPase (both expression and activity; Weigelt *et al.* 2009) in maturing pea embryos. The subcellular analysis was done by the method of non-aqueous fractionation and a mathematical estimation of the distribution.

On the whole embryo level the results of the present study were similar to that found previously in iAGP-3 (Weigelt *et al.* 2009) and as well in genus *Vicia* (Weber *et al.* 1995b; Weber *et al.* 2000b; Rolletschek *et al.* 2002). These studies have shown that the drastically reduced AGPase activity leads to a moderate starch reduction with concomitantly elevated concentrations of soluble sugars in legume embryos. In the active storage synthesis phase the activities of UGPase, PGM and PGI as well as their expression levels were increased in iAGP-3 (present study and Weigelt *et al.* 2009). These results indicate that the enhanced sugars/substrate concentrations activate these enzymes and that the repression of AGPase leads to higher rate of glycolysis.

In comparison to the pea embryos the potato tubers, which also have a high starch synthesis rate, the AGPase repression has showed slightly different effects. The reduced AGPase activity in potato tubers led to increased soluble sugars (sucrose and hexose-P) too, while the starch content was much more reduced compared to pea (up to 4-35%; Müller-Röber *et al.* 1992). Thereby the AGPase exerts relatively high control over the starch accumulation in developing potato tubers (control coefficient 0.55), in contrast to pea embryos (Sweetlove *et al.* 1999; Denyer *et al.* 1995). Furthermore, the antisense repression of the small subunit AGPase in potato did not affect the activities or expression (mRNA) of enzyme activities related to starch synthesis (PGM, SuSy, UGPase, iPPase, fructokinase, soluble and granule bound starch synthases, and starch branching enzyme) (Sweetlove *et al.* 1999; Müller-Röber *et al.* 1992). The increase in SPS expression (also in iAGP-3 embryos) and in selective activity (Fru6P), indicates the enhanced sucrose turnover in potato tubers and in pea embryos.

Subcellular localisation of AGPase and ADP-Glc

In the present study the AGPase was strongly confined to plastids in the embryo cells of Eiffel, which is in accordance with previous observations in legumes (Levi and Preiss 1978; MacDonald and ap Rees 1983; Denyer and Smith 1988). On the other hand the hypothesis of the existence of cytosolic ADP-Glc was supported by the strong confinement of ADP-Glc to the cytosol both in Eiffel and iAGP-3; though in dicotyledonous plants the ADP-Glc is supposed to be synthesized solely by the plastidial AGPase and to accumulate only in plastids. Interestingly the confinement of ADP-Glc in the cytosol and its concentration in iAGP-3 in the cytosol was much lower, being mainly plastidial, which is more in line with the result of NAF analyses of potato tubers (98% of ADP-Glc in plastids) (Tiessen *et al.* 2002, 2012). In contrast to dicots, the high cytosolic ADP-Glc concentration is typical for monocot cereals, which have a cytosolic AGPase in addition to the plastidial AGPase (Beckles et al. 2001b; Tiessen *et al.* 2012). The cytosolic ADP-Glc found in pea embryos raises the question about the source of ADP-Glc in the cytosol, when the AGPase is localized exclusively in plastids. This question was recently addressed by Bahaji *et al.* (2011), as the *Arabidopsis thaliana* AGPase knockout lines accumulate high rates of cytosolic ADP-Glc in leaves, even though the starch content is strongly reduced. Their study supports a hypothesis of alternative starch synthesis pathway in dicots, which has been under discussion for two decades (Pozueta-Romero *et al.* 1991a; Pozueta-Romero *et al.* 1991b; Baroja-Fernández *et al.* 2001; Baroja-Fernández *et al.* 2003; Baroja-Fernández *et al.* 2004; Muñoz *et al.* 2005; Baroja-Fernández *et al.* 2009; Bahaji *et al.* 2011). This hypothesis proposes that ADP-Glc is also synthesized in the cytosol of dicots, thereby the missing cytosolic AGPase is supposed to be substituted by SuSy and the ADP-Glc is transported via a yet unidentified adenylate transporter into plastids. Another indication for the cytosolic ADP-Glc synthesis in pea could be provided by the work of Beckles *et al.* (2001b). They compared the ratio of ADP-Glc and UDP-Glc in monocot and dicot seeds and found that the ADP-Glc to UDP-Glc ratio of about 0.5 is typical for species having a cytosolic AGPase besides the plastidial AGPase. In contrast, a ratio of about 0.03 was found to be typical for dicots, which were classified as having only a plastidial AGPase. They analyzed very young pea embryos during the stage when the AGPase activity is close to zero and the ADP-Glc to UDP-Glc ratio was found to be approximately 0.03 (Weigelt *et al.* 2009; Beckles *et al.* 2001b). In contrast the broad bean embryos analyzed at maturity had a ratio of 0.18, which is close to the mature pea embryos analyzed here (0.15 and 0.11 in wild type and iAGP-3, respectively). This shift may be due to a higher AGPase activity coupled with a higher starch synthesis rate in mature embryos when compared to young embryos or leaves analyzed in Beckles *et al.* (2001b). AGPase activity is developmentally changing, in

young embryos of pea the activity of AGPase is close to zero and the activity starts to rise starting from the mid seed filling phase (in Weigelt *et al.* 2009 from 25 DAP). Or this shift in the ADP-Glc to UDP-Glc ratio could be due to the synthesis of ADP-Glc in the cytosol.

The ADP-Glc synthesis in the cytosol and an ADP-Glc transporter would explain the relatively high ADP-Glc concentrations and the starch synthesis in plastids of the iAGP-3 embryos, where the AGPase activity was drastically reduced. When the ADP-Glc synthesis takes place in the cytosol of dicotyledonous species, there should exist an active ADP-Glc transporter, which until now has not been shown to play a role in starch synthesis or ADP-Glc transport in dicotyledonous plants (Pozueta-Romero *et al.* 1991a; Chen *et al.* 1998; Kirchberger *et al.* 2008). Examination of plastidial ATP/ADP transporter in pea roots revealed its relatively low affinity for ADP-Glc (K_m > 1mM) compared to ATP and ADP (K_m=10 µM and K_m=46 µM, respectively), thus having no relevance *in vivo* for the ADP-Glc transport (Schünemann *et al.* 1993). The situation can be similar in maturing pea embryos as the ADP-Glc concentrations in the cytosol are relatively low compared to ATP and the competition with ATP for binding and transport via the adenine nucleotide transporter is therefore unlikely.

On the envelope of plastids there are two types of adenine nucleotide transporters: NTT and Brittle1 (BT1). NTT is an ATP/ADP antiporter existing in all types of plastids throughout the plant kingdom (Linka *et al.* 2003). NTT mediates the ATP counter-exchange with ADP from the cytosol into plastids, and it does not transport ADP-Glc (Schünemann *et al.* 1993; Möhlmann *et al.* 1997; Tjaden *et al.* 1998). BT1 is a member of the huge mitochondrial carrier family (MCF) and has different functions in monocots and dicots (Leroch *et al.* 2005; Bowsher *et al.* 2007; Kirchberger *et al.* 2007). In monocots the BT1 counter-exchanges ADP-Glc and ADP as an antiport, the BT1 in dicots functions as a plastidial nucleotide uniport carrier protein that is strictly required to export newly synthesized adenylates from the chloroplast into the cytosol (Kirchberger *et al.* 2007; Kirchberger *et al.* 2008). In contrast, Bahaji *et al.* (2011 b, c) showed recently the simultaneous targeting of BT1 to plastids and mitochondria in *Arabidopsis thaliana* (*At*BT1). They showed that *At*BT1 functions as a uniporter in mitochondria, whereby the function of *At*BT1 in plastids still remains unknown. Another point to consider is that some members of the MCF transporter family can switch from uniport to antiport mode after treatment with reducing agents. These results suggest that minor modification of thiol groups are sufficient to induce changes in transport direction (Dierks *et al.* 1990); a change, which would be needed in mixotrophic cells of the pea embryo to support starch synthesis. In pea embryos cDNA of a BT1 homolog was isolated, which

showed some differences to known BT1 protein sequences (Kumpf 2012). As the functional analysis has still not been performed, the possible ADP-Glc transport in pea embryos is still unclear.

However it is possible, though unlikely, that the minor remaining AGPase activity in iAGP-3 embryos might be sufficient for starch synthesis. This remaining activity can be supported by elevated sucrose and Fru6P concentration in the plastids, as these compounds are known as activators of AGPase activity and expression (Tiessen *et al.* 2002; Salanoubat and Belliard 1989; Müller-Röber *et al.* 1990; Sokolov *et al.* 1998).

Is Susy the alternative source for ADP-Glc?

SuSy has very high sucrose degrading activity in plant tissues (also legume seeds) and is the main provider of substrates for starch synthesis via coupled reactions by UGPase and PGM in the cytosol (Figure 13). Furthermore its activity pattern during the legume seed development corresponds to starch accumulation as well as to starch synthase activity (Déjardin *et al.* 1997b; Heim *et al.* 1993). *In vitro* studies have shown that plant sucrose synthases can utilize several nucleotides (UDP, ADP, CDP, and GDP) to synthesize various nucleotide sugars, thus it may also synthesize ADP-Glc (Delmer 1972; Déjardin *et al.* 1997a). Therefore it is conceivable that SuSy is considered as an alternative cytosolic source for ADP-Glc (Pozueta-Romero *et al.* 1991b; Barratt *et al.* 2009; Muñoz *et al.* 2005, Baroja-Fernández *et al.* 2009, 2012, Bahaji *et al.* 2011). SuSy is usually described as a soluble cytosolic enzyme; however recent studies have shown a broader subcellular localization of SuSy. SuSy has been found to be connected to the plasma membrane and endoplasmatic reticulum and also to mitochondria and cell wall (Fujii *et al.* 2010; Barrero-Sicilia *et al.* 2011). SuSy in the seeds of Arabidopsis is associated with starch granules (Fallahi *et al.* 2008) and with the outer membrane of plastids (Núñez *et al.* 2008). In addition, proteome analysis using purified plastids *e.g.* amyloplasts from potato tuber or plastids from alfalfa roots have shown evidence for a plastidial SuSy (Stensballe *et al.* 2008; Daher *et al.* 2010). In present study, SuSy was mainly confined to the cytosol and only a small percent of activity contributed to plastids, which could also be due to compartmental contamination. In case the AGPase is 100% plastidial in pea embryos, SuSy should be considered to be a cytosolic enzyme based to their similar confinement either to plastids (94.0 ± 1.3 % for AGPase) and to the cytosolic compartment (96.4 ± 1.5 % for SuSy). At the same time a relatively high concentration of sucrose was confined to the plastids in this study, which is similar to that described in other studies using NAF (Farré *et al.* 2001, 2008; Benkeblia *et al.* 2007). The possible localization of SuSy and sucrose in the plastid envelope space or membrane could complicate the interpretation of the

results. Although no sucrose transporters have been found on the envelope of plastids, the outer membrane is permeable to sucrose (Heldt and Sauer 1971; Flügge and Benz 1984). Therefore it is likely that SuSy and sucrose can be found in the intermembrane space. Still, the relatively high sucrose concentrations found here in plastids of iAGP-3 embryos and in transgenic potato tubers expressing yeast invertase analyzed in Farré *et al.* (2008), contradict the conclusion of only cytosolic localization or a restriction to the envelope space. Furthermore the study with tobacco and potato showed noticeable uptake of sucrose into chloroplasts and its cleavage by invertase (Gerrits *et al.* 2001). It is currently not known how sucrose is translocated into plastids; it might be that the sucrose is transported into stroma of plastids by a specific sucrose transporter or maybe even by the maltose transporter (MEX). The maltose transporter (MEX) first described by Niittylä *et al.* (2004) has not yet been fully characterized, and whether it can transport sucrose in addition to maltose, as found for bacterial maltose transporters, is still unclear (Silva *et al.* 2005; Brunkhorst and Schneider 2005). The plastidial localization of SuSy was shown in one intriguing study identifying SuSy in a protein complex with starch synthase and AGPase (Hennen-Bierwagen *et al.* 2009). Thereby the potential primer for starch might be the SuSy products UDP-Glc or even ADP-Glc, especially under reduced AGPase activity in plastids, as several studies have shown some starch synthase isoforms with low affinity towards UDP-Glc or incorporation of a glucose unit from UDP-Glc to starch (Frydman and Cardini 1967; Denyer *et al.* 1996a; Valdez *et al.* 2008).

Role of UDP-Glc and UGPase in plastids?

In storage tissues (potato tubers) the UDP-Glc and the uridyl phosphates (UDP and UTP) have shown to largely localize both in cytosol and plastids (Farré *et al.* 2001). In plastids of pea embryo cells the concentration of UDP-Glc was much higher than ADP-Glc (Table 6), which might lead to the incorporation of UDP-Glc into starch chains under missing active AGPase. However, the situation in iAGP-3 was the opposite: the plastidial UDP-Glc concentration was lower than in Eiffel and also lower than the ADP-Glc concentration. This reduction could be due to the active use of UDP-Glc as a glucose primer by SS. Still this is not plausible, due to the low affinity of starch synthases towards UDP-Glc.

The source of UDP-Glc in plastids is unclear. Potentially there is a specific UDP-Glc translocator on the plastid envelope, or the UGPase may be located in the outer membrane of plastids causing the intermembrane space to be enriched with UDP-Glc, which is needed for membrane synthesis. Until recently UGPase has been considered to be an exclusively cytosolic enzyme, being either soluble or membrane bound (Kleczkowski 1994). Only some

evidence is available for plastidial UGPase (Kimura *et al.* 1992; Okazaki *et al.* 2009; Stensballe *et al.* 2008; Daher *et al.* 2010), which point to the plastidial source of UDP-Glc for plastid membrane synthesis. Recently a new UDP-galactose 4-epimerase (UGE; EC 5.1.3.2) isoform was discovered in rice, with the localization in plastids (Li *et al.* 2011a). UGE catalyzes the interconversion of UDP-Glc and UDP-galactose (UDP-Gal), whereby UDP-Gal is necessary for the production of galactolipids, the predominant component of photosynthetic membranes. Since these are degraded during the conversion of chloroplasts into amyloplasts, which could lead to a release of bulk UDP-Glc. As the starch granules are larger in maturing embryos of Eiffel, it may explain the higher UDP-Glc concentration in plastids of wild type Eiffel. Still, this scenario is not fully convincing.

The present study indicated a high plastidial activity of UGPase in both Eiffel and iAGP-3 embryos, therefore the plastidial UDP-Glc could be a product of plastidial UGPase. The UGPase catalyzes a reversible reaction between UDP-Glc + PPi and Glc1P + UTP. The catalytic properties of UGPase in pea seeds and potato tubers favor the synthesis of Glc1P over the UDP-Glc, supporting the idea of classical unidirectional starch synthesis (Turner and Turner 1958; Sowokinos *et al.* 1993). Also in present study the analysis of the mass action ratio and standard free energy change at the whole embryo level indicated that the Glc1P synthesis is favored in Eiffel (Table 8). In contrast, the UGPase reaction in iAGP-3 embryos seems to be close to equilibrium and rather favors UDP-Glc formation. On the subcellular level, the reaction of UGPase favors the Glc1P synthesis both in plastids and the cytosol in Eiffel; in iAGP-3 the UGPase reactions favors the UDP-Glc synthesis in plastids and Glc1P synthesis in the cytosol. This raises the question why the UDP-Glc synthesis is favored in iAGP-3 plastids and what is the fate of UDP-Glc. It might be that the UDP-Glc is used for membrane synthesis or for synthesis of trehalose 6-phosphate (T6P); thereby being a relevant regulator of starch synthesis and stress signaling in plastids (Kolbe *et al.* 2005; reviews of Ponnu *et al.* 2011; Liu *et al.* 2013). This evidence indicates that the retained high starch synthesis rate in iAGP-3 embryos may be due to the flexibility of plant cell metabolism in maintaining the genetically and developmentally regulated processes.

The role of PGM and Glc1P in starch synthesis

In present study the AGPase repression led to higher plastidial PGM (pPGM) activity in maturing pea embryos, which is in accordance with a two-fold higher expression level of pPGM in the maturing pea embryos of iAGP-3 than that of wild type (Weigelt *et al.* 2009). Starch synthesis and the growth of several dicotyledonous plants depend on pPGM (Caspar *et al.* 1985; Hanson and McHale 1988; Vriet *et al.* 2010). It plays an essential role in starch

accumulation also in pea embryos, as the mutation of pPGM causes a strong reduction of starch content (from 50% to 1-12% starch in DW of mature seed) (Harrison *et al.* 1998, 2000). In developing pea embryo cells the plastidial isoenzyme of PGM has 11 – 20 % of total activity of PGM (Forster and Smith 1993; Harrison *et al.* 2000), which is similar to the results in the present study (20.8 ± 3.2 % in Eiffel). In iAGP-3 the plastidial confinement of PGM was increased to 49.3 ± 7.4 %. PGM is of particular relevance for the starch accumulation in pea embryos because Glc6P is the main substrate imported into the plastids, and approximately 80% of Glc6P entering the plastids is directed to starch synthesis (Hill and Smith 1991). The flux to starch in pea embryos is favored by much higher PGM activities in plastids compared to glucose 6-phosphate dehydrogenase (G6PDH) (about 98 fold; Appendix 5); whose activity could otherwise be used to direct Glc6P to the oxidative pentose pathway (OPPP) or into glycolysis. Similar to other studies, the kinetics of PGM in the present study were determined using Glc1P and not Glc6P, although this does not allow for the description of the true reaction kinetics and equilibrium, especially as the current starch synthesis model prefers the unidirectional flux of Glc6P directed into starch. This contradicts the knowledge that PGM has a higher affinity towards Glc1P, which should then favor the backward reaction rather than the reaction in the direction of starch synthesis (Oesterhelt *et al.* 1996; Gao and Leary 2004; Lowry and Passounneau 1969). In pea embryos the Glc1P concentration (under the detection limit) is much lower than the Glc6P concentration, which *in vivo* and in combination with AGPase and iPPase leads to a unidirectional starch synthesis. The very low Glc1P concentration in wild type embryos could be interpreted as a sign for its rapid conversion via PGM and AGPase into starch. In iAGP-3 embryos the Glc1P concentration was increased, especially in the plastids, which might be the result of repressed AGPase activity and/or possibly the lower affinity of PGM towards Glc1P than towards Glc6P under the given concentrations. It is unclear what the fate of the formed Glc1P in plastid is: a) either it is converted back to Glc6P, b) used for starch synthesis, or c) used for UDP-Glc synthesis. The resynthesis of Glc6P due to feedback reactions of AGPase repressions could be inferred due to the elevated concentrations of Glc6P and Fru6P in plastids of iAGP-3. Thereby the Fru6P accumulation is interesting as Fru6P is known to be an AGPase activator (Preiss 1978). Fru6P may accumulate in plastids first due to high activities of PGI (which reversibly converts Glc6P and Fru6P) compared to Glc6PDH (about 99 fold difference). Secondly and most likely, the accumulation of Fru6P is due to the cycling via the pentose phosphate pathway (PPP), as in pea embryos the entering Glc6P has been shown to partition 80% into starch synthesis and 20% into oxidative PPP (OPPP), leaving glycolysis non relevant as the

Glc6P conversion into amino acids or fatty acids was shown to be neglectable (Forster and Smith 1993).

Potentially Glc1P is used for starch synthesis via starch phosphorylase (SP; E.C. 2.4.1.1; Turner and Turner 1957), especially in the case of the repressed AGPase and elevated pPGM activity, which causes the accumulation of Glc1P in iAGP-3 embryos. The reaction catalyzed by SP is reversible, but its function *in vivo* is considered to be only in phosphorylating the glucan chain, producing Glc1P (Zeeman *et al.* 2004; Kruger and ap Rees 1983). The low concentrations of Glc1P and high concentrations of inorganic P in amyloplasts could explain the unidirectional reaction of SP synthesizing the Glc1P in pea embryo cells (Kruger and ap Rees 1983). In iAGP-3 embryos the plastidial SP expression is upregulated in maturing embryos (25, 30, 35 DAP); which could indicate the enhanced starch degradation or *vice versa* the reversed reaction of starch phosphorylase elongating the glucan chain. The hypothesis that the repressed AGPase leads to enhanced starch degradation in maturing pea embryos is controversial due to parallel down and upregulation of several starch synthesis and degradation related genes (Weigelt *et al.* 2009). Furthermore the elevated starch degradation rate should also result in corresponding lower starch content. The main starch degradation pathway is the amylolytic starch degradation which leads to *β*-maltose synthesis. The *β*-maltose is concomitantly transported from plastids into the cytosol and can lead to Glc1P accumulation due to cytosolic SP which plays a role in the heteroglucan (*e.g.* maltotriose) metabolism, synthesizing Glc1P (Buchner *et al.* 1996; Lu *et al.* 2006; Niittylä *et al.* 2004; Lu and Sharkey 2006). But starch synthesis in iAGP-3 embryos was only moderately reduced and the cytosolic Glc1P concentration increased much less compared to plastidial Glc1P, therefore the role of plastidial SP in starch synthesis should be strongly considered. Several SP are known in pea; whereby Matheson and Richardson (1976, 1978) showed that SP efficiently synthesizes starch at saturating Glc1P *in vitro* (K_m = 4 mM), although *in vivo* the concentration of Glc1P is far from saturating and other enzymes such as PGM and AGPase have much higher affinity towards Glc1P. Matheson and Richardson (1976, 1978) suggested that one isoform of SP is confined to starch degradation while another isoform could contribute to starch synthesis. Furthermore the maintained ADP-Glc concentrations in pea plastids, even with AGPase repression, support the idea that the plastidial SP contributes to starch synthesis, as ADP-Glc is shown to inhibit the phosphorylysis activity of SP (Matheson and Richardson 1976, 1978; Kruger and ap Rees 1983). A similar conclusion was made by Buchner *et al.* (1996), proposing that the plastidial SP may act in starch synthesis as well as in the direction of degradation, as its activity and expression patterns follows those of starch

accumulation. Nonetheless, starch synthesis via the SP, using Glc1P, is contrary to the elevated concentrations of Glc6P and Fru6P in iAGP-3 plastids which rather support the feedback reactions. Thereby the Fru6P accumulation is interesting as Fru6P is known to be an AGPase activator and inhibitor of the starch phosphorylase glucan degrading activity (Preiss 1978; Matheson and Richardson 1978). This could explain the sustained levels of starch synthesis, possibly via the remaining AGPase activity or plastidial SP. These evidences indicate the strong preference of the flux towards starch synthesis and lower preference towards OPPP even in the plastids of iAGP-3 embryos. This leads to the conclusion that even if AGPase repression leads to the changes in the carbon flow in amyloplasts, the main end product of plastidial metabolism is starch in pea embryos.

The role of other sucrose cleavage related enzymes

Starch synthesis is affected by the substrate partitioning following the entrance of sucrose into the cell. Thereby invertases (Inv) catalyze the irreversible cleavage of sucrose into fructose and glucose, and, in addition to SuSy, are relevant for sucrose degradation and partitioning between different central carbohydrate pathways. During the seed filling phase of legume seeds Inv does not contribute to starch synthesis since during the seed filling phase the activity of Inv remains relatively low and unchanged, while the activity of SuSy increases dramatically and correlates with starch accumulation and AGPase activity (Edwards and ap Rees 1986; Weber *et al.* 1998). Otherwise, Inv have a crucial role in early seed development and in the developmental switch from the prestorage phase to the storage phase (Weber *et al.* 1995a, 1998; Weschke *et al.* 2003). Inv are generally considered to be localized in the cytosol (neutral), cell wall (acid) and in the apoplasm, and cover a wide array of functions in plant development (review by Ruan *et al.* 2010). The existence of plastidial Inv is recently is detected in several higher plants (Murayama and Handa 2007; Vargas *et al.* 2008). The present study indicates the existence and relevance of plastidial Inv in pea cotyledons. The functions of plastidial Inv are unclear, when it localizes in the stroma, where the sucrose is absent. Considering the remarkable concentration of sucrose in the plastids, the plastidial Inv together with plastidial HK can produce precursors for starch synthesis as well as for glycolysis and OPPP. In addition, considering only the passive sucrose uptake into the envelope space, the abundance of Inv together with HK in the plastid envelope membranes could support the synthesis of Glc6P and Fru6P from sucrose, following the transport into plastids via a Glc6P/Pi translocator (GPT).

Hexokinases and fructokinases (HK) have been localized in several recent studies in addition to the cytosol and mitochondria, in the plastid stroma (Olsson *et al.* 2003; Giese *et al.* 2005;

Kandel-Kfir *et al.* 2006; Damari-Weissler *et al.* 2006; Cho *et al.* 2006) and in the outer membrane of the plastid envelope (Wiese *et al.* 1999). Wiese *et al.* (1999) suggested that hexokinase bound to the outer envelope membrane is phosphorylating glucose exported from plastids, as a product of hydrolytic starch breakdown. Giese *et al.* (2005) and Cho *et al.* (2005) discussed the role of plastidial (stroma) hexokinase on starch recycling and or directing the glucose from starch degradation into OPPP. Cho *et al.* (2006) suggests that stromal hexokinase activity is needed in heterotrophic cells to utilize glucose transported into the amyloplast via the plastidial glucose transporter (pGlcT; Toyota *et al.* 2006). Although they assume that the stromal hexokinase is more likely involved in starch degradation than in starch synthesis. The present study supports the role of HK in plastids providing hexose 6-P for the starch synthesis as HK (glc and fru) activities were found to be localized equally in the cytosol and plastids. In particular, the fructose phosphorylating activity both in the stroma of plastids or on the outer membrane of plastids may be of relevance, as high concentrations of Fru6P, in addition to Glc6P, were found in plastids. On the other hand Fru6P is a product of the Calvin-cycle and could represent the carbon dioxide recycling in embryos as embryos of legumes as well as other green embryos are capable of refixating CO_2 (Furbank *et al.* 2004; Schwender *et al.* 2004). However, this can be negligible as the Rubisco activity, and expression, is rather low even in the late phase of maturation in pea embryos. In contrast, the phosphoenolpyruvate carboxylase (PEPCase) is considered to refixate some CO_2 in pea embryos (Hedley *et al.* 1975; Golombek *et al.* 1999). Hence, high levels of Fru6P cannot be explained as a result of the active Calvin-cycle in amyloplasts. Therefore Fru6P together with HK (fru) may play a higher regulatory role in starch synthesis than previously assumed (Figure 13).

In the present study a large amount of glucose and fructose was localized in plastids, which may be due to starch degradation or hexose transport from the cytosol into the plastids. Thus the highly elevated glucose concentration in iAGP-3 plastids could be an indicator for increased starch degradation or for the enhanced glucose uptake into plastids. There is evidence that the glucose could be transported into plastids if the glucose concentration is sufficiently high in the cytosol or in the envelope space (Möhlmann *et al.* 1995, Weber *et al.* 2000a, Fischer and Weber 2002; Servaites and Geiger 2002; Butowt *et al.* 2003; Toyota *et al.* 2006) (Figure 13). Thereby the putative glucose transporter (GT), which exports the glucose from starch degradation into the cytosol, could also transport glucose into plastids (Weber *et al.* 2000a; Servaites and Geiger 2002; Fischer and Weber 2002; Facchinelli and Weber 2011). In addition, the glucose transporter could transport fructose as well (Weber *et al.* 2000a). The

embryo metabolism and starch synthesis relies on imported sucrose; thereby the sucrose import during the day time may be even higher than at night. In this situation (high hexose concentration in the cytosol from degraded sucrose) the putative glucose transporter could import hexoses to the plastids (Figure 13). Thus the localization of HK in plastids would complete the possible route of imported glucose to starch.

Starch synthesis underlies a complex regulation. In order to understand and to modify the starch synthesis, the deep understanding of this pathway is required. Many questions remain open concerning the pathway and the importance of the participating enzymes and transport proteins. The uncertainties which were pointed out in the present study are illustrated in the following graph (Figure 13) together with some possibly relevant substrate routes of starch synthesis existing parallel to the accepted main pathway. Probably the most interesting question regards the source and re-localization of ADP-Glc, as the existence and functionality of ADP-Glc transporters in pea embryos have not yet been shown. Second, the HK in the outer envelope membrane might play a crucial role on directing the substrates towards starch synthesis, in addition to their known role in signalling (Wiese *et al.* 1999; Giese *et al.* 2005). The glucose transporter (GT; pGlcT) on the inner envelope membrane of plastids could possibly import the glucose and fructose from the cytosol or from the envelope intermembrane space into plastids, due to enhanced glucose concentrations in the iAGP-3 cytosol (Weber *et al.* 2000a). The localization and uptake of sucrose in plastids is one of most unclear topics; it is possible that sucrose moves into plastids via diffusion or via active transport (Gerrits *et al.* 2001) followed by the degradation by Inv (Murayama and Handa 2007; Vargas *et al.* 2008). In addition to that, the SuSy on the outer plastid envelope membrane together with the glucose transporter in the inner membrane of plastids could contribute to starch synthesis (Núñez *et al.* 2008; Weber *et al.* 2000a). A possible contribution of SP on the synthesis of Glc1P and starch should be considered together with starch degradation (Matheson and Richardson 1976, 1978; Buchner *et al.* 1996; Lu and Sharkey 2006). These, and other source routes, may play a more important role in starch synthesis than previously thought, either directly or via signalling.

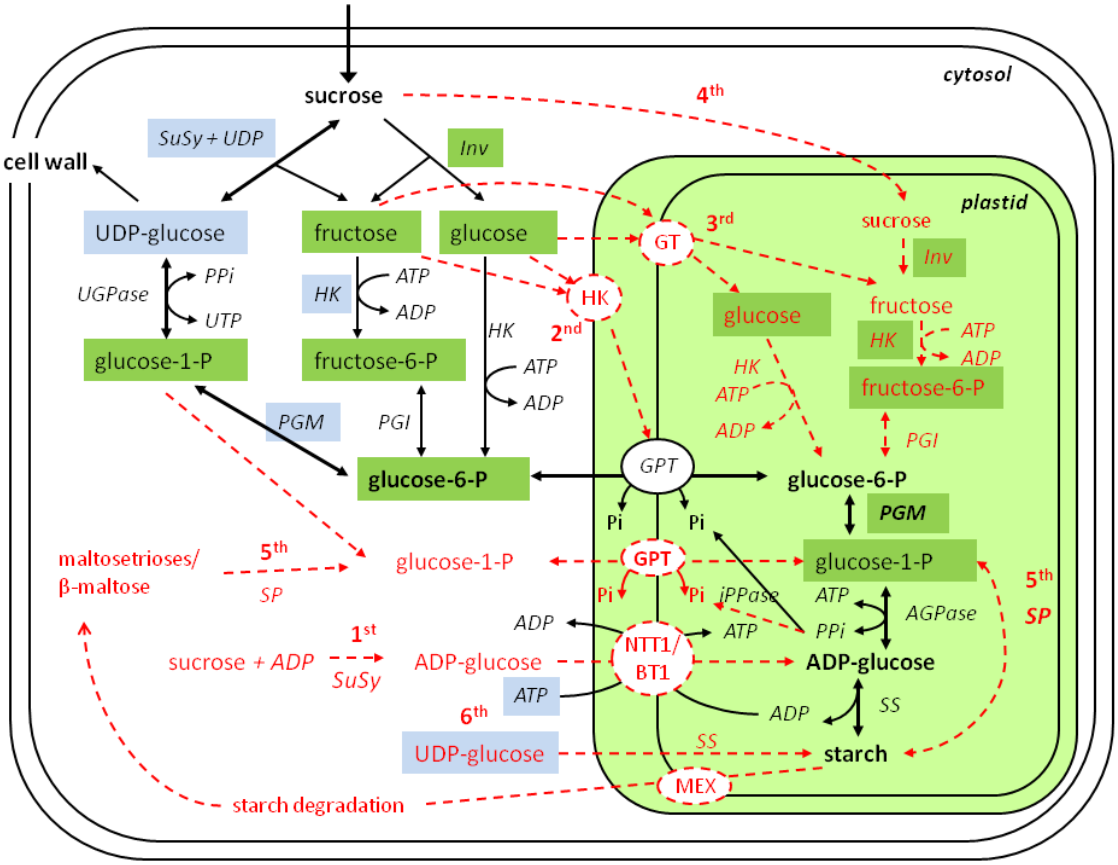


Figure 13. Possible alternative scenarios for starch synthesis from sucrose in the pea cotyledon cells:
The alternative scenarios are marked with red script and red dashed line. Significant changes in iAGP-3 pea embryos at the subcellular level are indicated by the color of the text box; green indicates an increase and blue a decrease.
1st ADP-Glc could be synthesized in cytosol by SuSy, followed by the transport into plastid through BT1 or NTT1 (Pozueta-Romero *et al.* 1991 a, b; Muñoz *et al.* 2005).
2nd Localization of HK in outer envelope membrane synthesizing hexose P, which can be transported into plastid via GPT (Wiese *et al.* 1999; Giese *et al.* 2005).
3rd Glucose transporter could import glucose and fructose into plastids (Weber *et al.* 2000a).
4th Possible uptake of sucrose *in vivo* into plastids could be via diffusion or via active transport (Gerrits *et al.* 2001) followed by degradation by Inv (Murayama and Handa 2007; Vargas *et al.* 2008).
5th SP and Glc1P could play a role in starch synthesis and starch degradation (Matheson and Richardson 1976, 1978; Buchner *et al.* 1996).
6th UDP-Glc could be possible glucose donor for starch synthesis (Frydman 1963; MacDonald and ap Rees 1985; Denyer *et al.* 1996a) and is either transported into plastids or synthesized in plastids (Li *et al.* 2011a).

Abbreviations: ADP, adenosine diphosphate; AGPase, ADP-Glc pyrophosphorylase; ATP, adenosine triphosphate; GT, glucose transporter; GPT, glucose 6-phosphate transporter; BT1, Brittle1; Inv, invertase; HK, hexokinase; iPPase, inorganic pyrophosphatase; MEX, maltose transporter; NTT1, nucleotide transporter; Pi, inorganic phosphate; PPi, inorganic pyrophosphate; PGI, phosphoglucoisomerase; PGM, phosphoglucomutase; SP, starch phosphorylase; SS, starch synthase; SuSy, sucrose synthase; UDP, uridine diphosphate; UGPase, UDP-Glc pyrophosphorylase; UTP, uridine triphosphate

Conclusions

The crucial role of AGPase in starch synthesis and accumulation can not be neglected. However, there is much evidence for a far more complex regulation and synthesis of starch, which possibly contributes to the stability of development and growth. The present study analyzed the effects of a reduced AGPase activity on starch synthesis at the subcellular level in maturing pea embryos, and gave insight into possible functions of several recently discovered plastidial enzymes within this context (e.g. plastidial Inv and HK). The possible involvement of hexose transporters in the starch synthesis of pea embryos was also discussed. The hexose transporters on the plastid envelope membrane seem to have a greater relevance in starch synthesis in pea embryos. Hexose transporters possibly not only export the glucose from starch degradation into the envelope space, but also participate in directing glucose back into the plastids stroma. Thereby the glucose transporter (GT) could be involved in the import of glucose from the cytosol into plastids or even in directing the products of the envelope membrane bound enzymes (SuSy, Inv) into plastids. The role of SP in the metabolism of iAGP-3 embryos is intriguing, as it is still unclear in which way starch synthesis is kept high under the drastic AGPase repression. Also the source of ADP-Glc in plastids remains an open question, as there may be a functional ADP-Glc transporter in pea embryos. The results presented here support the hypothesis of an alternative cytosolic source of ADP-Glc synthesized by SuSy. Further work should be done to understand how ADP-Glc and sugars, such as glucose, fructose and or even sucrose, are translocated into plastids, and how big their contribution is to starch synthesis.

Chapter 5: Starch synthesis in cotyledon cells of sucrose transporter (VfSUT1) overexpressed pea line

Results

Metabolite concentrations and enzyme activities in maturing pea embryos

Pea embryos of the parental line SUT wt and the transgenic line SUT7, with sucrose transporter SUT1 overexpression, were harvested at 30 DAP. Subsequent, embryos with a fresh weight of 400 ± 20 mg were selected for analysis. The embryos of SUT7 had slightly reduced dry matter content: 39.0 ± 0.9 % and 37.0 ± 0.8 % in SUT wt and SUT7, respectively (Student's *t*-test p=0.023). SUT7 and SUT wt embryos contained relatively similar concentrations of metabolites and enzymes (Table 9). Maturing embryos of SUT wt contained slightly higher concentrations of soluble sugars (sucrose, glucose and fructose) than SUT7 embryos. The concentrations of the other analyzed metabolites were similar between these two lines. In enzyme activities the only difference detected was the slight increase in UGPase activity (approximately 20%) in SUT7 embryos (Student's *t*-test p=0.042).

Table 9. Metabolites and enzymes in maturing embryos of the parent line SUT wt and sucrose transporter overexpressed line SUT7

Concentrations are given as the average of six embryos ± SE, except for adenylates (ADP-Glc, ATP, ADP, AMP) where three embryos were analyzed. Concentrations are given as nmol g^{-1} FW and enzyme activities are given as nmol min^{-1} g^{-1} FW.

	SUT wt	SUT7	t-test p-value
dry matter	39.0 ± 0.9 % FW	37.0 ± 0.8 % FW	**0.023**
starch	13.9 ± 0.6 % FW	13.5 ± 0.8 % FW	
	nmol min^{-1} g^{-1} FW	**nmol min^{-1} g^{-1} FW**	
AGPase	289.4 ± 19.0	284.9 ± 24.1	
iPPase	458.9 ± 31.7	628.0 ± 68.9	0.055
UGPase	**9869.5 ± 662.36**	**11964.4 ± 637.34**	**0.042**
SuSy	1568.0 ± 95.9	1464.0 ± 111.6	
Inv	96.9 ± 6.0	84.8 ± 7.5	
PGM	12650.0 ± 339.6	11400.0 ± 696.5	
PGI	9350.0 ± 242.4	9824.0 ± 621.7	
HK glc	52.7 ± 4.0	57.4 ± 3.3	
HK fru	140.9 ± 8.3	155.8 ± 17.8	
	SUT wt	**SUT7**	**t-test p-value**
	nmol g^{-1} FW	**nmol g^{-1} FW**	
sucrose	**84370.5 ± 2798.3**	**64772.9 ± 3812.6**	**0.004**
glucose	**399.856 ± 26.23**	**276.195 ± 34.2**	**0.025**
fructose	**680.1 ± 42.1**	**480.6 ± 60.7**	**0.034**
Glc6P	324.2 ± 10.9	294.1 ± 22.2	
Fru6P	62.7 ± 2.3	57.7 ± 5.2	
Glc1P	1.8 ± 0.8	2.0 ± 0.9	
UDP-Glc	211.6 ± 4.3	205.8 ± 11.9	
ADP-Glc	28.1 ± 3.0	28.3 ± 2.2	
AMP	9.6 ± 2.2	11.6 ± 1.7	
ADP	14.1 ± 2.0	15.7 ± 2.1	
ATP	65.7 ± 8.0	71.8 ± 5.8	
starch	770609.6 ± 31264.2	750221.5 ± 42043.2	

Histology and subcellular volumes

Similar to Eiffel and iAGP-3, the storage parenchyma cells of the maturing embryos contained large starch granules at 30 DAP for SUT wt and SUT7 line (Figure 14). Plastids in both lines were nearly 100% filled with starch. Starch granules occupied larger volume from the cotyledon tissue in SUT wt than in SUT7 (42.1 ± 0.4 % and 36.4 ± 0.4 %, Student's *t*-test $p<0.001$, n=70). The volume of the intercellular space was unchanged and made up 6.6 ± 0.3 % and 5.9 ± 0.3 % of cotyledon tissue in parental line and SUT7, respectively (n=59). The cell wall amounted from cotyledon parenchyma tissue 11.7 ± 0.1 % and 11.0 ± 0.1 % (n=22; $p=0.028$) in SUT wt and SUT7, respectively. Maturing pea embryo cells contained many small protein bodies and no distinguishable vacuole. In both pea lines the density of pea embryos was found to be approximately 1 g ml^{-1}, which was similar to that found for Eiffel and iAGP-3 in previous chapter (1.02 ± 0.01 g ml^{-1} SUT wt and 1.01± 0.01 g ml^{-1} in SUT7). Densities were measured only twice due to similar results on both repetitions; the total number of analyzed embryos was 28 for wild type and 31 for SUT7.

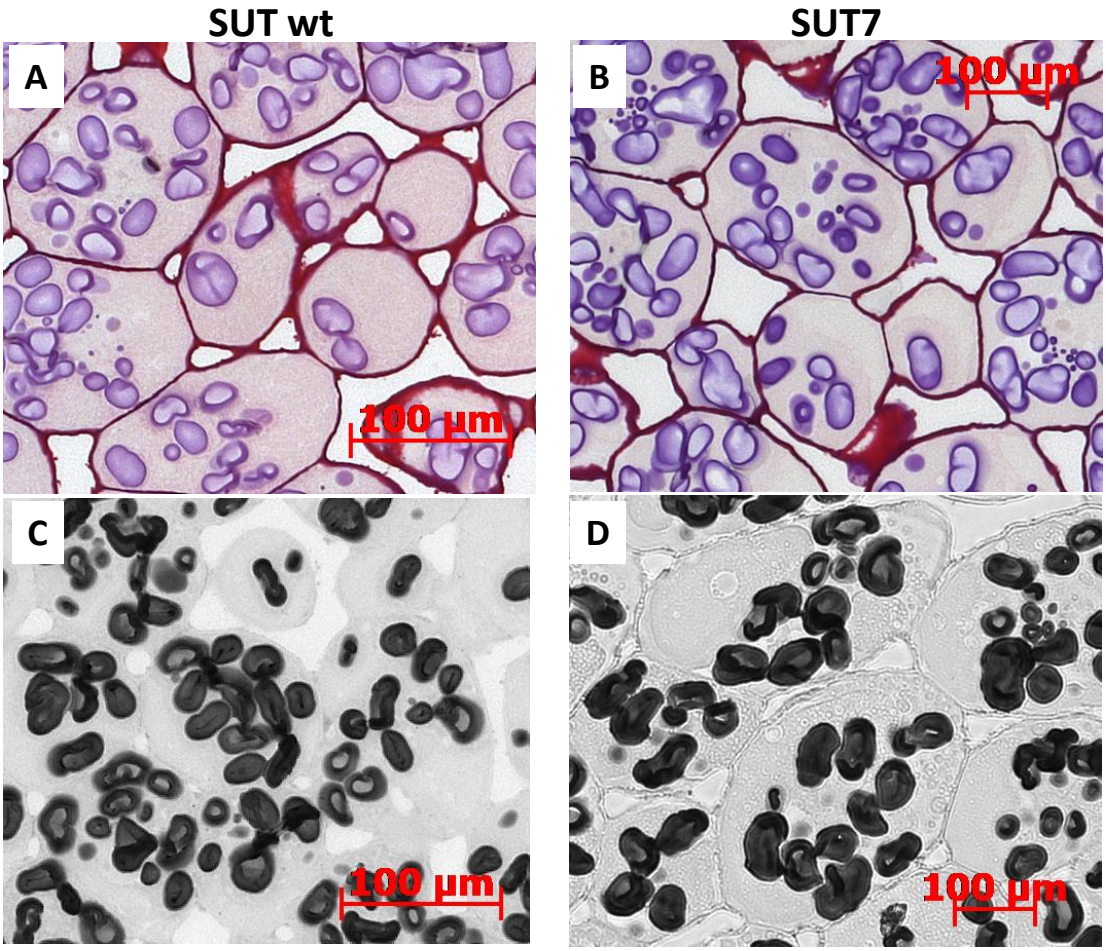


Figure 14. Cotyledon parenchyma of SUT wt (left A, C) and SUT7 (right B, D). Slides A and B (2.5 µm thin) were stained with basic fuchsin followed by brief counterstaining with crystal violet. Slides C and D were stained with Lugol's solution.

As discussed in Chapter 4, the measured volume of the cytosolic compartment and starch granule do not represent the actual reaction space. Therefor the mobile phase of plastids was estimated to be 10% of starch granule (Kosegarten and Mengel 1994). Also the volume of the cytosolic compartment was estimated (Table 10).

Table 10. Relative volumes of cell compartments in maturing cotyledons of the pea SUT wt and the transgenic line SUT7

	SUT wt	**SUT7**	
Data from this study	**% of tissue**		**t-test p-value**
starch granule	42.1 ± 0.4	36.4 ± 0.5	*p*=0.000
cell wall	11.7 ± 0.1	11.0 ± 0.0	*p*=0.028
intercellular space	6.6 ± 0.3	5.9 ± 0.3	*p*=0.143
cytosol + apoplasm	**11.23**	**16.73**	represents the non-plastidial compartment
Data from other publications			**references**
nucleus	3.5	3.5	Briarty *et al.* 1980, *Phaseolus vulgaris* cotyledon
ER	22	22	Briarty *et al.* 1980, *Phaseolus vulgaris* cotyledon
mitochondria	0.5	0.5	Farre *et al.* 2001, potato tuber
protein bodies	9.01	9.94	Weigelt *et al.* 2009, Craig *et al.* 1979, pea Colombek *et al.* 2001, faba bean Weichert *et al.* 2010, wheat

Non-aqueous fractionation of cells

The organelle marker distribution pattern in NAF pellets of SUT wt and SUT7 was similar to that of Eiffel and iAGP-3 (Figure 11; Figure 15). In the present study GAPDH, iPPase and starch were used as plastidial markers and both PEPCase and α-mannosidase as markers for the cytosolic compartment. The GAPDH and iPPase distribution pattern was very similar to that of AGPase and the PEPCase distribution pattern resembles the pattern of *α*-mannosidase and UGPase, this was seen when analyzing the correlations between markers (Table 11). GAPDH and PEPCase had significantly different distribution patterns (Student's *t*-test $p<0.05$). No significant difference (Student's *t*-test) was found between the cytosolic and vacuolar marker distribution (UGPase and *α*-mannosidase as well as between PEPCase and α-mannosidase) when analyzing either line.

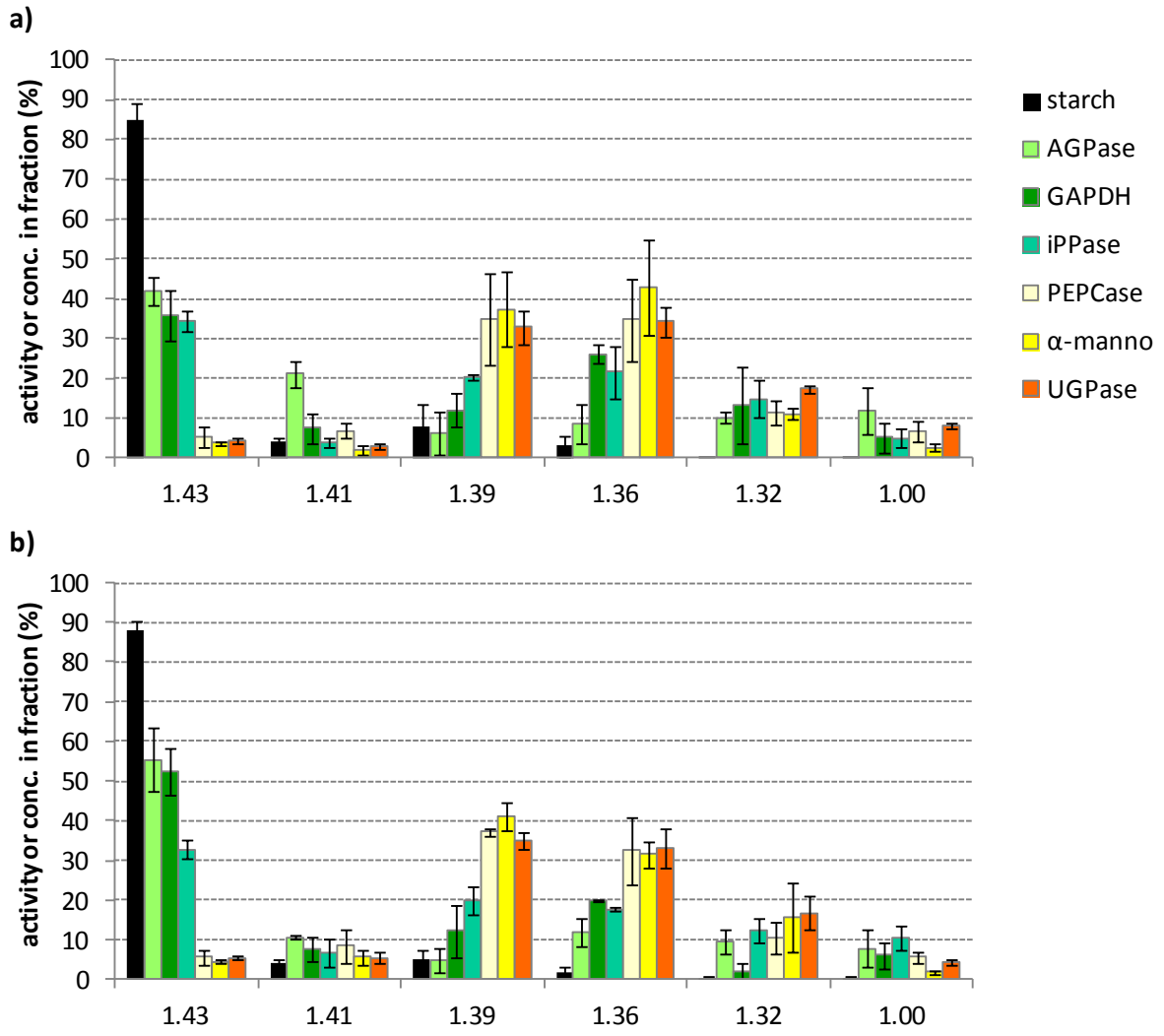


Figure 15. Subcellular distribution of organelle markers over sedimentation fractions of a) wild type pea embryos and b) SUT7, the sucrose transporter overexpressed transgenic line.
Data points are the means of three replicates of fractionation ± SE.

Table 11. Pearson's correlations coefficients (*r*) for subcellular compartment markers
Analysis was done using the relative activity or concentration distribution over fractions. Significant correlations (Student's *t*-test, α=0.05) are marked in bold, whereby negative correlations are in blue and positive correlations in green.

Pearson's *r*						
SUT wt	**AGPase**	**GAPDH**	**iPPase**	**PEPCase**	**α-manno**	**UGPase**
starch	**0.794**	**0.610**	**0.688**	-0.245	-0.225	-0.365
AGPase	**1**	0.348	**0.519**	-0.435	**-0.500**	**-0.589**
GAPDH	0.348	**1**	**0.518**	-0.047	0.116	0.044
iPPase	**0.519**	**0.518**	**1**	0.068	0.140	0.165
PEPCase	-0.435	-0.047	0.068	**1**	**0.943**	**0.902**
α-manno	**-0.500**	0.116	0.140	**0.943**	**1**	**0.944**
UGPase	**-0.589**	0.044	0.165	**0.902**	**0.944**	**1**
SUT7	**AGPase**	**GAPDH**	**iPPase**	**PEPCase**	**α-manno**	**UGPase**
starch	**0.934**	**0.913**	**0.762**	-0.322	-0.319	-0.355
AGPase	**1**	**0.905**	**0.676**	-0.321	-0.352	-0.350
GAPDH	**0.905**	**1**	**0.747**	-0.121	-0.116	-0.142
iPPase	**0.676**	**0.747**	**1**	0.055	0.072	0.107
PEPCase	-0.321	-0.121	0.055	**1**	**0.882**	**0.912**
α-manno	-0.352	-0.116	0.072	**0.882**	**1**	**0.949**
UGPase	-0.350	-0.142	0.107	**0.912**	**0.949**	**1**

Subcellular localization of starch synthesis related enzymes and metabolites

SUT wt and SUT7 had some significant differences considering subcellular localization of metabolites and enzyme activities (Figure 16). Similarly to the study with Eiffel and iAGP-3, the overall distribution favored the cytosolic confinement. Most of the metabolites and enzymes had significant confinement either to the cytosol or to plastids in both SUT wt and SUT7.

The activities of UGPase, SuSy, PGI, and PGM were dominantly localized to the cytosol (Figure 16). The activities of HK (glc and fru) and Inv were distributed relatively equally in both the cytosol and plastids (slightly more in the cytosol). The significant difference between SUT wt and SUT7 was the decreased confinement of UGPase to plastids: UGPase was confined 16% in wt and 5% in SUT7 to plastids.

The distribution of metabolites in the sucrose to starch pathway between plastids and the cytosol was largely similar among these two lines. The significant differences between SUT wt and SUT7 were in the distributions of AMP, Glc6P and Glc1P; thereby the plastidial confinement was increased for AMP, and decreased for Glc6P and Glc1P in SUT7. In both lines the sucrose, glucose, and fructose were dominantly localized to the cytosol. ADP-Glc and Fru6P were equally confined to cytosol and plastids in both lines.

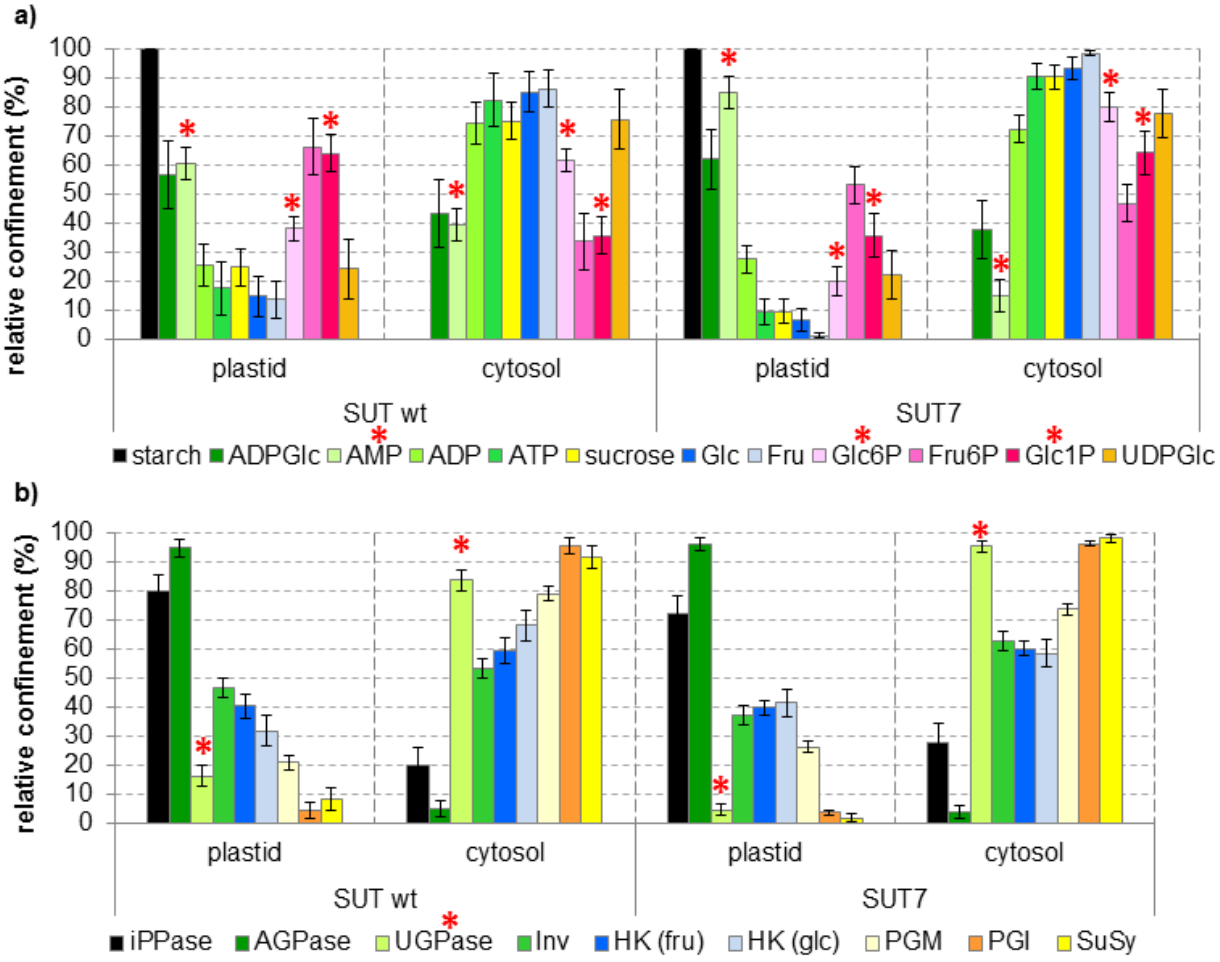


Figure 16. Relative subcellular confinement of metabolites (a) and enzyme activities (b) in the embryos of the SUT wt and the transgenic pea line SUT7 into plastidial and cytosolic compartment – calculated with BestFit analysis tool. Data represents the means of three replicates of fractionation ± SE. Data points with a star (*) have a significant difference (Student's *t*-test; α=0.05) between the wild type and SUT7.

Subcellular concentrations of starch synthesis related metabolites and enzymes

There were several significant differences between SUT wt and SUT7 in the relative and absolute subcellular metabolite concentrations and enzyme activities (Table 12). In SUT7 plastids the relative activity of PGM was significantly increased. From among the metabolites the relative plastidial concentration of AMP was increased in SUT7 embryos.

Changes in absolute concentrations and enzyme activities were mainly in the cytosolic compartment. The activities of AGPase and iPPase were significantly elevated in plastids of SUT7 compared to SUT wt. The plastidial absolute concentrations of starch and AMP were increased in SUT7. In contrast the plastidial concentration of Glc6P was decreased. In the cytosolic compartment of the SUT7 embryo cells most of the enzymes and metabolites showed a decreased activity and concentration compared to SUT wt.

Table 12. Subcellular relative distribution of metabolite concentrations and enzyme activity and subcellular concentration (mM) and enzyme activity (mM min^{-1}) levels

Data here represents three separate fractionations both for SUT wt and the SUT1 overexpressed line SUT7. The values in bold font mark the significant difference between these two pea lines; the increased values in SUT7 are highlighted in green and the decreased values in SUT7 are highlighted in blue. The concentration of starch is calculated as mM of glucose.

	Subcellular relative concentrations (%)		Subcellular concentrations (mM)	
SUT wt	**plastid**	**cytosol**	**plastid**	**cytosol**
AGPase	97.9 ± 1.4	2.1 ± 1.4	**6.54 ± 0.22**	0.13 ± 0.08
iPPase	90.6 ± 3.2	9.4 ± 3.2	**8.71 ± 0.70**	0.82 ± 0.26
UGPase	32.6 ± 6.3	67.4 ± 6.3	38.33 ± 9.42	73.53 ± 3.53
SuSy	17.5 ± 7.8	82.5 ± 7.8	3.17 ± 1.57	**12.78 ± 0.59**
Inv	69.5 ± 3.2	30.5 ± 3.2	1.08 ± 0.09	**0.46 ± 0.03**
HK glc	**53.2 ± 7.4**	**46.8 ± 7.4**	0.40 ± 0.07	**0.32 ± 0.03**
HK fru	63.3 ± 4.6	36.7 ± 4.6	1.35 ± 0.16	**0.75 ± 0.06**
PGI	9.8 ± 6.0	90.2 ± 6.0	10.00 ± 6.46	**79.51 ± 2.42**
PGM	**40.9 ± 3.8**	**59.1 ± 3.8**	63.16 ± 8.33	**88.99 ± 3.12**
starch	100.0 ± 0.0	0.0 ± 0.0	**1832.24 ± 0.00**	0.00 ± 0.00
ADPglc	73.5 ± 8.0	26.5 ± 8.0	0.38 ± 0.08	0.11 ± 0.03
AMP	**79.3 ± 4.1**	**20.7 ± 4.1**	**0.14 ± 0.01**	**0.03 ± 0.01**
ADP	43.5 ± 9.6	56.5 ± 9.6	0.09 ± 0.03	**0.09 ± 0.01**
ATP	27.6 ± 13.7	72.4 ± 13.7	0.27 ± 0.15	0.48 ± 0.06
sucrose	43.2 ± 9.2	56.8 ± 9.2	498.16 ± 135.90	**564.72 ± 50.90**
glucose	26.2 ± 11.3	73.8 ± 11.3	1.41 ± 0.71	**3.03 ± 0.26**
fructose	24.9 ± 10.4	75.1 ± 10.4	2.21 ± 1.10	**5.23 ± 0.41**
Glc6P	61.3 ± 4.3	38.7 ± 4.3	**2.94 ± 0.34**	**1.78 ± 0.13**
Fru6P	81.2 ± 6.7	18.8 ± 6.7	0.99 ± 0.16	0.19 ± 0.06
Glc1P	81.4 ± 4.5	18.6 ± 4.5	0.03 ± 0.00	0.01 ± 0.00
UDPglc	37.0 ± 14.1	63.0 ± 14.1	1.22 ± 0.56	**1.43 ± 0.21**
SUT7	**plastid**	**cytosol**	**plastid**	**cytosol**
AGPase	99.1 ± 0.6	0.9 ± 0.6	**7.53 ± 0.19**	0.07 ± 0.04
iPPase	91.4 ± 2.3	8.6 ± 2.3	**12.44 ± 1.16**	1.05 ± 0.25
UGPase	16.2 ± 6.8	83.8 ± 6.8	15.35 ± 7.04	68.17 ± 1.53
SuSy	6.8 ± 4.8	93.2 ± 4.8	0.74 ± 0.53	**8.59 ± 0.12**
Inv	72.3 ± 3.0	27.7 ± 3.0	0.87 ± 0.08	**0.32 ± 0.02**
HK glc	**75.3 ± 3.5**	**24.7 ± 3.5**	0.66 ± 0.08	**0.20 ± 0.02**
HK fru	74.8 ± 2.2	25.2 ± 2.2	1.71 ± 0.12	0.56 ± 0.03
PGI	14.4 ± 3.4	85.6 ± 3.4	9.91 ± 2.48	**56.56 ± 0.54**
PGM	**61.5 ± 2.6**	**38.5 ± 2.6**	82.03 ± 6.60	**50.31 ± 1.43**
starch	100.0 ± 0.0	0.0 ± 0.0	**2063.12 ± 0.00**	0.00 ± 0.00
ADPglc	85.6 ± 4.5	14.4 ± 4.5	0.48 ± 0.08	0.06 ± 0.02
AMP	**95.8 ± 1.8**	**4.2 ± 1.8**	**0.27 ± 0.02**	**0.01 ± 0.00**
ADP	61.2 ± 5.3	38.8 ± 5.3	0.12 ± 0.02	0.07 ± 0.00
ATP	25.1 ± 11.0	74.9 ± 11.0	0.18 ± 0.09	0.39 ± 0.02
sucrose	27.3 ± 10.3	72.7 ± 10.3	172.19 ± 75.67	**349.71 ± 16.44**
glucose	17.3 ± 11.1	82.7 ± 11.1	0.49 ± 0.32	**1.54 ± 0.07**
fructose	5.3 ± 3.5	94.7 ± 3.5	0.18 ± 0.12	**2.83 ± 0.03**
Glc6P	48.4 ± 8.7	51.6 ± 8.7	**1.60 ± 0.43**	**1.41 ± 0.09**
Fru6P	82.4 ± 3.7	17.6 ± 3.7	0.84 ± 0.11	0.16 ± 0.02
Glc1P	67.1 ± 7.6	32.9 ± 7.6	0.02 ± 0.00	0.01 ± 0.00
UDPglc	46.9 ± 11.3	53.1 ± 11.3	1.25 ± 0.50	0.96 ± 0.11

Energy state of starch synthesis in pea embryos

The energy change and the direction of starch synthesis related reactions was evaluated using the standard free energy change constants (ΔG`°, from http://equilibrator.weizmann.ac.il) and calculated mass action ratios (Q) and free energy change (ΔG).

There was no significant difference for Q and ΔG between these two lines at the whole embryo level (*t*-test, α=0.05, n=6) (Table 13 A). In general, the ΔG for whole embryos indicated that the preferred direction for reactions is towards starch synthesis. Exceptions were PGI and PGM guided reactions. The energy state of PGI reaction favored glycolysis (synthesis of Fru6P) in both pea lines. The PGM reaction was directed towards Glc1P synthesis instead of Glc6P. The calculated Q values for the whole embryo were mainly far from equilibrium, particularly remarkable were the Inv and HK (glc and fru) showing a strong forward reaction direction. At the whole embryo level only the pPGM and AGPase reaction was found to be very close to equilibrium.

At the subcellular level the biochemical energy parameters were relatively similar to those of the whole tissue level (Table 13 B) and showed that Inv and HK (glc and fru) are far from being in the equilibrium state, both in plastids and cytosol. Subcellular analysis indicated significant differences for Q between SUT wt and SUT7 in cytosolic Inv and UGPase as well as for SS (in plastids). In both pea lines the reactions of cytosolic SuSy, and the reactions of UGPase, AGPase and PGM in plastids were close to equilibrium. Interestingly the AGPase reaction in SUT wt plastids was much closer to equilibrium than in SUT7. In both lines the SuSy reaction was far from equilibrium in plastids and close to equilibrium in the cytosol. In contrast, the UGPase reaction in plastids was quite close to equilibrium, whereas in the cytosol the reaction was far from equilibrium. Similar to situation at the whole cell level, the plastidial and cytosolic PGM reactions seemed to be directed towards Glc1P synthesis instead of Glc6P in both lines.

Table 13. Bioenergetics of starch synthesis: Gibbs free-energy change ΔG, equilibrium constant ($K`_{eq}$) and mass action ratio (Q)
Values for standard free energy change for the biochemical standard state (ΔG`°) was taken from http://equilibrator.weizmann.ac.il , except the starch synthase (SS), which was taken from MetaCyc database.

A)Pea embryo biochemical energy parameters – mass action ratio Q, free energy change ΔG, equilibrium constant K'_{eq} and standard free energy change ΔG´° (constants for the biochemical standard state pH 7.0 and T=25°C)

Enzyme	SUT wt		SUT7		ΔG`° [kJ/mol]	$K`_{eq}$	reaction
	Q	ΔG	Q	ΔG			
SuSy	0.19 ± 0.01	-2.65 ± 0.16	0.17 ± 0.02	-3.03 ± 0.27	1.5	0.55	(UDPglc*fru)/(suc*UDP)
Inv	3.35 ± 0.40	-23.06 ± 0.31	2.17 ± 0.39	-24.35 ± 0.49	-25.9	34322	(glc*fru)/suc
HK (glc)	0.17 ± 0.03	-29.44 ± 0.33	0.29 ± 0.05	-28.32 ± 0.49	-24.9	22933	(Glc6P*ADP)/(glu*ATP)
HK (fru)	0.02 ± 0.00	-31.93 ± 0.34	0.03 ± 0.01	-30.83 ± 0.50	-22	7122	(Fru6P*ADP)/(fru*ATP)
PGI	0.19 ± 0.00	-4.27 ± 0.01	0.19 ± 0.00	-4.26 ± 0.04	-0.2	1.08	Fru6P/Glc6P
UGPase	0.05 ± 0.01	-5.78 ± 0.58	0.05 ± 0.01	-5.61 ± 0.59	1.6	0.52	(Glc1P*UTP)/(UDPglc*PPi)
PGM cyt	162.30 ± 53.32	3.94 ± 0.78	146.78 ± 52.18	3.64 ± 0.77	-7.8	23.22	Glc6/Glc1P
PGM pla	0.01 ± 0.00	-3.94 ± 0.78	0.01 ± 0.00	-3.64 ± 0.77	7.8	0.04	Glc1P/Glc6P
AGPase	1.92 ± 0.64	-0.16 ± 0.49	1.79 ± 0.60	-0.32 ± 0.51	-1.7	1.98	(ADPglc*PPi)/(ATP*Glc1P)
iPPase	17845132 ± 0.00	16.47 ± 0.00	17315265 ± 0.00	16.39 ± 0.00	-24.9	22933	(Pi*Pi)/PPi
SS	370603 ± 30632	-21.44 ± 0.20	419270 ± 21984	-21.10 ± 0.14	-53.15	2028658740	(starch*ADP)/ADPglc

B)The biochemical energy parameters calculated for plastidial and cytosolic compartment – mass action ratio Q, free energy change ΔG, equilibrium constant K´eq and standard free energy change ΔG´° (constants for the biochemical standard state pH 7.0 and T=25°C)

Enzyme	SUT wt				SUT7			
	Q		ΔG		Q		ΔG	
	plastid	cytosol	plastid	cytosol	plastid	cytosol	plastid	cytosol
SuSy	0.04 ± 0.01	0.27 ± 0.01	-5.86 ± 0.63	-1.86 ± 0.14	0.00 ± 0.00	0.24 ± 0.02	-3.16 ± 0.82	-2.12 ± 0.09
Inv	0.01 ± 0.00	***0.03*** ± ***0.00***	***-25.79*** ± ***3.37***	***-34.80*** ± ***0.11***	0.00 ± 0.00	***0.01*** ± ***0.00***	***0.00*** ± ***0.00***	***-36.76*** ± ***0.01***
HK (glc)	1.15 ± 0.30	0.12 ± 0.00	-7.28 ± 1.88	-30.22 ± 0.10	0.39 ± 0.32	0.14 ± 0.02	-3.88 ± 1.58	-29.47 ± 0.12
HK (fru)	0.25 ± 0.05	0.01 ± 0.00	-11.86 ± 2.17	-28.47 ± 2.33	0.26 ± 0.17	0.01 ± 0.00	-7.53 ± 1.94	-33.59 ± 0.19
PGI	0.36 ± 0.03	0.11 ± 0.01	-3.00 ± 0.23	-4.70 ± 0.46	0.80 ± 0.25	0.11 ± 0.01	-1.33 ± 0.30	-5.70 ± 0.14
PGM	0.01 ± 0.00	109.35 ± 1.29	-3.83 ± 0.03	3.83 ± 0.03	0.02 ± 0.01	97.90 ± 26.93	-2.94 ± 0.34	2.94 ± 0.34
AGPase	1.89 ± 0.35	3.82 ± 0.60	0.30 ± 0.22	0.95 ± 0.43	15.10 ± 6.53	0.86 ± 0.23	3.30 ± 0.60	-1.57 ± 0.25
iPPase	46866 ± 0	142001 ± 0	1.75 ± 0.00	4.49 ± 0.00	52597 ± 0	92481 ± 0	2.03 ± 0.00	3.43 ± 0.00
UGPase	0.54 ± 0.08	***0.02*** ± ***0.00***	-1.29 ± 0.53	***-8.31*** ± ***0.19***	0.27 ± 0.13	***0.04*** ± ***0.01***	-3.33 ± 0.53	***-6.45*** ± ***0.22***
SS	***378.6*** ± ***20.47***	0.00 ± 0.00	-38.58 ± 0.17	0.00 ± 0.00	***508.7*** ± ***17.28***	0.00 ± 0.00	-37.7 ± 0.03	0.00 ± 0.00

Discussion

For the plants the sucrose distribution and transport is crucial for the biomass and storage product formation as well as for the reproduction, thereby the sucrose translocation depends on several factors, *e.g.* sink strength and photosynthesis (Furbank *et al.* 2001; Bihmidine *et al.* 2013; Ludewig and Flügge 2013). The sucrose transport into the developing seeds is needed, as seeds are usually heterotrophic or mixotrophic. Sucrose import into the seed coat and embryos is changing throughout the pea seed development (Weber *et al.* 1997; Tegeder *et al.* 1999). Sucrose transporters and facilitators – SUTs, SUFs – are involved in the uptake of sucrose into the pea seed (Zhou *et al.* 2007, 2009). However, the importance of single sucrose transporter or sucrose facilitator on yield formation remains controversial, as sucrose is translocated in parallel via multiple transport proteins (Li *et al.* 2011b; Ishimaru *et al.*, 2007; Milne *et al.* 2013). The present study analyzed the effects of seed specific overexpression of the sucrose transporter (*Vf*SUT1) in pea embryos on enzyme activities and on metabolite concentrations in starch synthesis. Thereby an elevated starch synthesis rate was expeced.

The sucrose symporter SUT1 is well characterized in developing seeds of several monocot and dicot species. The pea *Ps*SUT1 and faba bean *Vf*SUT1 have highly similar sequences (97%), a similarly high affinity towards sucrose, high transport activity and are highly expressed in all tissues of the plant including the seeds. In the seeds, SUT1 is mainly localized in epidermal cells both in the seed coat and cotyledons and is the main SUT responsible for sucrose uptake into seeds, due to its high expression in seeds (Weber *et al.* 1997; Tegeder *et al.* 1999; Furbank *et al.* 2001; Sivitz *et al.* 2005; Zhou *et al.* 2007 and 2009; Scofield *et al.* 2007). This has been confirmed in the studies that analyzed the effect of overexpression or suppression of SUT1. For example the antisense suppression of *Os*SUT1 in rice led to the reduced growth and grain yield of rice (Scofield *et al.* 2002). In contrast to the repression of sucrose transport, the overexpression of sucrose transporters has led to an increased yield, *e.g.* the potato tubers expressing the rice sucrose transporters (*Os*SUT2 and *Os*SUT5) had higher biomass yield per plant though no enhancement in starch content (Sun *et al.* 2011). Overexpression of the barley *Hv*SUT1 in the endosperm of wheat led to a slight enhancement of the yield in field conditions with no increase in starch content, instead resulting in increased protein content (Weichert *et al.* 2010). Contrary to the findings in seeds, the effect of SUT1 on potato tuber yield and starch content is relatively low, as the repression of the *St*SUT1 and overexpression (using SUT1 from *Spinacia oleracia*) in potato tubers did not change the tuber yield and starch content (Kühn *et al.* 2003; Leggewie *et al.* 2003). The potato *St*SUT1 overexpression under the vicilin promoter in pea embryos has led to an

enhanced growth rate due to elevated uptake of sucrose, however the final seed dry weight was not changed (Rosche *et al.* 2002). The expression and synthesis of vicilin begins in the early cotyledon phase. However, the maximum expression peak and pattern of vicilin correlates with the mid cotyledon phase and with SuSy and AGPase activity, and the localization of vicilin synthesis in the embryo is confined to the cotyledon parenchyma (Abirached-Darmency *et al.* 2005; Colombek *et al.* 1999; Weber *et al.* 1996). Therefore the *St*SUT1 expression in pea should not interfere with the differentiation phase. However, the reasons why the elevation of *St*SUT1 did not increase the seed weight and yield remain unknown. One possibility is that the increased sucrose influx changed the sucrose to glucose ratio during the early cotyledon phase and affected the mitotic activity. This in turn may have led to seeds with reduced cell number and also earlier onset of maturation (Wobus and Weber 1999). A decreased cell number due to the early cessation of mitotic activity is doubtful, as vicilin accumulation occurs in pea cotyledon cells, which have already entered the cell expansion phase and are no longer mitotically active. Therefore the *St*SUT1 overexpression should not lead to smaller embryos due to decreased cell number (Corke *et al.* 1990).

In the present study most distinctive differences were found at the beginning of the starch synthesis pathway. *Vf*SUT1 expression in pea cotyledons led to an increased UGPase activity, a reduced soluble sugars concentrations and unchanged starch concentration in maturing SUT7 embryos (Table 9). The analysis of SUT7 and SUT wt support the existence of a sole plastidial AGPase in pea embryos and the possible ADP-Glc synthesis by SuSy in the cytosol as proposed in Chapter 4, because there were remarkable concentrations of ADP-Glc in cytosol in both pea lines with parallel confinement of AGPase solely into plastids (Table 12).

The overexpression of the sucrose transporter with *Vf*SUT1 under an unknown seed protein (USP) promoter in pea embryos was expected to enhance the sink strength and substrate availability for starch synthesis. Contrary to the expectations the overexpression of SUT1 led to slightly decreased concentrations of sucrose, glucose and fructose, and unchanged starch concentration. These results were similar to that found in pea embryos with the seed specific *St*SUT1 overexpression (Rosche *et al.* 2002), probably due to the fact that the USP promoter has similar action to the vicilin promoter used by Rosche *et al.* (2002). Both promototers cause the new transcript to be expressed dominantly during the early to mid seed maturation phase in cotyledon parenchyma (Bassüner *et al.* 1988; Bäumlein *et al.* 1991; Fiedler *et al.* 1993; Weber *et al.* 1996) and seem to have similar effects on seed growth. As stated above, the elevated sucrose influx may have led to an earlier maturation; however the reasons for this are still unclear.

It seems that SUT1 is not rate limiting for the uptake of sucrose into pea embryos, due to the high abundance and activity of the *Ps*SUT1 in epidermal cells (Weber *et al.* 1997; Tegeder *et al.* 1999; Zhou *et al.* 2007. This could explain also unchanged starch synthesis in maturing pea embryos by the overexpression of *Vf*SUT1 in embryo epidermal and cotyledon parenchyma cells. Three sucrose transporters have been identified in pea embryos: *Ps*SUT1, *Ps*SUF1, and *Ps*SUF4 (Zhou *et al.* 2009). Secondly, it seems reasonable to argue that the activities of sucrose translocating proteins (SUT, SUF) in the seed coats or even in leaves may have a stronger limiting character regulating the sink strength than the sucrose transporters in embryo (Zhou *et al.* 2007). Thirdly, the sucrose uptake correlates not only with the activity and expression of SUT, but the real sucrose uptake depends on sucrose concentration inside the embryo and its cleavage rate (Zhou *et al.* 2009). Thereby the increased sucrose concentration in embryo cells leads to a reduced uptake of sucrose, which can cause the reduction of *Ps*SUT1 expression (Zhou *et al.* 2009). Furthermore, the present study was done in a growth chamber under a controlled environment with optimal and constant growth conditions. The results may differ under limiting conditions occurring in the field; where the photosynthate availability is more affected by weather and day light length.

Elevation of sucrose concentrations activate the AGPase, and thereby increase the rate of starch synthesis, therefore the unchanged (slightly lower) starch content was unexpected in SUT7 (Sweetlove *et al.* 2002; Tiessen *et al.* 2003; Hendriks *et al.* 2003). It was also expected that SUT1 overexpression would lead to increased activity of SuSy, as sucrose can activate the SuSy (*e.g.* in rice; Tang *et al.* 2009). As the cleavage of sucrose is dominantly performed by SuSy, its activity should be limiting for starch synthesis and yield in pea embryos, as previously shown in potato tubers by repression and overexpression of SuSy (Zrenner *et al.* 1995; Baroja-Fernández *et al.* 2009). SuSy has a high activity in developing pea embryos and seed coats and its activity is strongly positively correlated to starch content and accumulation (Déjardin *et al.* 1997 a, b; Barratt *et al.* 2001). SuSy-overexpressing potato tubers have increased concentrations of starch, UDP-Glc and ADP-Glc and increased total yield (Baroja-Fernández et al. 2009). In contrast, the SuSy and UGPase in tobacco significantly influence and regulate the growth of plants, but their overexpression does not generally increase the partitioning of sugars into starch or cellulose (Coleman *et al.* 2006). Furthermore, the expression of SuSy is activated by osmotic stress, which may be caused by sucrose (Déjardin *et al.* 1999; Baud *et al.* 2004). As SuSy activity changes in pea embryos during their development (Edwards and ap Rees 1986; Dejardin *et al.* 1997b; Weigelt *et al.* 2009), the differences in SuSy activity and sucrose concentrations probably occurred during earlier

development, concomitantly with elevated SUT1 expression, and were not detectable in the maturation phase. This is indicated by the studies using pea embryos in the mid seed filling phase (20DAP) where the SUT1 overexpressed embryos had higher biomass and sucrose content (Junker, unpublished data; Table 14).

Table 14. Relative biomass composition of wild type and SUT1 overexpressed pea embryos in mid seed filling phase at 20 DAP.
Values represent mean ± SE of eight biological replicas for starch and for four biological replicas for sucrose and biomass.

	starch % in DW	**sucrose % in DW**	**biomass % in DW**
SUT wt	27.5 ± 2.5	21.4 ± 1.0	42.8 ± 2.3
SUT7	25.7 ± 0.1.3	29.6 ± 1.9	53.0 ±1.0
***t*-test *p*-value (α=0.05)**	0.54	0.02	0.01

The significantly enhanced (≈20 %) UGPase activity in SUT7 was unexpected, whereby this increase was mainly in cytosol. The subcellular analyses confined 16% of UGPase activity into plastids of wild type pea embryos and 5% in SUT7 embryos. Although UGPase is generally considered as a cytosolic enzyme (Kleczkowski 1994), strong evidence has recently emerged for the presence of plastidial UGPase (Kimura *et al.* 1992; Okazaki *et al.* 2009; Stensballe *et al.* 2008; Daher *et al.* 2010), for a more thorough discussion see Chapter 4. The UGPase activity in pea embryos is relatively high already in the early seed filling phase, but in the maturation phase its activity increases even nearly ten-fold (Edwards and ap Rees 1986; data for cultivar Eiffel and iAGP-3 in Appendix 5). It has been shown in the present and earlier studies that the UGPase reaction equilibrium in pea embryos is in favor of UTP and Glc1P formation (Turner and Turner 1958). This means that the UGPase reaction in pea embryos favors starch synthesis. The UGPase expression and activity is regulated metabolically, *e.g.* by phosphate concentration and sucrose (Kleczkowski *et al.* 2004). Thereby the elevated sucrose concentration activates the UGPase (Ciereszko *et al.* 2001). In the present study, at maturation, the sucrose concentration in SUT7 embryos was decreased. It might be possible that increased sucrose influx elevated the UGPase expression and the enhanced UGPase activity at 30 DAP reflects the UGPase stability. As the UGPase activity is very high, exceeding the activity needed for the flux through UGPase, especially in the maturing phase, its higher activity probably does not play a role in the sucrose partitioning to starch. The non-limiting role of UGPase on sucrose partitioning has been shown in studies with reduced UGPase (Zrenner *et al.* 1993; Meng *et al.* 2009). To summarise, sucrose could have caused the increased activities of UGPase, SuSy and AGPase in younger embryos, which could have influenced starch synthesis in the earlier seed filling phase. This change though was not detected for maturing pea embryos.

There might be other reasons for the lack of expected changes in the measured metabolite concentrations in maturing pea embryos. It could be that the SUT1 overexpression shifts the carbon flow into the cytosolic fatty acid production as shown in *Arabidopsis thaliana* embryos overexpressing a SUC5 gene belonging to the SUT1 glade of sucrose transporters (Pommerring *et al.* 2013) or the overflow of sucrose will be stored in raffinose oligosaccharides. The increased lipid accumulation can be neglected as there were no changes when analyzing the total lipid content of maturing embryos and furthermore the content of lipids is very low (approximately 0.8 % of fresh weight). A more reasonable explanation could be that the raffinose oligosaccharide concentration increased as the pea embryos accumulate a substantial amount of raffinose in maturing seeds when going into the dormant stage (Daveby *et al.* 1993). It is also possible that the SUT1 overexpression leads to an enhanced storage protein accumulation comparable to the wheat endosperm overexpressing the barley sucrose transporter (Weichert *et al.* 2010). Furthermore it might be that starch concentration and accumulation depends on the physical space of amyloplasts; the disruption of amyloplast membranes by starch granule expansion could initiate the cessation of seed storage accumulation and lead to desiccation and maturation (Bain and Mercer 1966; Horowitz 1983). Furthermore the amyloplasts in cotyledon parenchyma cells are by their nature half chloroplasts and thus photosynthetic (Bain and Mercer 1966; Flinn 1985; Smith *et al.* 1990; Rolletschek *et al.* 2003). The degradation of thylakoid membranes is associated to senescence; therefore the signals rising from the degradation might be involved in the regulatory network of seed maturation. The seed maturation underlies a complex regulation which involves the sugar and hormone signaling together with the expression of transcription factors; thereby the full understanding of the regulation is still missing (Weber *et al.* 2005; Santos-Mendoza *et al.* 2008). Obviously, seed size and starch concentration have different limitations besides the sucrose influx.

Conclusions

The overexpression of *Vf*SUT1 in pea cotyledon cells did not lead to the expected increase in starch content but in contrast led to a slightly decreased starch concentration during the maturing phase. In addition, also the concentrations of soluble sugars – sucrose, glucose, and fructose – were reduced in maturing embryos of the transgenic line SUT7. The changes caused by the overexpression of SUT1 need to be further analyzed. It might be that the starch concentration in pea embryos not only has substrate limitations but also physical and developmental restrictions. To summarize, seed metabolism and starch synthesis is robust and difficult to modify by enhancing or suppressing a single gene.

Chapter 6: General Discussion and Conclusion

Plant cells have a complex metabolism due to the separation of metabolic processes into distinguished organelles. Although the processes are separated, most of these are interdependent and underlay miscellaneous regulatory mechanisms. Furthermore, starch synthesis is regulated at different levels, starting with gene expression and ending with the regulation of enzyme activity. Thereby, simultaneous analysis of subcellular metabolism can give new insights to these complexities.

In the first chapter the non-aqueous fractionation (NAF) method was established for the developing pea embryos. Of specific interest was to study the starch metabolism during the seed filling phase of developing pea embryos. The seed filling phase of pea embryos starts when the developing cotyledons are fully differentiated, which is characterized by large changes in the concentration of soluble sugars and the shifting of sucrose synthase and invertase activities. The differentiation phase is characterized by a high hexose concentration and high invertase activity as well as high hexose transporter activity. During the transition, the active sucrose transport into embryos is initiated by the formation of special transport cells and the expression of the sucrose transporter (SUT) and the sucrose synthase activity increases in parallel with a reduction of the invertase activity (Borisjuk *et al.* 2003). The seed filling phase of pea embryos is characterized by the beginning of the accumulation of storage proteins and starch. Since during the seed filling phase the cells undergo temporarily and spatially structural and chemical changes, the separation of cell compartments is complicated (Borisjuk *et al.* 1995, 2003). The NAF of pea embryos was successful only using embryos in the late seed filling phase/maturing phase, most probably due to the more homogenous tissue containing dominantly large parenchyma cells. Large storage parenchyma cells lack the large central vacuoles, and are instead full of small dense protein bodies, which explains the separation of only two cell compartments – plastids and the so called cytosolic compartment (Hoh *et al.* 1995; Boyer *et al.* 1981; Gerhard and Heldt 1984; Stitt *et al.* 1989; Tiessen *et al.* 2012). Therefore NAF can be effectively used for the analysis of the plastidial and cytosolic (including mitochondria) carbohydrate metabolism in mature pea embryos.

Subcellular analysis was conducted in order to clarify the uncertainties regarding the localization of AGPase and ADP-glucose. Results of the subcellular analysis of maturing pea cotyledon cells strongly support the sole plastidial localization of AGPase and a remarkably high cytosolic pool of ADP-Glc. This result supports the idea of the possible synthesis of ADP-Glc in the cytosol via SuSy, as the ability of SuSy to synthesize ADP-Glc has been shown in several studies (Delmer 1972; Pozueta-Romero *et al.* 1991b; Baroja-Fernandez *et al.*

2003). The source of ADP for the ADP-glc synthesis via SuSy in the cytosol of pea embryo cells is probably the hexokinase catalyzed reaction, together with some reactions in the lower part of glycolysis. Furthermore ADP can be transported in counter action with ATP into the cytosol from amyloplasts. If the ADP in the cytosol is really used to synthesize ADP-Glc, the efficiency and high rate of starch synthesis can be explained. In maturing pea embryos the plastidial AGPase requires ATP and Glc6P to be transported from the cytosol, for ADP-Glc synthesis. ATP is counter-exchanged with ADP via the adenylate translocator (NTT1) in the plastid envelope. The hypothesis of possible ADP-Glc transport into plastids is supported by the existence of the Brittle1 homolog in pea (Kumpf 2012). Furthermore Schünemann *et al.* (1993) showed that the plastids from pea roots could transport ADP-Glc, which may also be true for the embryos. Still the studies of defective plastidial PGM (Harrison *et al.* 1998, 2000) contradict this hypothesis, as when ADP-Glc could be transported into plastids, the starch synthesis rate should have remained higher in these pPGM defective lines. Therefore it is more reasonable to suggest that Glc1P rather than cytosolic ADP-Glc might be used for starch synthesis by other enzymes then pAGPase is missing or defective (e.g. starch phosphorylase).

The study with elevated SUT1 expression in pea embryos indicates that the SUT1 activity is high in pea embryos and it is not rate limiting for storage synthesis. Furthermore the crucial role of the transitory phase in seed development is supported, as the SUT1 overexpression with USP promoter correlates with cell expansion and storage product accumulation. Thus it may have led to a shorter transitory phase and lower cell number.

Starch synthesis is very complex and in order to both understand and modify starch synthesis the simplistic view of this pathway is surely insufficient. The studies with AGPase repression and overexpression of SUT show that the central seed metabolism and fate of storage compound synthesis cannot be changed easily by enhancing or suppressing a single gene.

Abstract

Starch synthesis in plants is of high scientific and economic interest. Despite seeming to be thoroughly investigated, each year new findings regarding to its complexity and regulation are published. Recent observation of cytosolic ADP-Glc in dicotyledons species (*Solanum tuberosum*; Baroja-Fernandez *et al.* 2004) raised the question of whether ADP-Glc could be localized and synthesized in the cytosol of pea embryo cells. Therefore the transgenic pea line iAGP-3 (Weigelt *et al.* 2009) with a drastic reduction of ADP-Glc pyrophosphorylase (AGPase) activity was chosen for this analysis. The results of present study support the idea of localization of AGPase solely in the plastids. However, it appears that there is another source for ADP-Glc in the cytosol, in addition to AGPase in plastids, due to the equal localization of ADP-Glc in both plastids and the cytosol. The results of the present study indicate a higher relevance of several minor substrate routes on starch synthesis than previously thought; of which the most prominent is the glucose transport into plastids via a glucose transporter. In parallel a second study was performed with the sucrose transporter overexpressed pea line SUT7. Initially the SUT1 overexpression with *Vf*SUT1 was expected to enhance the starch content in embryos. However, the results actually indicate a slight reduction in starch and soluble sugar concentrations in maturing pea embryos. One reason may be the earlier maturation of SUT7 seeds, due to higher sucrose uptake and its rapid assimilation into starch. The regulatory changes are yet unknown.

Zusammenfassung

Umfassende Kenntnisse der Stärkesynthese in den Pflanzen sind von großem Bedeutung. Obwohl es bereits viele Erkenntnisse über die Stärkesynthese gibt, erscheinen jedes Jahr neue Publikationen über seine Komplexität und Regulierung. Neulich wurde gezeigt, dass ADP-Glc auch im Zytosol der zweikeimblättrigen Pflanzen vorkommt (*Solanum tuberosum*; Baroja-Fernandez *et al.* 2004). Deswegen wurde in dieser Arbeit die Frage gestellt, ob ADP-Glc auch im Zytosol der Parenchymazellen der Erbsenembryos lokalisiert ist und synthetisiert wird. Für diese Analyse wurde die transgene Erbsenlinie mit einer stark verringerten Aktivität der ADP-Glc-Pyrophosphorylase (AGPase) ausgewählt (iAGP-3; Weigelt *et al*. 2009). Die Ergebnisse unterstützen die anerkannte einzig plastidäre Lokalisation der AGPase. Die fast gleichmäßigen Verteilung der ADP-Glc Konzentration zwischen Plastiden und dem zytosolischen Kompartiment deutet jedoch auf, dass es eine andere Quelle für ADP-Glc im Zytosol gibt, zusätzlich zu der AGPase in den Plastiden. Die Ergebnisse der vorliegenden Studie weisen außendem daraufhin, dass viele bis jetzt als nicht relevant bezeichnete

Substratwege der Stärkesynthese eine höhere Relevanz haben könnten als bisher angenommen. Von diesen Substratwegen könnte z.B. der Glucosetransport über einen Glukosetransporter in die Plastiden von größerer Bedeutung sein.

Der zweite Teil der vorliegenden Studie wurde mit der Saccharosetransporter (SUT1) überexprimierenden Erbsenlinie SUT7 durchgeführt. Es wurde erwartet, dass die embryonenspezifische Überexpression von *SUT1* aus *Vicia faba* den Stärkegehalt in reifenden Erbsenembryonen erhöht. Doch die Ergebnisse zeigen eine leichte Abnahme des Stärkegehaltes und eine verringerte Konzentration an löslichen Zuckern in den reifenden Erbsenembryonen. Einer der Gründe hierfür könnte die frühere Reifung der Samen der SUT7 sein, aufgrund der erhöhten Saccharoseaufnahme und ihre rasche Assimilation in die Stärke in frühen Entwicklung. Die genauen regulatorischen Änderungen sind jedoch noch unbekannt.

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Eigenständigkeitserklärung

Hiermit erkläre ich, dass diese Arbeit bisher weder der Naturwissenschaftlichen Fakultät der Martin-Luther-Universität Halle Wittenberg noch irgendeiner anderen wissenschaftlichen Einrichtung zum Zweck der Promotion vorgelegt wurde.

Ich erkläre, dass ich mich bisher noch nie um einen Doktorgrad beworben habe.

Ich erkläre, dass ich die vorliegende Arbeit selbständig und ohne fremde Hilfe verfasst habe. Es wurden keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, und die den benutzten Werken wörtlich oder inhaltlich entnommen Stellen wurden als solche kenntlich gemacht.

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Appendices

Appendix 1 Western analysis of UGPase, AGPase, Rubisco localization in NAF fractions

SDS-PAGE electrophoresis modified after (Laemmli, 1970)

The separation gel (pH 8.8 with 1N HCl) final concentrations: 0.378M Tris, 0.1% SDS, 11.25% acrylamid, 0.05 % (v/v) tetramethylethylenediamine (TEMED) and 0.1% ammonium persulfate, 240mM HCl.

The stacking gel (pH 6.8 with 1N HCl) final concentrations: 0.123M Tris, 0.1% SDS, 11.25% acrylamid, 0.2% (v/v) tetramethylethylenediamine (TEMED) and 0.063% ammonium persulfate.

The electrode buffer (pH 8.3-8.6) contained: 0.25M Tris, 1.92M glycine, 0.1% SDS.

Semi-dry blot solutions

The anode solution I (pH 10.4 with 1M NaOH/1M HCl): 0.3M Tris, 20% methanol.

The anode solution II (pH 10.4 with 1M NaOH/1M HCl): 25 mM Tris, 20% methanol.

The cathode solution (pH 9.4 with 1M NaOH/1M HCl): 40mM capronic acid, 0.01% SDS, 20% methanol.

Immunodetection solutions

Tris buffered cooking salt solution with Tween (TBST) (filtered): 10mM Tris (pH 8 with HCl), 150mM NaCl, 0.05% Tween 20.

Blocking solution (filtered): 5% milk powder in TBST.

Antibody solutions: 0.5% BSA in TBST (filtered). Dilutions: AGPase 1:1000 (serum:TBST v:v), UGPase 1:3000, Rbcl 1:5000, IRDye 800CW coat anti-rabbit 1:15000.

Phosphate buffer cooking salt solution (PSB): 137mM NaCl, 1.8mM KH_2PO_4, 10.5mM $Na_2HPO_4*12H_2O$, 2.7mM KCl.

Appendix 2 Linear density gradient NAF protocol

Preparing the plant material

- Grind about 5 g fresh plant material twice for 3 minutes at 30 Hz using a swing mixer mill MM400 (Retsch GmbH, Haan, Germany) cooled with liquid N;
- take four small aliquots (≈40 mg) from the ground material;
- transfer the frozen ground material to a 50 ml plastic beaker, close the opening of beaker with paper tissue;
- lyophilize ≈100 hours at 0.52 mPa (-27˚C) in a freeze dryer Alpha 1- 2 LD Plus (Martin Christ, Osterode, Germany);
- weigh the dry samples and take four aliquots (≈20 mg);
- close the beakers quickly and place into plastic bags containing silica gel;
- store in boxes at -80˚C containing silica gel, or use immediately.

Preparing for gradient

- Suspend dried ground material with 15 ml tetrachlorethylene/heptane mix (66:34 v:v; ρ=1.3 g cm^{-3}) (solution A);
- sonicate for 30 seconds with 67 % power, 8 second pulses (Sonoplus HD 200, MS 73/D, Berlin, Germany) keeping the beaker in vessel filled with cooled (4˚C) heptane;
- take four 100 µl aliquots from this homogenate;
- filter the homogenate was through polyester net (20 µm) pre-wetted with solution A; wash the net once with 10 ml solution A;
- centrifuge the homogenate for 10 minutes at room temperature at 3200 g; discard the supernatant and resuspend the pellet in 3 ml solution A;
- resuspend pellet in 4 ml solution A (1.3 gcm-3);
- mix carefully and take aliquots (100 µl).

Gradient preparation

- form linear density gradient (30 mL, ρ = 1.43 – 1.62 g cm^{-3}) using Econo System Controller with a peristaltic pump Model EP-1 Econo Pump (Bio-Rad Laboratories Inc., Hercules, USA);
- use a 50ml polypropylene thickwall centrifuge tube (Beckman Coulter GmbH, Krefeld, Germany) and 11 cm long syringe needle placed with a tip on a bottom of tube;
- use two solutions: solution A and solution B (100% tetrachlorethylene);
- form gradient with flow rate 1.75 ml min^{-1} starting with 20% of solution A and 80% of B and ended with 100 % B (25 ml) and then an extra 5 ml of 100% B (total 30 ml);
- load the resuspended pellet carefully on top of gradient;
- centrifuge for 1 h at 5000 g with a swing out rotor (Sorvall DuPond HB-4) at 4˚C using centrifuge Sorvall RC5C Plus (Thermo Fisher Scientific Inc., Waltham, USA).;
- divide the gradient into ten fractions starting from top and transfer with Pasteur pipette into 15 ml tubes;
- dilute fractions with heptane 1:1 and centrifuge at 3200g for 5 min;
- discard supernatant and keep pellet;
- resolve the pellet with 2 ml 100% heptane and divide it into 4 equal aliquots and centrifuge at 3200g for 5 min; discard the supernatant;
- dry pellets in evaporator SpeedDry 2-33IR Rotational-Vacuum-Concentrator (Martin Christ, Osterode, Germany) for 15 minutes at 25˚C using 4.3 mbar vacuum;
- store the dry aliquots in -80˚C until further analysis.

Appendix 3 Confinement of metabolites and enzymes into two subcellular compartments — plastids and "cytosolic" compartment — according to BestFit analyzes done with cv. Eiffel and iAGP-3 embryos

Subcellular confinement (BestFit)	Eiffel							iAGP3							t-test between Eiffel and iAGP-3	
	plastid			cytosol			pla/cyt	plastid			cytosol			pla/cyt		
	average		sterr	average		sterr	t-test	average		sterr	average		sterr	t-test	plastid	cytosol
GAPDH	97.3	±	*0.2*	2.7	±	*0.2*	0.000	94.0	±	*1.4*	6.1	±	*1.4*	0.000	0.10	0.10
PEPCase	1.7	±	*0.9*	98.3	±	*0.9*	0.000	4.7	±	*1.7*	95.3	±	*2.0*	0.000	0.21	0.21
α-manno	12.9	±	*3.4*	87.1	±	*3.4*	0.000	15.5	±	*5.2*	84.5	±	*5.2*	0.000	0.69	0.69
iPPase	75.9	±	*2.3*	24.1	±	*2.3*	0.000	81.8	±	*4.4*	18.2	±	*4.4*	0.000	0.27	0.27
AGPase	94.0	±	*1.3*	6.0	±	*1.3*	0.000	26.4	±	*12.9*	73.6	±	*12.9*	0.027	**0.003**	**0.003**
UGPase	31.2	±	*9.7*	68.8	±	*9.7*	0.021	17.7	±	*7.5*	82.3	±	*7.5*	0.000	0.30	0.30
SuSy	3.6	±	*1.5*	96.4	±	*1.5*	0.000	2.2	±	*0.7*	97.8	±	*0.7*	0.000	0.41	0.41
PGM	20.8	±	*3.2*	79.2	±	*3.2*	0.000	**49.3**	**±**	***7.4***	**50.7**	**±**	***7.4***	**0.893**	**0.010**	**0.010**
PGI	5.7	±	*1.8*	94.3	±	*1.8*	0.000	1.9	±	*0.4*	98.1	±	*0.4*	0.000	0.09	0.09
HK (glc)	15.8	±	*3.3*	84.2	±	*3.3*	0.000	**32.4**	**±**	***13.3***	**67.6**	**±**	***13.3***	**0.091**	0.27	0.27
HK (fru)	31.1	±	*8.2*	68.9	±	*8.2*	0.008	**53.9**	**±**	***7.9***	**46.1**	**±**	***7.9***	**0.506**	0.07	0.07
Invertase	31.6	±	*4.9*	68.4	±	*4.9*	0.000	26.9	±	*4.8*	73.1	±	*4.8*	0.000	0.51	0.51
sucrose	8.5	±	*3.5*	91.5	±	*3.5*	0.000	9.1	±	*3.6*	90.9	±	*3.6*	0.000	0.90	0.90
starch	100.0	±	*0.0*	0.0	±	*0.0*	0.000	100.0	±	*0.0*	0.0	±	*0.0*	0.000	1.00	1.00
ADP-glucose	19.7	±	*0.9*	80.3	±	*0.9*	0.000	**61.6**	**±**	***20.5***	**38.4**	**±**	***20.5***	**0.454**	0.13	0.13
AMP	18.8	±	*6.8*	81.2	±	*6.8*	0.001	**44.0**	**±**	***19.5***	**56.0**	**±**	***19.5***	**0.679**	0.29	0.29
ADP	5.5	±	*1.5*	94.5	±	*1.5*	0.000	**33.7**	**±**	***12.7***	**66.3**	**±**	***12.7***	**0.118**	0.11	0.11
ATP	3.0	±	*1.3*	97.0	±	*1.3*	0.000	22.0	±	*8.4*	78.0	±	*8.4*	0.003	0.11	0.11
Glucose	23.1	±	*5.6*	76.9	±	*5.6*	0.000	8.5	±	*3.1*	91.5	±	*3.1*	0.000	0.054	0.054
Fructose	27.6	±	*8.1*	72.4	±	*8.1*	0.003	9.7	±	*4.2*	90.3	±	*4.2*	0.000	0.09	0.09
Glc6P	20.3	±	*6.7*	79.7	±	*6.7*	0.000	20.8	±	*5.6*	79.3	±	*5.6*	0.000	0.96	0.96
Fru6P	**60.8**	**±**	***7.9***	**39.2**	**±**	***7.9***	**0.080**	**39.5**	**±**	***7.2***	**60.5**	**±**	***7.2***	**0.066**	0.07	0.07
Glc1P	67.4	±	*5.2*	32.6	±	*5.2*	0.001	**52.9**	**±**	***9.9***	**47.1**	**±**	***9.9***	**0.688**	0.23	0.23
UDP-Glc	23.1	±	*5.4*	76.9	±	*5.4*	0.000	1.8	±	*0.8*	98.2	±	*0.8*	0.000	**0.010**	**0.010**
GAPDH+iPPase	90.8	±	*0.5*	9.2	±	*0.5*	0.000	91.7	±	*0.7*	8.3	±	*0.7*	0.000	0.33	0.33
PEPC+manno	5.1	±	*1.3*	94.9	±	*1.3*	0.000	5.7	±	*1.6*	94.3	±	*1.6*	0.000	0.78	0.78

Appendix 4 Correlation analysis for subcellular compartment markers for NAF made with embryos of pea cv. Eiffel and iAGP-3

Eiffel

Pearson's r	iPPase	AGPase	UGPase	α-manno	SuSy	starch	GAPDH
iPPase	1.00						
AGPase	0.65	1.00					
UGPase	0.38	-0.10	1.00				
α-manno	0.50	0.12	0.78	1.00			
SuSy	0.54	0.11	0.86	0.97	1.00		
starch	0.42	0.72	-0.36	-0.32	-0.32	1.00	
GAPDH	0.26	0.52	-0.33	-0.27	-0.26	0.43	1.00
PEPCase	0.14	-0.01	0.46	0.56	0.46	-0.32	-0.11
p-value	**iPPase**	**AGPase**	**UGPase**	**α-manno**	**SuSy**	**starch**	**GAPDH**
iPPase							
AGPase	0.000						
UGPase	0.013	0.525					
α-manno	0.001	0.451	0.000				
SuSy	0.000	0.504	0.000	0.000			
starch	0.006	0.000	0.020	0.040	0.041		
GAPDH	0.103	0.000	0.031	0.082	0.102	0.004	
PEPCase	0.379	0.955	0.002	0.000	0.002	0.039	0.508

iAGP-3

Pearson's r	iPPase	AGPase	UGPase	α-manno	SuSy	starch	GAPDH
iPPase							
AGPase	0.02						
UGPase	0.14	0.46					
α-manno	0.21	0.51	0.84				
SuSy	0.17	0.67	0.90	0.86			
starch	0.80	-0.24	-0.26	-0.19	-0.27		
GAPDH	0.83	-0.11	-0.17	-0.09	-0.15	0.82	
PEPCase	-0.01	0.79	0.83	0.69	0.91	-0.29	-0.14
p-value	**iPPase**	**AGPase**	**UGPase**	**α-manno**	**SuSy**	**starch**	**GAPDH**
iPPase							
AGPase	0.876						
UGPase	0.380	0.002					
α-manno	0.187	0.001	0.000				
SuSy	0.282	0.000	0.000	0.000			
starch	0.000	0.120	0.090	0.223	0.083		
GAPDH	0.000	0.482	0.290	0.582	0.346	0.000	
PEPCase	0.929	0.000	0.000	0.000	0.000	0.062	0.378

Appendix 5 Enzyme activities in six developmental points of pea embryos from cv. Eiffel and its transgenic line iAGP-3

average activity	iAGP-3						Eiffel					
	13 DAP 3±0.5 mg	15 DAP 20±2 mg	16 DAP 50±5 mg	21 DAP 300±20 mg	24 DAP 400±20 mg	30 DAP 500±20 mg	13 DAP 3±0.5 mg	15 DAP 20±2 mg	16 DAP 50±5 mg	21 DAP 300±20 mg	24 DAP 400±20 mg	30 DAP 500±20 mg
Alk Inv	269.0	227.3	327.7	**441.9**	**340.1**	109.9	353.8	240.6	309.4	**313.0**	**249.7**	79.2
HK (fru)	104.7	99.4	147.4	200.7	156.8	123.7	97.5	64.5	125.8	279.1	182.7	125.3
HK (glc)	41.8	51.2	74.9	42.8	51.6	77.5	49.3	40.4	40.5	51.2	71.1	88.7
SuSy	264.0	228.6	514.3	977.8	870.3	1093.7	347.61	263.71	473.49	869.71	639.40	1036.6
UGPase	424.1	463.4	431.3	**398.6**	**496.6**	4027.4	520.2	290.0	294.5	**325.1**	**339.8**	4101.7
AGPase	27.1	**3.7**	**17.3**	**36.0**	**33.7**	**4.61**	25.6	**26.0**	**37.4**	**142.7**	**171.0**	**317.60**
PGM	1885.1	2356.1	4748.3	6507.1	**5544.2**	**20615.7**	2103.06	2125.58	2617.82	5503.45	**3754.27**	**17504.5**
PGI	1304.0	1212.8	1931.9	3137.9	**4040.2**	12668.2	1420.4	843.5	1251.0	2598.9	**2752.8**	13390.8
iPPase	1746.7	2351.9	**2033.2**	**1093.5**	934.4	832.1	1674.5	1967.4	**1595.0**	**1280.8**	924.3	673.9
Glc6PDH	49.8	**75.6**	**135.1**	169.4	**245.5**		24.7	**23.3**	**40.6**	155.6	**165.7**	
sterr	iAGP-3						Eiffel					
Alk Inv	20.71	18.03	39.82	13.33	19.49	16.3	28.24	15.11	13.67	25.99	22.56	3.3
HK (fru)	14.22	16.30	41.35	22.19	17.34	6.9	8.14	5.27	10.31	13.54	13.20	5.0
HK (glc)	4.6	5.7	9.7	2.7	4.3	2.5	4.9	2.4	3.3	3.7	6.0	6.9
SuSy	36.0	22.8	34.9	123.5	64.7	64.6	22.7	23.8	78.0	30.7	63.6	31.0
UGPase	19.4	47.2	60.2	9.2	29.2	297.2	35.5	28.3	20.5	15.6	20.4	128.6
AGPase	4.7	5.4	4.0	1.6	2.4	2.26	2.5	4.0	4.3	7.2	12.7	30.98
PGM	134.0	174.5	776.9	213.0	485.8	718.2	279.6	212.6	283.6	628.5	399.4	1085.1
PGI	123.2	134.9	230.6	90.7	182.0	557.4	117.8	80.8	118.3	153.9	113.0	621.2
iPPase	96.72	129.62	81.30	42.29	31.62	68.0	126.3	125.8	86.5	42.9	51.2	21.5
Glc6PDH	5.6	6.0	22.8	6.5	15.3		6.0	4.8	6.4	13.8	12.2	
t-test: α=0.05; between Eiffel and iAGP-3												
Alk Inv	0.087	0.997	0.768	**0.009**	**0.008**	0.121						
HK (fru)	0.611	0.256	0.122	0.096	0.191	0.850						
HK (glc)	0.513	0.103	0.042	0.234	0.064	0.165						
SuSy	0.200	0.576	0.668	0.567	0.160	0.450						
UGPase	0.277	0.057	0.176	**0.040**	**0.009**	0.825						
AGPase	0.780	**0.007**	**0.034**	**0.000**	**0.000**	**0.000**						
PGM	0.948	0.931	0.128	0.373	**0.034**	**0.038**						
PGI	0.647	0.110	0.092	0.0500	**0.000**	0.405						
iPPase	0.761	0.160	**0.006**	**0.033**	0.926	0.068						
Glc6PDH	0.055	**0.000**	**0.025**	0.608	**0.011**							

Data represents the average of eight biological replicates both for Eiffel and iAGP-3. The significant differences (Student's t-test α=0.05) are marked in bold.

Enzyme activites are given as nmol*min^{-1}* g^{-1} FW.

Appendix 6 Confinement of metabolites and enzymes into two subcellular compartments — plastids and "cytosolic" compartment — according to BestFit analyzes done with maturing embryos of SUT wt and SUT7

Enzyme,	SUT wt		t-test		SUT7		t-test	t-test
metabolite	plastid	cytosol	pla/cyt		plastid	cytosol	pla/cyt	wt/SUT7
GAPDH	85.83 ± 6.32	14.17 ± 6.32	0.000		93.33 ± 4.60	6.67 ± 4.60	0.000	
PEPCase	3.50 ± 1.95	96.50 ± 1.95	0.000		3.17 ± 1.45	96.83 ± 1.45	0.000	
α-manno	0.83 ± 0.65	99.17 ± 0.65	0.000		0.33 ± 0.21	99.67 ± 0.21	0.000	
AGPase	95.00 ± 3.26	5.00 ± 3.26	0.000		96.17 ± 2.46	3.83 ± 2.46	0.000	
iPPase	79.83 ± 6.41	20.17 ± 6.41	0.000		72.00 ± 6.74	28.00 ± 6.74	0.001	
UGPase	16.33 ± 4.01	83.67 ± 4.01	0.000		4.67 ± 2.14	95.33 ± 2.14	0.000	0.035
SuSy	8.50 ± 4.20	91.50 ± 4.20	0.000		1.83 ± 1.33	98.17 ± 1.33	0.000	
Inv	46.67 ± 3.69	53.33 ± 3.69			37.17 ± 3.44	62.83 ± 3.44	0.000	
HK glc	31.83 ± 5.80	68.17 ± 5.80	0.001		41.50 ± 4.97	58.50 ± 4.97	0.036	
HK fru	40.33 ± 4.74	59.67 ± 4.74	0.016		39.83 ± 2.75	60.17 ± 2.75	0.000	
PGI	4.50 ± 2.91	95.50 ± 2.91	0.000		3.67 ± 0.92	96.33 ± 0.92	0.000	
PGM	21.00 ± 2.77	79.00 ± 2.77	0.000		26.17 ± 2.10	73.83 ± 2.10	0.000	
starch	100.00 ± 0.00	0.00 ± 0.00	0.000		100.00 ± 0.00	0.00 ± 0.00	0.000	
ADPGlc	56.67 ± 12.35	43.33 ± 12.35			62.00 ± 10.87	38.00 ± 10.87		
AMP	60.50 ± 6.10	39.50 ± 6.10	0.035		85.00 ± 6.15	15.00 ± 6.15	0.000	0.018
ADP	25.50 ± 7.60	74.50 ± 7.60	0.001		27.50 ± 5.00	72.50 ± 5.00	0.000	
ATP	17.50 ± 9.81	82.50 ± 9.81	0.001		9.33 ± 4.79	90.67 ± 4.79	0.000	
sucrose	24.83 ± 6.77	75.17 ± 6.77	0.000		9.67 ± 4.25	90.33 ± 4.25	0.000	
Glc	14.83 ± 7.44	85.17 ± 7.44	0.000		6.50 ± 4.27	93.50 ± 4.27	0.000	
Fru	13.67 ± 6.82	86.33 ± 6.82	0.000		1.33 ± 0.88	98.67 ± 0.88	0.000	
Glc6P	38.17 ± 4.35	61.83 ± 4.35	0.003		19.83 ± 5.38	80.17 ± 5.38	0.000	0.025
Fru6P	66.17 ± 10.52	33.83 ± 10.52			53.17 ± 6.82	46.83 ± 6.82		
Glc1P	64.17 ± 7.15	35.83 ± 7.15	0.019		35.67 ± 8.12	64.33 ± 8.12	0.032	0.025
UDPGlc	24.33 ± 11.08	75.67 ± 11.08	0.008		22.17 ± 8.87	77.83 ± 8.87	0.001	