Signaling Pathways in Legume Seed Development: Evidence for a Crosstalk between Trehalose 6-Phosphate and Auxin

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“My ambition is handicapped by lethargy.”

To Alexander

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Summary

Seed yield and seed quality are of significant interest for modern plant breeding and efficient food production. Regardless of its biological and economical importance, the molecular mechanisms regulating seed size and storage compound accumulation are still not well elucidated. In order to identify factors regulating such processes, seed material from different genetic and transgenic legume models was analyzed on the morphological, biochemical and transcriptional level.

Reciprocal crossings between different fava bean (*Vicia faba*) varieties previously demonstrated that hybrid vigor finds expression in mature embryo biomass and responds to parental genetic relatedness. Here, selfed and crossed seeds of two homozygous fava bean lines served as models for the investigation of the molecular mechanism underlying seed heterosis. The measurement of dry seed weight in reciprocal crosses revealed approximately 10% higher values over a period of 3 years when compared to inbred seeds. Transcript profiling indicated the stimulation of cell proliferation quite early in development delaying the onset of maturation in hybrid embryos, an effect, possibly enhancing assimilate uptake capacity and sink strength. Accordingly, higher metabolite fluxes increased the demand for assimilates, possibly leading to a shift in the size of available metabolite pools. As evidenced by transcriptional deregulation of a remarkable number of genes related to auxin functions, the growth advantage of crossed seeds over selfed seeds is potentially mediated by auxin effects.

Auxins regulate many aspects of plant development, although generally controlling cell proliferation in young tissues. Interestingly, developing seeds accumulate higher auxin levels than most other of the tissues of the plant, but its role during seed maturation and seed filling is still poorly understood. The characterization of the *tryptophan aminotransferase related2* (*tar2*) mutant in garden pea (*Pisum sativum*) revealed that auxin deficiency has detrimental effects not only on growth processes, but also on reserve starch accumulation in embryos. Mutation of *TAR2* largely impairs auxin synthesis at later stages of pea seed development, particularly that of the major auxin 4-chloroindole-3-acetic acid (4-Cl-IAA). Mature *tar2* seeds were strongly wrinkled when dry, smaller and contain significantly less starch on a per gram basis. Embryo-specific expression of wild type (WT) *TAR2* in homozygous *tar2* plants reversed the wrinkled phenotype and complemented seed size and starch content. Accordingly, treatment of growing *tar2-1* seeds with synthetic auxin reduced the wrinkling and increased starch content in mature seeds. Moreover, the activity of pivotal starch synthesis enzymes was reduced in mutant seeds, as was the expression of the corresponding genes. The presence of a number of auxin response elements in the promoter region

of those genes provides evidence that auxin participates directly in transcriptional regulation of starch synthesis in pea seeds. These findings considerably widen the scope for auxin action in plants and provide indications for a close interaction between auxin and sugar signaling pathways.

Carbohydrates are thought to play a crucial role in the regulation of seed development, and trehalose 6-phosphate (T6P) has been proposed as a key factor of sugar signaling that regulates plant growth in response to sugar availability. In the course of my studies, aiming on the modulation of T6P levels in pea embryos, it has become evident that T6P is required for normal seed maturation and germination. Embryo-specific expression the *Escherichia coli otsB* gene (USP::TPP) reduced the T6P content and had similar effects on seed development as did the mutation of *TAR2*. Accordingly, transgenic seeds were smaller, wrinkled, and contained significantly less starch. Transcript profiling and hormone measurements revealed that modulation of T6P affects auxin biosynthesis in growing embryos, since changes in the expression level of *TAR2* and in the amount of 4-Cl-IAA corresponded to the changes in T6P content. Through introduction of USP::TAR2 into USP::TPP lines, it was possible to bypass the repression of the innate *TAR2* gene and to restore seed size and starch content. This indicates that T6P facilitates its ability to promote embryo growth and reserve starch accumulation through transcriptional upregulation of auxin biosynthesis. Put together, results of this thesis suggest that both T6P and auxin participate and interact in a novel regulatory network that controls maturation and storage processes in developing legume seeds.

Zusammenfassung

Samen enthalten den von der Samenschale eingeschlossenen Pflanzenembryo, nebst einem Nährgewebe, und dienen der generativen Vermehrung und Ausbreitung von Samenpflanzen. Samenertrag und Samenqualität sind daher von entscheidendem Interesse für die moderne Pflanzenzüchtung und Nahrungsmittelproduktion. Ungeachtet ihrer wirtschaftlichen und biologischen Bedeutung sind die molekularen Mechanismen, welche die Samengröße als auch die Einlagerung von Reservestoffen kontrollieren, noch immer nicht gänzlich aufgeklärt. Mit dem Ziel neue regulatorische Faktoren dieser Reifeprozesse zu identifizieren, wurden umfassende morphologische, molekularbiologische und biochemische Studien an den Leguminosen Ackerbohne (*Vicia faba*) und Erbse (*Pisum sativum*) durchgeführt.

Reziproke Kreuzungen zwischen verschiedenen Ackerbohnensorten hatten vormals ergeben, dass die erhöhte Wuchskraft mischerbiger Samen gegenüber den reinerbigen ihre Ausprägung bereits in der Biomasse der reifen Samen findet und dabei durch die elterliche

genetische Verwandtschaft beeinflusst wird. Rein und mischerbige Samen zwei verschiedener Ackerbohnensorten dienten in der vorliegenden Arbeit als Untersuchungsmodelle, um die zugrundeliegenden molekularen Mechanismen der Samenheterosis zu entschlüsseln. Der Vergleich der mittleren Samengewichte dieser reziproken Kreuzungen ergab gegenüber den reinerbigen Samen eine Biomasseerhöhung von ungefähr 10% über einen Untersuchungszeitraum von drei Jahren. Umfassende Transkriptanalysen deuten dabei auf eine Stimulation der Zellproliferation während der frühen Entwicklungsphase des Hybridembryos hin, welche daraufhin zu einer Verzögerung der Reife und Speicherphase führt. Die daraus mögliche Folge ist eine erhöhte Assimilataufnahmekapazität und damit verbundene Steigerung des Speichervermögens. Die entsprechend höheren Metabolitflüsse steigern den Assimilatbedarf des wachsenden Hybridembryos und führten vermutlich zu einer Verlagerung des Metabolitgleichgewichts. Desweiteren deutet die transkriptionelle Degregulation einer beachtlichen Zahl auxinbezogener Gene darauf hin, dass der Wuchsvorteil der mischerbigen gegenüber den reinerbigen Embryonen möglicherweise durch die Wirkung von Auxin vermittelt wird.

Auxine steuern viele Aspekte der pflanzlichen Entwicklung, insbesondere die Zellproliferation in jungen Geweben. Interessanterweise akkumulieren reifende Samen weit höhere Auxinmengen als die meisten anderen Pflanzengewebe. Trotzdem ist die Funktion dieses Pflanzenhormons während der Samenfüllung weitgehend unbekannt. Durch die Charakterisierung einer Auxinbiosynthesemutation in der Modellpflanze Erbse konnte gezeigt werden, dass sich ein Auxinmangel nicht nur störend auf embryonale Wachstumsprozesse sondern auch nachteilig auf die Synthese der Speicherstärke auswirkt. Die Mutation des *TRYPTOPHAN AMINOTRANSFERASE RELATED2* (*TAR2*)-Gens beeinträchtigt in hohem Maße den Auxingehalt reifender Samen, insbesondere den des halogenierten Auxins 4-Chloroindole-3-Essigsäure (4-Cl-IAA). Die reifen *S*amen der *tar2*-Mutante sind stark gerunzelt, deutlich kleiner und enthalten weniger Stärke als jene des Wildtyps. Durch Komplementation der *tar2* Mutante, mittels embryospezifischer Expression des funktionalen *TAR2*-Gens, konnte der runzlige Samenphänotyp aufgehoben aber auch die Auswirkungen auf Samengröße als auch Stärkegehalt umgekehrt werden. Ähnliche Ergebnisse konnten durch eine äußere Auxinbehandlung reifender Mutantensamen erreicht werden. Die Ursache für den reduzierten Stärkegehalt reifender *tar2*-Samen lag in der verminderten Aktivität verschiedener Schlüsselenzyme der Stärkebiosynthese. Da ebenso die Expression der dazugehörigen Gene betroffen war und zudem eine Reihe möglicher auxinspezifischer *Response*-Elemente in den Promotorregionen dieser Gene identifiziert werden konnte, erscheint es sehr wahrscheinlich, dass Auxine direkt an der transkriptionellen Regulation der Stärkebiosynthese beteiligt sind. Somit erweitern diese Forschungsergebnisse das bekannte

Wirkspektrum dieses Pflanzenhormones wesentlich und weisen zudem auf eine direkte Interaktion zwischen Auxin-und Zuckersignalwegen hin.

Kohlenstoffhydrate stehen seither unter Verdacht, unmittelbar an der Steuerung der Samenentwicklung beteiligt zu sein. Seit einigen Jahren betrachtet man das Disaccharid Trehalose 6-Phosphat (T6P) als zentrales Signal für die zelluläre Verfügbarkeit von Zuckern, welches gemäß das Wachstum und die Entwicklung höherer Pflanzen reguliert. Durch eine embryospezifische Modulation des T6P-Gehalts in transgenen Erbsenpflanzen, wurde deutlich, dass T6P entscheidend an der Regulation der Samenreifung und Keimung beteiligt ist. Die embryospezifische Reduktion des T6P-Gehaltes rief hierbei vergleichbare phänotypische Veränderungen der Samen hervor wie der funktionale Verlust des *TAR2*-Gens. Ausgereifte USP::TPP Samen sind ebenfalls runzelig und das Gewicht als auch der Stärkegehalt dieser Samen sind deutlich reduziert. Eine Analyse der Transkript- und Hormonmengen wies dabei auf eine mögliche Störung der Auxinbiosynthese hin, da die *TAR2*-Transkriptmengen als auch der 4-Cl-IAA Gehalt in den transgenen Embryonen drastisch verringert waren. Um zu beweisen, dass die Repression des *TAR2*-Gens ursächlich für den Samenphänotyp der USP::TPP Pflanzen ist, wurden diese Pflanzen mit USP::TAR2 Linien gekreuzt. Dadurch wurde eine T6P-unabhängige Expression von *TAR2* in wachsenden Embryonen ermöglicht, in deren Folge eine Erhöhung des Stärkegehaltes und Embryogewichte erreicht werden konnte. Aus diesen Ergebnissen folgt, dass T6P über die transkriptionelle Anregung der Auxinbiosynthese das embryonale Wachstum als auch die Speicherstärkesynthese begünstigt. T6P fungiert dabei als ein übergeordneter Regulator der TAR2-abhängigen Auxinbiosynthese. Zusammenfassend kann man aus den vorliegenden Ergebnissen schlußfolgern, dass Auxin und T6P entscheidend an der normalen Samenentwicklung in Leguminosen beteiligt sind. Der Nachweis einer direkten Abhängigkeit beider Signalwege trägt dabei maßgeblich zu einem besseren Verständnis von Zucker-Hormon-Interaktionen in Pflanzen bei.

Chapter 1

General Introduction

In flowering plants, sexual reproduction occurs within floral organs. The newly formed generation starts as an embryo inside the growing seed, which arose from a fertilized ovule. The ovule of angiosperms is a multicellular entity that harbors an unfertilized egg cell, synergids, antipods and the large central cell. It is buried beneath several cell layers of the gynoecium, the female reproductive organ. After double fertilization of the haploid egg cell and the two polar nuclei of the central cell by two haploid male gametes, the diploid zygote and the primary endosperm are formed, respectively. The initial unequal division of the zygote generates two daughter cells one of which develops into the embryo proper, whereas the second cell forms the suspensor, a highly diverse tissue that pushes the embryo into the ovule lumen and keeps it connected with the maternal tissues. During embryogenesis, the basic embryo architecture is established and cotyledons as well as the shoot and root meristems are formed. Furthermore, accumulation of reserve compounds occurs to nourish the seedling during germination. Therefore, embryonic cells need to become specified and must differentiate into various cell types. Genetic studies mainly on Arabidopsis (*Arabidopsis thaliana*) (Hennig et al., 2004) as well as physiological approaches on legume and cereal seeds (Le et al., 2007) revealed that specific gene expression pattern and metabolic changes need to be spatially and temporally coordinated over the entire course of development in order to form a viable mature seed.

Legume seed development has been described in many studies, since many large-seeded species, such as fava bean or garden pea represent excellent models to study this issue in detail (reviewed in Weber et al., 2005). Embryogenesis roughly encompasses two major parts which will be illuminated in detail below (Fig. 1.1). The first part, the differentiation phase, is characterized by active cell division and embryo formation. During the second phase of embryo maturation, cotyledon cells are expanding and accumulate reserve compounds (e.g. oils, starch and proteins). The end of maturation is characterized by loss of water and the establishment of a quiescent state and various forms of dormancy.

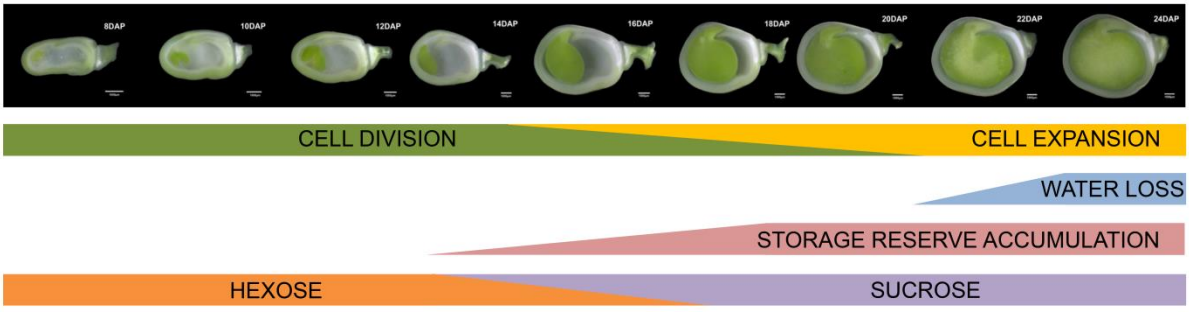

Figure 1.1. Schematic time course of developmental processes in growing garden pea embryos. Images represent longitudinal sections of developing pea seeds (8-24 DAP). Scale bars, 1mm.

Early embryo development

A major drawback for the study of early embryogenesis is the small size of young embryos and their location within the plant. The use of large-seeded legumes like fava bean allow the readily manipulation of young embryos, and for this reason many physiological and biochemical studies on early seed development are based on these species. Young fava bean embryos exhibit a high mitotic activity (Borisjuk et al., 1995). Few days after fertilization the globular embryo constitutes a radially symmetric meristem-like structure that consists of thin-walled and non-vacuolised cells. Subsequently, embryo symmetry changes from radial to bilateral and cotyledons are formed due to the unequal distribution of mitosis. The early pattern formation appears to be largely defined by maternal factors. It has been suggested that this early extrinsic control is exerted through seed coat-borne acid invertases (Weber et al., 1995). In fava bean, a cell wall-bound invertase (cwINV) is expressed in the unloading area of the young seed coat and cleaves the incoming sucrose in the apoplastic space between filial and maternal tissues. The liberated hexoses are possibly taken up by the embryo through via a hexose transporter (Weber et al., 1997). Jointly, these processes maintain a high hexose-containing environment that promotes the high cell division rate in the growing embryo. However, it is still unknown how a maternally generated sugar signal integrates into the control of cell division in the embryo. Our current understanding of the sugar-mediated control of cell division is largely based on sugar feeding experiments on cell suspension cultures and mutant seedlings. Supply of glucose closely correlates with the expression levels of several D-type cyclins (Riou-Khamlichi et al., 2000) and promotes the G2/M transition in Arabidopsis meristematic tissues by repressing the transcription of the negative regulator *TPR-DOMAIN SUPRESSOR OF STIMPY* (Skylar et al., 2011). Consequently, gene expression of central cell cycle components, required for the G2/M transition, is activated. These findings suggest the pivotal role of glucose signaling in regulating cell cycle progression, albeit feeding of glucose alone is not sufficient to trigger mitosis. Apparently, auxin signaling is also required for sustained mitotic activity (Wang and Ruan, 2013).

Further evidence for the cwINV-mediated regulation of cytokinesis during seed development comes from several studies on maize (*Zea mays*) kernels. In contrast to legume seeds, maize endosperm develops much more rapidly than the embryo, facilitated by a spatial gradient of glucose concentration between both tissues. Normal growth and sink strength in the developing endosperm depends on a high hexose environment that is maintained by *MINIATURE1* (*Mn1*) (Miller and Chourey, 1992). *Mn1* encodes a cwINV that is specifically expressed in the basal endosperm transfer layer, and loss of invertase activity in *mn1* mutants decreases hexose levels in the basal endosperm and causes a drastic reduction in endosperm size and weight (Cheng et al.,

1996). In order to prevent vigorous growth of the embryo, cleavage of sucrose into fructose and glucose is suppressed by expression of the invertase inhibitor ZmINVINH1, which localizes to a confined region that surrounds the embryo. By blocking cwINV activity in this cell layer, ZmINVINH1 has been suggested to serve as a barrier for hexose flow towards the embryo, at the same time it allows the hexose supply of the proliferating endosperm (Bate et al., 2004).

Genetic basis of seed size and sink strength

Before introducing the second part of seed development, the maturation phase, it should also be mentioned that the early extrinsic control of early embryo development substantially affects the final size and sink capacity of seeds. Reciprocal crossings between garden pea varieties with contrasting seed sizes revealed that dry seed weight and cell number are mainly determined by the maternal genotype, while the influence of the embryos own genotype seems negligible (Davies, 1975). By comparing large-seeded and small-seeded fava bean genotypes, it has been shown that larger seeds are the result of an extended period of cell division which is controlled by a seed coat-derived signal (Weber et al., 1996) It is very likely that a prolonged duration of cwINV activity in the seed coat accounts for the extension of the early cell division phase, which lastly increases sink capacity and seed size in the large-seeded varieties.

Nevertheless, F_1 seeds from some crossings between large-seeded and small seeded pea varieties showed a marked positive deviation from the maternal parent value (Davies, 1975). This suggests that the genetic constitution of the hybrid embryo, in other words the "intrinsic system", can significantly contribute to the determination of seed size. The phenomenon that hybrids (the progeny of crosses between diverse varieties or species) exhibit enhanced performance relative to the mean of their parents is referred to hybrid vigor or heterosis, and it is of particular interest for plant breeding to improve agricultural and yield-related traits in crop plants, such as biomass accumulation, speed of development, seed number or fertility (East, 1936; Birchler et al., 2010). This beneficial effect is confined to the F_1 generation and is already reduced in F_2 and subsequent generations. Since embryos are genetically distinct from that of their mother plant, particularly when different male gametes are introduced by cross-pollination, it is clear that embryos themselves can potentially exhibit heterosis when compared to the inbred. Accordingly, heterotic increments by 2% to 8% in mature seed size of fava bean due to cross fertilization versus selfing were reported by Duc et al. (2001). However, estimation of heterotic effects in embryos is hampered due to the fact that homozygous seeds of the male parent cannot grow on the same plant together with hybrid or homozygous seeds of the female parent. This drawback can be countered by producing hybrid seeds on both parent plants and taking the average as F_1 value to compare it with the average of

the homozygous seeds that are grown simultaneously on the same plants. Using this method, quantitative genetic analysis of embryo heterosis in fava bean revealed a mid parent-heterosis of 10.6 % on average for mature seed weight and 14.5 % in regard to seedling weight. In both traits, the genetic constitution of the embryo contributes considerably to the observed genetic variation (Dieckmann and Link, 2010). Nevertheless, the heterotic effects on embryo size are more articulated in crop plants. Meyer et al. (2007) demonstrated a strong cross breeding advantage of excised and *in vitro* cultured hybrid maize embryos reflected by 31% longer embryos compared to homozygous parental embryos. In Arabidopsis, the available data on seed heterosis are contradictory. Morphological analysis of reciprocal crosses of the Arabidopsis parental lines Col-0 and C24 indicates that seed size and seed weight are not altered in hybrid seeds (Meyer et al., 2012). A recent study that includes a larger number of parental combinations depicts a more intricate situation (Groszmann et al., 2014). Some parental combinations lead to larger mature F_1 embryos, but the differences between the various reciprocal hybrids suggest that embryo heterosis is likely influenced by maternal effects.

With regard to most plant species, growth changes released by heterotic effects basically result from differences in cell numbers. Cell size is usually not altered, although recent work on intraspecific Arabidopsis hybrids suggests that increase of both cell size and cell number contribute to enlarged cotyledons and rosette leaves (Groszmann et al., 2014). Nevertheless, the relative contribution of increased cell size largely differs between various F_1 hybrids and is generally lower than the contribution of mitotic activity. A seminal study focusing on changes in cell numbers provides new insights into the understanding of the molecular basis of heterosis in maize (Guo et al., 2010). Characterization of the maize *CELL NUMBER REGULATOR* (*ZmCNR*) family revealed that expression of Zm*CNR2* is negatively correlated with hybrid seedling vigor. Transgenic overexpression of the closely related *ZmCNR1* gene reduces plant and organ size and the strength of *ZmCNR1* expression was negatively correlated to vigor of transgenic plants. In contrast, reduction of *ZmCNR1* transcripts by either transgenic co-suppression or RNA interference caused a better growth performance due to the increase of cell number. There is a likelihood that the growth advantages of hybrid embryos also result from increased cell numbers (Meyer et al., 2004). Therefore, it will be interesting to further investigate whether similar molecular mechanisms also contribute to seed heterosis in crops like fava bean or maize.

Seed maturation

Initiation and control of storage processes

Once early embryogenesis is completed, mitotic growth decreases and the embryo passes through a fundamental switch from a meristem-like tissue into a highly differentiated storage organ. This transition phase is characterized by the initiation of a nutrient uptake system based on transfer cells, induction of storage-associated gene expression and the acceleration of growth, largely driven by cell expansion (Weber et al., 2005). Transfer cells are ubiquitous in plants and likely represent a morphological adaptation to facilitate nutrient transport (Offler et al., 2003). This cell type is characterized by secondary cell wall ingrowths which effectively increase the surface of the subjacent plasma membrane, enriched in assimilate transporters. Transfer cell formation in seeds is found in close proximity to maternal unloading tissues like the endospermic cell layer facing the nucellar projection in barley and cells of the basal endosperm in maize kernels (Weschke et al., 2000; Gómez et al., 2002). In some legumes, transfer cells are formed on the inner surface of the seed coat and in the epidermal cell layer of the cotyledons (Offler et al., 1989).

The requirement of transfer cells for normal embryo growth and maturation has been demonstrated by analysis of the *E2748* mutation in garden pea (Borisjuk et al., 2002). Although the gene product of *E2748* is not yet known, it is required for epidermal differentiation into transfer cells in embryos. The early development of mutant embryos is hardly distinguishable from that of the WT but further growth is arrested at the beginning of maturation phase, ultimately resulting in seed abortion. The loss of epidermal identity causes lower sucrose and starch levels in embryos, possibly because of the disturbed expression of the sucrose transporter (SUT1) and disruption of the symplastic continuity within the parenchyma.

Transfer cell-specific expression of SUT1 accounts for sucrose enrichment in the underlying tissues of the embryo (Weber et al., 1997). Increased uptake capacity of sucrose combined with declining cwINV activity in the seed coat leads to a switch from high-hexose to a high-sucrose state. Several lines of evidence suggest that increased sucrose accumulation functions as a trigger of storage associated processes (Koch, 2004). Sucrose feeding to young cotyledons leads to a disruption of the meristematic state and to the induction of cell expansion (Weber et al., 1996). Moreover, transcript levels of starch synthesis enzymes such as sucrose synthase (SUS) and ADP-glucose pyrophosphorylase (AGP) are up-regulated in response to sucrose (Heim et al., 1993; Weber et al., 1998). In order to demonstrate the effect of changed sucrose levels in storage cells of *Vicia narbonensis*, transgenic plants expressing a yeast-derived invertase have been generated (Weber et al., 1998). These transgenic seeds are wrinkled and contain lower amounts sucrose and starch, while hexose concentrations are increased. Further analysis revealed that changes in starch

content and transcript levels of SUS and AGP positively correlate with changes in sucrose concentration. These findings are consistent with the proposed function of sucrose in controlling storage associated differentiation and starch synthesis in maturing seeds. However, exogenous and endogenous modulation of sucrose levels will always influence the levels of other sugars like hexoses, hexose phosphates and nucleotide diphosphate sugars, an issue that complicates the interpretation of such experiments with regard to signaling functions. Therefore, the role of sucrose in mediating seed storage processes needs to be re-examined, particularly since the identification of the trehalose biosynthesis mutation *tps1* in Arabidopsis fundamentally changed the view on sugar signaling in seeds (Eastmond et al., 2002).

Storage processes

It is a typical feature of seeds to accumulate different kinds of reserve compounds during maturation .This repository is used later on to maintain seedling growth during germination until photosynthesis and autonomous growth are established. Usually, seeds contain a mixture storage compounds consisting of proteins and starch or oil (Bewley and Black, 1994). Depending on the plant species, the relative proportions vary considerably and contribute by up to 90% to the seed dry weight. Beyond the diversity of the storage compound mixtures, the tissues that serve as the repository for storage products differ as well. In monocots and endospermic dicots, storage cells are formed in the endosperm and the embedded nutrients get absorbed by the germinating seedling. Periplasmic seeds, like in some species of the *Amaranthaceae*, contain a diploid maternal food storage tissue that originates from the nucellus. In non-endospermic dicots, which include most of the crop legumes, cotyledons become filled and the endosperm is absorbed as the embryo grows inside the developing seed. At maturity, the endosperm has disappeared completely.

Dry seeds of important crop legumes of the *Fabeae*, including garden pea, fava bean and lentils (*Lens culinaris*), contain high amounts of carbohydrates (40-60%) and proteins (20-30%) inside of the embryo. In the past, garden pea served as a popular model plant to investigate reserve starch accumulation and storage protein deposition in seeds. Mutations that affect starch formation often result in the "wrinkling" of mature seeds (Kooistra, 1962). This distinct phenotypic effect enabled Mendel to carry out his fundamental experiments. In addition to Mendel´s famous mutation at the *rugosus* (*r*) locus (Bhattacharyya et al., 1990), other wrinkled mutants have been generated by Ethyl methanesulfonate induced mutagenesis and considerably contributed to the understanding of starch biosynthesis in heterotrophic seed tissues of dicots (Wang et al., 1990). In wrinkled seeds, impaired sucrose-to-starch conversion raises soluble sugar levels inside the embryo. As a consequence of the lowered osmotic potential, the water movement into storage cells increases and the embryo expands inside the seed coat. Reaching maturity, the mutant embryo dries and

shrivels more than the WT. Consequently, the overstretched seed coat wrinkles as the embryo shrivels inside.

Starch biosynthesis in maturing embryos depends on maternal sucrose supply via the seed coat. Once taken up by the embryo through SUT1, sucrose is gradually distributed within the embryonic parenchyma via symplastic transport, serving as the initial precursor for carbohydrate storage compounds (Borisjuk et al., 2002). As a first step, hexose phosphates are formed from imported sucrose by a series of reversible reactions in the cytosol of storage cells (Fig. 1.2). The sucrose synthase (SUS) catalyzes the readily reversible formation of UDP-glucose and fructose from UDP and sucrose, while sucrose breakdown activity prevails in maturing pea seeds (Craig et al., 1999). SUS activity is associated with sink strength and is abundant in heterotrophic tissues (Wang et al., 1993; Zrenner et al., 1995). Sucrose cleavage activity is allosterically inhibited by free hexoses and has a high K_m-value for sucrose (Ross and Davies, 1992). Efficient sucrose breakdown during the storage phase, therefore, depends on high sucrose levels and rapid removal of cleavage products. It appears that the initiation of reserve starch synthesis is driven by increased SUS activity, released by the metabolic switch from high to low hexose levels and the induction *SUS* gene expression during the transition phase.

Products of sucrose cleavage, UDP-glucose and fructose, are converted into hexose phosphates via UDP-glucose pyrophosphorylase and hexokinases, respectively. A state of equilibrium between the different kinds of hexose phosphates is maintained by activity of phosphoglucomutase (PGM) and phosphoglucoisomerase. The following steps of starch synthesis are carried out within the stroma of the amyloplast. Several lines of evidence suggest that glucose 6-phosphate (G6P) is the metabolite to be imported into the plastid (Kammerer et al., 1998). Sugar feeding experiments on isolated amyloplasts revealed that G6P is the only substrate capable to release physiologically relevant rates of starch synthesis (Hill and Smith, 1991). However, it has been shown that glucose 1-phosphate (G1P) is also translocated into amyloplasts of wheat endosperm as well as potato tubers (Tyson and Ap Rees, 1988; Kosegarten and Mengel, 1994). Thus, an alternative pathway based on direct glycosyl transfer from G1P to starch by plastidial phosphorylase (Pho) has been postulated (Fettke et al., 2010). However, loss of plastidic PGM in the starch-less *rug3* mutant implies that import of G6P into plastids is an indispensable step of starch biosynthesis in pea that cannot be bypassed by a potential import of G1P (Harrison et al., 1998). In legume seeds, however, no data are available whether plastidic G1P that is synthesized by plastidic PGM is utilized by Pho.

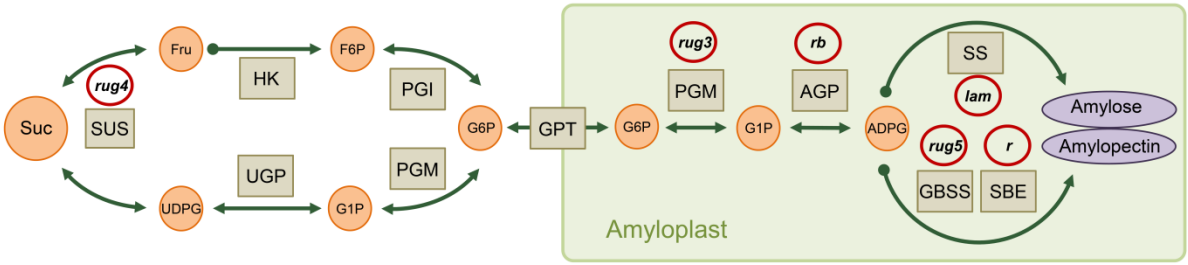

Figure 1.2. Overview of the starch biosynthetic pathway in storage cells of pea embryos. Squares symbolize enzyme activities and circles indicate metabolites. Mutations affecting the corresponding enzyme activities are indicated by red circular frames. Suc, sucrose; UDPG, UDP-glucose; Fru, fructose; F6P, fructose 6-phosphate; G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; ADPG, ADP-glucose; SUS, sucrose synthase; HK, hexokinase; UGP, UDPG pyrophosphorylase; PGI, phosphoglucoisomerase; , PGM, phosphoglucomutase; AGP, ADPG pyrophosphorylase; SS, starch synthase; GBSS, granule-bound SS; SBE, starch branching enzyme.

Nevertheless, ADP-glucose (ADPG) is considered to be the primary glycosyl donor for starch synthesis in plants. ADPG pyrophosphorylase (AGP) catalyzes the readily reversible synthesis of ADPG and inorganic pyrosphosphate from ATP and G1P (Preiss, 1988). As heterotetrameric enzymes, composed of two large "regulatory" and two small "catalytic" subunits, most plant AGPs are allosterically activated by 3-phosphoglycerate and inhibited by inorganic phosphate (Sowokinos, 1981; Sowokinos and Preiss, 1982; Okita et al., 1990). Post-translational redox modification of small AGP subunits in response to sugar availability also plays a role in regulating enzyme activity (Tiessen et al., 2002). However, it is less clear to which extent this allosteric regulation of AGP affects reserve starch biosynthesis in seeds. Several studies on legume embryos and barley endosperm revealed that, when compared to leaves, AGP is less sensitive to allosteric modulators (Hylton and Smith, 1992; Doan et al., 1999). Antisense inhibition of *AGP* genes as well as the mutation of the large AGP subunit (*rb*) considerably reduce AGP enzyme activity in pea embryos but starch content is only moderately reduced (Smith et al., 1989; Rolletschek et al., 2002; Weigelt et al., 2009). Low flux control coefficients of AGP to starch, as quantified with the aid of these embryos, suggest that AGP exerts low control on starch biosynthesis, whereas a substantial proportion of control lies with AGP in leaves (Neuhaus and Stitt, 1990; Denyer et al., 1995). However, the starch less phenotype of Arabidopsis *adg-1* mutant, which completely lacks AGP activity, suggests that plastidic ADPG synthesis is indispensable for starch synthesis in numerous plant species (Lin et al., 1988).

For the sake of completeness, it should be mentioned that graminaceous plants evolved an alternative pathway of ADPG synthesis inside the cytosol of endospermal storage cells, facilitated

by extraplastidial isoforms of AGP (Denyer et al., 1996). This relocation of ADPG synthesis requires a specific transporter that mediates the import of cytosolic ADPG into the amyloplast to feed starch synthesis (Shannon et al., 1998). Several lines of evidence suggest that ADPG is produced in the cytosol of Arabidopsis and potato leaf cells (Baroja-Fernández et al., 2004; Bahaji et al., 2011). Targeted expression of a bacterial ADPG-cleaving enzyme either in plastids or in cytosol revealed that ADPG degradation in the cytosol reduces the starch and total ADPG content in source leaves. Since AGP activity is exclusively confined to plastids, ADPG synthesis from ADP and G1P through cytosolic SUS has been postulated to be supportive to starch synthesis (Muñoz et al., 2005). However, such alternative pathway apparently plays a minor role in legume seeds, as evidenced by nearly starch-less seeds in the *rug3* pea mutant (Harrison et al., 1998).

ADPG is incorporated into the growing glucan chain by activity of starch synthases (SSs) and starch branching enzymes (SBEs). In pea embryos, both soluble and granule-bound forms of SSs exist (Preiss, 1988). The concerted action of SSs and SBEs determines the proportion of amylose and amylopectin in the growing starch granule. Both components differ with respect to the degree of branching, amylose consisting of basically linear linked glucose units with α 1,4-glycosidic bonds and amylopectin being a highly branched polymer due to additional α 1,6-glycosidic bonds every 24 to 30 glucose units (Martin and Smith, 1995). Loss of single SS and SBE isoforms in various garden pea mutants result in considerable changes of starch composition and starch granule morphology. Mutations that specifically eliminate the major isoforms of SS (*rug5* locus) and SBE (*r* locus) largely affect amylopectin synthesis (Edwards et al., 1988; Craig et al., 1998). The resultant phenotype of both mutations is characterized by increased amylose proportion and highly convoluted starch granules. In contrast, the "waxy" starch mutant *lam* exhibits a low amylose phenotype by loss of a granule-bound SS isoform that is required for the formation of the amylose fraction (Denver et al., 1995; Bogracheva et al., 1999).

Starch granules are formed and grow inside the amyloplast to a final size of about 2 μm to 100 μm. These structures serve as a long-term storage form of carbohydrates. The starch granule is a semi-crystalline structure that consists of about 20-30% of amylose, even though this fraction is not essentially needed to build up a granule. The basic framework of the granule is defined by the packing of amylopectin polymers. Radial arrangement of glucan chains is believed to cause the formation of alternating repeats of crystalline and amorphous lamellae, referred to as growth rings (Buléon et al., 1998). With the onset of germination, glucan chains are phosphorylated to be accessible for starch degrading enzymes (Smith et al., 2005). Mainly ß-amylases and isoamylases are required to release maltose from the non-reducing end of the glucan chain. Maltose is the major product of starch degradation and serves as a precursor for sucrose re-synthesis as soon as it is exported from the plastids.

As an alternative to starch, many plant species accumulate lipids, mainly composed of triacylglycerols (TAGs), as the major storage form of fixed carbon. The production of TAGs takes place in two compartments, the synthesis of acyl chains from acetyl-CoA occurs in the plastid and the subsequent lipid assembly and storage in oil bodies are done in the endoplasmatic reticulum (ER). Most of the plastidial acetyl-CoA necessary for fatty acid (FA) synthesis is derived from sucrose, which was previously metabolized by SUS and glycolysis. The glycolytic product phosphoenolpyruvate (PEP) appears to be the metabolite transported from cytosol into the plastids via specific PEP-transporter (Fischer et al., 1997; Schwender et al., 2003). Inside the stroma, PEP is metabolized by plastid-localized pyruvate kinases (PKps), thus providing the pyruvate for acetyl-CoA production (Baud et al., 2007). The final formation of TAGs in the ER is facilitated by sequential transfer of FA chains to glycerol 3-phosphate (Ohlrogge and Browse, 1995). The TAGs are then deposited in small subcellular oil bodies of around 1 μm in diameter. Seed oil bodies contain a matrix of TAGs surrounded by phospholipids and a layer of unique proteins, termed oleosins. The latter are thought to lend mechanical stability to the oil body (Huang, 1996).

While the storage form for fixed carbon can be very different in seeds, virtually all plant species accumulate seed storage proteins (SSPs) as the major storage forms for nitrogen compounds like amino acids. Legume seeds typically contain high amounts of SSPs that are deposited inside of protein storage vacuoles (PSVs). Traditionally, the major SSPs are grouped into different classes depended on extractability in water (albumins), dilute saline (globulins) and alcohol/water mixtures (prolamins) (Osborne, 1924). The globulin storage proteins have been studied in most detail since the vast majority of legume SSPs goes into this family. According to their sedimentation coefficients, globulins can be divided into two groups, namely legumins (11S) and vicillins (7S). Mature legumins account for about 70% of fava bean seed protein and are made up of six subunit pairs each consisting of one type A and one type B subunit. Synthesized as precursor proteins, subunits are proteolytically cleaved after disulfide bond formation. Vicillins of fava bean and garden pea are trimeric proteins lacking cysteine residues and hence cannot form disulfide bridges (Müntz, 1996). The extent of post-translational processing including glycosylation and proteolytical cleavage of vicillin precursors determines the composition of single subunits (Shewry et al., 1995).

The initial steps of SSP synthesis occur in the rough ER. Precursors are imported co-translationally into the ER lumen and are transported into the PSV by vesicle-mediated trafficking, where the precursors are converted into mature proteins by proteolytic processing at specific cleavage sites (Hara-Nishimura et al., 1991). In fava bean and Arabidopsis, prolegumins are cleaved at a conserved Asn-Gly peptide bond by an asparaginyl-endopeptidase, referred as vacuolar

processing enzymes (VPEs). The VPE gene family of Arabidopsis includes four genes. Knock-out of the entire VPE function in Arabidopsis *αvpe βvpe γvpe δvpe* quadruple mutants elicits a shift from correctly processed peptides to alternatively cleaved forms at sites other than the conserved Asn positions targeted by VPEs (Gruis et al., 2004). Interestingly, alternative processing does not affect the successful packaging of these abnormal SSPs.

SSP accumulation in seeds depends on nitrogen uptake and availability in the form of amino acids, which are delivered by maternal tissues and are further processed after uptake by the embryo. In garden pea, mainly Gln, Ala and Thr are unloaded by the seed coat (Lanfermeijer et al., 1992). At later stages asparagine is released. Uptake of amino acids into the embryo is passive at early stages. Later on, much of the control of N-uptake is likely exerted by expression of the amino acid transporter (AAP) and peptide transporters in parenchyma cells of the embryo (Miranda et al., 2003; Tegeder and Rentsch, 2010). Overexpression of *Vf*AAP1 in pea cotyledons results in elevated N-content and stimulated globulin synthesis due to improved amino acid supply (Rolletschek et al., 2005). Work on Arabidopsis *aap1* mutants revealed that impairment of *AAP1* expression in seeds leads to amino acid accumulation and an increased number of protein bodies in mature seed coats/endosperm (Sanders et al., 2009). Accordingly, protein levels in mutant embryos are reduced, indicating that AAP1 functions in amino acid uptake into the embryo.

ABA signaling and genetic regulation of seed maturation

Apart from storage product accumulation, seed maturation comprises the suppression of precocious germination, loss of water and simultaneous acquisition of desiccation tolerance. Many plant species establish various degrees of endogenous dormancy at the final stage of seed maturation, although most crop legumes are considered to be non-dormant (Baskin and Baskin, 2004; Smýkal et al., 2015). Maturation thus constitutes a "break" of seedling development. In other words, this phase allows the entry into quiescent state that facilitates the resistance towards unfavorable environmental situations and the resuming of seedling growth under optimal conditions (germination).

Abscisic acid (ABA) is found in seeds of many plant species. The hormone is necessary to maintain the embryo in a developmental mode until it is fully developed and sufficient amounts of storage compounds are formed. ABA also prevents precocious germination of the developing embryo. Generally, ABA levels increase when seed dry mass increases and decline when seed water content decreases (Eeuwens and Schwabe, 1975; McWha, 1975). In *Phaseolus vulgaris* the embryo is non-dormant at maturity, but *in vitro* experiments on excised embryos revealed that germination capacity is dependent on embryo age and is inversely correlated to ABA content (Prevost and Le Page-Degivry, 1985). The delay in germination extends in parallel to the increase in ABA content.

In some species, feeding of exogenous ABA to excised embryos inhibits germination (Kermode et al., 1989). Embryos from different maize mutants that germinate prior to dispersal are either ABA deficient or less sensitive to ABA (McCarty, 1995). Thus, ABA appears to play an essential role in preventing precocious germination. However, a direct role of ABA in establishing dormancy is questionable. Often there is no clear relationship between the degree of dormancy and ABA content in the mature seed (Walton, 1980). In some cases, non-dormant seeds contain high levels of ABA or embryo dormancy is imposed before ABA levels increase. Thus, high ABA concentrations in seeds are not necessarily associated with dormancy and vice versa. Moreover, attempts to artificially induce dormancy in non-dormant seeds by treatment with ABA were not successful. These observations imply that ABA on its own is not an essential dormancy factor.

The isolation of ABA-insensitive mutants in Arabidopsis led to the identification of regulatory components in the ABA signaling pathway, some of which are transcription factors that appear to be important factors of ABA- or seed-specific gene expression, namely ABA INSENSITIVE3 (ABI3), ABI4, ABI5, LEAFY COTYLEDON1 (LEC1), LEC2 and FUSCA3 (FUS3). For example, both *ABI3* and the orthologous *VIVIPAROUS1* (*VP1)* gene in maize encode B3-domain transcription factors specifically expressed in seeds (McCarty et al., 1991; Giraudat et al., 1992). Maturing embryos of the severe Arabidopsis *abi3-6* mutant allele remain green, fail to impose dormancy and display germinative events prior to dessication (Nambara et al., 1994). Based on transgenic approaches and expression analysis, ABI3/VP1 family is thought to mediate the ABA-associated stimulation of storage protein accumulation and synthesis of osmoprotectants like LEA proteins and heat shock proteins (Nambara et al., 1992; Bies-Etheve et al., 1999; Rojas et al., 1999). Furthermore, they play a role in dormancy inception and repress the post-germinative gene expression. ABI3 is involved in the combined control of ABA-mediated gene expression in seeds, likely by formation of a regulatory complex together with ABI4 and ABI5. The effects on seed development caused by mutations in *ABI4* and *ABI5* gene loci widely resemble those elicited by weaker *abi-3* alleles (Finkelstein, 1994). By using yeast two-hybrid assays, it has been show that homodimers of the bZIP transcription factor ABI5 physically interact with the B1 domain of ABI3 via two conserved charged domains (Nakamura et al., 2001). Binding of ABI5 to ABA-responsive promoter elements presumably aids the targeting of ABI3 to RY elements and to the transcription machinery. ABI3 and ABI5 can act antagonistically or synergistically on gene expression, depending on the gene that is regulated by both transcription factors (Delseny et al., 2001).

In a concerted fashion along with ABI3, ABI4 and ABI5, the transcription factors LEC1, LEC2 and FUS3 promote crucial processes at later stages of embryo development including acquisition of desiccation tolerance, induction of dormancy and repression of germination-related

processes. LEC1 is expressed quite early in embryo development and promotes embryonic growth by stimulating the expression of LEC2 and FUS3 (Suzuki and McCarty, 2008). Although *lec1* and *fus3* embryos occasionally exhibit vivipary, mutant embryos respond normally to ABA (Keith et al., 1994; Meinke et al., 1994). However, LEC1 and FUS3 positively regulate the abundance of ABI3 in seeds and a synergistic action of these three components is thought to regulate various events including sensitivity to ABA, anthocyanin accumulation and expression of 12S storage proteins (Parcy et al., 1997). Notably, none of the individual ABI transcription factors seem to interact physically with LEC1 and FUS3 (Brocard-Gifford et al., 2003).

It appears that FUS3 and LEC2 also regulate gibberellic acid (GA) biosynthesis in seeds. In Arabidopsis mutants defective in the respective genes, the GA pathway is misactivated, resulting in increased levels of bioactive GAs (Curaba et al., 2004). GAs primarily promote cell expansion and are generally considered as critical for the promotion and maintenance of germination, thereby acting antagonistically to ABA. There is a growing body of evidence that the ABA/GA ratio is important in controlling germination as well as the maintenance and loss of dormancy. For example, induced depletion of GA in an ABA-deficient maize mutant (*vp5*) results in secondary suppression of vivipary and acquisition of desiccation in developing kernels (White et al., 2000). It is speculated that relatively high native GA levels in mutant maize kernels induce precocious germination since the ABA/GA ratio is distorted due to the absence of ABA. The reduction of GA by transgenic manipulation re-establishes the ABA/GA ratio appropriate to prevent germination. Another family of B3 transcription factors, referred as *VAL* clade, has been shown to suppress embryonic functions in order to permit the switch into germination. These transcription factors are closely related to the AFL (ABI3/LEC2/FUS3) clade and are required to repress the LEC1/AFL transcription factor network in germinating seeds. As revealed by analysis of the *val1 val2* double mutant in Arabidopsis, VAL function possibly involves the regulation GA-synthesis in seeds (Suzuki et al., 2007).

Trehalose 6-phosphate and SUCROSE NON-FERMENTING1-RELATED KINASE in plant signaling

Besides hormones, carbohydrates are thought to play a crucial role in the control of seed development. During the past decade, several studies on the Arabidopsis *tps1* mutant revealed that the trehalose biosynthetic pathway, in particular the intermediate T6P, is indispensable for normal seed development (Eastmond et al., 2002; Schluepmann et al., 2003; Gómez et al., 2006). Down to this day, it became clear that T6P controls multiple aspects of plant growth and metabolism in response to sugar availability. Lack of suitable facilities to detect trace amounts of T6P and

trehalose initially led to the false assumption that the ability to form trehalose is merely confined to resurrection plants, which accumulate considerable amounts of trehalose as an osmoprotectant (Paul et al., 2008). However, transgenic integration of bacterial and yeast trehalose metabolic genes into tobacco and potato plants, aiming on the improvement of drought resistance, elicited phenotypes which pointed to an altered regulation of sugar metabolism (Goddijn et al., 1997; Romero et al., 1997). Soon after several functional genes, encoding trehalose 6-phosphate synthases (TPSs) and trehalose 6-phosphate phosphatases (TPPs), had been identified in Arabidopsis, the publication of the full genomic sequence of Arabidopsis confirmed the existence of trehalose metabolism in plants (Blazquez et al., 1998; Vogel et al., 1998; Kaul et al., 2000).

In an analogous manner to the sucrose synthesis in plants, trehalose is produced through the sequential action of TPS and TPP (Fig. 1.3A). In a first step, the intermediate T6P and UDP are formed by TPS from UDPG and G6P. T6P is then dephosphorylated by TPP yielding trehalose and inorganic phosphate. Finally, free trehalose is hydrolyzed into two glucose molecules by a single trehalase (TRE) isoform. Notably, genomes of plants contain a surprisingly large number of genes encoding TPS and TPP isoforms (Leyman et al., 2001; Ge et al., 2008). For example, the genome of Arabidopsis contains eleven *TPS*, ten *TPP* genes but only a single *TRE* gene (Fig. 1.3B). As revealed by yeast complementation assays, all *TPP* genes encode functional TPPs, while only *TPS1* seems to encode a catalytically active TPS in Arabidopsis (Vandesteene et al., 2010; Vandesteene et al., 2012). *TPS* and *TPP* genes display a wide expression range throughout the development of plants. Non-active class II *TPS* are subjected to a high degree of regulation on the transcriptional level by light, diurnal rhythm, starvation and hormones as well as on the post-translational level via phosphorylation and protein-protein interactions with 14-3-3 proteins (Ramon et al., 2009). A recent study demonstrated that class II TPS are capable to interact physically with different members of class I and II TPS and possibly have regulatory functions (Zang et al., 2011). Promoter reporter lines and transcriptional analyses revealed that individual TPPs display distinct tissue-specific expression patterns during development and in response to different environmental stimuli (Li et al., 2008; Vandesteene et al., 2012). A number of developmental processes in plants are regulated by trehalose metabolism, such as seed maturation, cell shaping, floral transition and inflorescence architecture (Eastmond et al., 2002; Satoh-Nagasawa et al., 2006; Chary et al., 2008; Wahl et al., 2013).

Loss of TPS1 in Arabidopsis results in embryo lethality due to the arrested embryo growth at the transition from late torpedo or early cotyledon stage (Eastmond et al., 2002). Furthermore, rate of cell division and concentration of storage compounds are diminished in seeds homozygous for *tps1* (Gómez et al., 2006). Early embryo development appears to be less affected by the mutation, indicating that TPS1 and the related synthesis of T6P are not essential for the regulation

of embryo morphogenesis, but it is an indispensable factor regulating the transition into maturation phase of embryo development. TPS1 is also required for vegetative growth and floral transition, as evidenced by the seed-specific rescue of *tps1* or the generation of weaker *tps1* mutant alleles (Gómez et al., 2010).

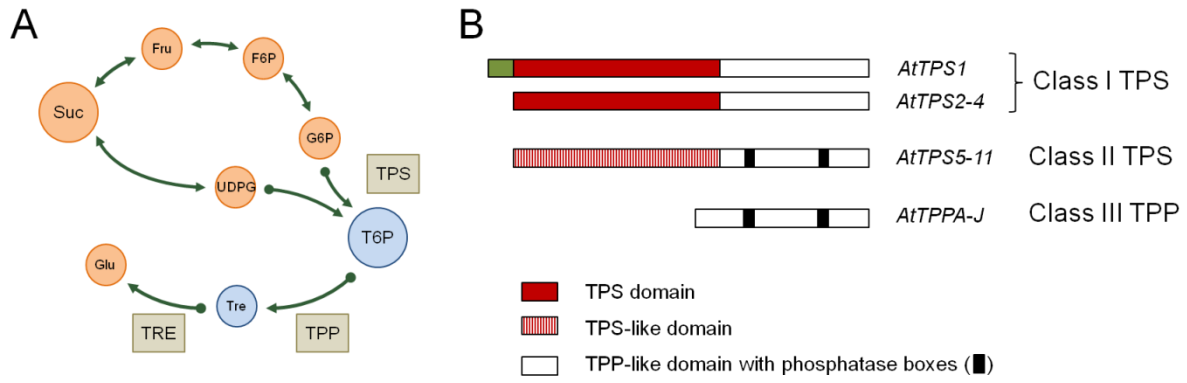

Figure 1.3. (**A**) Overview of the trehalose metabolic pathway in plants. Squares represent enzyme activities and circles indicate metabolites. Suc, sucrose; UDPG, UDP-glucose; Fru, fructose; F6P, fructose 6-phosphate; G6P, glucose 6-phosphate; T6P, trehalose 6-phosphate; Tre, trehalose; Glu, glucose; TPS, trehalose 6-phosphate synthase; TPP, trehalose 6-phosphate phosphatase; TRE, trehalase. (**B**) Domain structures of plant TPS and TPP genes, illustrated by the example of Arabidopsis.

Evidence that the intermediate T6P has signaling functions in plants comes from transgenic plants with altered trehalose metabolism. Ectopic expression of bacterial TPS, TPP or trehalose 6-phosphate phosphohydrolase (*TPH*) in Arabidopsis resulted in contrasting plant phenotypes that can clearly be attributed to the changes in T6P content (Schluepmann et al., 2003). Developing rosettes of plants expressing TPS contain more T6P, grow smaller and display dark green leaves. In contrast, plants expressing TPP or TPH exhibit larger leaves with bleached areas. Furthermore, floral transition is delayed due to the reduction of T6P levels. The fact that the expression of TPP or TPH elicits similar phenotypic changes but has different effects on trehalose content counters the argument that trehalose is the active signaling component.

The target sites of T6P action are largely unknown. Several studies suggest a role of T6P in post-translational regulation of pivotal enzymes involved in primary metabolism (Kolbe et al., 2005; Figueroa et al., 2015). Kolbe et al. (2005) demonstrated that T6P regulates the redox modification of AGP. Transgenic plants expressing a bacterial TPS displayed increases in the redox activation of AGPB whereas plants expressing bacterial TPP showed the opposite effect. In addition, short-term feeding of T6P to intact isolated chloroplasts promotes monomerization of the small AGP

subunits, providing evidence that T6P is synthesized in the cytosol and acts on chloroplast metabolism possibly by import into plastids. The discovery of two TPP isoforms localized in the stroma of chloroplasts substantiates suchlike hypothesis (Krasensky et al., 2014), even though it has recently been shown that AGP redox modification by T6P has little influence on the rate of starch synthesis (Martins et al., 2013). The T6P content is closely correlated to sucrose content across a range of physiological treatments (Lunn et al., 2006), leading to the suggestion that T6P acts as a sucrose-specific signal. Recently, the T6P-sucrose nexus, a homeostatic mechanism that maintain sucrose levels in a suitable range for particular physiological and developmental demands, has been postulated (Yadav et al., 2014).

A ubiquitous mechanism that adapts the cellular response to the energy status in plants is controlled by the SUCROSE NON FERMENTING 1 RELATED KINASE (SnRK1). As it was shown for monocots and dicots, application of various amounts of T6P to protein extracts affects considerably the *in vitro* activity of SnRK1 (Zhang et al., 2009; Martínez-Barajas et al., 2011). Immunoprecipitation of SnRK1 from Arabidopsis seedling extracts eliminates the T6P sensitivity of purified SnRK1, indicating that the specific inhibition of SnRK1 by T6P depends on an unknown intermediary factor (Zhang et al., 2009). In plants, SnRK1 is activated under low sugar conditions and energy depletion as released by darkness or hypoxia (Baena-González et al., 2007). Activated SnRK1 stimulates catabolic processes and inhibits anabolism in order to maintain energy homeostasis. For instance, processes like reserve compound mobilization are up-regulated by SnRK1, whereas energy demanding processes like cell proliferation are suppressed. Thus, the SnRK1-mediated activation of low energy and low carbon conditions are opposite to the effects activated by T6P (Gazzarrini and Tsai, 2014). Several lines of evidence propose a role of SnRK1 in the regulation of seed maturation. In garden pea, embryo-specific repression of SnRK1 profoundly affects various maturation processes (Radchuk et al., 2006). *SnRK1*-antisense embryos accumulate more sucrose likely due to reduced sucrose conversion into storage compounds. Occasionally, a proportion of these seeds exhibit precocious germination and green cotyledons at maturity. This phenotype is reminiscent to mutant seeds of the severe *abi3-6* allele in Arabidopsis (Nambara et al., 1994). This is in line with the finding that ABA content and transcripts of *ABI3* are reduced in *PsSnRK1* antisense seeds. Moreover, SnRK1 possibly acts as a positive regulator of FUS3 in Arabidopsis, as it is capable to directly phosphorylate this transcription factor (Tsai and Gazzarrini, 2012). Via this mechanism SnRK1 likely promotes ABA synthesis and prevents induction of germination processes. Recently, it has been shown that Ser/Thr protein phosphorylases (PP2C) dephosphorylate and inactivate SnRK1 (Rodrigues et al., 2013). ABA inhibits PP2C activity and, in turn, it positively regulates SnRK1 by preventing the inactivation by

PP2Cs. These findings implicate the requisite of SnRK1 in the ABA-mediated regulation of seed maturation.

Several lines of evidence suggest a crosstalk between T6P, SnRK1 and ABA. Detailed examination of the Arabidopsis *tps1* mutant revealed that TPS1 is required to promote germination and seedling growth. Interestingly, only a proportion of 30% to 40% of *tps1* seeds were capable to germinate after stratification, leading to the suggestion that dormancy is increased in mutant seeds (Gómez et al., 2006). Furthermore, germination of weaker *tps1* alleles is hypersensitive to ABA (Gómez et al., 2010). On the contrary, constitutive expression of *TPS1* produces glucose- and ABA-insensitive phenotypes (Avonce et al., 2004). Some TPP mutants in Arabidopsis, particularly *tppg,* are also less sensitive to ABA (Vandesteene et al., 2012). All together, these studies imply that T6P might be involved in the control of germination by inhibiting SnRK1 and decreasing ABA sensitivity.

Crosstalk between auxin and sugar signaling

In recent years, evidence has emerged that the interaction between sugar and auxin signaling pathways regulates plant growth and development. Auxin biosynthesis in plants is quite complex and different pathways have been postulated, including the indole-3-acetamide (IAM) pathway, the indole-3-pyruvic acid (IPA) pathway and two pathways based on indole-3-acetaldoxime and indole-3-acetonitrile found in the *Brassicaceae* (Mano and Nemoto, 2012). Several lines of evidence suggest that auxin biosynthesis, distribution and response are regulated by changes in sugar levels. Incubation of Arabidopsis seedlings on varying concentrations of glucose and sucrose increased the levels of IAA and those of the IAA precursor anthranilate and tryptophan (Sairanen et al., 2012). The sugar-induced IAA accumulation requires the induction of several genes associated to auxin biosynthesis. This involves the negative regulation by members of the PHYTOCHROME INTERACTING FACTOR (PIF) transcription factor family. It has been shown that the capacity of glucose to induce IAA biosynthesis was heightened in Arabidopsis *pif1 pif3 pif4 pif5* quadruple mutant line, whereas the response to glucose was reduced in plants overexpressing PIF5. Besides its function in auxin synthesis, glucose also seems to influence IAA signaling and distribution. As revealed by microarray analysis, the presence of 3% glucose in growth media is sufficient to modulate the expression of 62% of IAA associated genes in Arabidopsis seedlings, including auxin receptor, transporter and auxin response factors (Mishra et al., 2009). Moreover, the Arabidopsis *glucose insensitive2* mutant also exhibits insensitivity to auxin (Moore et al., 2003).

Latest insights on the regulation of apical dominance in garden pea are another exciting example for a crosstalk between sugars and auxin, as they counter the prevalent perception that

bud outgrowth after decapitation is mediated by changes in apical auxin supply. It has been shown that bud release already happens before the auxin content decreases in the stem and that sugars increase inside the buds within a time frame that is correlated to bud release (Mason et al., 2014). Furthermore, application of sucrose to buds of intact plants initiates bud release and suppresses the branching repressor *BRANCHED1*. Based on these findings a model has been proposed that centers sugar demand of the shoot tip as a crucial factor that maintains apical dominance. Removal of the apical sugar demand by decapitation increases sugar availability in the axillary buds. This effect eventually causes bud release independently of auxin. Possibly in response to the changes in sugars status, auxins exert influence at later stages of branch growth.

The analysis of the maize *miniature1* mutant, lacking basal endosperm-specific cwINV, provides evidence that auxins regulate kernel development in response to sugar supply (LeClere et al., 2010). Disturbed conversion of sucrose into hexoses results in pleiotropic effects and decreases IAA content in mutant kernels. Reduced IAA levels correspond to the reduction of *ZmYUCCA* transcripts, a key enzyme which is involved in Trp-dependent auxin synthesis and is essential for endospermal cell proliferation (Bernardi et al., 2012). It has been concluded that developing seeds possibly modulate growth processes by altering auxin biosynthesis in response to varying sugar concentrations. Nevertheless, the molecular mechanisms underpinning sugar and auxin signaling interactions are still poorly known. Downstream components of sucrose and hexose signaling pathways, like hexokinase or T6P, may also be involved in auxin regulation and remain to be investigated.

Scope of the thesis

The aim of my study was to identify molecular components and mechanisms regulating major processes of seed development such as transition into maturation, deposition of storage compounds, embryo growth and germination. To this end, combinatorial analyses including biochemical, transcriptional and histological approaches were applied on seed material from different genetic and transgenic legume models. Based on the conclusions drawn from the following chapters, I propose a model which integrates the sugar signal T6P and the classic plant hormone auxin into a developmental pathway that regulates major maturation processes in legumes seeds.

Chapter two focuses on the identification of physiological and molecular mechanisms underlying embryo heterosis. Selfed and reciprocally crossed embryos of two homozygous fava bean lines were grown on the same mother plant and subjected to transcriptional and compositional analyses. As revealed by comparing the mean values between crossed and selfed embryos, increased sink strength of hybrid embryos results from a stimulation of cell proliferation possibly controlled by auxin signaling. Furthermore, depletion of intermediary metabolite pools reflects the enhancement of storage capacity consequently leading to larger seeds.

Chapter three reports about the *tar2* auxin biosynthesis mutation in garden pea, which reduces the size and starch content of mature seeds. Embryo-specific expression of functional *TAR2* in *tar2* mutant background complemented the starch content and reversed the wrinkled phenotype. Furthermore, application of a synthetic auxin also increased starch content and partially rescued the seed phenotype. The reduced starch synthesis in mutant seeds is attributable to reduced expression and activity of key starch synthetic enzymes. The results indicate that reserve starch accumulation and cotyledon growth in garden pea seeds depends on auxin.

Chapter four illuminates the role of T6P signaling during pea seed development. Embryo-specific depletion of T6P in transgenic USP::TPP pea plants results in wrinkled seeds, reduced starch content and smaller embryos. Profiling of transcripts, metabolites and enzyme activities revealed that the reduced starch accumulation is largely attributable to impaired activity of AGP. Furthermore, our evidence indicates that the TAR2-dependent auxin biosynthesis is repressed on the gene level. Introduction of the USP::TAR2 transgene into USP::TPP plants largely reversed the wrinkled seed phenotype, as it bypasses the transcriptional suppression of the native *TAR2* gene.

These findings suggest that normal starch accumulation and embryo growth in garden pea seeds relies on the control of *TAR2* gene expression by T6P.

The results presented in **chapter five** provide evidence that modulation of T6P profoundly affects the germination of garden pea seeds. Furthermore, measurements of soluble sugar levels in growing embryos of different wrinkled garden pea mutants suggest that T6P may convey the availability of hexose phosphates.

In **chapter six**, I summarize the key results and conclusions drawn from the different chapters and discuss them with respect to generality and interrelation. Moreover, I make suggestions for future research and forthcoming studies.

Chapter 2

Hybrid embryos of *Vicia faba* develop enhanced sink strength, which is established during early development

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Chapter 3

Evidence that auxin is required for normal seed size and starch synthesis in pea

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Chapter 4

Trehalose 6-phosphate promotes seed filling by activating auxin biosynthesis

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Chapter 5

Additional results

Modulation of trehalose 6-phosphate affects seed germination

Trehalose 6-phosphate signals hexose phosphate availability

Chapter 6

Synthesis

Auxins regulate maturation and sink capacity in legume seeds

In flowering plants, the seed represents the reproductive unit, composed of three compartments: seed coat, endosperm and the embryo. Correct coordination of processes required for the formation of these compartments depends on the spatio-temporal transmission and perception of different signals such as hormones and sugars. The plant hormone auxin controls and coordinates numerous aspects of seed development, including cell division, endosperm growth and early pattern formation in the embryo. Even though seeds accumulate higher auxin levels than any other tissue of the plant, very limited data are available on auxin-related mutants affecting seed development. In maize kernels, endosperm synthesizes nearly 100 to 500-fold higher levels of IAA relative to vegetative tissues (Jensen and Bandurski, 1994). Several studies on the maize auxin biosynthesis mutant, *defective endosperm18,* imply that auxin is essential for normal cell proliferation in the endoperm. The dry weight of mutant endosperm is approximately 60% of the WT weight and can be partly normalized by application of the synthetic auxin naphtalene-acetic acid (Torti et al., 1986). Recently, it has been shown that the mutation of the endosperm-specific *ZmYUCCA1* gene impairs a critical step of IAA biosynthesis in *de18* kernels (Bernardi et al., 2012).

In garden pea and its close relatives, seeds also produce high levels of auxin during maturation, in particular the halogenated auxin 4-Cl-IAA predominates (Lam et al., 2015). As revealed by exogenous application to maize coleoptiles, 4-Cl-IAA is much more effective in stimulating growth than IAA (Karcz and Burdach, 2002), but its biological role in legume seeds is slowly being clarified. Reinecke et al. (1995) demonstrated that seed-borne 4-Cl-IAA regulates pea fruit development. Removal of seeds from two days old pods causes poor pericarp growth and abscission, an effect that can be reversed by application of 4-Cl-IAA. It appears that 4-Cl-IAA and gibberellins (GA) act together in regulating pea fruit growth, since 4-Cl-IAA treatment is sufficient to replace impaired GA biosynthesis in de-seeded pods, which has been shown to be important for pericarp elongation (van Huizen et al., 1995; Ozga and Reinecke, 1999).

One key result of this thesis is that impaired 4-Cl-IAA synthesis in the garden pea *tar2-1* mutant resulted in a dramatic perturbation of seed maturation (**chapter 3**). *TAR2* encodes a functional aminotransferase capable of synthesizing indole-3-pyruvic acid and 4-chloroindole-3-pyruvic acid in parallel, both accordingly yielding IAA and 4-Cl-IAA due to physicochemical conversion (Tivendale et al., 2012). In growing pea seeds, transcript abundance of *TAR2* coincides with developmental changes in 4-Cl-IAA content, both being relatively high during the maturation phase. Mutation of *TAR2* largely affects the level of 4-Cl-IAA, while the effect on IAA was

relatively small. This indicates that TAR2 is primarily responsible for 4-Cl-IAA synthesis in growing pea seeds. The results presented in **chapter 3** show that mutation of *TAR2* caused smaller and wrinkled seeds. Furthermore, starch content of *tar2-1* seeds was severely reduced due to decreased activity of starch synthesis enzymes. Particularly, AGP was strongly repressed on the enzymatic and transcriptional level. 4-Cl-IAA apparently controls the activity of this enzyme by promoting gene expression of the corresponding genes, as evidenced by a number of putative auxin response elements in the promoter sequences of several AGP genes. Furthermore, it was possible to reverse starch deficiency and wrinkling of *tar2-1* seeds by introducing a transgene that facilitates the seed-specific expression of the *TAR2* coding sequence. Application of the synthetic auxin 2,4-D to developing *tar2-1* seeds also reduced wrinkling and significantly increased the starch content compared to the untreated control. These findings suggest that increased biosynthesis of 4-Cl-IAA during maturation is required for normal growth and storage processes in the embryo. As a novel mode of auxin action in plants, 4-Cl-IAA appears to be involved in the coordination of carbon partitioning between sucrose and starch.

The conclusions that have been drawn from *tar2-1* mutants partly correspond to previous results from our study, focused on the analysis of molecular mechanisms underlying mid-parent heterosis in *Vicia fava* hybrid embryos (**chapter 2**). The vigorous growth of crossed embryos led to a shift of metabolite profiles and increases in storage capacity when compared to inbred embryos. In plants, the superior growth of hybrids often results from changes in cell number rather than changes in cell size (Birchler et al., 2010; Guo et al., 2010). Our transcript analysis indicated that the heterotic increment of fava bean embryos might also be due to activated cell proliferation. Furthermore, several genes related to auxin function were up-regulated, indicating that the stimulation of auxin functions promotes the establishment of a higher cell number in hybrid embryos during early development. This eventually causes a higher sink capacity once the embryo is entering the maturation phase. A previous study on fava bean corroborates this assumption, since differences in final embryo size were associated with the duration of mitotic activity. In embryos of large-seeded fava bean varieties, the initiation of storage activities is delayed when compared to the small-seeded varieties (Weber et al., 1996). In consequence, duration of the early cell proliferation phase is extended and higher cell numbers are established. This effect possibly increases sink strength and the capacity to generate larger seeds. However, our studies on *tar2-1* mutant seeds provide evidence that auxin also controls cotyledon expansion and reserve starch accumulation at later stages of embryo development. This raises the possibility that the auxin-mediated control of cell elongation and storage product accumulation is also contributive to growth vigor in hybrid embryos of the closely related fava bean.

In conclusion, auxin emerges as a central regulator of legume seed maturation. Garden pea and closely related species possibly evolved a second auxin pathway that regulates specific processes during seed maturation. It is a very intriguing finding that auxins are apparently involved in the metabolic control of reserve starch accumulation in garden pea and it will be interesting to investigate whether auxins have similar functions in other plant species. Similar to pea seeds, starch is the major reserve compound in maize kernels deposited in the storage cells of the endosperm. However, an effect of auxin deficiency on reserve starch biosynthesis has not yet been demonstrated. Therefore, the maize *de-18* mutant represents an exciting research object in order to investigate the influence of auxin on the molecular regulation of starch metabolism in cereals (Bernardi et al., 2012).

T6P links sugar availability with auxin functions

Besides its primary role in metabolism, sugars have been suggested to act as signaling compounds that regulate metabolism, developmental transitions and growth in plants (Hanson and Smeekens, 2009). In the last decade, evidence has emerged on the role of T6P in regulating diverse aspects of plant development, such as embryo development, floral architecture and primary metabolism (Lunn et al., 2014). As previously implied by modulation of T6P levels in Arabidopsis plants and potato tubers, expression of bacterial TPS and TPP in garden pea embryos led to perturbations of cotyledon growth and had contrasting effects on primary carbon metabolism (Fig. 6.1). This is in agreement with a proposed model in which T6P controls the utilization of carbohydrates for growth (Schluepmann et al., 2003; Debast et al., 2011). Consistent with these approaches, USP::TPP embryos accumulated more sucrose, UDPG and hexose phosphates. This effect was apparently the result of impaired sugar utilization due to retarded embryo growth as well as decreased accumulation of reserve starch during maturation. Ectopic expression of TPS released the opposite effects on seed carbon metabolism and severely reduced the levels of various soluble sugars. Nevertheless, dry weight and starch content of mature USP::TPS seeds were unaltered.

It is interesting to note that modulation of T6P caused similar changes in soluble sugar levels, regardless of the plant species and the plant tissue to which it applies, however, the impact on starch levels differs a lot. The effect of T6P modulation on starch metabolism in pea seeds is largely consistent with those observed in Arabidopsis leaves, suggesting that T6P promotes carbon partitioning into starch (Kolbe et al., 2005). In contrast, modulation of T6P levels in potato tubers does not change the carbon flux into reserve starch (Debast et al., 2011). Starch was even reduced on a per gram basis in tubers with elevated T6P content. It has been proposed that T6P deficiency mainly affects the carbon consumption for tuber growth rather than carbon partitioning between

the central metabolic pathways. The reason for the differences between these models is unclear. Plants may have evolved different targets for T6P in order to regulate processes that are required for the specific function and purpose of single tissues und plant organs. The positive regulation of reserve starch accumulation by T6P may represent a specific characteristic of pea embryos and similar mechanisms may also exist in other plant species with starch-rich seeds.

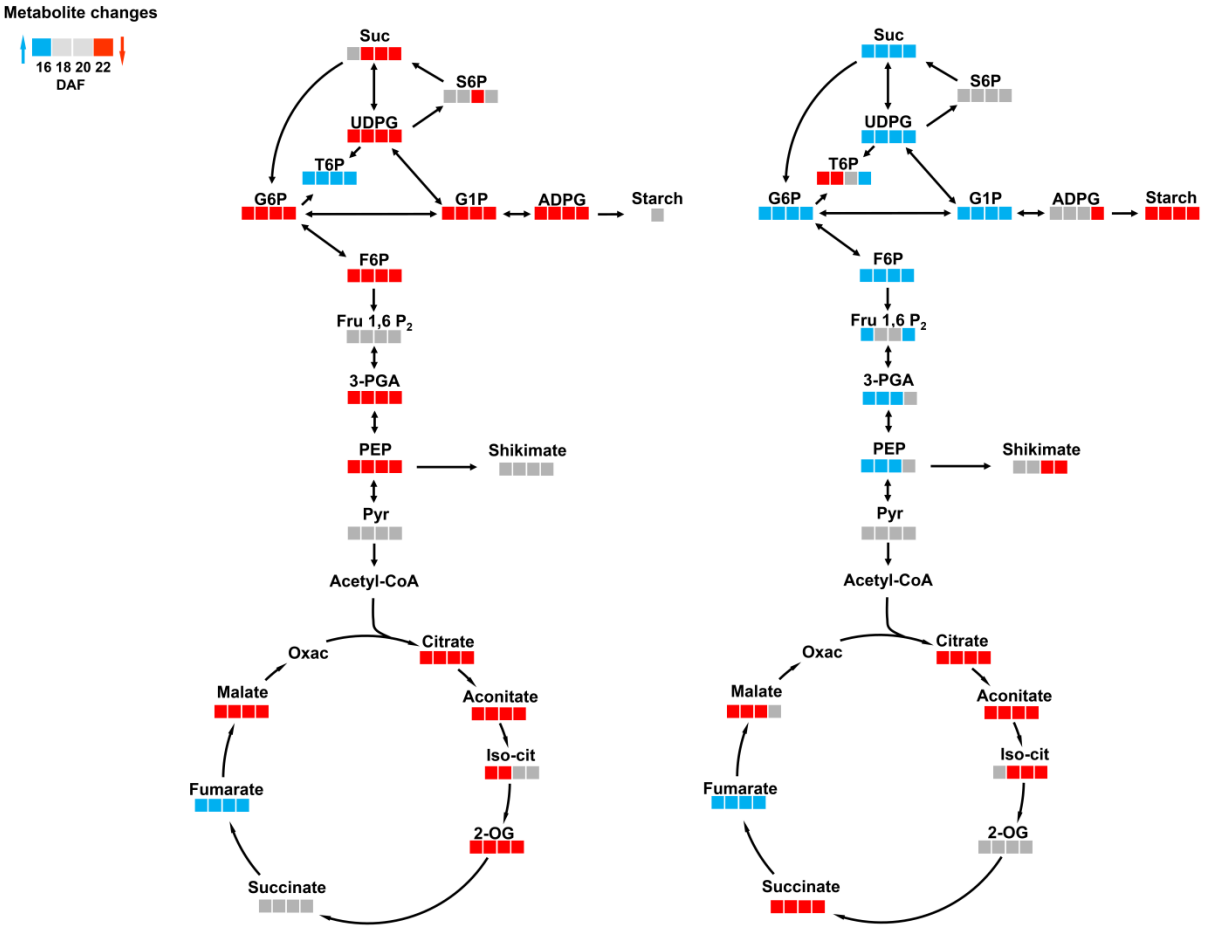

Figure 6.1. Comparison of metabolic changes between USP::TPS (left) and USP::TPP (right) embryos as analyzed by LC-MS. Data are derived from **chapter 4** (Fig. 1E and 2A, fig. S4 and S7). Red, significantly reduced metabolite levels with respect to the WT; blue, significantly increased metabolite levels with respect to the WT. Numbers 16, 18, 20 and 22 refer to DAP.

Nevertheless, many plants accumulate oils as the major seed storage compound, and starch contents are accordingly very low in those seeds. Although there is a broad diversity in how different species allocate fixed carbon within the seed, basically the same metabolic pathways for energy storage and usage can be used by all plants. These pathways are possibly differentially stimulated and regulated by elemental signals, such as T6P and hormones, in order to achieve specific patterns of carbon partitioning. However, the knowledge about the role of T6P in oil-rich

seeds is still rudimentary and further analyses of weaker Arabidopsis *tps1* mutants may provide new insights into the regulation of FA metabolism and lipid storage in seeds (Gómez et al., 2010). It has been shown that the FA concentration is significantly lowered in Arabidopsis *tps1* embryos, whereas sugars and starch accumulate (Gómez et al., 2006). It is not yet clear whether these changes are side effects of perturbed embryo growth or the result of altered carbon flow into fatty acids. The findings presented in **chapter 4** clearly indicate that T6P controls the utilization of incoming sucrose by regulating activity of key enzymes involved in primary and storage metabolism of pea seeds. Particularly, the regulation of AGP by T6P appears to be of general importance in plants, as similar mechanisms also exist in potato tubers and Arabidopsis leaves (Wingler et al., 2000; Kolbe et al., 2005; Lunn et al., 2006). Recently, it has been concluded by the use of ethanol-inducible TPS expression in Arabidopsis that high T6P decreases sucrose content by diverting photoassimilates away from sucrose in favor of nitrate assimilation, organic acid and starch synthesis (Martins et al., 2013; Figueroa et al., 2015). These effects are likely facilitated by posttranslational activation of specific enzymes like PEPC and nitrate reductase (Figueroa et al., 2015). It is therefore very likely that T6P also promotes FA synthesis in lipid-rich seeds by modulating the conversion of sucrose into FAs, thereby it possibly modifies the activities of specific enzymes involved in FA metabolism, for instance PKps. Previous studies indicated that PKps are essential for FA production in maturing Arabidopsis seeds (Baud et al., 2007). Loss of PKp1 and PKp2 causes FA deficiency and a wrinkled seed phenotype which is quite comparable to those of the starch mutants in garden pea. Therefore, PKps and other key regulators of FA synthesis like the WRINKLED1 transcription factor might be considered as potential targets for T6P and promising objects for future studies (Baud et al., 2009).

Much within the scope of T6P signaling aims on the transcriptional modulation of developmental and metabolic pathways (Satoh-Nagasawa et al., 2006; Zhang et al., 2009; Wahl et al., 2013). However, the perception of T6P and the connecting elements between T6P and its target genes are largely unknown. An exception is SnRK1, a central regulatory hub of energy and carbon metabolism which is negatively regulated by T6P (Zhang et al., 2009), a mechanism that appears to be also present in pea seeds (**chapter 5**). In recent years, a signaling network between sugars and auxins has been implicated to regulate growth by modulating cell proliferation and cell expansion (Wang and Ruan, 2013). One important finding of this thesis is the identification of a molecular mechanism in which T6P regulates embryo growth and starch accumulation by promoting the synthesis of auxin (**chapter 4**). Our results indicate that T6P exerts control over auxin biosynthesis by transcriptional activation of *TAR2*. Expression of *TAR2* was dramatically reduced in USP::TPP embryos and it was possible to bypass this restrictive effect by introduction of the USP::TAR2 transgene into USP::TPP plants. As a result of this combination, the wrinkled phenotype of

USP::TPP seeds has largely been rescued by the T6P-independent expression of *TAR2*. All together, these findings represent an exciting novelty, since they provide strong evidence that the regulatory function of T6P depends on the interaction with a specific plant hormone pathway.

The general validity of such mechanism in plants, however, might be called into question, since the ability to form halogenated auxin is limited to only a view legume species within the clade of the *Fabaceae* (Lam et al., 2015). Nevertheless, several previous discoveries that have been made on sugar-auxin interactions allow to be discussed with regard to T6P signaling. To elaborate, kernel growth in maize appears to be regulated by auxins in response to sugar levels. Lack of cwINV in *miniature1* results in reduced kernel mass and reduces the concentrations of hexoses and IAA in developing kernels (LeClere et al., 2010). It has been shown that the conversion of sucrose into hexoses through cwINV is required to drive expression of *ZmYUCCA1*, a gene encoding a key IAA-biosynthetic enzyme. Interestingly, the control of *ZmYUCCA1* appears to be dependent on the activity of hexokinases and thus, on the availability of G6P, a direct precursor of plant T6P synthesis. Recently, evidence emerged that both YUC and tryptophan aminotransferases (TAA1/TAR) act together in the IPA pathway (Mashiguchi et al., 2011). Members of the TAA1/TAR family produce IPA, while YUCs are believed to convert IPA into IAA. This close cooperation of both families raises the possibility that, similar to TAR2 in pea seeds, the expression of specific TAA1/TAR and YUC genes is controlled by T6P in order to adapt auxin biosynthesis to sugar availability. The scope of such regulation may largely differ between single plant species and might be tissue-specific or depended on environmental conditions.

Apart from seeds, it can be assumed that the concerted action of T6P and auxin signaling pathways controls branching and apical dominance in plants. It is commonly accepted that auxin plays a substantial role in the regulation branching and apical dominance. However, a recent study on garden pea revealed that apical dominance is maintained by excessive sugar demand of the shoot tip (Mason et al., 2014). The resulting limitation of sugar availability in the axillary buds eventually suppresses the initiation of bud out growth. The decapitation of the plant relieves the sugar demand of the shoot tip and releases branching due to changed sugar supply in the buds. These findings take up the lively debate whether auxin is directly involved in the regulation of bud release, because endogenous auxin levels in axillary buds do not correlate with the degree of bud inhibition. Consistent with this, exogenously applied IAA to buds of decapitated plants did not prevent initial bud growth but greatly reduced branch lengths after buds have already commenced growth (Morris et al., 2005). Apparently, auxin exerts influence on the transition into sustained branch growth. It therefore seems plausible that bud outgrowth after decapitation is possibly mediated by T6P in response to increased sugar supply. The rise of T6P levels in parallel with those of sucrose may promote endogenous auxin synthesis to facilitate branch growth.

Direct evidence that T6P is involved in the regulation branching comes from the identification of the defective TPP gene underlying the *ramosa3* mutation in maize, causing additional long branches at the basis of the ears (Satoh-Nagasawa et al., 2006). *RA3* is expressed at a restricted area at the base of axillary primordia, indicating that cell-specific T6P degradation is required to regulate identity and determinacy of the meristem. Loss of TPP activity possibly leads to elevation of T6P levels in the primordial tissues, eventually causing the abnormal outgrowth of branches. This perturbation of the T6P signal might be attributable to the deregulation of the ramosa pathway (McSteen, 2006), which eventually results in an undesired activation of auxin pathways and abnormal branch growth.

As a prospect for the future, it would be exciting to intensify the studies in the field of plant architecture by developing strategies aimed on the modulation of T6P in axillary meristems. There are already a number of genes known to be specifically expressed in the primordial tissues, whose promoters might be used for the expression of bacterial trehalose synthesis genes in axillary meristems (Vollbrecht et al., 2005; Aguilar-Martínez et al., 2007) Furthermore, a closer investigation of the promoter regions of T6P "responsive" genes like TAR2 may allow the identification of T6P-responsive DNA-elements, which might pave the way to discover transcription factors that specifically bind to these elements. Eventually, this may lead to a better understanding of T6P signaling pathways or even to the discovery of new T6P receptors.

Is T6P a specific signal for G6P availability?

In plants, nearly all cellular functions and metabolic pathways are ultimately linked to the central metabolites UDPG and G6P, both providing carbon skeletons for synthesis of cell wall compounds and storage products. T6P is directly synthesized from UDPG and G6P through TPS. Therefore, the amount of T6P might act as an immediate indicator for the availability of UDPG and G6P (Paul et al., 2008). As a consequence of this putative interrelation, T6P possibly regulates cellular mechanisms to maintain the pool size of these metabolites in an adequate metabolic range. Several years ago, it has been demonstrated that such kind of mechanism regulates the first steps of glycolysis in yeast. Based on the results of *in vitro* studies, it became clear that T6P negatively regulates the synthesis of G6P by inhibiting hexokinase activity (Blázquez et al., 1993). Our results provide evidence that a negative regulation of G6P pool size by T6P may also exists in plants. On the one hand, diminishment of T6P in USP::TPP embryos caused the suppression of major metabolic pathways directly drawing from G6P pools such starch production (**chapter 4**) and SPS-depended synthesis of sucrose (**chapter 5**). On the other hand, metabolic processes feeding into these pools like sucrose breakdown through SUS were stimulated (**chapter 4**). In other words, T6P

seems to control both, the activation of UDPG/G6P consumption as well as the deactivation of *de novo* synthesis. I therefore propose that T6P is likely conveying the pool sizes of UDPG and/or G6P in order to keep the metabolite levels in an appropriate physiological range.

Nevertheless, several previous studies indicate that T6P acts as a sucrose-specific signal (Lunn et al., 2006; Debast et al., 2011; Martínez-Barajas et al., 2011). In a recent study, a homeostatic mechanism has been postulated which centers the ratio between T6P and sucrose as a critical parameter to maintain sucrose levels within a range that is appropriate for the cell type and developmental requirements (Yadav et al., 2014). Although the close correlation between sucrose and T6P seems compelling, I suggest that the proposed T6P-sucrose-nexus is inconclusive for the following reasons. Yadav et al. (2014) demonstrated that feeding of increasing concentrations of sucrose to C-starved Arabidopsis seedlings leads to continuously raising sucrose levels followed by the increase of T6P. However, an adjustment of the sucrose levels, as proposed by the authors, is not clearly visible. In contrast, when sucrose was fed to higher concentrations, the sharp rise in UDPG and G6P was arrested as soon as a certain level is reached. This implies that the levels of these metabolites might be adjusted to avoid too high metabolite concentrations. In the same study, sucrose and the glucose analogue 2-deoxyglucose were applied together to Arabidopsis seedlings. Possibly due to the sequestration of P_i in 2-deoxyglucose 6-phosphate, levels of UDPG and G6P were decreased or remained unaltered in this experiment. However, sucrose levels increased to the same extent as if sucrose was supplied alone, while the strong rise of T6P disappeared. This implies that the shortage of UDPG and G6P may account for the depletion of T6P synthesis. Since the absence of a T6P response in DOG-treated plants is not in accordance with the increase of sucrose, these results do not fit well with the postulated role of T6P in balancing and signaling sucrose levels. Hence, it is apparently not yet clear whether T6P signaling merely regulates sucrose levels, but also levels of UDPG and G6P in plants.

A deciding limitation of such correlation studies is that direct application of G6P and UDPG is impracticable, due to the restricted absorption capacity by living cells. To circumvent this drawback, I decided to examine the T6P content in growing embryos of various wrinkled pea mutants (Fig. 6.2). Due to specific mutations of single starch biosynthesis enzymes, those mutants displayed large differences in G6P and UDPG content, while sucrose was raised in any event (**chapter 5**). In pea embryos, starch biosynthesis is dependent on sucrose uptake, and for the most part, synthesis of the intermediates UDPG and G6P thus relies on the breakdown of maternally delivered sucrose (see also Fig. 1.2). Metabolite measurements on embryos of the *rug4* mutant revealed that the disruption of sucrose breakdown resulted in a severe reduction of UDPG and a comparatively small decrease in G6P. Although *rug4* embryos contained around 1.6 times more sucrose then the WT, the excess of sucrose did not result in a significant elevation of T6P levels.

On the contrary, T6P levels were tremendously raised in embryos of the *rb* and *r* mutants, although the increase in sucrose was in a similar range to that in *rug4*. Since mutations at the *r* and *rb* loci affect plastidic steps of starch synthesis, the utilization of cytosolic G6P is reduced. Accordingly, G6P levels increased dramatically in both mutants as did T6P, whereas UDPG was either moderately elevated in *rb* or unaltered in *r* embryos. By comparing the metabolic pattern of all three mutants, it becomes clear that T6P levels changed independently from those of sucrose, but there was a close linkage to G6P, evidenced by highest positive correlation (r=0.896) between T6P and G6P. Taken together, it appears that T6P controls the relation between G6P consumption and synthesis in response to the availability of G6P in the cytosol. Lower correlation coefficients between sucrose and T6P (r=0.646) as well as UDPG and T6P (r=0.215) supports the conclusion that levels of those metabolites are not conveyed by T6P.

We are only beginning to understand the basis of the interrelations between T6P and other sugars like sucrose or G6P. Synthesis and degradation of T6P seem to be tightly controlled in plants, and both may contribute to the modulation of the T6P signal. Further studies are needed to ascertain whether all trehalose synthesis enzymes are indeed involved in the regulation T6P levels (Lunn et al., 2014). Nevertheless, the catalytic properties of the yeast TPS may provide some indication how a homeostatic mechanism between T6P and G6P might be maintained in plant cells. The catalytic domains of plant TPS and yeast TPS are closely related and thus both possibly exhibit similar kinetic properties (Avonce et al., 2006; Lunn, 2007). Characterization of yeast TPS revealed that the K_m value for UDPG (0.5 mM) is similar to that of the intracellular concentration, whereas the K_m value of G6P (3.5 mM) exceeds the intracellular concentration. Therefore, the availability of G6P has been suggested to be the modulating factor of *in vivo* T6P synthesis in yeast (Vandercammen et al., 1989). If this also applies for plant TPS, T6P synthesis would rapidly increase in response to a large rise of cytosolic G6P levels and might give an explanation for the strong linkage between T6P and G6P in mutant pea seeds.

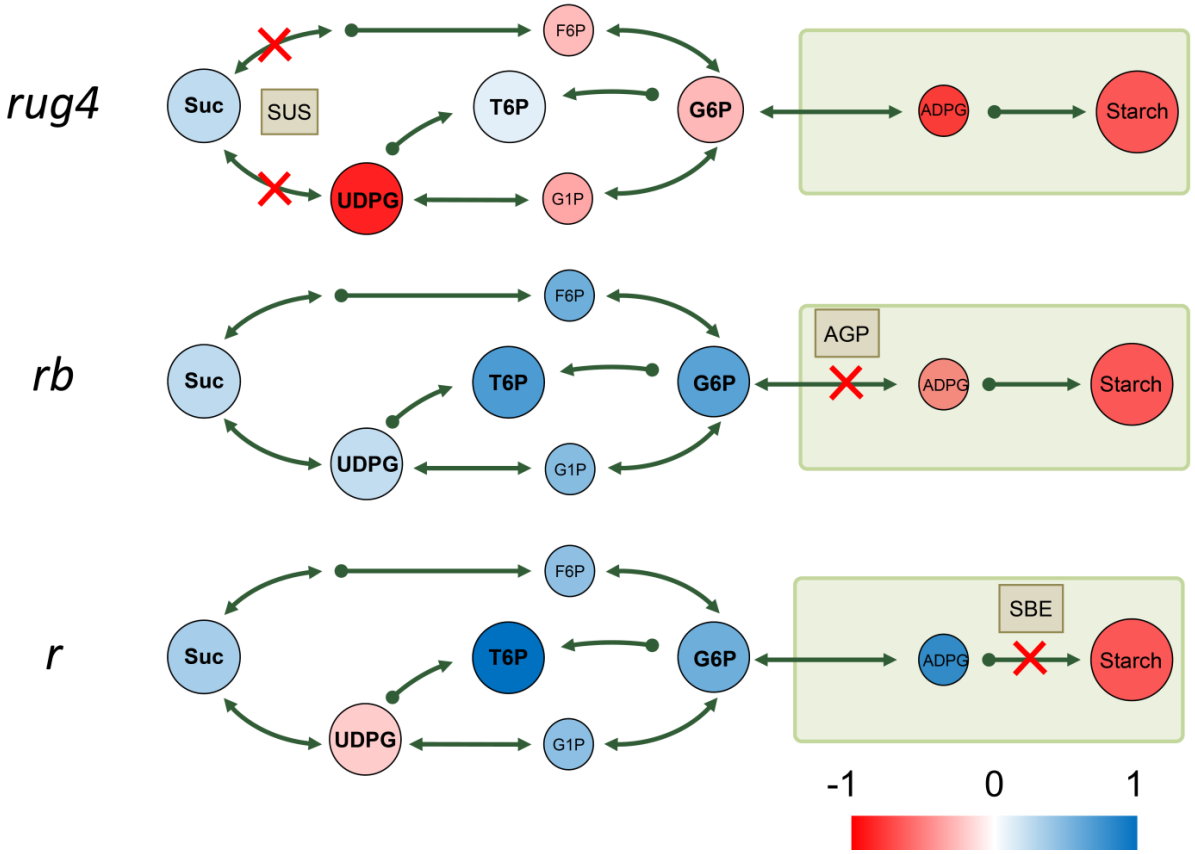

Figure 6.2. Comparison between T6P and soluble sugar levels in 24-day-old *rug4*, *rb* and *r* mutant embryos. Data are derived from Fig. 5.5 and 5.6. Red, relative reduction of metabolite levels with respect to the WT; blue, relative increase of metabolite levels with respect to the WT. Enzymatic steps affected in specific mutants are indicated by red crosses.

In order to disentangle the relationships between T6P and other sugar species further studies are needed. The garden pea has the advantage to produce large seeds allowing the readily isolation and manipulation of the embryo. Furthermore, there already exists a range of mutants and transgenic models that affect carbohydrate metabolism in pea seeds. According to previous short-term feeding experiments (Yadav et al., 2014), C-starved embryos from these models may be exposed to the treatment with different sugars and inhibitors in order to test the resultant response of T6P. The application of plant permeable and light-activated precursor of T6P and G6P may further strengthen the evidential value of such feeding experiments (Griffiths et al., 2016). Moreover, it will be important to analyze the catalytic properties of plant trehalose synthesis enzymes, which will possibly gain a better understanding of how certain metabolites influence the level of T6P as well as its synthesis in plants.

T6P is a positive regulator of seed germination

Elevation of T6P levels in pea embryos strongly affected very late embryo development. At maturity, USP::TPS seeds appeared hydrated and embryos remained green. Furthermore, transgenic seeds germinated prematurely, evidenced by a marked root outgrowth and impaired seedling formation upon imbibition (**chapter 5**). These effects are reminiscent to various Arabidopsis mutants affected in B3-domain transcription factors (Finkelstein, 2013). Particularly, the striking similarity to the severe seed phenotype of the *abi3-6* mutant provide indication that precocious germination of USP::TPS seeds is the result of either T6P-mediated insensitivity towards ABA or suppression of ABA-related functions (Nambara et al., 1992). On the contrary, depletion of T6P had the opposite effect on germination, since seed germination was severely delayed and germination capacity was reduced in USP::TPP seeds. It appears that reduction of T6P imposes a type of physiological dormancy in those seeds. In general, garden pea embryos are characterized by the lack of endogenous or physiological dormancy. Only wild pea progenitors exhibit physical seed dormancy facilitated by a hard and thickened seed coat, which prevents water uptake and soaking of the embryo (Smýkal et al., 2015). Cultivated garden pea varieties are generally capable to germinate over a wide range of physical and environmental conditions, since selection towards the reduction of seed coat impermeability led to the loss of physical dormancy. Put together, our evidence indicates that that T6P is required to release seed germination in garden pea.

The plant hormone ABA plays a central role in maintaining and inducing endogenous seed dormancy in many plant species. As in garden pea, seeds of many other crop legumes are non-dormant at maturity. However, ABA profiling in growing garden bean (*Phaseolus vulgaris*) revealed that changes in ABA content are inversely correlated to germinability of *in vitro* grown embryos (Prevost and Le Page-Degivry, 1985). It has been shown that germinative growth of excised garden bean embryos decreased in parallel with the rise in their ABA content. It appears that ABA apparently prevents germination without necessarily imposing dormancy. T6P has previously been implicated in the regulation of ABA signaling during germination. Seedling growth of transgenic Arabidopsis overexpressing TPS1 is less sensitive towards ABA in presence of 2.5 μM ABA (Avonce et al., 2004). The loss of a single TPP isoform in Arabidopsis also gains insensitivity towards ABA (Vandesteene et al., 2012). In contrast, seeds from weaker *tps-1* mutants are hypersensitive to ABA (Gómez et al., 2010). Several lines of evidence suggest that the influence of T6P on germination and ABA signaling is mediated by SnRK1 (Tsai and Gazzarrini, 2014). Similar to the effects in USP::TPS seeds, embryo-specific repression of SnRK1 in transgenic pea results in precocious germination and greenish seeds (Radchuk et al., 2006). Moreover, *ABI3* expression is repressed and maturing seeds contain less ABA. In Arabidopsis, SnRK1 positively regulates the

synthesis of ABA by phosphorylation of FUS3 (Tsai and Gazzarrini, 2012). In a feedback manner, ABA inactivates PP2Cs and relieves the negative regulation of SnRK1 activity by the latter (Rodrigues et al., 2013). The results presented in **chapter five** demonstrate that application of T6P inhibited SnRK1 activity in protein extracts from growing pea embryos. This is consistent with previous studies in Arabidopsis and wheat (Zhang et al., 2009; Martínez-Barajas et al., 2011), indicating that modulation of T6P levels in USP::TPS and USP::TPP embryos may also affect the *in vivo* inhibition of SnRK1. This effect possibly influences the sensitivity towards ABA and thus also the germination behavior of transgenic pea seeds.

Taken together, T6P acts as a trigger of seed germination that releases outgrowth of the embryonic shoot and root. High T6P levels in USP::TPS embryos seem to interfere with the acquisition of seed rest during late maturation stages. Therefore, it can be expected that T6P levels decline towards the end of maturation in order to impose a quiescent state, otherwise the seed would germinate precociously. Our measurements of T6P content in growing embryos substantiate suchlike hypothesis, since T6P levels were high at early maturation phase, but declined strongly in the further course of maturation, being lowest at the latest stage (**Chapter 4,** Fig. 1 A). Once seeds have acquired maturity and desiccation tolerance, T6P levels may raise again after imbibition, possibly conveying the commencing release of soluble sugars from starch degradation as soon as dry seeds are left to soak in water. Such increase in T6P potentially triggers the initiation of seedling growth by modulating hormone signaling.

It has long been recognized that seed germination is regulated by the plant hormones ABA and GA. Both hormones act antagonistically with each other (Cadman et al., 2006; Finkelstein et al., 2008). Furthermore, brassinosteroids and jasmonates have also been suggested to play a role during germination (Steber and McCourt, 2001; Dave et al., 2011). Future research on changes in the hormonal balance and response of germinating USP::TPS and USP::TPP seeds will possibly give indications on how T6P integrates into the existing hormonal network of germination control.

Conclusions

The initiation and maintenance of seed maturation and seed filling are believed to be regulated by a network of various signals including hormones and sugars. However, the interrelations and molecular connections between sugar and hormone signaling are poorly understood. Correspondingly, the central issue addressed by my thesis is the identification of possible relationships between the central sugar signal T6P and the classic plant hormone auxin, both previously implicated in the control of seed development (Eastmond et al., 2002; LeClere et al., 2010).

The results presented here indicate that both T6P and auxin are pivotal factors of a regulatory system, for the purpose to coordinate sugar metabolism with developmental and cellular processes in maturing and germinating garden pea embryos (Fig. 6.3). The transmission of sugar availability via T6P provides a convenient way of metabolic regulation in plants, since its synthesis draws directly from UDPG and G6P pools at the center of primary carbon metabolism. The strong correlation between T6P and G6P levels in embryos of different wrinkled pea mutants suggests that T6P participates in a homeostatic mechanism that controls the pool size of G6P in the embryo. It appears that the rise of T6P is an indication for elevated G6P levels, potentially promoting G6P consuming processes, such as cotyledon growth and starch accumulation, to ensure that G6P levels are kept at the optimal concentration range for the cell. For the same purpose, processes that feed into the G6P pool are suppressed by T6P, as exemplified by negative regulation of sucrose breakdown through SUS. As soon as G6P reaches the desired level, T6P decreases, thereby relieving the signaling effect. Via this mechanism T6P facilitates the efficient allocation of incoming sucrose into reserve starch and other energy consuming processes like embryo growth.

Perception and transmission of T6P requires the integration of plant hormones and other signaling components. A previously identified mechanism that implies the inhibition of SnRK1 by T6P apparently also exists in growing pea seeds and may exert influence over sucrose metabolism and the release of germination. Furthermore, our evidence indicates that T6P interacts with hormonal pathways, because it was shown that T6P promotes auxin biosynthesis via the TAR2 pathway. The resulting effect on auxin levels very likely accounts for the stimulation of cotyledon expansion and starch accumulation in maturing seeds, as evidenced by various auxin responsive elements present in the promoter regions of different starch biosynthesis genes, in particular those encoding AGP. Since it could be shown that T6P acts as an upstream regulator of auxin in maturing seeds, these findings significantly contribute to a better understanding of the signaling connections between sugar metabolism and hormonal pathways in plants.

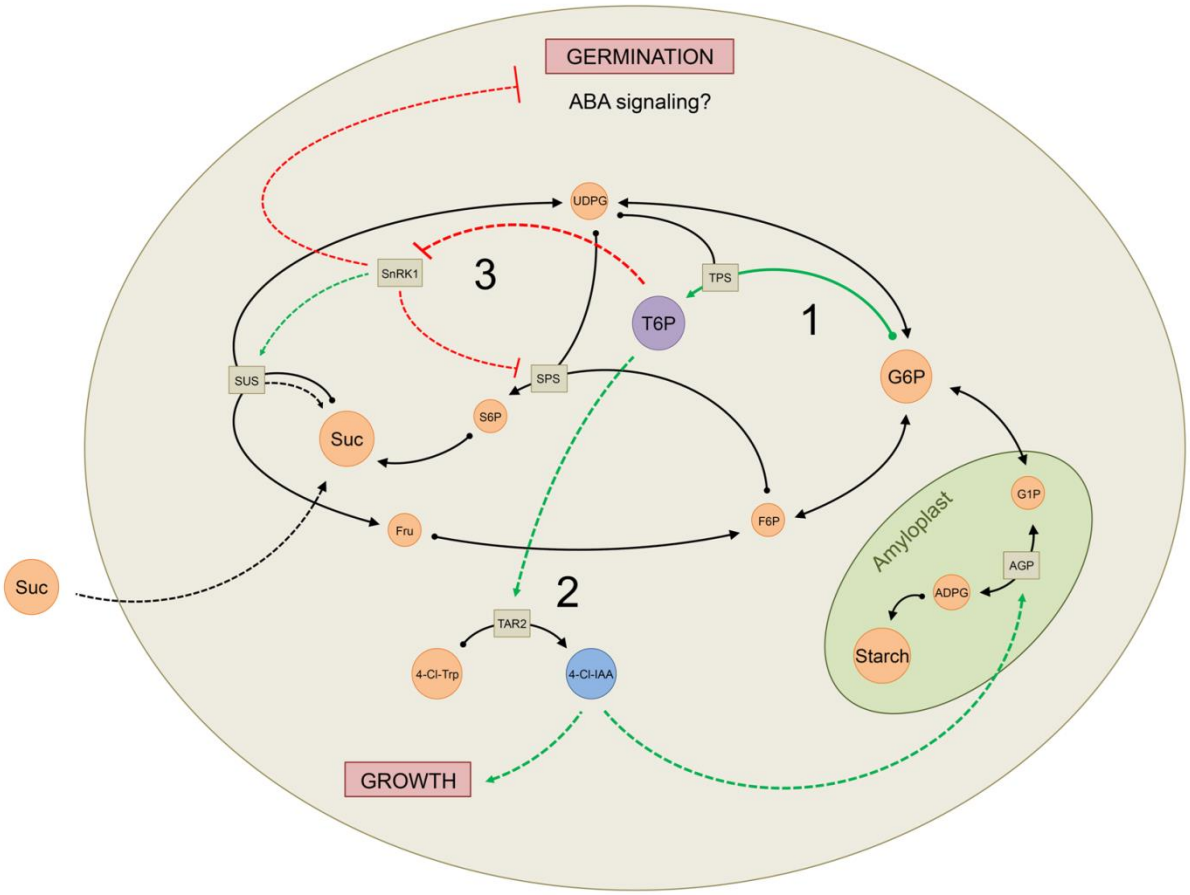

Figure 6.3. Functional context of T6P and auxin pathways in garden pea embryos. As a signaling metabolite, T6P is in close vicinity to the G6P pool, which it may respond to and regulate (1). T6P promotes cotyledon growth and reserve starch synthesis by activating TAR2 on the gene level. This gives rise to the synthesis of the auxin 4-Cl-IAA which possibly facilitates storage cell differentiation and starch accumulation, for example by positive regulation of AGP (2). Furthermore, T6P controls a variety of other developmental and metabolic processes such as germination and sucrose metabolism. It is possible that the regulation of these processes involves the post-translational modulation of SnRK1 activity by T6P (3).

References

Aguilar-Martínez JA, Poza-Carrión C, Cubas P (2007) Arabidopsis BRANCHED1 acts as an integrator of branching signals within axillary buds. The Plant Cell **19:** 458-472

Avonce N, Leyman B, Mascorro-Gallardo O, Dijck P, Thevelein JM, Iturriaga G (2004) The Arabidopsis Trehalose-6-P Synthase AtTPS1 Gene Is a Regulator of Glucose, Abscisic Acid, and Stress Signalling. Plant Physiol **136**

Avonce N, Mendoza-Vargas A, Morett E, Iturriaga G (2006) Insights on the evolution of trehalose biosynthesis. BMC Evolutionary Biology **6:** 1-15

Baena-González E, Rolland F, Thevelein JM, Sheen J (2007) A central integrator of transcription networks in plant stress and energy signalling. Nature **448:** 938-942

Bahaji A, Li J, Ovecka M, Ezquer I, Muñoz FJ, Baroja-Fernández E, Romero JM, Almagro G, Montero M, Hidalgo M (2011) Arabidopsis thaliana mutants lacking ADP-glucose pyrophosphorylase accumulate starch and wild-type ADP-glucose content: further evidence for the occurrence of important sources, other than ADP-glucose pyrophosphorylase, of ADP-glucose linked to leaf starch biosynthesis. Plant and cell physiology **52:** 1162-1176

Baroja-Fernández E, Muñoz FJ, Zandueta-Criado A, Morán-Zorzano MT, Viale AM, Alonso-Casajús N, Pozueta-Romero J (2004) Most of ADP· glucose linked to starch biosynthesis occurs outside the chloroplast in source leaves. Proceedings of the National Academy of Sciences of the United States of America **101:** 13080-13085

Baskin JM, Baskin CC (2004) A classification system for seed dormancy. Seed science research **14:** 1-16

Bate NJ, Niu X, Wang Y, Reimann KS, Helentjaris TG (2004) An invertase inhibitor from maize localizes to the embryo surrounding region during early kernel development. Plant Physiology **134:** 246-254

Baud S, Wuilleme S, Dubreucq B, De Almeida A, Vuagnat C, Lepiniec L, Miquel M, Rochat C (2007) Function of plastidial pyruvate kinases in seeds of Arabidopsis thaliana. The Plant Journal **52:** 405-419

Baud S, Wuillème S, To A, Rochat C, Lepiniec L (2009) Role of WRINKLED1 in the transcriptional regulation of glycolytic and fatty acid biosynthetic genes in Arabidopsis. The Plant Journal **60:** 933-947

Bernardi J, Lanubile A, Li Q-B, Kumar D, Kladnik A, Cook SD, Ross JJ, Marocco A, Chourey PS (2012) Impaired auxin biosynthesis in the defective endosperm18 mutant is due to mutational loss of expression in the ZmYuc1 gene encoding endosperm-specific YUCCA1 protein in maize. Plant physiology **160:** 1318-1328

Bewley JD, Black M (1994) Seeds. *In* Seeds. Springer, pp 1-33

Bhattacharyya MK, Smith AM, Ellis TN, Hedley C, Martin C (1990) The wrinkled-seed character of pea described by Mendel is caused by a transposon-like insertion in a gene encoding starch-branching enzyme. Cell **60:** 115-122

Bies-Etheve N, da Silva Conceicao A, Koornneef M, Léon-Kloosterziel K, Valon C, Delseny M (1999) Importance of the B2 domain of the Arabidopsis ABI3 protein for Em and 2S albumin gene regulation. Plant molecular biology **40:** 1045-1054

Birchler JA, Yao H, Chudalayandi S, Vaiman D, Veitia RA (2010) Heterosis. The Plant Cell **22:** 2105-2112

Blázquez MA, Lagunas R, Gancedo C, Gancedo JM (1993) Trehalose-6-phosphate, a new regulator of yeast glycolysis that inhibits hexokinases. FEBS letters **329:** 51-54

Blazquez MA, Santos E, Flores Cl, Martínez-Zapater JM, Salinas J, Gancedo C (1998) Isolation and molecular characterization of the Arabidopsis TPS1 gene, encoding trehalose-6-phosphate synthase. The Plant Journal **13:** 685-689

Bogracheva TY, Cairns P, Noel T, Hulleman S, Wang T, Morris V, Ring S, Hedley C (1999) The effect of mutant genes at the r, rb, rug3, rug4, rug5 and lam loci on the granular structure and physico-chemical properties of pea seed starch. Carbohydrate Polymers **39:** 303-314

Borisjuk L, Walenta S, Rolletschek H, Mueller-Klieser W, Wobus U, Weber H (2002) Spatial analysis of plant metabolism: sucrose imaging within Vicia faba cotyledons reveals specific developmental patterns. The Plant Journal **29:** 521-530

Borisjuk L, Wang TL, Rolletschek H, Wobus U, Weber H (2002) A pea seed mutant affected in the differentiation of the embryonic epidermis is impaired in embryo growth and seed maturation. Development **129:** 1595-1607

Borisjuk L, Weber H, Panitz R, Manteuffel R, Wobus U (1995) Embryogenesis of Vicia faba L.: histodifferentiation in relation to starch and storage protein synthesis. Journal of Plant Physiology **147:** 203-218

Brocard-Gifford IM, Lynch TJ, Finkelstein RR (2003) Regulatory networks in seeds integrating developmental, abscisic acid, sugar, and light signaling. Plant Physiology **131:** 78-92

Buléon A, Colonna P, Planchot V, Ball S (1998) Starch granules: structure and biosynthesis. International journal of biological macromolecules **23:** 85-112

Cadman CS, Toorop PE, Hilhorst HW, Finch-Savage WE (2006) Gene expression profiles of Arabidopsis Cvi seeds during dormancy cycling indicate a common underlying dormancy control mechanism. The Plant Journal **46:** 805-822

Chary SN, Hicks GR, Choi YG, Carter D, Raikhel NV (2008) Trehalose-6-phosphate synthase/phosphatase regulates cell shape and plant architecture in Arabidopsis. Plant Physiology **146:** 97-107

Cheng W-H, Taliercio EW, Chourey PS (1996) The miniature1 seed locus of maize encodes a cell wall invertase required for normal development of endosperm and maternal cells in the pedicel. The Plant Cell **8:** 971-983

Craig J, Barratt P, Tatge H, Dejardin A, Handley L, Gardner CD, Barber L, Wang T, Hedley C, Martin C (1999) Mutations at therug4locus alter the carbon and nitrogen metabolism of pea plants through an effect on sucrose synthase. The Plant Journal **17:** 353-362

Craig J, Barratt P, Tatge H, Déjardin A, Handley L, Gardner CD, Barber L, Wang T, Hedley C, Martin C (1999) Mutations at therug4locus alter the carbon and nitrogen metabolism of pea plants through an effect on sucrose synthase. The Plant Journal **17:** 353-362

Craig J, Lloyd JR, Tomlinson K, Barber L, Edwards A, Wang TL, Martin C, Hedley CL, Smith AM (1998) Mutations in the gene encoding starch synthase II profoundly alter amylopectin structure in pea embryos. The Plant Cell **10:** 413-426

Curaba J, Moritz T, Blervaque R, Parcy F, Raz V, Herzog M, Vachon G (2004) AtGA3ox2, a key gene responsible for bioactive gibberellin biosynthesis, is regulated during embryogenesis by LEAFY COTYLEDON2 and FUSCA3 in Arabidopsis. Plant Physiology **136:** 3660-3669

Dave A, Hernandez ML, He Z, Andriotis VM, Vaistij FE, Larson TR, Graham IA (2011) 12-oxo-phytodienoic acid accumulation during seed development represses seed germination in Arabidopsis. Plant Cell **23:** 583-599

Davies DR (1975) Studies of seed development in Pisum sativum. Planta **124:** 297-302

Debast S, Nunes-Nesi A, Hajirezaei MR, Hofmann J, Sonnewald U, Fernie AR, Börnke F (2011) Altering trehalose-6-phosphate content in transgenic potato tubers affects tuber growth and alters responsiveness to hormones during sprouting. Plant physiology **156:** 1754-1771

Delseny M, Bies-Etheve N, Carles C, Hull G, Vicient C, Raynal M, Grellet F, Aspart L (2001) Late embryogenesis abundant (LEA) protein gene regulation during Arabidopsis seed maturation. Journal of Plant Physiology **158:** 419-427

Denyer K, Barber L, Burton R, Hedley C, Hylton C, Johnson S, Jones D, Marshall J, Smith A, Tatge H (1995) The isolation and characterization of novel low-amylose mutants of Pisum sativum L. Plant, Cell & Environment **18:** 1019-1026

Denyer K, Dunlap F, Thorbjornsen T, Keeling P, Smith AM (1996) The major form of ADP-glucose pyrophosphorylase in maize endosperm is extra-plastidial. Plant Physiology **112:** 779-785

Denyer K, Foster J, Smith AM (1995) The contributions of adenosine 5'-diphosphoglucose pyrophosphorylase and starch-branching enzyme to the control of starch synthesis in developing pea embryos. Planta **197:** 57-62

Dieckmann S, Link W (2010) Quantitative genetic analysis of embryo heterosis in faba bean (Vicia faba L.). Theoretical and applied genetics **120:** 261-270

Doan DN, Rudi H, Olsen O-A (1999) The allosterically unregulated isoform of ADP-glucose pyrophosphorylase from barley endosperm is the most likely source of ADP-glucose incorporated into endosperm starch. Plant Physiology **121:** 965-975

Duc G, Moessner A, Moussy F, Mousset-Déclas C (2001) A xenia effect on number and volume of cotyledon cells and on seed weight in faba bean (Vicia faba L.). Euphytica **117:** 169-174

East EM (1936) Heterosis. Genetics **21:** 375

Eastmond PJ, Dijken AJH, Spielman M, Kerr A, Tissier AF, Dickinson HG, Jones JDG, Smeekens SC, Graham IA (2002) Trehalose-6-phosphate synthase 1, which catalyses the first step in trehalose synthesis, is essential for Arabidopsis embryo maturation. Plant J **29**

Edwards J, Green JH, Ap Rees T (1988) Activity of branching enzyme as a cardinal feature of the Ra locus in Pisum sativum. Phytochemistry **27:** 1615-1620

Eeuwens C, Schwabe W (1975) Seed and pod wall development in Pisum sativum, L. in relation to extracted and applied hormones. Journal of Experimental Botany **26:** 1-14

Fettke J, Albrecht T, Hejazi M, Mahlow S, Nakamura Y, Steup M (2010) Glucose 1-phosphate is efficiently taken up by potato (Solanum tuberosum) tuber parenchyma cells and converted to reserve starch granules. New Phytologist **185:** 663-675

Figueroa CM, Feil R, Ishihara H, Watanabe M, Kölling K, Krause U, Höhne M, Encke B, Plaxton WC, Zeeman SC (2015) Trehalose 6-phosphate coordinates organic and amino acid metabolism with carbon availability. The Plant Journal

Finkelstein R (2013) Abscisic acid synthesis and response. The Arabidopsis Book**:** e0166

Finkelstein R, Reeves W, Ariizumi T, Steber C (2008) Molecular aspects of seed dormancy. Plant Biology **59:** 387

Finkelstein RR (1994) Mutations at two new Arabidopsis ABA response loci are similar to the abi3 mutations. The Plant Journal **5:** 765-771

Fischer K, Kammerer B, Gutensohn M, Arbinger B, Weber A, Häusler RE, Flügge U-I (1997) A new class of plastidic phosphate translocators: a putative link between primary and secondary metabolism by the phosphoenolpyruvate/phosphate antiporter. The Plant Cell **9:** 453-462

Gazzarrini S, Tsai AY-L (2014) Trehalose-6-phosphate and SnRK1 kinases in plant development and signaling: the emerging picture. Frontiers in plant science **5:** 119

Ge L-F, Chao D-Y, Shi M, Zhu M-Z, Gao J-P, Lin H-X (2008) Overexpression of the trehalose-6-phosphate phosphatase gene OsTPP1 confers stress tolerance in rice and results in the activation of stress responsive genes. Planta **228:** 191-201

Giraudat J, Hauge BM, Valon C, Smalle J, Parcy F, Goodman HM (1992) Isolation of the Arabidopsis ABI3 gene by positional cloning. The Plant Cell **4:** 1251-1261

Goddijn OJ, Verwoerd TC, Voogd E, Krutwagen RW, De Graff P, Poels J, van Dun K, Ponstein AS, Damm B, Pen J (1997) Inhibition of trehalase activity enhances trehalose accumulation in transgenic plants. Plant physiology **113:** 181-190

Gómez E, Royo J, Guo Y, Thompson R, Hueros G (2002) Establishment of Cereal Endosperm Expression Domains Identification and Properties of a Maize Transfer Cell–Specific Transcription Factor, ZmMRP-1. The Plant Cell **14:** 599-610

Gómez LD, Baud S, Gilday A, Li Y, Graham IA (2006) Delayed embryo development in the ARABIDOPSIS TREHALOSE-6-PHOSPHATE SYNTHASE 1 mutant is associated with altered cell wall structure, decreased cell division and starch accumulation. The Plant Journal **46:** 69-84

Gómez LD, Gilday A, Feil R, Lunn JE, Graham IA (2010) AtTPS1-mediated trehalose 6-phosphate synthesis is essential for embryogenic and vegetative growth and responsiveness to ABA in germinating seeds and stomatal guard cells. The Plant Journal **64:** 1-13

Griffiths CA, Sagar R, Geng Y, Primavesi LF, Patel MK, Passarelli MK, Gilmore IS, Steven RT, Bunch J, Paul MJ (2016) Chemical intervention in plant sugar signalling increases yield and resilience. Nature **540:** 574-578

Groszmann M, Gonzalez-Bayon R, Greaves IK, Wang L, Huen AK, Peacock WJ, Dennis ES (2014) Intraspecific Arabidopsis hybrids show different patterns of heterosis despite the close relatedness of the parental genomes. Plant physiology**:** pp. 114.243998

Gruis D, Schulze J, Jung R (2004) Storage protein accumulation in the absence of the vacuolar processing enzyme family of cysteine proteases. The Plant Cell **16:** 270-290

Guo M, Rupe MA, Dieter JA, Zou J, Spielbauer D, Duncan KE, Howard RJ, Hou Z, Simmons CR (2010) Cell Number Regulator1 affects plant and organ size in maize: implications for crop yield enhancement and heterosis. The Plant Cell **22:** 1057-1073

Hanson J, Smeekens S (2009) Sugar perception and signaling—an update. Current opinion in plant biology **12:** 562-567

Hara-Nishimura I, Inoue K, Nishimura M (1991) A unique vacuolar processing enzyme responsible for conversion of several proprotein precursors into the mature forms. FEBS letters **294:** 89-93

Harrison C, Hedley C, Wang T (1998) Evidence that the rug3 locus of pea (Pisum sativum L.) encodes plastidial phosphoglucomutase confirms that the imported substrate for starch synthesis in pea amyloplasts is glucose-6-phosphate. Plant journal **13:** 753-762

Heim U, Weber H, Bäumlein H, Wobus U (1993) A sucrose-synthase gene of Vicia faba L.: expression pattern in developing seeds in relation to starch synthesis and metabolic regulation. Planta **191:** 394-401

Hennig L, Gruissem W, Grossniklaus U, Köhler C (2004) Transcriptional programs of early reproductive stages in Arabidopsis. Plant Physiology **135:** 1765-1775

Hill LM, Smith AM (1991) Evidence that glucose 6-phosphate is imported as the substrate for starch synthesis by the plastids of developing pea embryos. Planta **185:** 91-96

Huang A (1996) Oleosins and oil bodies in seeds and other organs. Plant Physiology **110:** 1055

Hylton C, Smith AM (1992) The rb mutation of peas causes structural and regulatory changes in ADP glucose pyrophosphorylase from developing embryos. Plant Physiology **99:** 1626-1634

Jensen PJ, Bandurski RS (1994) Metabolism and synthesis of indole-3-acetic acid (IAA) in Zea mays (levels of IAA during kernel development and the use of in vitro endosperm systems for studying IAA biosynthesis). Plant physiology **106:** 343-351

Kammerer B, Fischer K, Hilpert B, Schubert S, Gutensohn M, Weber A, Flügge U-I (1998) Molecular characterization of a carbon transporter in plastids from heterotrophic tissues: the glucose 6-phosphate/phosphate antiporter. The Plant Cell **10:** 105-117

Karcz W, Burdach Z (2002) A comparison of the effects of IAA and 4-Cl-IAA on growth, proton secretion and membrane potential in maize coleoptile segments. Journal of experimental botany **53:** 1089-1098

Kaul S, Koo HL, Jenkins J, Rizzo M, Rooney T, Tallon LJ, Feldblyum T, Nierman W, Benito MI, Lin X (2000) Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. nature **408:** 796-815

Keith K, Kraml M, Dengler NG, McCourt P (1994) fusca3: a heterochronic mutation affecting late embryo development in Arabidopsis. The Plant Cell **6:** 589-600

Kermode AR, Dumbroff E, Bewley JD (1989) The role of maturation drying in the transition from seed development to germination VII. Effects of partial and complete desiccation on abscisic acid levels and sensitivity in Ricinus communis L. seeds. Journal of Experimental Botany **40:** 303-313

Koch K (2004) Sucrose metabolism: regulatory mechanisms and pivotal roles in sugar sensing and plant development. Current opinion in plant biology **7:** 235-246

Kolbe A, Tiessen A, Schluepmann H, Paul M, Ulrich S, Geigenberger P (2005) Trehalose 6-phosphate regulates starch synthesis via posttranslational redox activation of ADP-glucose pyrophosphorylase. Proceedings of the National Academy of Sciences of the United States of America **102:** 11118-11123

Kooistra E (1962) On the differences between smooth and three types of wrinkled peas. Euphytica **11:** 357-373

Kosegarten H, Mengel K (1994) Evidence for a glucose 1-phosphate translocator in storage tissue amyloplasts of potato (Solanum tuberosum) suspension-cultured cells. Physiologia Plantarum **91:** 111-120

Krasensky J, Broyart C, Rabanal FA, Jonak C (2014) The redox-sensitive chloroplast trehalose-6-phosphate phosphatase AtTPPD regulates salt stress tolerance. Antioxidants & redox signaling **21:** 1289-1304

Lam HK, McAdam SA, McAdam EL, Ross JJ (2015) Evidence that chlorinated auxin is restricted to the Fabaceae but not to the Fabeae. Plant physiology **168:** 798-803

Lanfermeijer FC, van Oene MA, Borstlap AC (1992) Compartmental analysis of amino-acid release from attached and detached pea seed coats. Planta **187:** 75-82

Le BH, Wagmaister JA, Kawashima T, Bui AQ, Harada JJ, Goldberg RB (2007) Using genomics to study legume seed development. Plant physiology **144:** 562-574

LeClere S, Schmelz EA, Chourey PS (2010) Sugar levels regulate tryptophan-dependent auxin biosynthesis in developing maize kernels. Plant Physiology **153:** 306-318

Leyman B, Van Dijck P, Thevelein JM (2001) An unexpected plethora of trehalose biosynthesis genes in Arabidopsis thaliana. Trends in plant science **6:** 510-513

Li P, Ma S, Bohnert HJ (2008) Coexpression characteristics of trehalose-6-phosphate phosphatase subfamily genes reveal different functions in a network context. Physiologia Plantarum **133:** 544-556

Lin T-P, Caspar T, Somerville C, Preiss J (1988) Isolation and characterization of a starchless mutant of Arabidopsis thaliana (L.) Heynh lacking ADPglucose pyrophosphorylase activity. Plant Physiology **86:** 1131-1135

Lunn JE (2007) Gene families and evolution of trehalose metabolism in plants. Functional Plant Biology **34:** 550-563

Lunn JE, Ashton AR, Hatch MD, Heldt HW (2000) Purification, molecular cloning, and sequence analysis of sucrose-6F-phosphate phosphohydrolase from plants. Proceedings of the National Academy of Sciences **97:** 12914-12919

Lunn JE, Delorge I, Figueroa CM, Van Dijck P, Stitt M (2014) Trehalose metabolism in plants. Plant J **79:** 544-567

Lunn JE, Feil R, Hendriks JH, Gibon Y, Morcuende R, Osuna D, Scheible W-R, Carillo P, Hajirezaei M-R, Stitt M (2006) Sugar-induced increases in trehalose 6-phosphate are correlated with redox activation of ADPglucose pyrophosphorylase and higher rates of starch synthesis in Arabidopsis thaliana. Biochemical Journal **397:** 139-148

Mano Y, Nemoto K (2012) The pathway of auxin biosynthesis in plants. Journal of experimental Botany**:** ers091

Martin C, Smith AM (1995) Starch biosynthesis. The plant cell **7:** 971

Martínez-Barajas E, Delatte T, Schluepmann H, de Jong GJ, Somsen GW, Nunes C, Primavesi LF, Coello P, Mitchell RA, Paul MJ (2011) Wheat grain development is characterized by remarkable trehalose 6-phosphate accumulation pregrain filling: tissue distribution and relationship to SNF1-related protein kinase1 activity. Plant physiology **156:** 373-381

Martins MCM, Hejazi M, Fettke J, Steup M, Feil R, Krause U, Arrivault S, Vosloh D, Figueroa CM, Ivakov A (2013) Feedback inhibition of starch degradation in Arabidopsis leaves mediated by trehalose 6-phosphate. Plant physiology **163:** 1142-1163

Mashiguchi K, Tanaka K, Sakai T, Sugawara S, Kawaide H, Natsume M, Hanada A, Yaeno T, Shirasu K, Yao H (2011) The main auxin biosynthesis pathway in Arabidopsis. Proceedings of the National Academy of Sciences **108:** 18512-18517

Mason MG, Ross JJ, Babst BA, Wienclaw BN, Beveridge CA (2014) Sugar demand, not auxin, is the initial regulator of apical dominance. Proceedings of the National Academy of Sciences **111:** 6092-6097

McCarty DR (1995) Genetic control and integration of maturation and germination pathways in seed development. Annual review of plant biology **46:** 71-93

McCarty DR, Hattori T, Carson CB, Vasil V, Lazar M, Vasil IK (1991) The Viviparous-1 developmental gene of maize encodes a novel transcriptional activator. Cell **66:** 895-905

McSteen P (2006) Branching out: the ramosa pathway and the evolution of grass inflorescence morphology. The Plant Cell **18:** 518-522

McWha J (1975) Changes in abscisic acid levels in developing grains of wheat (Triticum aestivum L.). Journal of Experimental Botany**:** 823-827

Meinke DW, Franzmann LH, Nickle TC, Yeung EC (1994) Leafy cotyledon mutants of Arabidopsis. The Plant Cell **6:** 1049-1064

Meyer RC, Törjék O, Becher M, Altmann T (2004) Heterosis of biomass production in Arabidopsis. Establishment during early development. Plant Physiology **134:** 1813-1823

Meyer RC, Witucka-Wall H, Becher M, Blacha A, Boudichevskaia A, Dörmann P, Fiehn O, Friedel S, Von Korff M, Lisec J (2012) Heterosis manifestation during early Arabidopsis seedling development is characterized by intermediate gene expression and enhanced metabolic activity in the hybrids. The Plant Journal **71:** 669-683

Meyer S, Pospisil H, Scholten S (2007) Heterosis associated gene expression in maize embryos 6 days after fertilization exhibits additive, dominant and overdominant pattern. Plant molecular biology **63:** 381-391

Miller ME, Chourey PS (1992) The maize invertase-deficient miniature-1 seed mutation is associated with aberrant pedicel and endosperm development. The Plant Cell **4:** 297-305

Miranda M, Borisjuk L, Tewes A, Dietrich D, Rentsch D, Weber H, Wobus U (2003) Peptide and amino acid transporters are differentially regulated during seed development and germination in faba bean. Plant Physiology **132:** 1950-1960

Mishra BS, Singh M, Aggrawal P, Laxmi A (2009) Glucose and auxin signaling interaction in controlling Arabidopsis thaliana seedlings root growth and development. PLOS one **4:** e4502

Moore B, Zhou L, Rolland F, Hall Q, Cheng W-H, Liu Y-X, Hwang I, Jones T, Sheen J (2003) Role of the Arabidopsis glucose sensor HXK1 in nutrient, light, and hormonal signaling. Science **300:** 332-336

Morris SE, Cox MC, Ross JJ, Krisantini S, Beveridge CA (2005) Auxin dynamics after decapitation are not correlated with the initial growth of axillary buds. Plant Physiology **138:** 1665-1672

Muñoz FJ, Baroja-Fernández E, Morán-Zorzano MT, Viale AM, Etxeberria E, Alonso-Casajús N, Pozueta-Romero J (2005) Sucrose synthase controls both intracellular ADP glucose levels and transitory starch biosynthesis in source leaves. Plant and Cell Physiology **46:** 1366-1376

Müntz K (1996) Proteases and proteolytic cleavage of storage proteins in developing and germinating dicotyledonous seeds. Journal of Experimental Botany **47:** 605-622

Nakamura S, Lynch TJ, Finkelstein RR (2001) Physical interactions between ABA response loci of Arabidopsis. The Plant Journal **26:** 627-635

Nambara E, Keith K, McCourt P, Naito S (1994) Isolation of an internal deletion mutant of the Arabidopsis thaliana ABI3 gene. Plant and cell physiology **35:** 509-513

Nambara E, Naito S, McCourt P (1992) A mutant of Arabidopsis which is defective in seed development and storage protein accumulation is a new abi3 allele. The Plant Journal **2:** 435-441

Neuhaus HE, Stitt M (1990) Control analysis of photosynthate partitioning. Planta **182:** 445-454

Offler C, Nerlich S, Patrick J (1989) Pathway of photosynthate transfer in the developing seed of Vicia faba L. Transfer in relation to seed anatomy. Journal of Experimental Botany **40:** 769-780

Offler CE, McCurdy DW, Patrick JW, Talbot MJ (2003) Transfer cells: cells specialized for a special purpose. Annual review of plant biology **54:** 431-454

Ohlrogge J, Browse J (1995) Lipid biosynthesis. The Plant Cell **7:** 957

Okita TW, Nakata PA, Anderson JM, Sowokinos J, Morell M, Preiss J (1990) The subunit structure of potato tuber ADPglucose pyrophosphorylase. Plant physiology **93:** 785-790

Osborne TB (1924) The vegetable proteins.

Ozga JA, Reinecke DM (1999) Interaction of 4-chloroindole-3-acetic acid and gibberellins in early pea fruit development. Plant growth regulation **27:** 33-38

Parcy F, Valon C, Kohara A, Miséra S, Giraudat J (1997) The ABSCISIC ACID-INSENSITIVE3, FUSCA3, and LEAFY COTYLEDON1 loci act in concert to control multiple aspects of Arabidopsis seed development. The Plant Cell **9:** 1265-1277

Paul MJ, Primavesi LF, Jhurreea D, Zhang Y (2008) Trehalose metabolism and signaling. Annu. Rev. Plant Biol. **59:** 417-441

Preiss J (1988) Biosynthesis of starch and its regulation. The biochemistry of plants **14:** 181-254

Prevost I, Le Page-Degivry MT (1985) Inverse correlation between ABA content and germinability throughout the maturation and the in vitro culture of the embryo of Phaseolus vulgaris. Journal of experimental botany **36:** 1457-1464

Radchuk R, Radchuk V, Weschke W, Borisjuk L, Weber H (2006) Repressing the expression of the SUCROSE NONFERMENTING-1-RELATED PROTEIN KINASE gene in pea embryo causes pleiotropic defects of maturation similar to an abscisic acid-insensitive phenotype. Plant Physiology **140:** 263-278

Ramon M, De Smet I, Vandesteene L, Naudts M, Leyman B, Van Dijck P, Rolland F, Beeckman T, Thevelein JM (2009) Extensive expression regulation and lack of heterologous enzymatic activity of the Class II trehalose metabolism proteins from Arabidopsis thaliana. Plant, cell & environment **32:** 1015-1032

Reinecke DM, Ozga JA, Magnus V (1995) Effect of halogen substitution of indole-3-acetic acid on biological activity in pea fruit. Phytochemistry **40:** 1361-1366

Riou-Khamlichi C, Menges M, Healy JS, Murray JA (2000) Sugar control of the plant cell cycle: differential regulation of Arabidopsis D-type cyclin gene expression. Molecular and Cellular Biology **20:** 4513-4521

Rodrigues A, Adamo M, Crozet P, Margalha L, Confraria A, Martinho C, Elias A, Rabissi A, Lumbreras V, González-Guzmán M (2013) ABI1 and PP2CA phosphatases are negative regulators of Snf1-related protein kinase1 signaling in Arabidopsis. The Plant Cell **25:** 3871-3884

Rojas A, Almoguera C, Jordano J (1999) Transcriptional activation of a heat shock gene promoter in sunflower embryos: synergism between ABI3 and heat shock factors. The Plant Journal **20:** 601-610

Rolletschek H, Hajirezaei M-R, Wobus U, Weber H (2002) Antisense-inhibition of ADP-glucose pyrophosphorylase in Vicia narbonensis seeds increases soluble sugars and leads to higher water and nitrogen uptake. Planta **214:** 954-964

Rolletschek H, Hosein F, Miranda M, Heim U, Götz K-P, Schlereth A, Borisjuk L, Saalbach I, Wobus U, Weber H (2005) Ectopic expression of an amino acid transporter (VfAAP1) in seeds of Vicia narbonensis and pea increases storage proteins. Plant Physiology **137:** 1236-1249

Romero C, Bellés JM, Vayá JL, Serrano R, Culiáñez-Macià FA (1997) Expression of the yeast trehalose-6-phosphate synthase gene in transgenic tobacco plants: pleiotropic phenotypes include drought tolerance. Planta **201:** 293-297

Ross HA, Davies HV (1992) Purification and characterization of sucrose synthase from the cotyledons of Vicia faba L. Plant physiology **100:** 1008-1013

Sairanen I, Novák O, Pěnčík A, Ikeda Y, Jones B, Sandberg G, Ljung K (2012) Soluble carbohydrates regulate auxin biosynthesis via PIF proteins in Arabidopsis. The Plant Cell **24:** 4907-4916

Sanders A, Collier R, Trethewy A, Gould G, Sieker R, Tegeder M (2009) AAP1 regulates import of amino acids into developing Arabidopsis embryos. The Plant Journal **59:** 540-552

Satoh-Nagasawa N, Nagasawa N, Malcomber S, Sakai H, Jackson D (2006) A trehalose metabolic enzyme controls inflorescence architecture in maize. Nature **441:** 227-230

Schluepmann H, Pellny T, Dijken A, Smeeekens S, Paul M (2003) Trehalose 6-phosphate is indispensable for carbohydrate utilization and growth in Arabidopsis thaliana. PNAS **100**

Schwender J, Ohlrogge JB, Shachar-Hill Y (2003) A flux model of glycolysis and the oxidative pentosephosphate pathway in developing Brassica napus embryos. Journal of Biological Chemistry **278:** 29442-29453

Shannon JC, Pien F-M, Cao H, Liu K-C (1998) Brittle-1, an adenylate translocator, facilitates transfer of extraplastidial synthesized ADP-glucose into amyloplasts of maize endosperms. Plant Physiology **117:** 1235-1252

Shewry PR, Napier JA, Tatham AS (1995) Seed storage proteins: structures and biosynthesis. The plant cell **7:** 945

Skylar A, Sung F, Hong F, Chory J, Wu X (2011) Metabolic sugar signal promotes Arabidopsis meristematic proliferation via G2. Developmental biology **351:** 82-89

Smith AM, Bettey M, Bedford ID (1989) Evidence that the rb locus alters the starch content of developing pea embryos through an effect on ADP glucose pyrophosphorylase. Plant Physiology **89:** 1279-1284

Smith AM, Zeeman SC, Smith SM (2005) Starch degradation. Annu. Rev. Plant Biol. **56:** 73-98

Smýkal P, Vernoud V, Blair MW, Soukup A, Thompson RD (2015) The role of the testa during development and in establishment of dormancy of the legume seed. Advances in Seed Biology**:** 206

Sowokinos JR (1981) Pyrophosphorylases in Solanum tuberosum II. Catalytic properties and regulation of ADP-glucose and UDP-glucose pyrophosphorylase activities in potatoes. Plant Physiology **68:** 924-929

Sowokinos JR, Preiss J (1982) Pyrophosphorylases in Solanum tuberosum III. Purification, physical, and catalytic properties of ADPglucose pyrophosphorylase in potatoes. Plant Physiology **69:** 1459-1466

Steber CM, McCourt P (2001) A role for brassinosteroids in germination in Arabidopsis. Plant Physiol **125:** 763-769

Suzuki M, McCarty DR (2008) Functional symmetry of the B3 network controlling seed development. Current opinion in plant biology **11:** 548-553

Suzuki M, Wang HH-Y, McCarty DR (2007) Repression of the LEAFY COTYLEDON 1/B3 regulatory network in plant embryo development by VP1/ABSCISIC ACID INSENSITIVE 3-LIKE B3 genes. Plant Physiology **143:** 902-911

Tegeder M, Rentsch D (2010) Uptake and partitioning of amino acids and peptides. Molecular Plant **3:** 997-1011

Thormählen I, Meitzel T, Groysman J, Öchsner AB, von Roepenack-Lahaye E, Naranjo B, Cejudo FJ, Geigenberger P (2015) Thioredoxin f1 and NADPH-dependent thioredoxin reductase C have overlapping functions in regulating photosynthetic metabolism and plant growth in response to varying light conditions. Plant physiology **169:** 1766-1786

Tiessen A, Hendriks JH, Stitt M, Branscheid A, Gibon Y, Farré EM, Geigenberger P (2002) Starch synthesis in potato tubers is regulated by post-translational redox modification of ADP-glucose pyrophosphorylase a novel regulatory mechanism linking starch synthesis to the sucrose supply. The Plant Cell **14:** 2191-2213

Tivendale ND, Davidson SE, Davies NW, Smith JA, Dalmais M, Bendahmane AI, Quittenden LJ, Sutton L, Bala RK, Le Signor C (2012) Biosynthesis of the halogenated auxin, 4-chloroindole-3-acetic acid. Plant physiology **159:** 1055-1063

Torti G, Manzocchi L, Salamini F (1986) Free and bound indole-acetic acid is low in the endosperm of the maize mutantdefective endosperm-B18. Theoretical and applied genetics **72:** 602-605

Tsai AY-L, Gazzarrini S (2014) Trehalose-6-phosphate and SnRK1 kinases in plant development and signaling: the emerging picture. Frontiers in Plant Science **5**

Tsai AYL, Gazzarrini S (2012) AKIN10 and FUSCA3 interact to control lateral organ development and phase transitions in Arabidopsis. The Plant Journal **69:** 809-821

Tyson R, Ap Rees T (1988) Starch synthesis by isolated amyloplasts from wheat endosperm. Planta **175:** 33-38

van Huizen R, Ozga JA, Reinecke DM, Twitchin B, Mander LN (1995) Seed and 4-chloroindole-3-acetic acid regulation of gibberellin metabolism in pea pericarp. Plant physiology **109:** 1213-1217

Vandercammen A, François J, HERS HG (1989) Characterization of trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase of Saccharomyces cerevisiae. European Journal of Biochemistry **182:** 613-620

Vandesteene L, López-Galvis L, Vanneste K, Feil R, Maere S, Lammens W, Rolland F, Lunn JE, Avonce N, Beeckman T (2012) Expansive evolution of the trehalose-6-phosphate phosphatase gene family in Arabidopsis. Plant physiology **160:** 884-896

Vandesteene L, Ramon M, Le Roy K, Van Dijck P, Rolland F (2010) A single active trehalose-6-P synthase (TPS) and a family of putative regulatory TPS-like proteins in Arabidopsis. Molecular plant **3:** 406-419

Vogel G, Aeschbacher JM, Boller T, Wiemken A (1998) Trehalose-6-phosphate phosphatases from Arabidopsis thaliana: identification by functional complementation of the yeast tps2 mutant. Plant J **13**

Vollbrecht E, Springer PS, Goh L, Buckler IV ES, Martienssen R (2005) Architecture of floral branch systems in maize and related grasses. Nature **436:** 1119-1126

Wahl V, Ponnu J, Schlereth A, Arrivault S, Langenecker T, Franke A, Feil R, Lunn JE, Stitt M, Schmid M (2013) Regulation of flowering by trehalose-6-phosphate signaling in Arabidopsis thaliana. Science **339:** 704-707

Walton DC (1980) Biochemistry and physiology of abscisic acid. Annual Review of Plant Physiology **31:** 453-489

Wang F, Sanz A, Brenner ML, Smith A (1993) Sucrose synthase, starch accumulation, and tomato fruit sink strength. Plant Physiology **101:** 321-327

Wang L, Ruan Y-L (2013) Regulation of cell division and expansion by sugar and auxin signaling. Frontiers in plant science **4:** 163

Wang TL, A. H, Harwood A, Welham TJ, Fiarwood WA, Faulks R, Fiedley CL (1990) An analysis of seed development in *Pisum sativum*. XIII. The chemical induction of storage product mutants. Plant Breeding **105:** 311—320

Weber H, Borisjuk L, Heim U, Buchner P, Wobus U (1995) Seed coat-associated invertases of fava bean control both unloading and storage functions: cloning of cDNAs and cell type-specific expression. The Plant Cell **7:** 1835-1846

Weber H, Borisjuk L, Heim U, Sauer N, Wobus U (1997) A role for sugar transporters during seed development: molecular characterization of a hexose and a sucrose carrier in fava bean seeds. The Plant Cell **9:** 895-908

Weber H, Borisjuk L, Wobus U (1996) Controlling seed development and seed size in Vicia faba: a role for seed coat-associated invertases and carbohydrate state. The Plant Journal **10:** 823-834

Weber H, Borisjuk L, Wobus U (2005) Molecular physiology of legume seed development. Annu. Rev. Plant Biol. **56:** 253-279

Weber H, Buchner P, Borisjuk L, Wobus U (1996) Sucrose metabolism during cotyledon development of Vicia faba L. is controlled by the concerted action of both sucrose-phosphate synthase and sucrose synthase: expression patterns, metabolic regulation and implications for seed development. The Plant Journal **9:** 841-850

Weber H, Heim U, Golombek S, Borisjuk L, Manteuffel R, Wobus U (1998) Expression of a yeast-derived invertase in developing cotyledons ofVicia narbonensisalters the carbohydrate state and affects storage functions. The Plant Journal **16:** 163-172

Weber H, Heim U, Golombek S, Borisjuk L, Wobus U (1998) Assimilate uptake and the regulation of seed development. Seed Science Research **8:** 331-346

Weigelt K, Kuster H, Rutten T, Fait A, Fernie AR, Miersch O, Wasternack C, Emery RJ, Desel C, Hosein F, Muller M, Saalbach I, Weber H (2009) ADP-glucose pyrophosphorylase-deficient pea embryos reveal specific transcriptional and metabolic changes of carbon-nitrogen metabolism and stress responses. Plant Physiol **149:** 395-411

Weschke W, Panitz R, Sauer N, Wang Q, Neubohn B, Weber H, Wobus U (2000) Sucrose transport into barley seeds: molecular characterization of two transporters and implications for seed development and starch accumulation. The Plant Journal **21:** 455-467

White CN, Proebsting WM, Hedden P, Rivin CJ (2000) Gibberellins and seed development in maize. I. Evidence that gibberellin/abscisic acid balance governs germination versus maturation pathways. Plant Physiology **122:** 1081-1088

Wingler A, Fritzius T, Wiemken A, Boller T, Aeschbacher RA (2000) Trehalose induces the ADP-glucose pyrophosphorylase gene, ApL3, and starch synthesis in Arabidopsis. Plant Physiology **124:** 105-114

Xue-Fei D, Na C, Li W, Xiao-Cui Z, Bo Q, Tian-Lai L, Guo-Liang Z (2012) The SnRK Protein Kinase Family and the Function of SnRK1 Protein Kinase. International Journal of Agriculture & Biology **14**

Yadav UP, Ivakov A, Feil R, Duan GY, Walther D, Giavalisco P, Piques M, Carillo P, Hubberten H-M, Stitt M (2014) The sucrose–trehalose 6-phosphate (Tre6P) nexus: specificity and mechanisms of sucrose signalling by Tre6P. Journal of experimental botany**:** ert457

Zang B, Li H, Li W, Deng XW, Wang X (2011) Analysis of trehalose-6-phosphate synthase (TPS) gene family suggests the formation of TPS complexes in rice. Plant molecular biology **76:** 507-522

Zhang Y, Primavesi LF, Jhurreea D, Andralojc PJ, Mitchell RA, Powers SJ, Schluepmann H, Delatte T, Wingler A, Paul MJ (2009) Inhibition of SNF1-related protein kinase1 activity and regulation of metabolic pathways by trehalose-6-phosphate. Plant Physiology **149:** 1860-1871

Zrenner R, Salanoubat M, Willmitzer L, Sonnewald U (1995) Evidence of the crucial role of sucrose synthase for sink strength using transgenic potato plants (Solanum tuberosum L.). The Plant Journal **7:** 97-107

Appendix

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Education and research

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10/2001-03/2008 Diploma in Biology at the Martin-Luther-University of Halle-Wittenberg. Diploma thesis: „Die Rolle von P_{II} und der cytosolischen $NADP^+$-abhängigen Isocitrat-Dehydrogenase in der Aminosäurebiosynthese während der Samenentwicklung von *Pisum sativum* L."
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"*Molecular and functional analysis of trehalose metabolism during pea seed development.*"
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"*Molecular genetics of heterosis of the plant's earliest stage, the embryo, using the large seeded faba bean as model.*"
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Supervision

07.06.2011 Dirk Stephani. Diploma thesis: „*Protein-Protein-Interaktionsstudien an pflanzlichen Trehalose-6-Phosphatsynthasen und –Phosphatasen*", Naturwissenschaftliche Fakultät I, Pflanzenphysiologie, Universität Halle

11.06.2013 Marco Zietz. Bachelor thesis: „*Exploring the functions of X8 domain-containing proteins using co-expression and protein-protein interaction studies*", Naturwissenschaftliche Fakultät I, Pflanzenphysiologie, Universität Halle

Research experience abroad

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List of publications

Rajaraman J., Douchkov D., Lück S., Hensel G., Nowara D., Pogoda M., Rutten T., **Meitzel T.**, Brassac J., Höfle C., Hückelhoven R., Klinkenberg J., Trujillo M., Bauer E., Schmutzer T., Himmelbach A., Mascher M., Lazzari B., Stein N., Kumlehn J., & Schweizer P., (2018) Evolutionarily conserved partial gene duplication in the Triticeae tribe of grasses confers pathogen resistance. *Genome Biology 19:116*.

McAdam E. L.*, **Meitzel T**.*, Quittenden L. J., Davidson S. E., Dalmais M., Bendahmane A. I., Thompson R., Smith J. J., Nichols D. S., Urquhart S., Gelinas-Marion A., Aubert G., & Ross J. J., (2017) Evidence that auxin is required for normal seed size and starch synthesis in pea. *New Phytologist*, *216*, 193–204
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Thormählen I., **Meitzel T.**, Groysman J., Öchsner A. B., von Roepenack-Lahaye E., Naranjo B., .Cejudo F. J., & Geigenberger P., (2015). Thioredoxin f1 and NADPH-dependent thioredoxin reductase C have overlapping functions in regulating photosynthetic metabolism and plant growth in response to varying light conditions. *Plant physiology*, *169*, 1766-1786.

Meitzel T., Radchuk R., Nunes-Nesi A., Fernie A. R., Link W., Weschke W., & Weber H., (2011). Hybrid embryos of Vicia faba develop enhanced sink strength, which is established during early development. *The Plant Journal*, *65*, 517-531.

Riebeseel E., Häusler R.E., Radchuk R., **Meitzel T.**, Hajirezaei M.R., Emery R., Küster H., Nunes-Nesi A., Fernie A.R., & Weschke W., (2010) The 2-oxoglutarate/malate translocator mediates amino acid and storage protein biosynthesis in pea embryos. *The Plant Journal, 61*, 350-363

Conference contributions

Oral presentations

Meitzel T., "*Activation of auxin biosynthesis by trehalose 6-phosphate is required for normal seed filling in pea (Pisum sativum)"* International Plant Molecular Biology (IPMB) 2018 Congress, Montpellier, France, August 05 – 10, 2018

Meitzel T., "*Activation of auxin biosynthesis by trehalose 6-phosphate is required for normal seed filling in pea (Pisum sativum)"* Plant Biology Europe (PBE) 2018 Congress, Copenhagen, Denmark, June 18 – 21, 2018

Poster

Meitzel T., Radchuk, R.., Weschke, W., Weber, H. (2012) "*Seed-specific modulation of trehalose 6-phosphate levels in pea*", Plant Science Student Conference 2012, IPK Gatersleben, Germany.

Ross, J., **Meitzel T.**, Thompson, T., Dalmais, M., Bendahmane, A., Quittenden, L., McAdam, E., Cook, S., Smith, J. (2016) "*A role for auxin during the later stages of legume seed development*" 22nd International Conference on Plant Growth Substances, Victoria University in the University of Toronto, Toronto, Ontario, Canada June 21 – 25, 2016.

Meitzel T., Radchuk, R., Ross, J., McAdam, E., Weber, H. (2016) "*A trehalose 6-phosphate-auxin crosstalk regulates embryo maturation in pea*", Plant Biology Europe EPSO/FESPB 2016 Congress, Prague, Czech Republic, June 21 – 25, 2016

Meitzel T., Radchuk, R., Ross, J., McAdam, E., Weber, H. (2017) "*Trehalose 6-phosphate-auxin crosstalk regulates embryo maturation in pea*", Plant Biotech Denmark Annual meeting, University of Copenhagen, Copenhagen, Denmark, February 1 – 2, 2017

Eigenständigkeitserklärung

Hiermit erkläre ich, dass die Arbeit mit dem Titel „**Signaling Pathways in Legume Seed Development: Evidence for a Crosstalk between Trehalose 6-Phosphate and Auxin**“ bisher weder bei der Naturwissenschaftlichen Fakultät I Biowissenschaften der Martin-Luther-Universität Halle-Wittenberg noch einer anderen wissenschaftlichen Einrichtung zum Zweck der Promotion vorgelegt wurde. Darüber hinaus erkläre ich, dass ich die vorliegende Arbeit eigenständig und ohne fremde Hilfe verfasst sowie keine anderen als die im Text angegebenen Quellen und Hilfsmittel verwendet habe. Textstellen, welche aus verwendeten Werken wörtlich oder inhaltlich übernommen wurden, wurden von mir als solche kenntlich gemacht. Ich erkläre weiterhin, dass ich mich bisher noch nie um einen Doktorgrad beworben habe.

Halle (Saale), 08.05.2017

Tobias Meitzel