Characterization of potato plants overexpressing plastidic transporters involved in starch metabolism

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CONTENTS

1. INTRODUCTION

1. 1. History of the cultivated potato

The cultivated potato is one of the most important world food crops. Potato was first cultivated in the Andes Mountains of Peru and Bolivia. Spanish conquerors introduced potato in Europe as early as 1565 (Thornton, 1980) from Northern Colombia. In 17th and 18th centuries, potato population was increased in other parts of Europe (Bradshaw and Mackeny, 1994) and introduced to China, India, Japan and parts of Africa. Potato plants being originally adapted to short day conditions of tropical highlands were restricted to cooler climates. After a few centuries of natural and unconscious selection, potato plants were able to produce tubers under long day photoperiodic conditions and became a popular crop in the world.

1. 2. Taxonomy and characteristics of potato

Cultivated potato (*Solanum tuberosum L.*) belongs to the family *Solanaceae* and genus *Solanum* (Correll, 1962). This genus consists of about 2,000 species. Potato is one of as many as 235 tuber-bearing *Solanum* species recognized to date, of which 228 are wild and seven are cultivated (Hawkes, 1990). Potato is an annual herbaceous plant. It has a series of ploidy levels, based on a haploid number of 12 chromosomes (Dodds, 1962). The cultivated potatoes are autotetraploid (4n=48) (Ross, 1986). Potato plants undergo several developmental stages during their life cycle involving stolon formation, tuberisation, tuber filling, dormancy and tuber sprouting (Ewing and Struik, 1992). According to the classical hypothesis (Gregory, 1956), a not defined substance is synthesized in leaves under short day conditions and transported to the stolons, where it triggers tuber formation. During tuberisation, the tuber is highly metabolically active (Ewing and Struik, 1992), and two major biochemical changes occur: accumulation of starch and formation of storage proteins (Appeldoorn et al., 1997; Li, 1985). The most abundant of these proteins is a 40kD glycoprotein called patatin, which is a lipid acyl hydrolase (Racusen, 1983; Rosahl et al., 1987; Andrew et al., 1988; Jefferson et al., 1990). Potato tubers serve as sink organs where assimilates accumulate. The size of tubers differs with age and cultivar. Commercial potato cultivars are all reproduced vegetatively as clones. This necessarily means that once a culture is produced, it is genetically stable in perpetuity (Shepard et al., 1980).

1. 3. Importance of potato

Potato is a source of food in almost every nation of the world. The reason is, first, it ranks fourth in terms of total world crops after wheat, maize and rice (FAO production yearbook, 1999), and it is grown in many countries (Kruger, 1997; Maldonado et al., 1998). Second, potato is an especially rich source of energy. There are high-quality proteins (2% of fresh weight) and substantial amounts of essential vitamins, minerals and trace element in potato tubers. However, the most important value of potato tubers is their high starch content (Tarn et al., 1992). Starch can account for 80% of the dry weight of mature potato tubers (Kruger, 1997). Moreover, potato can be used in a wide variety of processed, livestock feed and industrial applications. Besides the great economic impact, research on potato has many advantages: it is easily transformable and therefore amenable to genetic manipulation, and it can be rapidly propagated both in tissue culture and through cuttings (Jackson, 1999). Overall, potato tubers are the agronomic product of potato cultivation while starch is the major component of the potato tubers. High starch yield is an important target in potato breeding.

1.4. Metabolic pathway relevant to starch synthesis

Starch is the major reserve carbohydrate of higher plants and is found as a water-insoluble granule. Granules usually contain two D-glucose homopolymers: amylose and amylopectin. Amylose contains 600-3000 1,4-α-glucosyl residues with a 1,6-α-glucosyl branch every 1000 residues and makes up about 30% of starch (Kruger, 1997; Martin and Smith, 1995; Hovenkamp-Hermelink et al., 1988). This proportion may vary considerably with the plant species and variety (Detherage et al., 1955) and also with the plant organ, the developmental age of that organ, and the growth condition of the plant (Shannon and Garwood, 1984). Amylopectin contains 6000-60000 glucosyl residues with an average of one 1,6-α-glucosyl linkage every 20 to 26 units and makes up about 70% of starch (Kruger, 1997; Martin and Smith, 1995). In nearly all types of plants, starch occurs in two forms, the transitory starch in chloroplasts of leaves (Mares et al., 1978; Levi and Preiss, 1976; Robinson and Preiss, 1987; Preiss, 1991), and the storage starch in amyloplasts of specialized heterotrophic tissues (MacDonald et al., 1983; Mohabir et al., 1988; Ziegler and Beck, 1989).

Starch biosynthesis starts from photosynthetic carbon fixation. Photosynthetically

fixed carbon can be metabolized in different ways.

First, within the chloroplast, CO_2 and H_2O are combined with ribulose-1,5-bisphosphate to form 3-phosphoglycerate (3-PGA) in a reaction catalyzed by Rubisco (Ribulose-1,5-bisphosphate carboxylase/oxygenase). 3-PGA is further converted to 1,3-bisphosphoglycerate through use of ATP in a reaction catalyzed by 3-phosphoglycerate kinase. 1,3-bisphosphoglycerate is reduced to glyceraldehyde-3-phosphate (GAP) through use of NADPH in a reaction catalyzed by NADP:glyceraldehyde-3-phosphate dehydrogenase. GAP is converted via triose phosphate isomerase to dihydroxyacetone-3-phosphate (DHAP) in an isomerization reaction. DHAP undergoes aldol condensation with GAP in a reaction catalyzed by aldolase to give fructose-1,6-bisphosphate (Fru1,6P_2). Fructose-6-phosphate (Fru6P) is formed from Fru1,6P_2 in a reaction catalyzed by fructose-1,6-bisphosphatase. Fru6P is further converted to glucose-6-phosphate (Glc6P) by glucose phosphate isomerase (PGI). Glucose-1-phosphate (Glc1P) is formed from Glc6P in a reaction catalyzed by phosphoglucomutase (PGM). ADP-glucose pyrophosphorylase (AGPase; Preiss 1988) catalyzes the conversion of Glc1P and ATP to ADP-glucose (ADPG) and inorganic pyrophosphate (PPi). The glucose moiety of ADPG is then transferred to starch by starch synthases. Alternatively, photosynthetically fixed carbon is exported as triose phosphate (trioseP) or 3-PGA into the cytoplasm in exchange with inorganic phosphate via the triose phosphate/phosphate translocator (TPT). Fru1,6P_2 is also formed from trioseP, and then Fru6P, Glc6P and Glc1P are produced by the same sequence of reactions in the cytoplasm as in the chloroplast. UDP-glucose (UDPG) is formed from Glc1P and UTP in a reaction catalyzed by UDP-glucose pyrophosphorylase (UGPase) (Zrenner et al., 1993). It donates the glucose moiety on Fru6P to yield sucrose-6-phosphate in a reaction catalyzed by sucrose-phosphate synthase (SPS). Finally, sucrose-6-phosphate is hydrolyzed to sucrose by sucrose-6-phosphate phosphatase. Sucrose moves from the photosynthesizing cells in the mesophyll to the vicinity of the sieve elements in the smallest veins of the leaf mainly through the symplast via the plasmodesmata or through the apoplast (Frommer and Sonnewald, 1995) for short distance transport. Sucrose is transported into the sieve elements and companion cells for sieve element loading. The uptake of sucrose into companion cells is mediated by an H^+-sucrose co-transporter (SUT) (Hitz et al., 1986; Lemoine et al., 1988; Hecht et al, 1992) from the apoplast against a concentration gradient into the conduction complex. The symport of sucrose and protons is driven

by a plasma membrane H^+-ATPase activity which maintains an electrochemical proton gradient across the membrane. Finally, sucrose being inside the sieve element is translocated through long-distance transport from source to sink organs by a pressure flow mechanism and is unloaded in storage tissues from the phloem either via symplasmic connections or via the apoplast (Oparka et al., 1992). In potato tubers, it is mainly used to synthesize starch.

Amyloplasts are plastids present in storage cells that accumulate starch (MacDonald et al., 1983; Mohabir et al., 1988). In potato tubers, starch biosynthesis takes place in amyloplasts. Since it is unable to generate hexose phosphates from C3-compounds owing to the absence of Fru1,6P_2ase activity (Entwistle et al., 1990; Flügge, 1999), it relies on the import of cytosolically generated hexose phosphates that are formed from sucrose delivered from source tissues. Translocated sucrose arriving at potato tubers is hydrolyzed to glucose and fructose by an invertase (Hawker et al., 1965; Russell et al., 1982), or it is converted by sucrose synthase (SuSy; Zrenner et al., 1995) to fructose and UDPG. Glucose and fructose are phosphorylated in developing tubers to Glc6P and Fru6P, respectively (Renz et al., 1993), and UDPG is converted to Glc1P via a pyrophosphate (PPi) dependent reaction catalyzed by UGPase. After interconversion of hexose-phosphates, Glc6P enters the amyloplast by the glucose 6-phosphate/phosphate translocator (Hill et al., 1991; Schott et al., 1995; Naeem et al., 1997; Kammerer et al., 1998; Wischmann et al., 1999). Since ATP can't be generated in amyloplasts, uptake of ATP is required as a driving force for starch synthesis. ATP is synthesized in mitochondria, is subsequently transported into the cytosol, and from there into amyloplasts via an ATP/ADP translocator (Heldt, 1969; Schuenemann et al., 1993; Kampfenkel et al., 1995; Tjaden et al., 1998a, b; Geigenberger et al., 2001). Within amyloplasts, Glc1P is formed from Glc6P. ADPG is synthesized from Glc1P and ATP by the AGPase reaction. The final polymerizing steps are catalyzed by different classes of starch synthases, soluble, or granule-bound isoforms, which incorporate glucose moieties of ADPG into the elongating glucan chains of the granule (Martin and Smith, 1995; Smith et al., 1997). A lot of starch is accumulated in mature potato tubers (Figure 1.1).

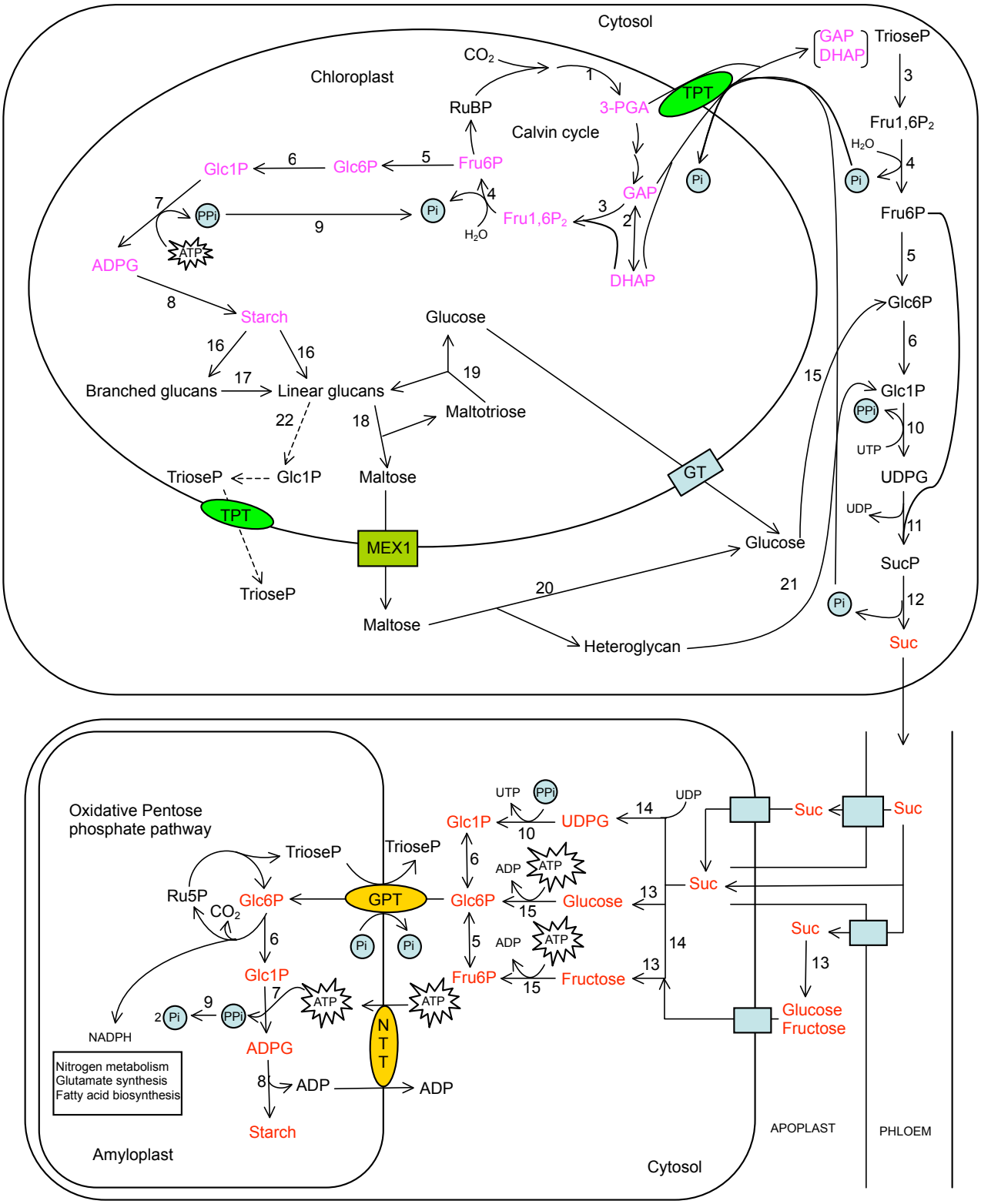


Figure 1.1: Pathway of starch synthesis and degradation in plants

Preiss, 1991; Flügge, 1999; Taiz & Zeiger, 2002; Smith et al., 2005

TrioseP, Triose phosphate; 3-PGA, 3-phosphoglycerate; TPT, Triose phosphate/phosphate translocator; GAP, Glyceraldehyde 3-phosphate; DHAP, Dihydroxyacetone phosphate; RuBP, ribulose 1,5-bisphosphate; Fru1,6P_2, Fructose1,6-bisphosphate; Fru6P, Fructose 6-phosphate; Glc6P, Glucose 6-phosphate; Glc1P, Glucose 1-phosphate; ADPG, ADP-Glucose; ATP, Adenosine triphosphate; ADP, Adenosine diphosphate; UTP, Uridine triphosphate; UDP, Uridine diphosphate; UDPG, UDP-Glucose; SucP, Sucrose 6-phosphate; Suc, Sucrose; GPT, Glucose 6-phosphate/phosphate translocator; NTT, ATP/ADP transporter; GT, Glucose translocator; MEX1, Maltose translocator; SUT, Sucrose translocator; Pi, Inorganic phosphate; PPi, Inorganic pyrophosphate; GWD, Glucan, water dikinase; PWD, Phosphoglucan, water dikinase; Ru5P, Ribulose 5-phosphate

Enzymes:

1, Rubiso; 2, Triose phosphate isomerase; 3, Aldolase; 4, Fructose1,6-bisphosphatase;
5, phosphoglucoisomerate; 6, Phosphoglucomutase; 7, ADP-Glucose pyrophosphorylase; 8, Starch synthase and starch branching enzyme; 9, Pyrophosphatase; 10, UDP-Glucose pyrophosphorylase; 11, Sucrose phosphate synthase; 12, Sucrose phosphate phosphatase; 13, Invertase; 14, Sucrose synthase; 15, Hexokinase; 16, Glucan water dekinase; Phosphoglucan water dekinase; 17, Debranching enzyme (ISA3); 18, ß-amylase; 19, Disproportionating enzyme (DPE1); 20, Cytosolic transglucosidase (DPE2); 21, Cytosolic glucan phosphorylase ?; 22, plastidic glucan phosphorylase (PHS1)

1.5. Starch degradation

Starch is degraded during the dark in leaves and during tuber sprouting, respectively. The pathway of starch degradation is not as clear as the starch synthesis pathway in all organs of plants. However, some groups identified it in *Arabidopsis* leaves at night recently (Figure 1.1) (Mikkelsen et al., 2004; Niittylae et al., 2004; Koetting et al., 2005; Smith et al., 2005). In chloroplasts at night, glucan release from starch granules remains unknown since α-amylases are not required in this process (Yu et al., 2005; Smith et al., 2005). The glucan water dikinase and the phosphoglucan water dikinase add phosphate groups to amylopectin (Ritte et al., 2002; Mikkelsen et al., 2004; Baunsgaard et al., 2005; Koetting et al., 2005). Linear glucans are formed from branched glucans in a reaction catalyzed by debranching enzyme (ISA3). The degradation of linear glucans in the Arabidopsis chloroplast usually proceeds via β-amylase rather than glucan phosphorylase (Zeeman et al., 2004). Maltose is derived from linear glucans in a reaction catalyzed by β-amylase, and subsequently is exported to the cytosol by a maltose transporter (MEX1). Maltose is the main starch breakdown product (Weise et al., 2004). Plastidic starch phosphorylase is not involved in transitory starch breakdown (Zeeman et al., 2004; Weber et al., 2005). In addition,

glucose is formed from maltotriose in a reaction catalyzed by the disproportionating enzyme (DPE1), and then it is transported to the cytosol by the glucose transporter (GT). In the cytosol, exported maltose is further metabolized via a transglucosylation reaction to produce glucose and a glucosylated acceptor molecule. Both glucose residues derived from cytosolic maltose and glucose exported from chloroplasts are finally fed into the hexose phosphate pool, and then go to sucrose and cellular metabolism. This pathway of starch degradation in *Arabidopsis* leaves was suggested to be the same or similar in leaves of other plants and nonphotosynthetic tissues in which starch is stored transiently, but not to be possible for any other plant organ, e.g. some starch-storing organs in which starch is a major, long-term reserve compound (Smith et al., 2005). In sprouting potato tubers, starch degradation occurs in amyloplasts, but the pathway of degradation of soluble glucan derived from starch remains unclear (Duwenig et al., 1997; Lloyd et al., 2004; Takaha et al., 1998).

1.6. Transgenic plants

1.6.1. Genetic modification (GM) technology

Because of the continuously increasing need for food and raw materials due to a growing world population and long-term reduction of land suitable for growing crops, it is increasingly becoming a task for biological research to increase yield of crops and their food content. Prior to genetic engineering, most of the genetic variation is created through crossing (Huffman, 2004). The advent of genetic modification (GM) technology in the early 1970s allowed further development of the genetic variation (Royal Society of London, 2000). GM technology can generate more useful and productive crop varieties containing new combinations of genes. A feature of GM technology is that it involves the introduction of one or, at most, a few well-defined genes rather than the introduction of whole genomes or parts of chromosomes as in traditional plant breeding. Additionally, GM technology can introduce genes from diverse organisms. Desirable genes may provide features such as higher yield or improved quality by modification of metabolic pathways. On the other hand, together with suppression of endogenous gene expression, transgenic plants can be used to study regulation of gene expression (Gatehouse, 1997). Without molecular tools such as transgenic plants, little of the current awareness of gene function would have been obtained (Bowles and Klee, 2001).

1.6.2. Methods of transformation

Stable transformation is the heritable change in a cell or organism brought about by the uptake and establishment of introduced DNA. There are three main methods of transforming plant cells and tissues: the Agrobacterium-mediated transfer (Hooykas and Schilperoot, 1992; Zambryski, 1988; Hinchee et al., 1993), direct gene uptake through protoplasts (PEG-mediated, electroporation) (Negrurtiu et al., 1987; Fromm et al., 1985) and uptake of naked DNA into intact tissue (particle gun) (Gordon-Kamm et al., 1990). Agrobacterium-mediated gene transfer is well established in solanaceous species for obtaining transgenic plants and is now in use in laboratories throughout the world. However, Agrobacterium does not infect all host plants with equal efficiency, and will not infect monocots at all (Avigad and Dey, 1997). Genetic engineering of cereal crops is thus not possible by this route at present, although it is possible to force the bacterium to infect some monocot tissues by chemical treatment. In order to distinguish between the transformed and untransformed tissues efficiently, e.g. antibiotic resistance genes have been used as selective markers in the process of genetic modification. Kanamycin (Toepfer et al., 1988) is one of the most commonly used selection markers for plant transformation.

1.6.3. Transgenic potato plants

Potatoes were one of the first crop plants in which transgenic plants were successfully regenerated (An et al., 1986; Shanin and Simpson, 1986). Potato transformation has since become well developed, and now offers a real alternative approach for cultivar improvement. The advantage of this approach is that it theoretically allows the incorporation of single genes into otherwise elite clones to achieve cultivar improvement (Conner and Christey, 1994).

Agrobacterium-mediated transformation using binary vectors is the preferred transformation method for potato (Sheerman and Bevan, 1988; Stiekema et al., 1998; Wenzler et al., 1989; Conner et al., 1991). In virtually all transgenic potato plants developed to date, kanamycin resistance has been commonly used as marker system for selection of transformed cells and their subsequent regeneration into complete plants. In addition, other selectable marker systems like hygromycin (Becker, 1990) and methotrexate (Jacobs et al., 1994) have been successfully used for potato transformation.

1.7. Promoters

Promoters have been cloned and widely used for both biotechnological application and basic research in plants. Promoters determine the spatio-temporal expression as well as the strength of expression.

1.7.1. Cauliflower mosaic virus 35S (CaMV 35S) promoter

The CaMV 35S promoter was obtained from the virus that causes cauliflower mosaic disease in several vegetables (Odell et al., 1985). This promoter is upregulated by cell division, and thus is expressed at some time in a life of all cells. Since the CaMV 35S promoter was the first promoter showing a strong expression in almost all plant tissues, that is not greatly influenced by environmental conditions and developmental state, it became almost universal to constitutively express foreign genes in genetically engineered plants (Kuipers et al., 1997). However, this "constitutive" and strong promoter also has several drawbacks. The gene of interest is also expressed in tissues and at times when it is not necessary or even unwanted.

1.7.2. Patatin promoter B33

Since the CaMV 35S promoter has some drawbacks, a second generation of promoters became available in the beginning of the 1990's. These promoters were somewhat better adapted to particular requirements (Matsuoka and Sanada, 1991; Jefferson et al., 1990; Chen and Beachy, 1987). Several potato tuber promoters were isolated such as patatin (Wenzler et al., 1989: Koester-Toepfer et al., 1989), starch phosphorylase (St-Pierre and Brisson, 1995; von Borcke and Kruger, 1999), ADP-glucose pyrophosphorylase (Mueller-Roeber et al., 1994), granule bound starch synthase (GBSS) (Dai et al., 1996; Rohde et al., 1990; Visser et al., 1991) and sucrose synthase (Fu and Park, 1995). In genetically engineered potato plants, the patatin promoter was most often chosen to drive gene expression.

Patatin is a family of glycoproteins with an apparent molecular weight of 40 kD from potato (*Solanum tubersoum* L.) tubers. It is present in all cultivars so far examined and accounts for 30-40% of the total soluble protein (Rosahl et al., 1986; Paiva et al., 1983; Racusen, 1983; Racusen et al., 1980). Patatin appears to serve as a storage protein, and it also has esterase activity (Rosahl et al., 1987). Based on structural considerations two classes of patatin genes are distinguished by the presence (class II) or absence (class I) of a 22-bp sequence within the 5'-untranslated region (Mignery et

al., 1988). Class I accounts for 98-99% of the patatin mRNA in tubers (Mignery et al., 1988), but normally is not expressed in leaves, roots or stems (Paiva et al., 1983; Rosahl et al., 1986). However, it can be induced to express at high levels in these organs (when they were detached) by high exogenous concentrations of sucrose (Racusen, 1983; Paiva et al., 1983; Wenzler et al., 1989; Rocha-Sosa et al., 1989; Kim et al., 1994; Müller-Röber et al., 1992). The B33 class I gene is highly expressed in vascular tissue at early stages of tuber development and in both vascular and parenchyma tissue during later stages of development (Liu et al., 1991; Sonnewald et al., 1989; Kuehn et al., 2003) The class I patatin gene contains promoter repeat sequences, which are critical for developmental and metabolic regulation (Fu et al., 2001). The B33 class I gene carries a strong tuber-specific promoter (Rocha-Sosa et al., 1989; Liu et al., 1990; Jefferson et al., 1990; Kim, et al., 1994). Therefore, class I patatin promoter B33 is considered to be a tuber-specific, sucrose-inducible promoter.

1.7.3. Bidirectionalization of polar promoters

It is often necessary to introduce multiple genes into plants for metabolic engineering and trait stacking (Ye et al., 2000). In order to minimize or avoid repeated use of a single promoter that may cause transcriptional gene silencing (De Wilde et al., 2000), a polar plant promoter was first bidirectionalized in *Arabidopsis*. To get a bidirectional plant promoter, a unidirectional promoter may be fused with either a homogeneous or heterogeneous minimal promoter at its 5' end in opposite orientation. One bidirectionalized promoter can direct the expression of two genes, one on each end of the promoter (Xie et al., 2001).

1.8. Plastidic translocators

Plastids are plant-specific organelles, which are able to perform many specialized functions that are essential for plant growth and development. All plastids of higher plants are double membrane organelles. Three types of membrane transporters enhance the movement of solutes across membranes: channels, carriers and pumps. In plastids, the outer membrane contains different channel-like proteins that act as metabolite-regulated selectivity filters (Boelter and Soll, 2001). The inner envelope membrane is the actual permeability barrier between the plastid and the surrounding cytosol and is the site of numerous metabolite translocators that coordinate the metabolism in both compartments (Flügge, 1999). These metabolite translocators are

carriers. Metabolite exchange between plastids and the surrounding cytosol has been identified to be achieved by four known subfamilies of plastidic phosphate translocators and different other nonphosphate transporters, e.g. the adenylate translocator. The controlled exchange of metabolites between plastids and the cytosol depends on the activity of transport proteins (Weber, 2004).

1.8.1. Phosphate translocators

Plastidic phosphate translocators exchange various phosphorylated C3-, C5- and C6-carbon compounds with inorganic phosphate between plastids and cytosol. All phosphate translocators belong to the TPT / nucleotide sugar transporter (NST) superfamily. Functionally known plastidic phosphate translocators can be grouped into four classes: the triose phosphate/phosphate translocator (TPT) (Flügge et al., 1989), the phosphoenolpyruvate/phosphate translocator (PPT) (Fischer et al., 1994, 1997), the glucose 6-phosphate/phosphate translocator (GPT) (Kammerer et al., 1998) and the xylulose 5-phosphate/phosphate translocator (XPT) (Eicks et al., 2002). These four distinct subfamilies have been identified and characterized until now. All of them are antiporters and they share inorganic phosphate as common substrate, but have different spectra of counter exchange substrates to fulfill the metabolic needs of individual cells and tissues (Flügge et al., 2003). The TPT functions predominantly in photosynthetic tissues (Flügge et al., 1989), GPT in roots and reproductive organs (Kammerer et al., 1998), whereas PPT appears to be ubiquitously expressed (Fischer et al., 1997). Moreover, XPT's possible function is to provide the plastids' pentose phosphate pathways with cytosolic carbon skeletons in the form of Xul-5-P (Eicks et al., 2002). However, GPT and PPT are main and important transporters in potato tubers.

1.8.1.1. Glucose 6-phosphate/phosphate translocator (GPT)

The glucose 6-phosphate/phosphate translocator was purified from maize endosperm (Flügge, 1995; Kammerer et al., 1998). The corresponding cDNA was isolated from maize endosperm, pea roots, potato tubers and other heterotrophic tissues. GPT proteins of different higher plants share a high degree of identity with each other. GPT expression is mainly restricted to heterotrophic tissues. In potato plants, only tubers had high levels of GPT mRNA (Kammerer et al., 1998). The GPT can accept inorganic phosphate, triose phosphate, 3-phosphoglycerate (3-PGA), and glucose 6-

phosphate as counter-exchange substrates (Kammerer et al., 1998). Other hexose phosphates, such as Glc1P and Fru6P, are not transported by the GPT (Kammerer et al., 1998; Schott et al., 1995; Flügge et al., 2003). The main physiological function of the GPT is the import of Glc6P into plastids of heterotrophic tissue. Inside the plastids, Glc6P can serve two different functions. First, Glc6P is a precursor for starch biosynthesis during which inorganic phosphate is released. Second, it is a substrate for the oxidative pentose phosphate pathway (OPPP) yielding triose phosphate (Kammerer et al., 1998; Flügge, 1998, 2003; Weber, 2004). The GPT is considered to be important in controlling the supply of carbon precursors for starch synthesis.

1.8.2. Plastidic ATP/ADP translocator (NTT)

Non-green plastids depend on the supply of energy for the synthesis of starch and fatty acids (Hill and Smith, 1991; Kang and Rawsthorne, 1994; Kleppinger-Sparace et al., 1992; Moehlmann et al., 1994). ATP represents the universal energy currency of all living cells. In heterotrophic cells, the bulk of ATP is synthesized in mitochondria, subsequently transported into the cytosol, and from there into various organelles. Due to both size and charge, adenylates do not cross biomembranes freely (Neuhaus and Emes, 2000). ATP import into all types of plastids is mediated by a specific ATP/ADP translocator – the plastidic nucleotide transporter (NTT) (Heldt, 1969; Neuhaus et al., 1993a; Schuenemann et al., 1993; Linka et al., 2003). NTT was first found in the inner envelope membrane of spinach chloroplasts (Heldt, 1969). However, NTTs exist in all higher and lower plants analyzed so far (Linka et al., 2003). All orthologs of the NTT, e.g. the two isoforms of *Arabidopsis* or a potato ortholog, exhibit similar transport properties with respect to substrate specificity and substrate affinity (Moehlmann et al., 1998; Tjaden et al., 1998a, 1998b; Linka et al., 2003). It has been identified that NTTs represent a second type of eukaryotic adenylate transporters, which is not related to the adenylate translocator from mitochondria (Klingenberg, 1989; Schuenemann et al., 1993; Neuhaus et al., 1997). Generally, mitochondrial adenylate carriers (AACs) serve to supply the cytosol with ATP synthesized at the cristae site of the mitochondria through oxidative phosphorylation in strict counter exchange with cytosolic ADP (Vignais, 1976; Klingenberg, 1989). By contrast, the NTT catalyzes the uptake of ATP in exchange for organellar ADP. It is also an antiporter. ATP uptake into isolated plastids occurs in a counter-exchange mode (Heldt, 1969; Schuenemann et al., 1993; Neuhaus et al., 1997; Tjaden et al., 1998b).

Therefore, the main function of NTT is the supply of storage plastids with ATP (Schuenemann et al., 1993; Kang and Rawsthorne, 1994; Neuhaus and Emes, 2000). In potato plants, the highest accumulation of NTT mRNA is found in tubers. NTT may have a high flux control coefficient within the starch metabolic pathway (Tjaden et al., 1998a; Geigenberger et al., 2001).

1.9. Outline of the thesis

Potato is one of the most important crops in the world. Starch is the major component of potato tubers (John, 1992). Carbon and energy are essential substrates for starch synthesis in amyloplasts of potato tubers. Transporters play an important role in plant cellular metabolism. GPT transports carbon from the cytosol to amyloplasts, while NTT transports energy to amyloplasts. The aim of this project is to increase starch content and yield of potato tubers. To achieve this goal, pea GPT and AtNTT genes would be overexpressed in potato plants individually or together using either the CaMV 35S promoter, the patatin promoter B33 or a bidirectionalized B33 promoter.

Tjaden et al. (1998) created plants that overexpressed an *Arabidopsis* NTT in potato under control of the CaMV 35S promoter. The CaMV 35S promoter is a powerful promoter that strongly mediates gene expression in almost all plant tissues. The CaMV 35S promoter was used to overexpress pea GPT in different potato plants.

The patatin promoter B33 is a tuber-specific, sucrose-inducible promoter. It strongly mediates gene expression in tubers, and normally no expression in other organs. However, it can be induced to express in other detached organs by high exogenous concentrations of sucrose. Patatin promoter B33 was chosen to overexpress pea GPT and AtNTT in different potato plants.

In order to avoid possible transcriptional gene silencing when multiple genes were introduced to plants simultaneously, plasmids with a bidirectionalized patatin promoter B33 were constructed. One contained GUS and GFP reporter genes; another one contained pea GPT and AtNTT genes. Transgenic plants harboring these plasmids were analyzed on molecular level.

All transgenic plants were grown in a greenhouse and analyzed for their (I) tuber yield and (II) starch content of the tubers. Furthermore, a detailed analysis including soluble sugars, phosphorylated intermediates, transport activities, $^{14}CO_2$ feeding and photosynthesis measurements in leaves and/or tubers was performed.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals and Enzymes

Restriction enzymes were purchased from MBI Fermentas (St. Leon-Rot, Germany), Promega (Mannheim, Germany), Invitrogen (Karlsruhe, Germany); Taq-polymerase were purchased from Qiagen GmbH (Hilden, Germany), Takara (Gennevilliers, Frankreich) and Promega and used with the 10x buffer supplied, unless otherwise stated. Laboratory reagents were obtained from Roche (Mannheim, Germany), Fluka (Buchs, CH), Merck (Darmstadt, Germany) and Sigma (Muechen, Germany), unless otherwise stated. dNTP were from Pharmacia. Filter papers were obtained from Whatwan. Radioisotopes [α-^{32}P]-dATP (10 µCi/µl), [^{32}P]-orthophosphate (10 µCi/µl) and $^{14}CO_2$ (56 mCi/mmol) were purchased from Amersham Buchler and ICN (Braunschweig, Germany). Reagents for tissue culture were purchase from Duchefa (Haarlem, Netherlands). Biochemical enzymes were obtained from Roche and Sigma.

2.1.2. Commercial Kits

QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany)
MiniElute Gel Extraction Kit (50) (Qiagen GmbH, Hilden, Germany)
QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany)
MinElute PCR Purification Kit (Qiagen GmbH, Hilden, Germany)
QIAgen plasmid Mini, Midi and Maxi. Kit (Qiagen GmbH, Hilden, Germany)
Quantum Prep Plasmid miniprep Kit (BioRad, München, Germany)
Big Dye Terminator v1.1 cycle Sequencing Kit (Applied Biosystems, Foster City, USA)

2.1.3. Plant material

Solanum tuberosum cv. Desirée
ATP/ADP transporter overexpressors (JT62; Tjaden et al. 1998)

2.1.4. Bacterial strains and cloning vectors

E.coli

DH5α

supE44 ΔlacU169 (ϕ80, lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 rel A1

(Hanahan, 1983)

Agrobacterium tumefaciens

C58C1 (GV2260)

Cloning vectors

TOPO TA cloning vector (Invitrogen) for cloning and sequencing

PGEM-T easy (Promega) for cloning and sequencing

pBluescriptII (SK^-) (Stratagene) for plasmid amplification

B33-pBin19 (Flügge group) for plant transformation

Microscope

Nikon Eclipse E800

Filter GFP (R)-BP EX 460-500; DM 505; BA 510-560

2.1.5. Primers

AtNTT:

primer NTT1 AAG GAT CCA TGG AAG CTG TGA TTC AAA CC

primer NTT2 AAG TCG ACT TAT AAG TTG GTG GGA GCA GAT TTC

35S minimal promoter:

primer 1 AAG AGC TCT CGC AAG ACC CTT CCT CTA TAT AAG G

primer 2 AAA CTA GTA GGC CTC GTG TCC TCT CCA AAT GAA ATG

CaMV terminator:

primer 3: AAA CTA GTA CCG GTT AAT TCG GGG GAT CTG GAT TTT AG

primer 4: AAG AAT TCG ATG TGT CGA TCG ACA AGC TCG

“35S minimal promoter + CaMV terminator” cassette

primer 5: AAG AAT TCT CGC AAG ACC CTT CCT CTA TAT AAG G

primer 6 (4): AAG AAT TCG ATG TGT CGA TCG ACA AGC TCG

GUS in construct A:

primer 7: AAG GAT CCA TGT TAC GTC CTG TAG AAA CCC C

primer 8: AAG TCG ACT CAC ACG TGG TGG TGG TGG

GFP in construct A:

primer 9: AAA CTA GTA TGG TAG ATC TGA CTA GTA AAG G

primer 10: AAA CCG GTT CAC ACG TGG TGG TGG TGG

GUS in construct B:

primer 11: AAA CTA GTA TGT TAC GTC CTG TAG AAA CCC C

primer 12: AAA CCG GTT CAC ACG TGG TGG TGG TGG

GFP in construct B:

primer 13: AAG GAT CCA TGG TAG ATC TGA CTA GTA AAG G

primer 14: AAG TCG ACT CAC ACG TGG TGG TGG TGG

600 bp GUS:

primer 11: AAA CTA GTA TGT TAC GTC CTG TAG AAA CCC C

primer 11’: AAA TTG GCC ACC ACC TGC CAG TCA AAC

pea GPT:

primer 15: AAA CTA GTA TGA TTT CCT CGT TGA GAC AAC C

primer 16: AAA CCG GTC TAT TGT TTT GCC TGT GAG TAT AAG

2.1.6. Media

Luria-Bertani (LB)

10 g/l	Tryptone
5 g/l	Yeast extract
5 - 10 g/l	NaCl
15 g/l	Agar for plating

Add distilled water and adjust pH to 7.0 with NaOH, bring to 1 liter with distilled water. Autoclave was at 120°C for 20 min. For plating, 15 g agar was added before autoclaving. After autoclaving, cool to 50°C, and then pour the plates either directly without adding antibiotic, or alternatively, with ampicillin added to a final concentration of 100 µg/ml or kanamycin added to final concentration of 25 µg/ml.

White and blue selection medium (1 liter)

1000 ml LB with 15 g agar

2 ml	50 mg/ml Amplicillin
2 ml	20 mg/ml X-gal
40 µl	200 mg/ml IPTG

Make the LB plating medium as above, supplement with ampicillin, X-gal and IPTG when the medium is cooled to 50°C, and then pour the plates.

YEB

5,0 g/l	Beef extract
1,0 g/l	Yeast extract
5,0 g/l	Peptone (Bacto)
5,0 g/l	Sucrose
0,5 g/l	$MgSO_4*7H_2O$
15 g/l	Bacto agar for plating

Add distilled water to 1 liter. Autoclave was at 120°C for 20 min. For plating, 15 g agar was added before autoclaving. After autoclaving, cool to 50°C, and then pour the

plates. In the case of potato, carbenicillin was added to final concentration of 100 µg/ml (dissolved in 1 M Tris-HCl pH 8,0), rifampicin added to final concentration of 150 µg/ml and kanamycin added to final concentration of 100 µg/ml before pouring the plates.

2.1.7. Buffers and Solutions

10 x stop (loading) buffer

20% (w/v)	Ficoll 400
100 mM	EDTA
1,0% (w/v)	SDS
0,05% (w/v)	Bromphenolblue
0,05% (w/v)	Xyleneblue
40 mM	Tris-HCl pH 7,5

50 x TAE buffer

2 M	Tris-HCl pH 7,5
50 mM	EDTA

Ethidiumbromide solution (EtBr)

5% (w/v)	Ethidiumbromide in methanol

1 x TE

10 mM	Tris-HCl pH 8,0
1 mM	EDTA

0,1% (v/v) DEPC water

DEPC in water, left overnight with stirring at room temperature, and then autoclaved.

P : C : I

phenol : chloroform : isoamyl alcohol 25 : 24 : 1

C : I

chloroform : isoamyl alcohol 24:1

Stock solutions

Name	Stock concentration (mg/ml)	Dissolve	Store
Amplicillin	50	H_2O	-20°C
Kanamycin	25	H_2O	-20°C
Rifampicin	30	DMSO	RT
HygromycinB	410 (commercial)	Hepes	4°C
Cefotaxime	-	H_2O	4°C
Carbenicillin	50	1 M Tris pH 8	-20°C
NAA	1	1 M NaOH	4°C
BAP	1	H_2O	4°C
Zeatin	1	1 M NaOH	-20°C
GA3	1	H_2O	-20°C

2.2. Methods

2.2.1. DNA extraction from agarose gel

PCR fragments, which were used for cloning or labeling as probes, were gel-extracted using QIAquick Gel Extract Kit. The extraction procedures followed the supplier's protocol. The concentration of extracted DNA was estimated as in section 2.2.8.

2.2.2. Plasmid DNA extraction from *E.coli*

Solution I

25 mM Tris-HCl pH 8,0

10 mM EDTA

Solution II

0,2 M NaOH

1% (w/v) SDS

Solution III

3 M KOAc pH 4,8

Plasmid DNA minipreparations were based on the alkaline lysis method (Birnboim and Doly, 1979) with some modifications.

5 ml sterile LB medium was inoculated with a single bacterial colony and incubated at 37°C with shaking overnight. 2 ml of a saturated culture was transferred to a 2 ml eppendorf tube and the cells were precipitated at 13000 rpm for 5 min in a microcentrifuge (Eppendorf 5417). The supernatant was discarded. The pellet was resuspended in 100 µl solution I and incubated at room temperature for 5 min. 200 µl solution II was added and incubated at room temperature for another 5 min (denature bacterial protein, chromosomal and plasmid DNA). 150 µl solution III was added and incubated on ice for 10 min to neutralize the mixture. The mixture was centrifuged at 13000 rpm for 10 min (chromosomal DNA, bacterial proteins and SDS were in pellet, plasmid DNA in supernatant). The supernatant was treated with following procedures: 400 µl P:C:I was added and spun down at 13000 rpm for 5 min; 400 µl C:I was added and spun down at 13000 rpm for 5 min; 1ml absolute EtOH was added and spun down at 13000 rpm for 30 min. The pellet was washed with 70% EtOH two times, dried at room temperature for 10 min and dissolved in 40 µl bidest-H_2O.

In order to eliminate the contamination of RNA, 5 µl RNaseA boiled (10 mg/ml) was added to the minipreparation product and incubated at 37°C for 30 min. The mixture was purified with P:C:I and C:I and precipitated by absolute EtOH. The pellet was washed by 70% EtOH and dissolved in bidest-H_2O. This plasmid can be used for digestion and sequencing.

Qiagen midi- and maxi-plasmid Kit were used to prepare plasmid of low copy DNA for sequencing and plant transformation. The processes followed the supplier's protocols.

2.2.3. Nucleic acid manipulation

2.2.3.1. A-tailing of PCR products

The pGEM-T easy and TOPO TA cloning vectors need an A-tailed PCR product for ligation. Since Taq polymerase has the function of adding deoxyadenosine at the end, it was not necessary to add the A-tailing in PCR product. Otherwise, A-tailing was

done in 40 µl volume as follows before ligation:

1 µg purified PCR product
4 µl (2 mM) dATP
4 µl 10 x buffer
1 µl (1 U/µl) Taq

The mixture was incubated at 70°C for 30 min. To precipitate the product, 4 µl 3 M NaOAc (pH 5,2), 100 µl 100% EtOH was added and left at –20°C for 20 min. The mixture was centrifuged at 13000 rpm for 30 min at room temperature and washed with 70% EtOH two times, and then dissolved in 10 µl H_2O.

2.2.3.2. Dephosphorylation

The removal of the 5'-terminal phosphates from the dsDNA ends prevents vector self-ligation and improves ligation results. After the vector was digested with restriction enzyme in 15 µl volume at 37°C for 30 min, the mixture was heated to 65° for 15 min to stop the reaction. 2,5 µl 10 x SAP buffer and 1 µl SAP (1 U/µl) was added to 25 µl volume and incubated at 37°C for 10 min for dephosphorylated reaction. SAP was completely and irreversibly inactivated by heating at 65°C for 15 min. 2,5 volumes EtOH and 1/10 volume 3 M NaOAc was added to precipitate the pellet. The pellet was dissolved in 50 µl bidest-H_2O.

2.2.3.3. Ligation

A molar ratio 3:1 of insert : vector was adopted to calculate the amount of PCR product for the ligation reaction according to the following formula:

PCR product (ng) = 3 x (50 - 200 ng vector) x (size of PCR product in kb) / size of vector (kb).

Ligation was done in 20 µl as follows:

1 µl vector (concentration optimized)
1 µl fragment (concentration optimized)
4 µl 10 x ligation buffer
1 µl (3 U/µl) T4 DNA ligase

The mixture was incubated at 12°C overnight before transformation.

2.2.4. Transformation

2.2.4.1. Transformation of *E.coli*

2.2.4.1.1. Preparation of TSS-competent host cells

The methods were according to the methods of Chung et al. (1993) with some modifications.

TSS buffer

3 g	PEG 8000
1,2 ml	1 M $MgCl_2$
1,5 ml	DMSO
30 ml	LB
pH 6,5 - 6,8	

PEG and $MgCl_2$ were diluted in 25 ml LB and pH was adjusted to 6,5 - 6,8. The volume was filled to 28,5 ml with LB. The solution was sterilized by filter and then DMSO was added to the solution.

A glycerol stock of *E.coli* strain DH5α was streaked on LB medium and incubated at 37°C overnight. A single colony was picked and cultured overnight in 5 ml of LB liquid medium followed by subculture of 600 µl in 100 ml LB. The culture was shaken at 37°C overnight. When optical density (OD) was in the range of 0,3 - 0,4, the culture was transferred to two clean and cold tubes and centrifuged at 4°C, 2500 g for 5 min. The cells were resuspended in 3 - 5 ml cold TSS buffer. The cell suspension was divided into 100 µl –aliquot and frozen in liquid nitrogen and stored at –80°C.

2.2.4.1.2. Transformation

For each transformation, 100 µl competent cells of *E.coli* strain DH5α, thawed on ice, were mixed with 1 - 5 µl of ligation mixture and incubated on ice for 30 min. The cells were incubated at 42°C for 90 sec and subsequently put back on ice for another 1 - 2 min. 900 µl LB was added and incubated at 37°C for 1 h. The culture was spun down at 14000 rpm for 1 min and 800 µl supernatant was discarded. 100 - 200 µl bacteria were plated on LB medium containing the appropriate antibiotic and were

incubated at 37°C for 14 - 20 h.

2.2.4.1.3. Screening

Single colonies were picked out with a sterile tip and dipped in a PCR master mix to amplify the target insert of the plasmid using appropriate primer sets as in section 2.2.9.1. In parallel, the single colony was also streaked on a LB plate. Once the correct PCR product was obtained, the corresponding colony was propagated for plasmid DNA isolation and sequence.

2.2.4.2. Transformation of *A. tumefaciens*

2.2.4.2.1. Preparation of electro-competent cells

MGL buffer

5 g	Bacto peptone
2,5 g	Yeast extract
5 g	NaCl
5 g	Mannitol
1,16 g	Na-glutamate
0,25 g	KH_2PO_4
0,1 g	$MgSO_4*7H_2O$
1 mg	Biotin

add distilled water to 1 liter, and then autoclave.

Agrobacteria were cultured in 5 ml MGL buffer containing rifampicin (150 µg/ml) and carbenicillin (100 µg/ml) for 1 - 2 days at 28°C with 200 rpm shaking until saturation and then were diluted with 100 ml MGL buffer to an OD_{600}=0,04 - 0,08. When bacteria were cultured to an OD_{600}=0,5 (ca 4 h), cells were centrifuged at 5000 rpm, 4°C for 5 min (Rotor GS, Sorball RC5B) and resuspended in 40 ml 1 mM Hepes (pH 7,0). Following centrifugation at 4°C, 5000 rpm for 5 min, cells were resuspended in 40 ml 1 mM Hepes / 10% Glycerin (v/v). After another centrifugation, cells were resuspended in 2 ml 1 mM Hepes / 10% Glycerin (v/v). The suspension was divided into two eppendorf tubes and centrifuged for 30 sec. The sediment was

resuspended in 200 µl Hepes / 10% Glycerin (v/v) of each eppendorf tube. The cell suspension was divided into 50 µl –aliquot and frozen in liquid nitrogen and stored at –80°C.

2.2.4.2.2. Transformation

50 µl competent cells of *A.tumefaciens* strain GV 2260, thawed on ice, were mixed with 100 - 200 ng (about 2 µl) plasmid DNA and incubated on ice for 2 min. The mixture was transferred to a 0,2 cm electroporation cuvette (pre-cooled on ice) avoiding bubbles. Electroporation was done at 25 µF, 400 Ω, 2,5 kV on an electroporator (Bio-Rad). 1 ml YEB was added immediately and removed to a 2 ml Eppendorf tube and incubated at 28°C for 2 h with shaking. The culture was spun down at 14000 rpm for 1 min and 800 µl supernatant was discarded. 100 - 200 µl bacteria were plated on YEB medium containing the appropriate antibiotic and were incubated at 28°C for 2 days.

Single colonies were checked by colony PCR as in sections 2.2.4.1.3 and 2.2.9.1. A positive colony was cultured on a new YEB plate at 28°C for another 2 days for plant transformation.

2.2.4.3. Transformation and regeneration of transgenic potato plants

3MS medium (1 liter)

4,6 g	MS244
30,0 g	Sucrose
3,0 g	Gelrite
pH 5,6 - 5,8	

2MS solid medium (1 liter)

4,6 g	MS244
20,0 g	Sucrose
3,0 g	Gelrite
pH 5,6 - 5,8	

Add distilled water and adjust pH 5,6 - 5,8 with 2M NaOH, bring to 1 liter with

distilled water. Autoclave was at 120°C for 20 min. For plating, 3,0 g Gelrite was added before autoclaving. After autoclaving, cool to 50°C, and then pour the jars with or without antibiotic.

CG-76 solid medium (200 ml)

0,88 g	MS245
3,2 g	Glucose
0,6 g	Gelrite
pH 5,6 - 5,8	
1 ml	1 mg/ml NAA
20 µl	1 mg/ml BAP
100 mg	Cefotaxime

CG-75 solid medium (1 liter)

4,4 g	MS245
16,0 g	Glucose
3,0 g	Gelrite
pH 5,6 - 5,8	
20 µl	1,0 mg/ml NAA
2 ml	1,0 mg/ml Zeatin
20 µl	1,0 mg/ml GA3
500 mg	Cefotaxime

Add distilled water and adjust pH to 5,6 - 5,8 with 2 M NaOH, bring to appropriate volume with distilled water. Add 3,0 g/l Gelrite and subsequently autoclave at 120°C for 20 min. For CG-76 medium, when medium was cooled to 50°C, pour the plates with NAA added to final concentration of 5 g/l, BAP added to final concentration of 0,1 g/l and cefotaxime added to final concentration of 500 mg/l. For CG-75 medium, pour the jars with NAA added to final concentration of 0,02 mg/l, Zeatin added to final concentration of 2,0 mg/l, GA3 added to final concentration of 0,02 mg/l and cefotaxime to final concentration of 500 mg/l.

A single colony of *A. tumefaciens* containing the construct to be transformed into the plants was drawn a line on a YEB plate and incubated at 28°C for two days. A lot of

Agrobacterium was transferred to an Eppendorf tube containing 1 ml 3MS liquid medium (Murashige and Skoog, 1962) and mixed well. The sterile plant material was grown on 2MS solid medium for 4 - 6 weeks. Potato leaves were cut and placed in 12 ml 3MS liquid medium. 1 ml bacterial culture was added and incubated with potato leaves in the dark for two days at room temperature. Leaves were then removed carefully from solution, and laid topside down on CG-76 solid medium with 7,5 mg/l hygromycin or 50 mg/l kanamycin and incubated in a tissue-chamber (22°C, 16 / 8 h of light / dark) for one week to select for growth of transgenic cells. Then leaves were transferred to CG-75 solid medium with 7,5 - 15 mg/l hygromycin or 50 mg/l kanamycin, left in the same tissue-chamber. Between 3 - 4 weeks following incubation, calli formed at the periphery of the leaves, and shoots subsequently appeared. Every three weeks the leaves were placed on fresh CG75 medium. Once shoots were 0,5 - 1,0 cm in size, they were removed and placed into jars (11cm x 10cm) containing 60 - 80 ml 3MS solid medium with 30 mg/l hygromycin or 100 mg/l kanamycin, left in the same tissue-chamber. When sufficient root formation had occurred, the plantlets were removed to pots and transferred to a greenhouse for further growth and analysis.

2.2.5. Plant growth conditions

Potato plants were grown in soil of RHP15 (Klasmann) and watered daily under a greenhouse conditions as follows: the temperature was not less than 20°C during the day, and about 17 – 18°C during the night. The additional light (100-200 µmol photons m^{-2} s^{-1}) was given from 5:00 am to 21:00 pm (16 h) every day, but just when the sunshine was not efficient.

2.2.6. Isolation of plant genomic DNA for PCR

Genomic DNA quick-extraction buffer

200 mM	Tris-HCl pH7.5
250 mM	NaCl
25 mM	EDTA
0,5% (w/v)	SDS

Autoclave

Two pieces of potato leaf discs (r=0,4 cm) were used to isolate genomic DNA after the plants had been transferred from tissue culture to a greenhouse for four weeks. The discs were macerated in a 1.5 ml Eppendorf tube at room temperature with a homogenizer, and then 400 µl quick-extraction buffer was added and the sample was vortexed for 5 sec. The mixture was incubated at room temperature for at least one hour until all samples had been extracted. 100 µl 3M KOAc was added to the extract and incubated at room temperature for 5 min and centrifuged for 2 min at 13000 rpm. 300 µl supernatant was transferred to a new Eppendorf tube containing 300 µl isopropanol and incubated at room temperature for at least 2 min. A centrifugation at 13000 rpm for 5 min followed. The pellet was dried at room temperature and dissolved in 50 µl 1xTE.

2.2.7. Isolation of plant total RNA

2.2.7.1. Isolation of total RNA from potato leaves

Total RNA extraction buffer (with distilled water)

8 M	Guanidinium-HCl pH 7.0
20 mM	EDTA
20 mM	MES
0,7% (v/v)	β-Mercaptoethanol, added directly before extraction

0,3 - 0,5 g potato leaves were ground with mortar and pestle in liquid nitrogen. 3 volumes (g/ml) extraction buffer was added to the fine powder. 700 µl mixture was transferred to an Eppendorf tube containing 700 µl P:C:I and centrifuged for 5 minutes at 12000 rpm at room temperature. The total RNA was precipitated by mixing the supernatant with 0,7 volume absolute ethanol, 1/20 volume 1M HAc. The pellet was washed with 3M NaOAc (pH 5.2) and 75% EtOH, finally the pellet was dissolved in DEPC-treated water and incubated at 65°C for 20 min to dissolve in water. After another centrifugation, the supernatant was taken to a new Eppendorf tube and stored at –20°C.

2.2.7.2. Isolation of total RNA from potato tubers

The process of isolating total RNA from potato tubers was modified according to the methods of isolating total RNA from potato leaves. There is much more starch in tubers than in leaves, so the fine powder with quick-extraction buffer should be centrifuged at 14000 rpm for a short time to discard the starch before transferring the supernatant to the P:C:I solution. RNA was dissolved in 500 µl DEPC-H_2O after precipitation and incubated at 65°C for 10 min. After that a centrifugation at 13000 rpm for 10 min was carried out. Supernatant was moved to a new tube containing 550 µl LiCl (5 M), mixed and left at 4°C overnight. The mixture was centrifuged at 13000 rpm for another 10 min at room temperature. The pellet was treated as in section 2.2.7.1.

2.2.8. Concentration measurement of DNA and RNA

The quality and quantity of DNA was measured by comparison of band-intensity on ethidium bromide stained agarose gels with a DNA molecular weight standard, or alternatively, by the absorbance at 260 nm (Pharmacia LKB Ultrospec III). The quality and quantity of RNA were measured by the absorbance at 260 nm.

2.2.9. PCR amplification analysis

2.2.9.1. PCR amplification of plasmid DNA

PCR amplification from plasmid or colony (*E.coli* and *Agrobacterium*) template was performed in 50 µl volume as follows:

10 ng plasmid DNA or a single colony
5 µl 10 x Taq buffer
2 µl (10 mM) dNTPs (dATP, dGTP, dCTP, dTTP)
1 µl (10 pM) each primer
1 µl (1 U/µl) Taq DNA polymerase

Amplification was carried out under the following conditions :

3 min at 94°C initial denaturation
5 sec at 94°C denaturation
30 sec at 55°C annealing
45 sec at 72°C extention

The last three stages were cycled 30 times, followed by a longer extention of 2 min at

72°C to ensure all reaction had been completed.

2.2.9.2. PCR amplification assays of transformed plants

Genomic DNA was taken (section 2.2.6) to perform the PCR reaction in 25 µl volume as follows:

0,5 µl genomic DNA
1 µl (10 pM) each primer
2,5 µl 10 x Ex Taq^{TM} buffer
2 µl (2,5 mM each) dNTPs
0,1 µl (1 U/µl) Taq (Takara)

Amplification was carried out under the same conditions as in section 2.2.9.1.

2.2.10. Reverse transcription PCR amplification analysis

2.2.10.1. Reverse transcription

The following reaction was set up in 50 µl volume

5 µg total RNA
10 µl 5 x reverse transcriptase buffer
2 µl (10 U/µl) DNase I

The mixture was incubated at 37°C for 30 min, 5 µl 25 mM EDTA was added and heated to 65°C for 10 min to stop the reaction.

The following reaction was set up in 50 µl volume:

12,5 µl samples of above reaction
7,5 µl 5 x reverse transcriptase buffer
2 µl (500 µg/µl) oligo dT(12-15)
10 µl (2,5 mM) dNTPs
5 µl (0,1 M) DTT
1 µl (300 U/µl) Reverse transcriptase (SUPERSCRIPT^{RT}, GIBCO, BRL)

The solution was incubated at 42°C for 1 h. The reaction was stopped by heating to 70°C for 15 min. The mixture was quick-chilled on ice. It can be used directly or stored at –20°C for further use.

2.2.10.2. PCR amplification

PCR amplification from cDNA first strand templates was performed in 25 µl volume:

2,5 µl cDNA (from last step)
2,5 µl (320 mM) NH_4^+
0,75 µl (50 mM) Mg^{2+}
0,75 µl (10 mM each) dNTPs
0,25 µl (1 U/µl) home Taq
0,75 µl (10 pM) each primers

Amplification was carried out under the same conditions as in section 2.2.9.1.

2.2.11. RNA Northern blot analysis

10 x running buffer (with distilled water, stored at 4°C)

2 M	MOPS
0,05 M	NaOAc
0,01 M	EDTA

Hybridization stock solution (A+B)

A, 1 M NaH_2PO_4, pH7,2 + 2 mM EDTA
B, 14% SDS
Autoclave

20 x SSC

3 M	NaCl
300 mM	sodium citrate

For RNA work, an electrophoresis tank was designated as being for RNA work only and kept free from RNase contamination. 40 µg total RNA was denatured in 7,5 µl 10 x running buffer, 9 µl formaldehyde, 30 µl formamide at 65°C for 10 min and then 2 µl 2 x stop buffer and 1 µl EtBr (1 mg/ml) were added for loading on gel. The denatured RNA samples were electrophoresed in a 1,5% (w/v) agarose gel containing 6% (v/v) formaldehyde, 1 x running buffer. RNA was visualized under UV light and a photograph was taken. The samples were then blotted onto a Hybond nylon

membrane (Amersham) as described by Brown and Mackey (current protocols in molecular biology, 1997). The RNA was fixed to the membrane by incubation at 80°C for 2 h, or alternatively, by irradiation at 120,000 µJoules cm^{-2} for 1 min using a UV cross-linker (Stratagene). Prehybridization and hybridization of filters were carried out in the solution containing 0,5 M NaH_2PO_4, pH 7,2; 1 mM EDTA, 7% (w/v) SDS. Prehybridization was at 68°C for at least 1 h, and hybridization was at 58°C overnight. Following hybridization, the filter were washed as follows:

1 x 5 min in (6 x SSC; 0.5% (w/v) SDS) at room temperature

1 x 5 min in (6 x SSC; 0,5% (w/v) SDS) at 58°C

2 x 5 min in (4 x SSC; 0,5% (w/v) SDS) at 58°C

2 x 5 min in (2 x SSC; 0,5% (w/v) SDS) at 58°C

The filters were sealed in plastic bags and exposed to a radiation sensitive plate at room temperature in a cassette.

2.2.12. Radioactive labeling of DNA probes

Solution I

1,25 M	Tris-HCl pH 8,2
125 mM	$MgCl_2$
2% (w/v)	β-Mercaptoethanol
0,5 M	dCTP, dGTP, dTTP each

Sloution II

2 M	Hepes/NaOH pH 6,6

Solution III

3,6 µg/µl	Hexadeoxynucleotides (dN_6)

Mix A solution (5 x labeling buffer)

100 solution I / 250 solution II / 100 solution III

The labeling was carried out in 50 µl reaction volume:

50 - 200 ng DNA fragment

10 µl mix A solution

x µl H_2O

The mixture was incubated at 95°C for 10 min and immediately chilled on ice for 5 min. The following was added:

2 µl (1 µg/µl) BSA

2 µl (2 U/µl) Klenow enzyme (Roche, Mannheim)

4 µl (10 µCi/µl) [α-^{32}P] dATP

Leave the labeling reaction at 37°C for at least 1 h. The probe was denatured again before it was used for hybridization.

To check the labeling efficiency, 1 µl of 50 µl of the above reaction solution was taken to a new eppendorf tube, 100 µl H_2O, 150 µl TCA (20% (w/v)) and 5 µl salmon sperm DNA (10 mg/ml) were added and incubated on ice for 5 - 10 min and centrifuged at 13000 rpm for 2 min. The supernatant was transferred to a new eppendorf tube and measured by a Geiger counter. Meanwhile, the pellet was also measured. The labeling efficiency can be calculated from the ratio of the supernatant to pellet.

The separation of labeled DNA-fragments from unincorporated nucleotides was carried out using a MicroSpin S-200 HR Column (Amersham Pharmacia Biotech, Buckinghamshire, UK). The process followed the supplier's protocol.

2.2.13. Extraction and measurement of potato tubers' starch, sugar and phosphorylated intermediates

2.2.13.1. Extraction and measurement of potato tubers' starch

Buffer A

60 µl	333 mM Hepes/NaOH pH 7.5
10 µl	16 mM NADP
10 µl	40 mM ATP
10 µl	200 mM $MgCl_2$

0,5 ml 80% EtOH was added to an Eppendorf tube containing thin potato tuber slices, which had been stored at –80°C. The tube was incubated at 75°C for 30 min. 1 ml 0,2 M KOH was added to potato slices after ethanol was discarded. The slices were disrupted and incubated at 95°C for 45 min. Appropriate volume of 1M HAc was then added to the slices to reach pH 5,2 - 5,5 (adjust pH before). About 8U amyloglucosidase and 90 U α-Amylase were used to digest starch at room temperature for 16 h and 37°C for another 3 h. Before measurement, the solution was spun down and Lugol's solution (I_2 / KI) was added to sediment to check the digestion.

The extract was diluted (1:10) with water, and 10 µl dilution was mixed with 90 µl buffer A and 100 µl H_2O for the measurement. The measurement was accomplished by a spectrafluorometer (TECAN). The absorption was detected at 340 nm, this is the wave length where NADPH in contrast to NADP has an absorption maximum. Hexokinase (HK) (0,15 U) and glucose-6-phosphate-dehydrogenase (G6PDH) (0,1 U) were applied together to start the reaction (Figure 2.1).

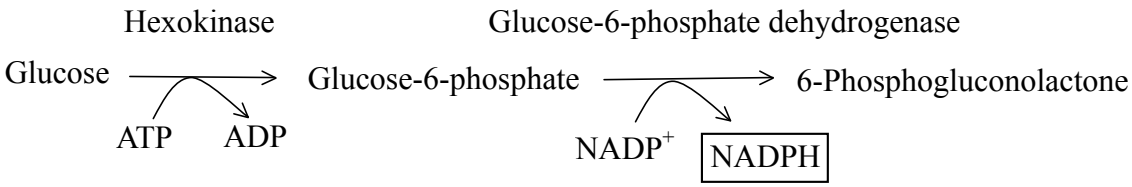


Figure 2.1: Enzyme reactions for starch measurements

The calculation of the NADPH concentration was according to Lambert-Beer's formula: $\Delta c=\Delta E\cdot\pi\cdot r^2/(V_p\cdot\varepsilon)$. $\varepsilon=6{,}22\ cm^{-2}\cdot\mu mol^{-1}$; V_p: the sample's volume for measurement; r: radius of the well in 96 well plate; ΔE: the absorption difference.

For 1 mol NADPH synthesized 1 mol glucose was in the solution, the starch content in the sample can then be given as µmol hexose unit per gram of the fresh weight.

2.2.13.2. Extraction and measurement of potato tubers' sugar

0,5 ml 80% EtOH was added to an Eppendorf tube containing a piece of potato tubers, which had been stored at –80°C. It was incubated at 75°C for 30 min, and then the solution was transferred to a new tube. Another 0,5 ml 80% EtOH was added to this piece of potato tubers and incubated at 75°C for 30 min again. The solution was collected to the first part. 20 µl extract was mixed with 90 µl buffer A and 90 µl H_2O.

This mixture was used to measure glucose, fructose and sucrose at 340 nm. The enzymes in the reaction were hexokinase (0,15 U), glucose-6-phosphate-dehydrogenase (0,1 U), phosphoglucose isomerase (PGI) (70 mU) and invertase (120 U). 6-phosphogluconolactone was formed from glucose by the reactions catalyzed by hexokinase and glucose-6-phosphate dehydrogenase. In this process, NADPH was produced from NADP, so the glucose concentration can be calculated as described above. Addition of PGI to the reaction led to NADPH production being the equivalent of the fructose amount. The terminal addition of invertase to the reaction yielded in another increase of NADPH that is the equivalent of the sucrose being in the reaction (Figure 2.2).

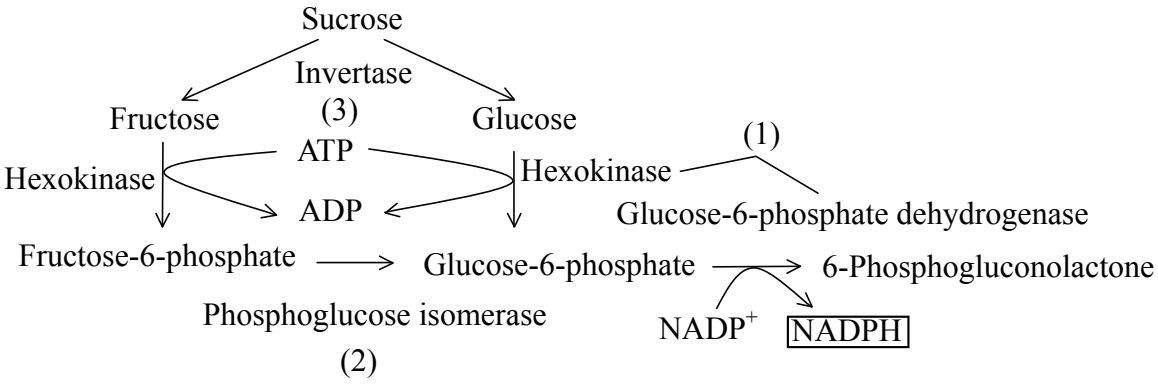


Figure 2.2: Enzyme reactions for sugar measurements

(1), (2), (3): the order of enzymes added

2.2.13.3. Extraction and measurement of potato leaves' sugar and starch

Potato leaves' sugar was extracted according to 2.2.13.2, and then the leaves were for starch extraction. The total solution was dried (in speedVac) for 3 - 4 hours. The sugars were dissolved in 200 µl distilled water. The starch of potato leaves was extracted according to 2.2.13.1 from the dried leaves. 4 U amyloglucosidase and 40 U α-Amylase were used to digest leaf starch since leaves contain less starch than tubers. The measurement of potato leaves' sugar and starch was performed according to sections 2.2.13.1 and 2.2.13.2.

2.2.13.4. Extraction and measurement of potato leaves' and tubers' phosphorylated intermediates

Buffer I

60 µl	333 mM Tris-HCl pH 8,1
10 µl	100 mM $MgCl_2$
10 µl	16 mM NADP
10 µl	200 µM Glc1,6 bP

Buffer II

60 µl	333 mM Hepes / NaOH pH7
10 µl	200 mM $MgCl_2$
10 µl	40 mM ATP
10 µl	600 µM NADH

Buffer III

60 µl	166 mM Hepes / NaOH pH 7,5
10 µl	20 mM ADP
10 µl	12 mM NAD
10 µl	40 mM NaH_2PO_4

Three pieces of potato leaves (r=0,4 cm) or about 100 mg tuber slices were stored at –80°C in an Eppendorf tube. They were ground for a short time by a homogenizer. 100 µl 1 M $HClO_4$ was added and ground well. 100 µl 0,1 M $HClO_4$ was added to the mixture and incubated on ice for 5 min. The mixture will be neutral after adding the right volume of 5 M K_2CO_3 (pH should be adjusted before extraction). It was incubated on ice for another 5 min, and then some active carbon was added to the extract and mixed well. The samples were put into liquid nitrogen immediately and stored at –80°C.

The spectrafluorometer (TECAN) was applied to measure the phosphorylated intermediates. The fluorescence was detected at 460 nm. The samples were taken out of –80°C and thawed at room temperature. They were centrifuged for 5 min at 13000 rpm. 40 µl or 50 µl (from leaves or tubers) supernatant was mixed with 90 µl buffer I and 70 µl or 60 µl H_2O for phosphorylated hexoses measurement. Glucose-6-

phosphate dehydrogenase (0,1 U) was added to the measured solution to produce 6-phosphogluconolactone and NADPH from Glc6P and NADP. Phosphoglucose isomerase (PGI) (70 mU) was added to the reaction to form NADPH being the equivalent of the Fru6P amount. Finally, phosphoglucomutase (PGM) (40 mU) was added to the former reaction to produce NADPH that is the equivalent of the Glc1P amount (Figure 2.3). A calibration curve was created by using 0,04 mM NADPH on each 96 well plate. Different amounts of NADPH were applied in this process: 0, 0,5 nmol, 1 nmol, 1,5 nmol and 2 nmol. According to this liner, Glc6P, Fru6P and Glc1P can be calculated.

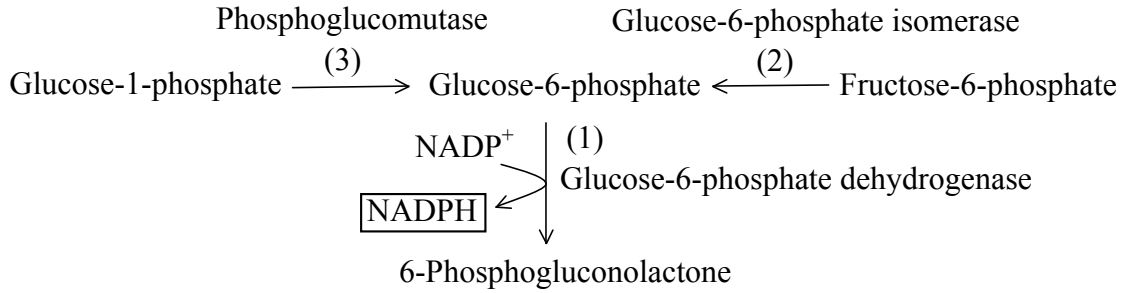


Figure 2.3: Enzyme reactions for phosphorylated hexose measurements
(1), (2), (3): the order of enzyme added.

40 µl sample was mixed with 90 µl buffer II and 70 µl H_2O for 3-PGA measurement. Phosphoglycerate kinase (PGK) (0,6 U) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (160 mU) were added together to the measured solution to consume NADH being the equivalent of the 3-phosphoglycerate (Figure 2.4).

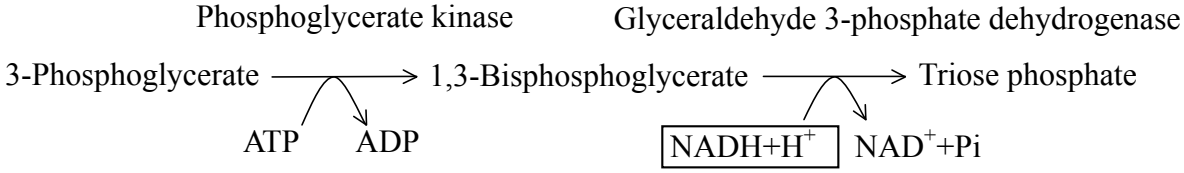


Figure 2.4: Enzyme reactions for 3-PGA measurement

40 µl sample was mixed with 90 µl buffer III and 70 µl H_2O for triose phosphate measurement. Phosphoglycerate kinase (0,6 U), glyceraldehyde 3-phosphate dehydrogenase (160 mU) and triose phosphate isomerase (TIM) (10 U) were added

together to the solution to produce NADH being the equivalent of the triose phosphate.

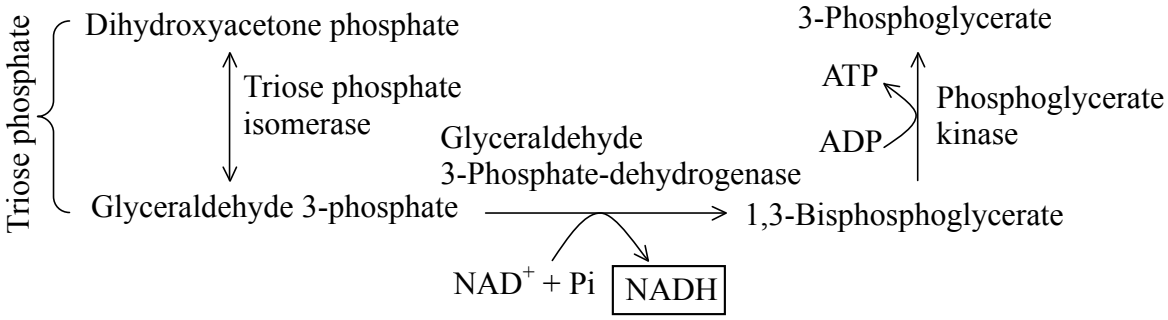


Figure 2.5: Enzyme reaction for triose phosphate measurement

The calibration curve was created by using 0,04 mM NADH on each 96 well plate. Different amounts of NADH were applied in this process: 0, 0,5 nmol, 1 nmol, 1,5 nmol and 2 nmol. According to this liner, 3-phosphoglycerate and triose phosphate can be calculated.

2.2.14. Measurement of transporters' activities

2.2.14.1. Measurement of glucose-6-phosphate/phosphate transloctor's activity

The method was according to the method of Flügge (1994) with some modifications.

2.2.14.1.1. Purification of phospholipids

It is necessary to separate free fatty acids from phospholipid for transporter experiments. The method was based on the method of Krämer (1977) with some modifications. 30 g L-α-phophatidylcholine (Asolectin TypIV-S, Sigma) was dissolved in approximate 3 volumes of chloroform (maximum 100 ml) and stirred about 10 min at room temperature. 540 ml ice-cold acetone was added after it was dissolved completely and continuously stirred at room temperature for another 2 h. The solution was left at 4°C overnight to precipitate the phospholipids. The supernatant was decanted, and sediment was dissolved in 100 ml diethyl ether. The ether was extracted in a rotary evaporator and lipid was dried on filter paper until no more ether smell can be detected. The lipid was aliquoted and frozen at –20°C.

2.2.14.1.2. Preparation of liposomes

2 x liposome buffer I

200 mM	Tricine-KOH pH 7,8
60 mM	Substrate (Phosphate; Glc6P; 3-PGA, respectively)
60 mM	K-gluconate

2 x liposome buffer II

200 mM	Tricine-KOH pH 7,8
120 mM	K-gluconate

Phosphate, glucose-6-phosphate and 3-PGA were used as substrates for GPT measurements, respectively. K-gluconate was used as substrate of negative control for measurement because GPT does not transport it through the membrane. Every 2 x liposome buffer I contained only one substrate (3 kinds of 2 x liposome buffer I). 22 % (w/v) phospholipid was added to 1,2 ml 2 x liposome buffer I or buffer II, respectively. They were sonified with 30% duty cycle, output control 5 by Branson Sonifier 250 for 10 min on ice. The liposomes should appear as a clear yellow suspension after sonification. The liposomes were kept on ice until they were used.

2.2.14.1.3. Extraction of proteins and preparation of proteoliposomes

Extraction buffer

0,2 M	Tricine-KOH pH 7,8
10 mM	EDTA
4 mM	DTT
6 mM	Ascorbate
0,1% (w/v)	BSA (fat free)
0,1% (w/v)	Polyclar AT (insoluble PVP)

200 - 500 mg frozen material (-80°C) was ground with mortar and pestle in liquid nitrogen. 3 ml fresh extraction buffer was added and mixed well with the fine powder. 32 µl PMSF (30 mg/ml, dissolved in EtOH) and 6 µl protease inhibitor cocktail

(1:500 dilution) were then added and homogenized until the material thawed. The extract was transferred to Eppendorf tubes and centrifuged at 4°C, 2500 rpm for 2 min. The supernatant was transferred to new Eppendorf tubes, and then 40 µl 2% Triton X-100 (v/v) was added to 1 ml supernatant.
500 µl extract was mixed with 500 µl liposomes nicely to form proteoliopsomes, and frozen in liquid nitrogen quickly. The final concentration of phospholipid and substrate in proteoliposomes was 11% and 30 mM, respectively. It can be stored at –80°C for a few days.

2.2.14.1.4. Transport experiment

PD10 buffer

150 mM	Na-gluconate
50 mM	K-gluconate
10 mM	Tricine-KOH pH 7,8

Dowex buffer

200 mM	Na-Acetate

PD10 columns (Sephadex G-25) were used to separate the outer substrates from liposomes. The columns were rinsed with PD10 buffer for three times before using.
Proteoliposomes were thawed on ice, and then sonified with 25% duty cycle, output control 5 for 30 pulses at room temperature. All proteoliposomes were loaded to the PD10 columns. About 2 ml PD10 buffer was applied to run the proteoliposomes to the bottom of the columns. Proteoliposomes were eluted by the addition of 950 µl PD10 buffer. PD10 columns were washed with bidest water and kept in 0,1% NaN_3.

1ml Dowex (AG1-X8) was filled into glass columns. Dowex was used to separate [^{32}P]- and [^{31}P]- phosphate inside the proteoliposomes (transported) from the phosphate that had not been transported into the proteoliposomes. The anions were bound to Dowex if they were not transported into the proteoliposomes. The Dowex columns were rinsed with Dowex buffer for three times before using.
1,5 µCi [^{32}P] orthophosphate was mixed with 0,2 M NaH_2PO_4 (or 0,2M KH_2PO_4) and

water to 50 µl. 45 µl was taken out to mix with 850 µl proteoliposomes eluted from PD10 columns. 200 µl of this mixture was loaded to different Dowex columns at different time points (for example: 15, 35, 55, 75 sec). Elution was done with 0,5 ml and 1 ml of Dowex buffer. The elution was measured by a scintillation counter. To check the labeling efficiency, 10 µl of 4 mixtures (residue from loading mixture to Dowex columns) was mixed with 1,5 ml Dowex buffer respectively and measured by a scintillation counter. Dowex columns were washed with 1 M HCl.

2.2.14.2. Measurement of ATP/ADP translocator's activity

2.2.14.2.1. Extraction of proteins and preparation of proteoliposomes

Incubation buffer

50 mM	Hepes/NaOH pH 7,5
1 mM	EDTA
1 mM	KCl
1 mM	$MgCl_2$
4 mM	Cystein
1 M	Sorbitol

Liposome buffer

100 mM	Tricin-NaOH pH 7,5
30 mM	K-gluconate

Resuspension buffer

10 mM	Tricin-NaOH pH 7,5
150 mM	K-gluconate

10 - 25 g potato tuber material without peel was cut into small pieces and put into 75 ml of incubation buffer for 30 min. The material was homogenized in a Waring Blender, and filtered through Miracloth. The supernatant was centrifuged at 4°C, 4000 g for 5 min. Resuspend the pellet in 4 ml of liposome buffer. To destroy all compartments, the suspension was homogenized in a glass tissue grinder with

pumping 15 – 20 times up and down, and then centrifuged at 4°C, 400 g for 3 min to get rid of starch. The supernatant was centrifuged at 4°C, 100,000 g for 20 min to precipitate the membranes. The pellet was resuspended in 500 µl resuspension buffer. The protein content was determined by spectro-photometer (Pharmacia LKB, Novaspec II). 50 µg samples were mixed with 150 µl resuspension buffer. 15 µl Triton X-100 (10%) was added and vortexed. 50 µl of the above mixture was pre-loaded with either 100 mM ADP or 120 mM K-gluconate (as control) liposomes, vortexed and quickly frozen in liquid N_2.

2.2.14.2.2. Transport experiment

Proteoliposomes (liposomes and extract) were thawed on ice, and then sonified with 25% duty cycle, output control 5 for 20 pulses on ice and loaded onto equilibrated (with resuspension buffer) NAP5 columns. Elute with 2 x 550 µl resuspension buffer and use for transport experiment.

The proteoliposomes were either incubated with 1 mM bongkrekic acid (10 µl for 400 µl proteoliposomes) at 30°C for 2 min or not to inhibit the mitochondrial ADP/ATP transporter, and then 100 µl were incubated together with 100 µl radioactively labelled 100 µM [^{32}P]-ATP at 30°C for 1 min. During this time the radiolabelled ATP was transported into the proteoliposomes. The whole liquild is transferred to columns of the anion exchange resin Dowex to get rid of the non–transported labelled ATP. Elution was done with 3ml of Dowex buffer. The elution was measured by a scintillation counter.

2.2.15. $^{14}CO_2$ feeding experiments

$^{14}CO_2$ feeding experiments were done in a special installation (Figure 2.6).

2 layers of whatman papers were placed at the bottom of chamber. 5 ml $NaHCO_3$ (1 M, pH 9,1) was used to wet the papers. 4 leaf discs (Ø=1,5 cm) of each plant were cut and kept in wet. They were moved to the chamber subsequently (quickly). Close the chamber and give light (200 µmol m^{-2} s^{-1}) for 10 min. The light was removed and 20 µl (0,2 mCi) [^{14}C]-$NaHCO_3$ (56 mCi/mmol) was injected to the chamber. 3 min later, the light was given back for 30 min. Remove the light and transfer leaves to tubes containing 1 ml pre-heated 80% EtOH. The chamber was cleaned with 10 ml 2 M NaOH.

The extraction of starch and sugar were the same as described in section 2.2.13.3.

Sugars were dissolved in 300 µl H_2O after the ethanol was evaporated in a vacuum centrifuge (SpeedVac). 200 µl sugar solution mixed with 2 ml Rotiszint was measured in the scintillation counter. 400 µl starch solution mixed with 2 ml Rotiszint was measured in the scintillation counter.

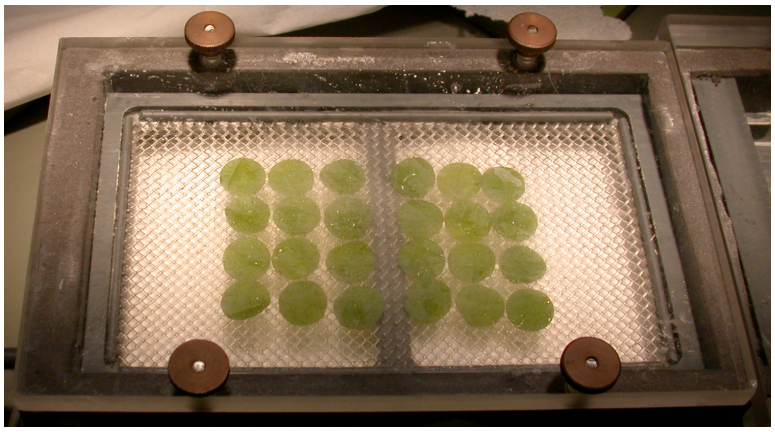


Figure 2.6 Installation of $^{14}CO_2$ labeling of detached leaf disces

2.2.16. Photosynthesis measurement

Photosynthetic parameters were measured in following procedure.

One leaf being attached to the plant was in dark for 10 min. Light (PFD=827 µmol m^{-2} s^{-1}) and CO_2 either from ambient air containing 350 ppm CO_2 or from a special gas mixture containing 1450 ppm CO_2 and 2% O_2 were given to this leaf simultaneously for 15 min. The CO_2 concentration was increased when concentration of CO_2 and assimilation got to stable values each time. Meanwhile, electron transport was measured for this leaf using a P-A-M. The assimilation and electron transport versus concentration of CO_2 can be calculated, respectively.

2.2.17. Histochemical detection of GUS activity

GUS staining buffer

0,1 M	Na_3PO_4 pH 7,0
10 mM	EDTA
0,5 mM	Potasiumferricyancide $[K_3Fe(CN)_6]$
0,5 mM	Potasiumferrocyancide $[K_4Fe(CN)_6]*3H_2O$

0,1% (v/v)	Triton X-100
1 mM	X-Gluc

One piece of potato tuber hand section (r=0,4 cm) was incubated in 450 µl GUS staining buffer and vacuum infiltrated for 15 min. The treatment was repeated 2 - 3 times, and subsequently the pieces were incubated at 37°C in dark overnight. When the tissue had an intense blue colour, the reaction was terminated by replacing the solution with 80% EtOH at 60°C and stored at 4°C to take pictures.

3. RESULTS

The starch content and yield of tubers are important traits in potato plants, and starch represents the major storage product in potato tubers (John, 1992). Carbon and energy are substrates for starch synthesis in amyloplasts of potato tubers. The glucose 6-phosphate/phosphate translocator can import carbon, while the ADP/ATP translocator imports energy into the amyloplast. To increase starch content and yield of potato tubers, pea GPT and AtNTT were transformed to potato plants under control of different promoters using *Agrobacterium tumefaciens* mediated gene transfer. All transgenic plants were characterized at molecular and physiological levels.

3.1. Comparison of the starch content in tubers of different size

In order to know if tuber starch content is related to tuber size, tubers with a fresh weight ranging from 3,5 g to 26,5 g were analyzed for their starch content (2.2.13.1). The results showed that the starch content was not dramatically influenced by tuber size, despite there was a slight trend that smaller tubers contained more starch (Figure 3.1). Tubers of approximately the same weight should be analyzed in further studies.

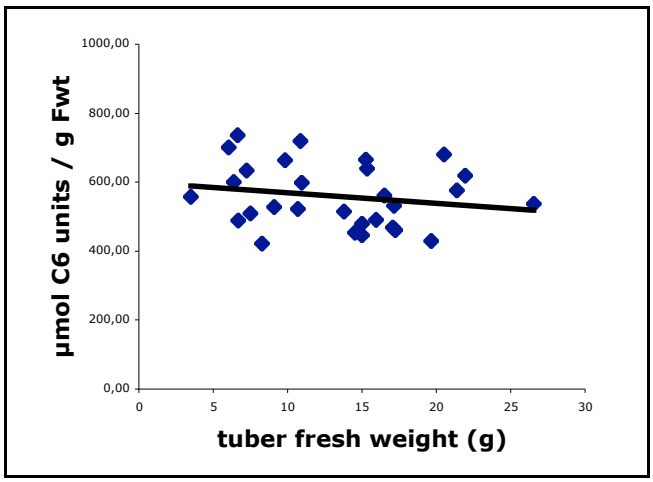


Figure 3.1: Relation between potato tuber size and starch content
Values are given in µmol C6 units g^{-1} Fwt.

3.2. Comparison of the expression level of an introduced gene in transgenic potato tubers of different size

To check if tuber size influences the expression level of an introduced gene, a Northern blot analysis with total RNA isolated from tubers of different weight of one line overexpressing pea GPT was conducted. The results show that pea GPT

transcript level, although not stable, was not affected by tuber size (Figure 3.2).

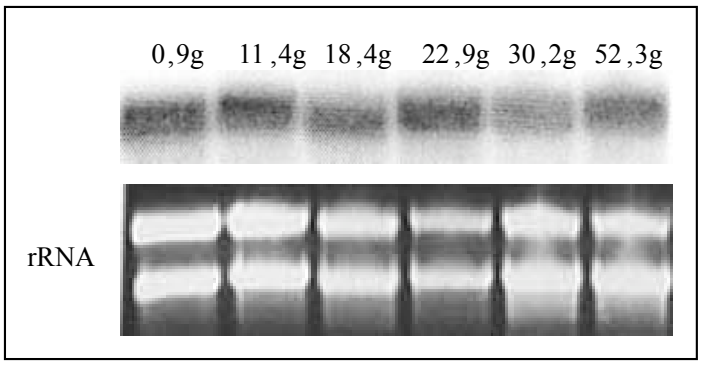


Figure 3.2: Northern blot analysis of potato tubers of different size

Samples were taken from tubers overexpressing pea GPT (SA62SG 17). 40 µg denatured total RNA was loaded in each lane, EtBr staining showed equal RNA loading. Probe: 1,6 kb fragment of pea GPT.

3.3. Overexpression of AtNTT using the patatin promoter B33 in potato plants (BA)

AtNTT is an important transporter for starch biosynthesis in amyloplasts of potato tubers. Tjaden et al. (1998a) created potato plants that overexpressed *Arabidopsis* NTT1 under control of the CaMV 35S promoter. One of three lines examined (JT62 lines) accumulated increased amounts of tuber starch, whereas decreased tuber yield was observed in these plants compared with the wild type. Moreover, we observed that these plants were smaller than wild type, and younger leaves were yellowish and crimple under some growth conditions (Figure 3.30). To avoid the leaf phenotype and to increase tuber yield, the patatin promoter B33 was used to express AtNTT mainly in tubers. The resulting plants were named BA.

3.3.1. Cloning of AtNTT into the B33-pBin19 vector

AtNTT was amplified from cDNA (1,9 kb) of *Arabidopsis thaliana* flowers with primers NTT1 and NTT2 containing BamHI and SalI restriction enzyme sites. Amplification was performed using the proof reading Pfu DNA polymerase to reduce the advent of errors that may have arisen due to the PCR. The purified AtNTT PCR fragment was cloned into TOPO TA vector and subsequently sequenced. The AtNTT fragment was excised with BamHI and SalI from the TOPO/AtNTT plasmid and cloned into BamHI and SalI sites of the transformation vector B33-pBin19 (Figure 3.3).

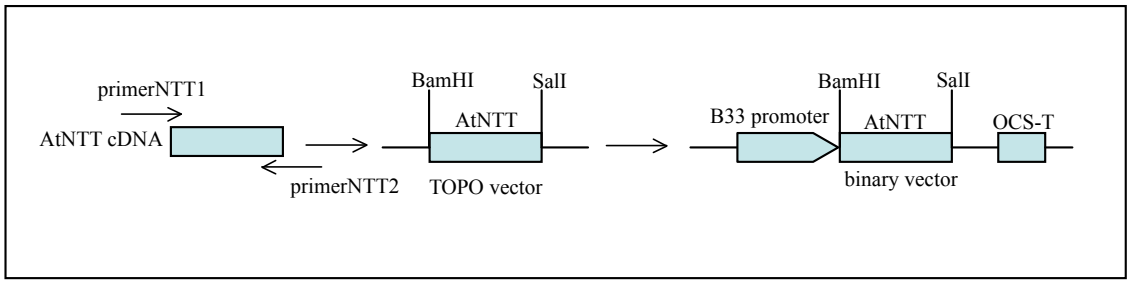


Figure 3.3: Construction of the B33-pBin19-AtNTT plasmid

OCS-T: terminator; AtNTT: *Arabidopsis* ATP/ADP translocator.

3.3.2. Analysis of AtNTT expression levels in potato tubers of BA plants

The *Agrobacterium tumefaciens* strain GV2260 was transformed with the B33-pBin19-AtNTT construct by electroporation (2.2.4.2.2). The resulting Agrobacterium strain was used to transform potato plants (2.2.4.3). 30 independent kanamycin resistance transformed plants were regenerated from tissue culture and transferred to a greenhouse. By use of AtNTT gene specific primers, we were able to amplify a PCR product of the expected size (about 1,9 kb) on genomic DNA prepared from BA leaves but not on genomic DNA prepared from the wild type (data not shown). The accumulation of AtNTT mRNA in various lines of BA plants was monitored by Northern blot experiments. Several lines of BA plants exhibited strong accumulation of AtNTT mRNA in tubers. In contrast to this, no AtNTT mRNA accumulated in tuber tissue from wild-type plants. Lines 8, 12, 24 were used for subsequent analysis (Figure 3.4).

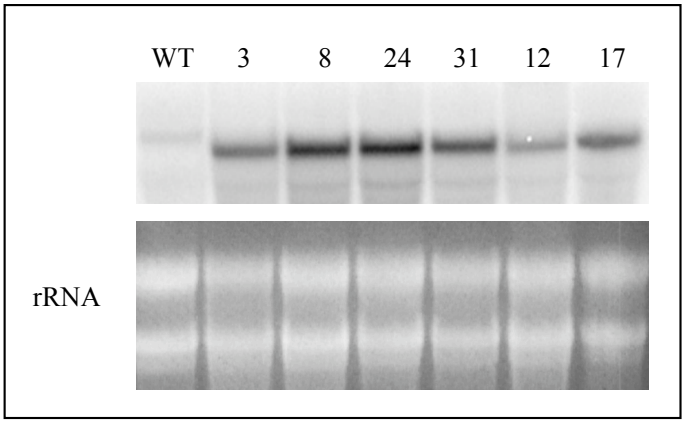


Figure 3.4: Northern blot analysis of potato tubers of BA and wild-type plants

40 µg denatured total RNA was loaded in each lane, EtBr staining showed equal RNA loading. Probe: 1,9 kb fragment of AtNTT; WT: wild type; 3, 8, 24, 31, 12, 17: BA lines.

3.3.3. Analysis of BA tuber yield

Since a reduction of tuber yield was observed in plants that overexpressed AtNTT under control of the CaMV 35S promoter (Tjaden et al., 1998a), it is essential to know how about the tuber yield in BA plants. Three batches of BA and wild-type plants were grown in a greenhouse. To measure tuber fresh weight, tubers were harvested after 8, 10 and 12 weeks of growth in a greenhouse, respectively. In these three independent experiments, tuber yield differs between the batches, whereas no apparent changes were observed between BA and wild-type plants (Figure 3.5 A, B and C).

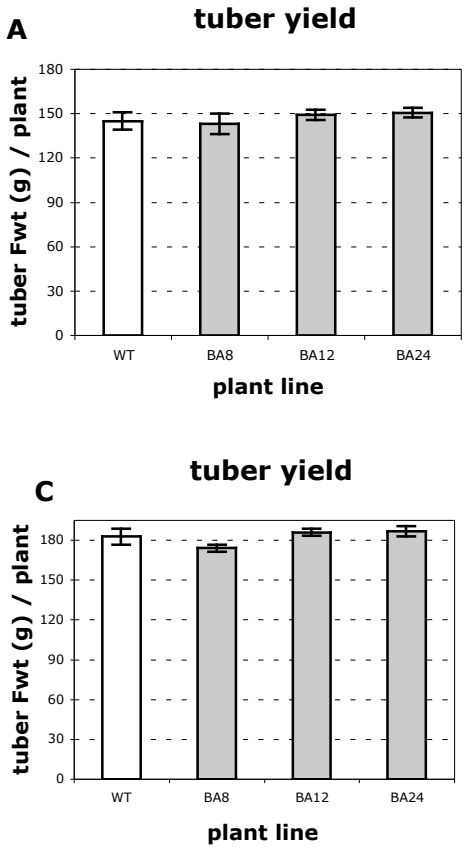


Figure 3.5: Tuber yield of BA and wild-type plants

A: plants grown for 8 weeks; B: plants grown for 10 weeks; C: plants grown for 12 weeks;

The data given are means of five plants from each line at each harvest time (n=5), and data are means ± standard error (SE). Results are given as tuber fresh weight (g) per plant.

3.3.4. Analysis of starch and soluble sugar content in tubers of BA plants

Increased tuber starch was detected in JT62 plants (Tjaden et al., 1998a). BA tuber tissue used for the measurement of soluble sugars and starch was taken after 8, 10 and 12 weeks of growth in the greenhouse (2.2.13.1 & 2.2.13.2). There was no marked difference in the amount of starch between BA tubers and wild-type plants in either

experiment (Figure 3.6 A-C). Moreover, levels of glucose and sucrose were not strongly changed in BA tubers compared with wild-type plants in three measurements, while that of fructose was close to the detection limit. One of three measurements is shown in Figure 3.6 D.

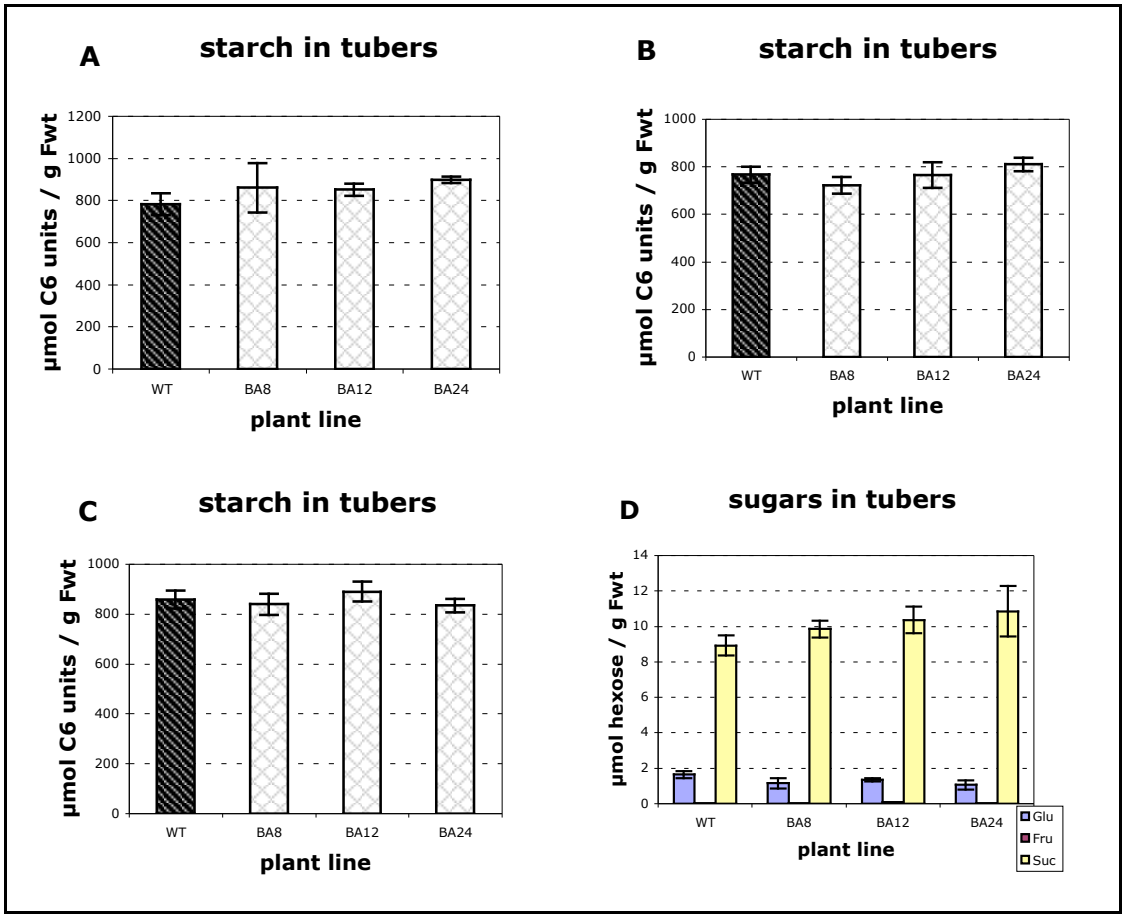


Figure 3.6: Starch and soluble sugar content in tubers of BA and wild-type plants
A-C starch content of tubers (8 weeks (A), 10 weeks (B) and 12 weeks (C) of growth); D: content of soluble sugars in tubers (10 weeks of growth)
The data given are means ± SE of five plants of each line in every experiment (n=5). Values are given in µmol C6 units g^{-1} Fwt and µmol hexose g^{-1} Fwt.

3.3.5. Determination of phosphorylated intermediates in tubers of BA plants

In order to know if there was impact on phosphorylated intermediates from tubers with increased AtNTT expression, Glc6P and Fru6P were measured in BA tubers after 8 and 12 weeks of growth in a greenhouse, respectively (2.2.13.4). There were no significant changes in the levels of both Glc6P and Fru6P from corresponding tuber extracts between 8 and 12 weeks. In Figure 3.7 (8 weeks), no marked difference

in Fru6P level was observed between BA and wild type plants, whereas two of three BA lines examined exhibited decreased Glc6P compared with wild-type plants.

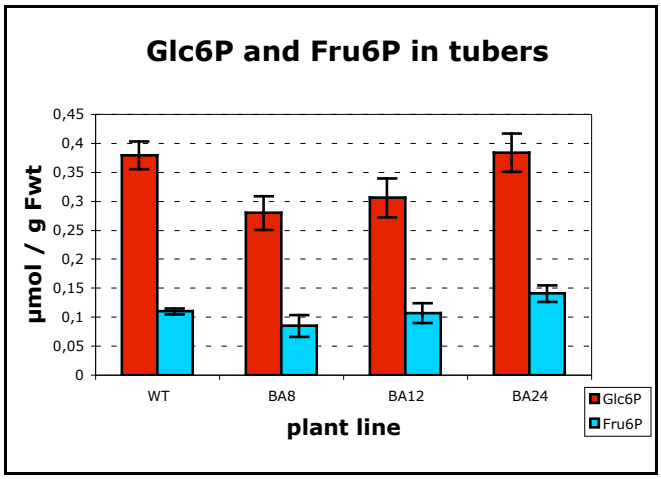


Figure 3.7: Phosphorylated intermediates in tubers of BA and wild-type plants
Results are means ± SE of five independent samples from five plants of one line (n=5). Values are given in µmol g^{-1} Fwt.

BA plants overexpressing only the NTT under control of the tuber-specific B33 promoter did not exhibit increased tuber starch and yield compared with wild-type plants.

3.4. Overexpression of pea GPT using the CaMV 35S promoter in potato plants (EW)

To assess the impact of GPT on starch biosynthesis in amyloplasts of potato tubers, the pea GPT was overexpressed under control of the CaMV 35S promoter. These plants were named EW.

3.4.1. Analysis of pea GPT expression levels in EW plants

Expression levels of pea GPT in EW plants were determined by PCR and Northern blot analysis. Total RNA from leaf and tuber tissues was loaded and several lines exhibited strong accumulation of pea GPT mRNA in both leaves and tubers. In contrast, as expected no pea GPT mRNA accumulated in leaves or tubers of wild-type plants. Lines 11, 14 and 15 were used in the following experiments (Figure 3.8 A and B).

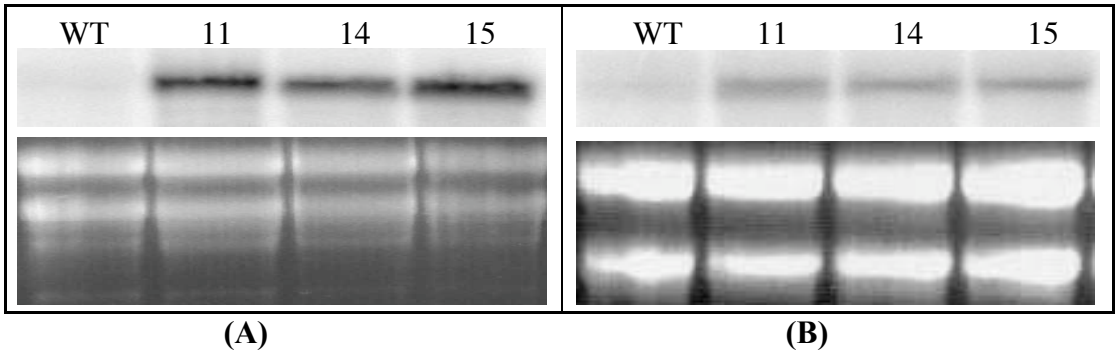


Figure 3.8: Northern blot analysis of EW and wild-type plants

A: Northern blots of potato leaves; B: Northern blots of potato tubers

40 µg total RNA was loaded in each lane, EtBr staining showed equal RNA loading. Probe: 1,6 kb fragment of pea GPT; WT: wild type; 11,14,15: EW lines.

3.4.2. Expression analysis of potato endogenous GPTs in tubers of EW plants

Since pea GPT was introduced into potato plants, it was worth to note if there was an effect on endogenous potato GPT expression levels (StGPT1 and StGPT2). To address this question, transcript levels of StGPT1 and StGPT2 were analyzed in tubers of three EW lines and wild-type plants by Northern blot experiments. As shown in Figure 3.9, there was no effect on transcript levels on either StGPT1 or StGPT2 in EW plants (Figure 3.9).

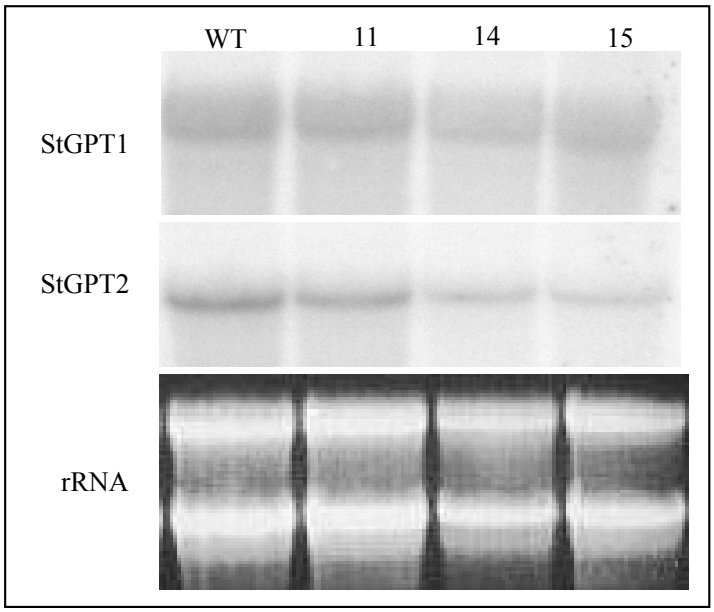


Figure 3.9: Expression analysis of endogenous potato GPTs in tubers of EW and wild-type plants

40 µg denatured total RNA was loaded in each lane; EtBr staining indicated equal RNA loading. Probe: StGPT1 and StGPT2 cDNA; WT: wild-type; 11, 14, 15: EW lines.

3.4.3. Time course of starch and soluble sugar content in leaves of EW plants

For gaining information on alterations in the dynamics of carbohydrate metabolism, diurnal variations in the contents of starch and soluble sugars were investigated. For this experiment, four independent plants of each line were examined. The samples were taken every 4,5 h from morning (9:30 am) to evening (11:00 pm). In order to reduce the diversity caused by sampling of different parts of plants in each line, the samples were taken according to the model in Figure 3.10.

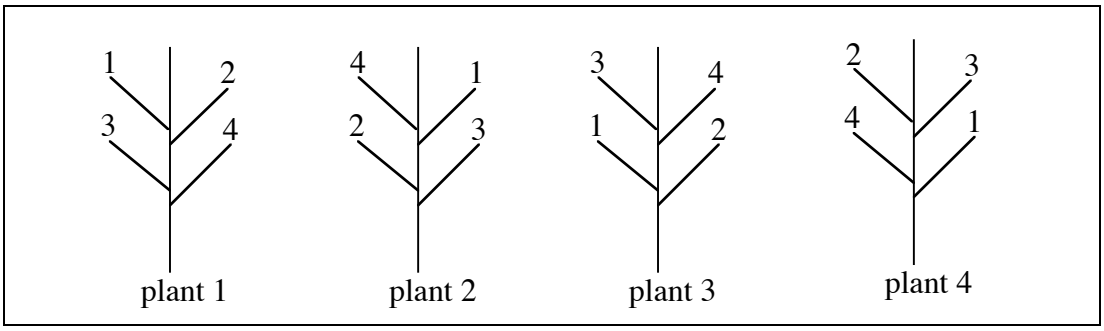


Figure 3.10: Model of taking leaf discs in a time course experiment

One line included 4 independent plants for examination. Three leaf discs (r=0,4 cm) were taken from different parts of plants and frozen in liquid nitrogen immediately. 1: taking leaf discs at 9:30 am; 2: taking leaf discs at 2:00 pm; 3: taking leaf discs at 6:30 pm; 4: taking leaf discs at 11:00 pm.

All EW lines examined had increased leaf starch content compared with the wild-type at all time points. There was no clear increase of starch content detectable during the day in EW plants (Figure 3.11). Moreover, first, there was no marked difference in glucose between EW and wild-type plants (Figure 3.12 A); Secondly, there was no significant difference in fructose between EW11,15 and the wild-type, while EW14 exhibited a different trend (Fugure 3.12 B). Furthermore, wild-type plants exhibited decreased sucrose from 2:00 pm to the evening, whereas an increase in sucrose was observed in EW plants from 2:00 pm to the evening (Figure 3.12 C).

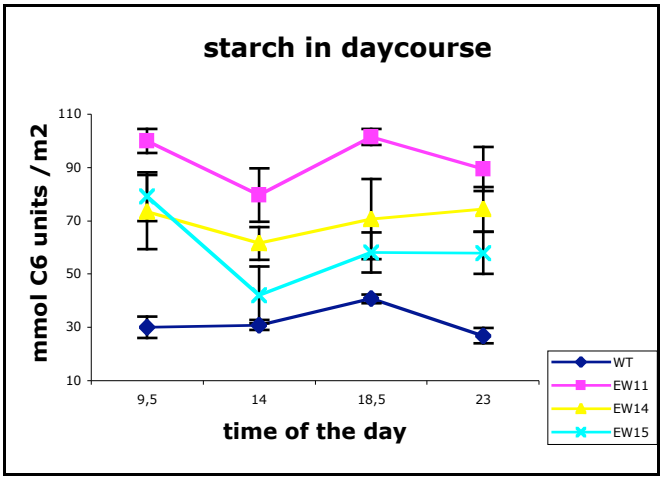
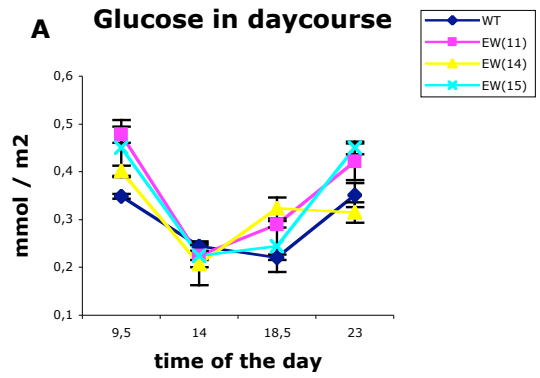
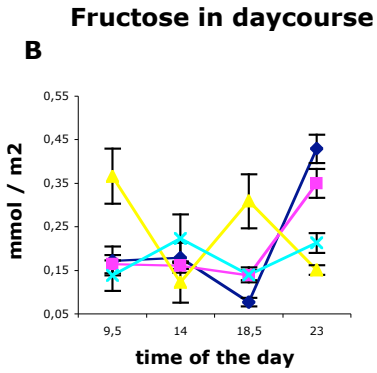


Figure 3.11: Diurnal changes in leaf contents of starch in EW and wild-type plants

Data points are means ± SE of four independent samples from four plants of a line (n=4). Each sample represents 3 leaf discs (r=0,4 cm) taken from one plant. Results are given as mmol C6 units m^{-2}





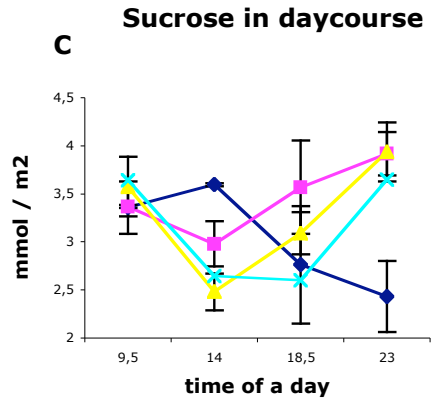


Figure 3.12 : Diurnal variation in leaf contents of soluble sugars in EW and wild-type plants

A: glucose ; B: fructose; C: sucrose;

Data points are means ± SE of four independent samples from four plants of one line (n=4). Each sample represents 3 leaf discs (r=0,4 cm) taken from one plant. Results are given as mmol hexose m^{-2}.

3.4.4. Time course of the phosphorylated intermediates in leaves of EW plants

With the purpose of measuring phosphorylated intermediates in leaves (2.2.13.4),

samples were taken from plants based on the model in Figure 3.10. Results are shown in Figure 3.13 A to E. During the time course, EW leaves exhibited increased Glc6P, Fru6P and Glc1P levels compared with wild type; particularly in the dark period the increase was dramatic. Increased 3-PGA was observed at 2:00 pm in EW leaves, whereas there were no marked changes between EW and wild-type plants at other time points. Furthermore, all EW plants examined exhibited a reduction of TrioseP compared with the wild type at all time points.

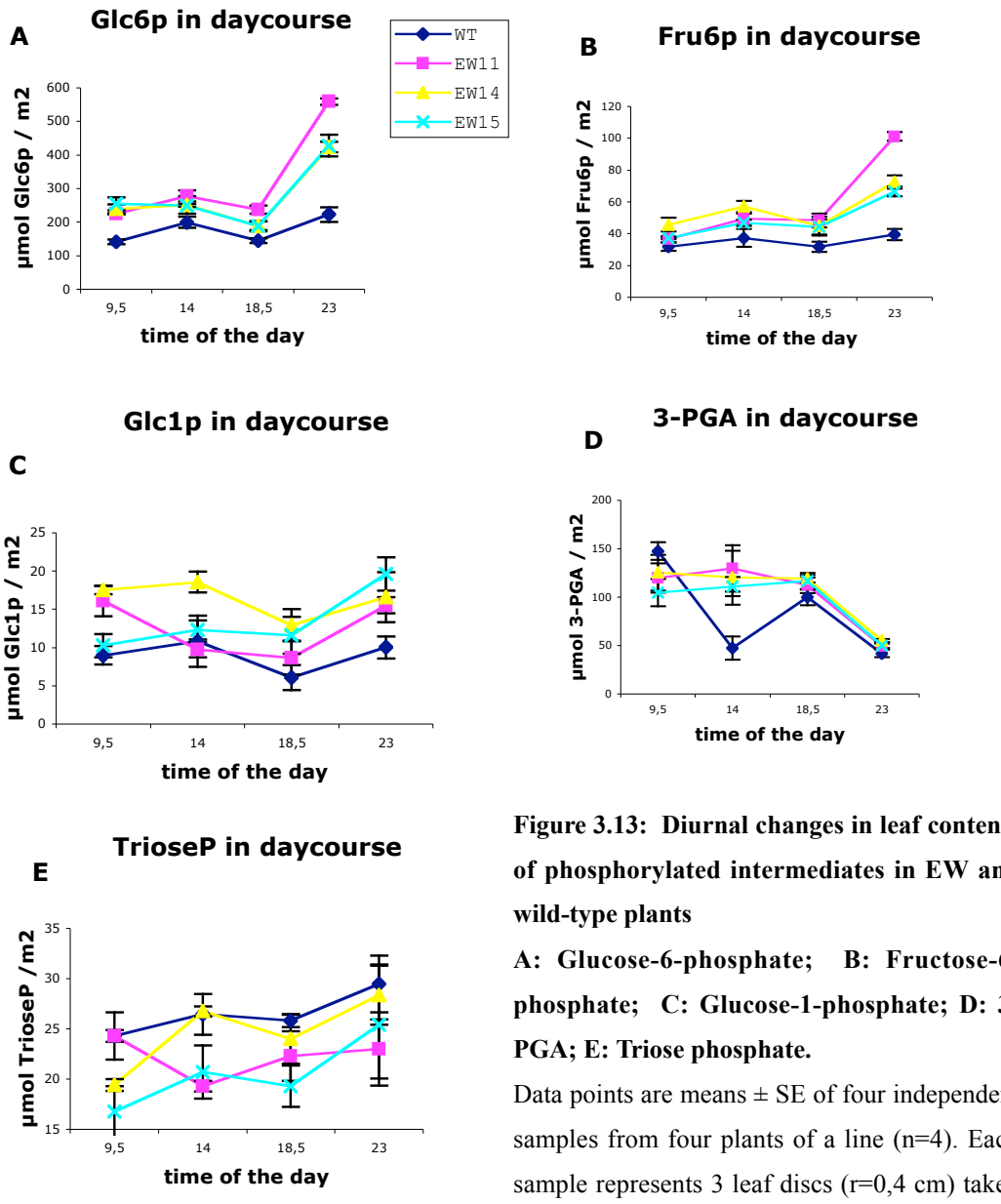


Figure 3.13: Diurnal changes in leaf contents of phosphorylated intermediates in EW and wild-type plants

A: Glucose-6-phosphate; B: Fructose-6-phosphate; C: Glucose-1-phosphate; D: 3-PGA; E: Triose phosphate.

Data points are means ± SE of four independent samples from four plants of a line (n=4). Each sample represents 3 leaf discs (r=0,4 cm) taken from one plant. Values are given in µmol m^{-2}.

3.4.5. Partitioning of $^{14}CO_2$ into starch and sucrose of EW leaves

To analyze the increased starch contents in EW leaves in more detail, the fluxes of [^{14}C] CO_2 into starch and sucrose were determined (2.2.15). The results showed that more carbon was directed into starch biosynthesis. The percentage of ^{14}C incorporated into starch increased from 43% in the wild type to about 51% in EW plants (Figure 3.14).

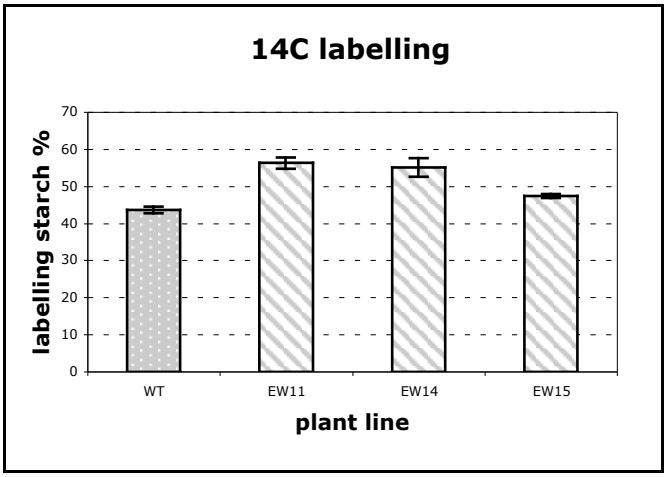


Figure 3.14: Incorporation of $^{14}CO_2$ into starch and soluble components

Results are means ± SE of 3 different samples from 3 plants of each line (n=3). For one sample, 4 leaf discs (d=1,5 cm) were taken and analyzed from one plant. Leaf discs were treated with approx. 5% $^{14}CO_2$ in the gas phase (specific activity 0,14 MBq $mmol^{-1}$) for 30 min at PFD of approx. 200 µmol m^{-2} s^{-1}. Values represent the starch labeling percentage of total labeling. WT: wild type; 11,14,15: EW lines.

3.4.6. Analysis of yield, starch and soluble sugar content in tubers of EW plants

Since increased starch content was observed in EW leaves, it is interesting to know if there was also increased starch content and yield of EW tubers. For this purpose, tuber fresh weight was measured, and starch and soluble sugar contents of tubers were determined after three months of growth in the greenhouse (2.2.13.1 & 2.2.13.2). Similar results were obtained in four independent experiments. EW plants revealed no apparent changes in total fresh weight and tuber starch compared with the wild-type (Figure 3.15). There was no difference in glucose, while sucrose decreased to a different extent in different EW lines compared to wild-type tubers. Fructose was below the detection limit (Figure 3.16).

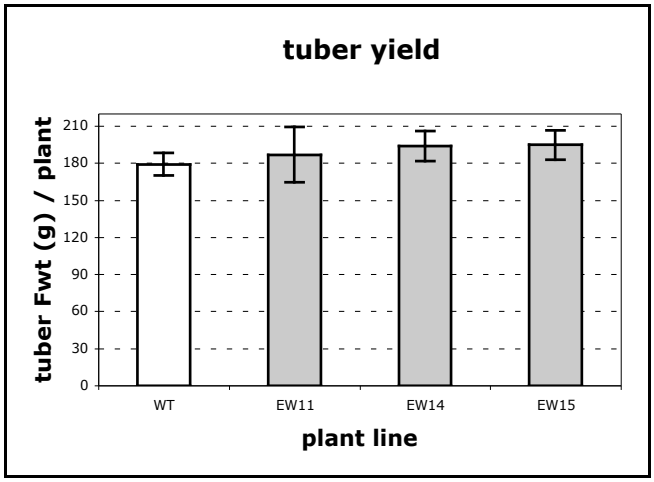


Figure 3.15: Tuber yield of EW and wild-type plants

These plants were examined for four independent batches. The results represent one of four experiments. The data given are means ± SE of five plants from each line (n=5). Results are given in tuber fresh weight (g) per plant.

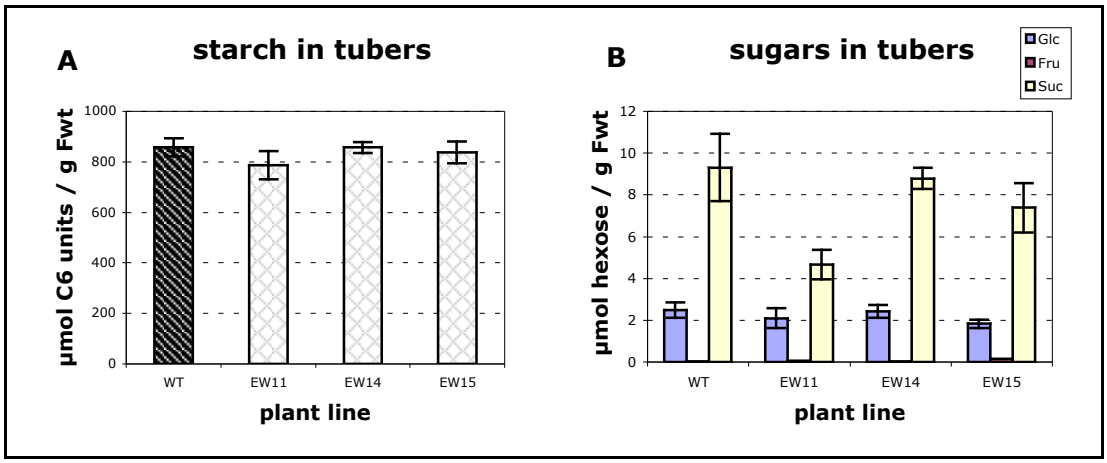


Figure 3.16: Amount of starch and soluble sugars in tubers of EW and wild-type plants

A: starch content of tubers; B: soluble sugar contents of tubers

Amount of starch and soluble sugars of these plants were examined in four independent experiments. The results represent one of four measurements. Results are means ± SE of five plants from each line (n=5). Values are given in µmol C6 units g^{-1}Fwt and µmol hexose g^{-1}Fwt .

3.4.7. Determination of phosphorylated intermediates in tubers of EW plants

Glc6P can be imported into amyloplast from the cytosol by GPT and can be used to synthesize starch in potato tubers. Therefore, it is important to know the levels of phosphorylated intermediates in EW tubers. The samples were taken to detect Glc6P and Fru6P when tubers were dug out after 12 weeks of growth in the greenhouse (2.2.13.4). There was no marked difference in Glc6P and Fru6P between all EW lines

examined and wild-type plants (Figure 3.17).

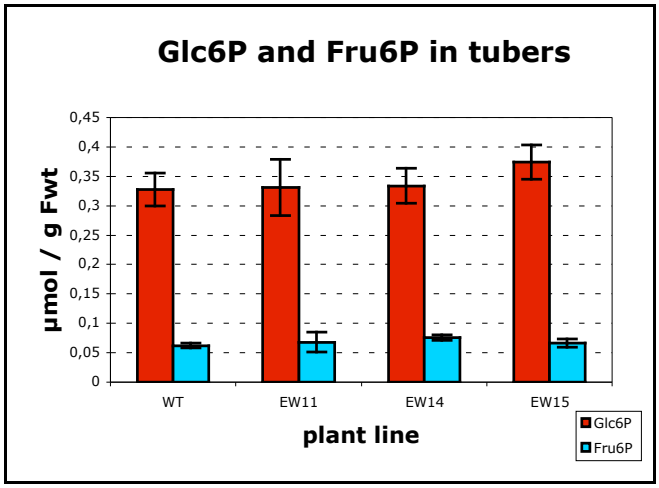


Figure 3.17: Phosphorylated intermediates in tubers of EW and wild-type plants

Results are means ± SE of five independent samples from five plants of one line (n=5). Each sample represents 3 tuber discs (r=0,4 cm) taken from one plant. Values are given in µmol g^{-1}Fwt.

3.4.8. Photosynthesis of EW plants

The photosynthesis measurements were performed with EW and wild-type plants. Four plants of EW line 14 and wild-type were chosen to measure parameters of photosynthesis in the morning and afternoon after 10 weeks of growth in a greenhouse (2.2.16.). There were no differences in the rates of CO_2 assimilation and photosynthetic electron transport at different CO_2 concentrations between EW and wild type plants (Figure 3.18 and 3.19).

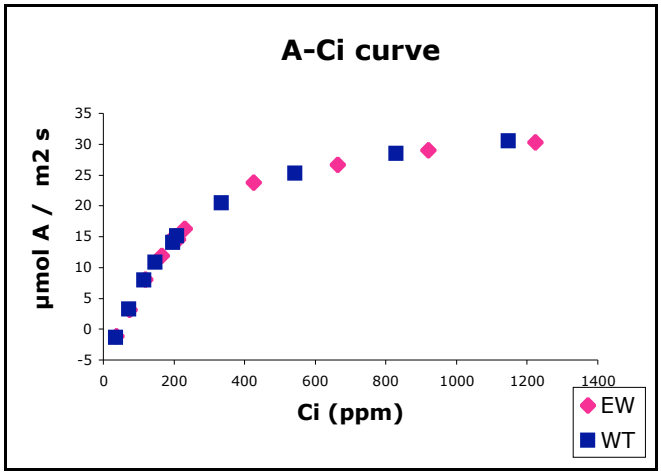


Figure 3.18: Assimilation-Ci curve of EW and wild type plants

The PFD was 827 µmol $m^{-2}s^{-1}$; A: assimilation; Values are given in µmol $m^{-2}s^{-1}$.

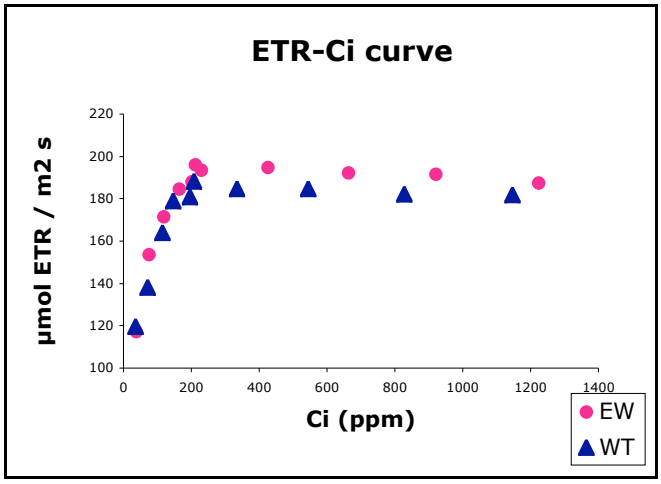


Figure 3.19: Electron transport-Ci curve of EW and wild-type plants
The PFD was 827 µmol $m^{-2}s^{-1}$; ETR: electron transport; Values are given in µmol $m^{-2}s^{-1}$.

EW plants exhibited increased starch in leaves, but not in tubers compared with wild-type plants. Therefore, the patatin promoter B33 was considered to generate plants overexpressing the GPT specifically in tubers.

3.5. Overexpression of pea GPT using the patatin promoter B33 in potato plants (BG)

EW plants did not exhibit increased tuber starch content and yield compared with wild-type plants. Since the patatin promoter B33 strongly drives gene expression in potato tubers, plants overexpressing pea GPT under control of the patatin promoter B33 were constructed to analyze whether both starch content and yield of tubers were increased. These plants were named BG.

3.5.1. Analysis of pea GPT expression levels in tubers of BG plants

40 hygromycin resistant independently transformed plants were regenerated in tissure-culture and transferred to the greenhouse. Expression levels of pea GPT in these plants were determined by PCR and Northern blot analysis. By use of pea GPT specific primers, the expected PCR products (1,6 kb) could be amplified on genomic DNA from BG plants examined but not from wild-type plants (data not shown). The accumulation of pea GPT mRNA in BG plants was determined by Northern blots. Several lines of BG plants exhibited differently increased accumulation of pea GPT mRNA in tubers. In contrast, no pea GPT mRNA accumulated in wild-type tubers.

Three lines (1, 6 and 12) were selected for further studies (Figure 3.20).

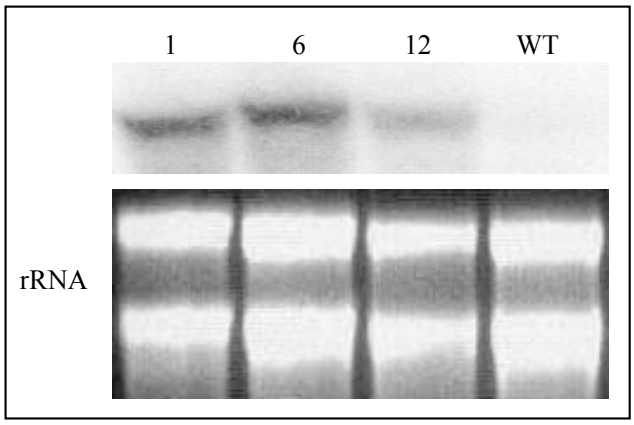


Figure 3.20: Northern blot analysis of potato tubers of BG and wild-type plants

40 µg denatured total RNA was loaded in each lane, EtBr staining indicated equal RNA loading. Probe: 1,6 kb fragment of pea GPT; WT: wild type; 1, 6, 12: BG lines.

3.5.2. Expression analysis of endogenous potato GPTs in tubers of BG plants

In order to find out if there was an effect on the expression of endogenous potato GPTs when pea GPT gene was introduced into potato plants under control of the patatin promoter B33, the accumulation of StGPT1 and StGPT2 mRNA was examined in BG and wild-type tubers by Northern blot analysis. There were no significant changes in transcript levels of both StGPT1 and StGPT2 in BG lines 1 and 6, whereas both StGPT1 and StGPT2 transcript amounts were decreased in BG lines 12, 16 and 19 compared to wild-type plants (Figure 3.21).

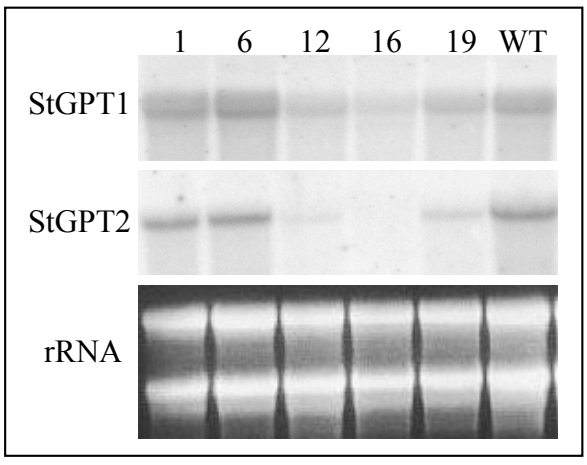


Figure 3.21: Expression analysis of potato endogenous GPTs in tubers of BG and wild-type plants

40 µg denatured total RNA was loaded in each lane, EtBr staining showed equal RNA loading. Probe: StGPT1 and StGPT2 cDNA; WT: wild type; 1, 6, 12, 16, 19: BG lines.

3.5.3. Analysis of the GPT activity in tubers of BG plants

For the sake of quantifying the activity of the plastidic glucose-6-phosphate transporter in BG lines and wild-type plants, proteins from whole-tissue tuber homogenates were reconstituted in liposomes to yield proteoliposomes (2.2.14.1). These were pre-loaded with phosphate, Glc6P or 3-PGA, respectively. The results indicated that there was increased GPT activity in all BG lines examined compared with the wild type, the increase being outstanding for line BG1. The uptake levels of Pi, Glc6P and 3-PGA increased by up to 1,3-fold, 2,0-fold and 1,7-fold in proteoliposomes of BG1 tubers compared to those of the wild type (Figure 3.22).

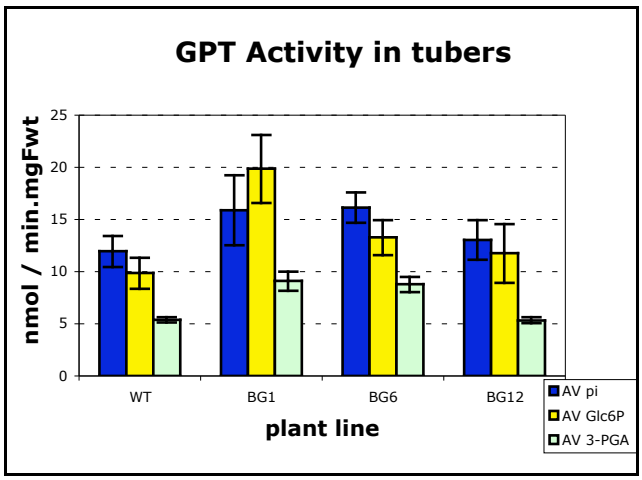


Figure 3.22: GPT activity in tubers of BG and wild-type plants

All values are means ± SE of measurements made on four extracts from four plants (n=4). The results are given as nmol $min^{-1}mg^{-1}$ Fwt.

3.5.4. Analysis of BG tuber yield

Potato tubers were harvested after 8, 10 and 12 weeks of growth in a greenhouse to determine the tuber weight. In these independent batches, there was no big difference in tuber yield between BG lines and the wild type in plants of different age (Figure 3.23).

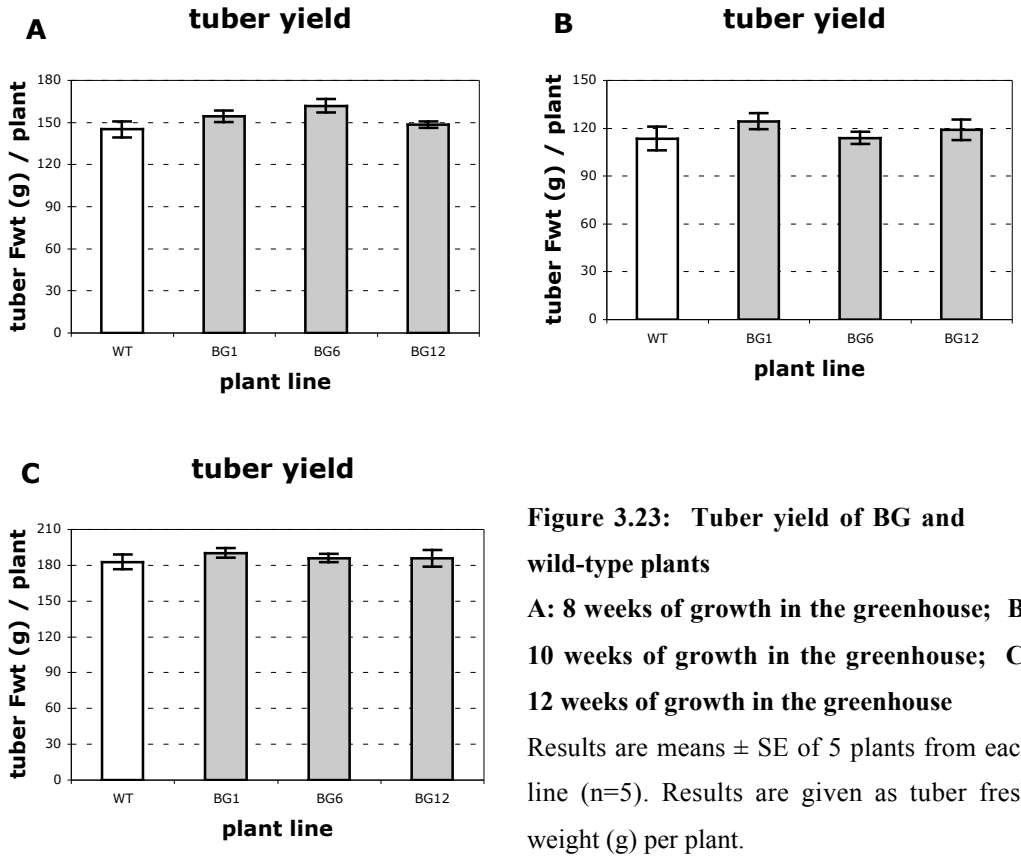


Figure 3.23: Tuber yield of BG and wild-type plants

A: 8 weeks of growth in the greenhouse; B: 10 weeks of growth in the greenhouse; C: 12 weeks of growth in the greenhouse

Results are means ± SE of 5 plants from each line (n=5). Results are given as tuber fresh weight (g) per plant.

3.5.5. Analysis of starch and soluble sugar contents in tubers of BG plants

Although BG lines examined did not exhibit increased tuber yield compared with wild-type, starch and soluble sugar contents were still interesting in these tubers (2.2.13.1 & 2.2.13.2). Samples were taken from 8, 10 and 12 week-old tubers. There was no marked difference in tuber starch between BG plants and wild-type, whereas increased starch content was detected in 12 weeks compared with 8 and 10 weeks (no big difference between 8 and 10 weeks) (Figure 3.24). Moreover, in plants of different age, there were no strong differences in the levels of glucose and sucrose between BG and wild-type tubers, while fructose was below the detection limit. One result is shown in Figure 3.25.

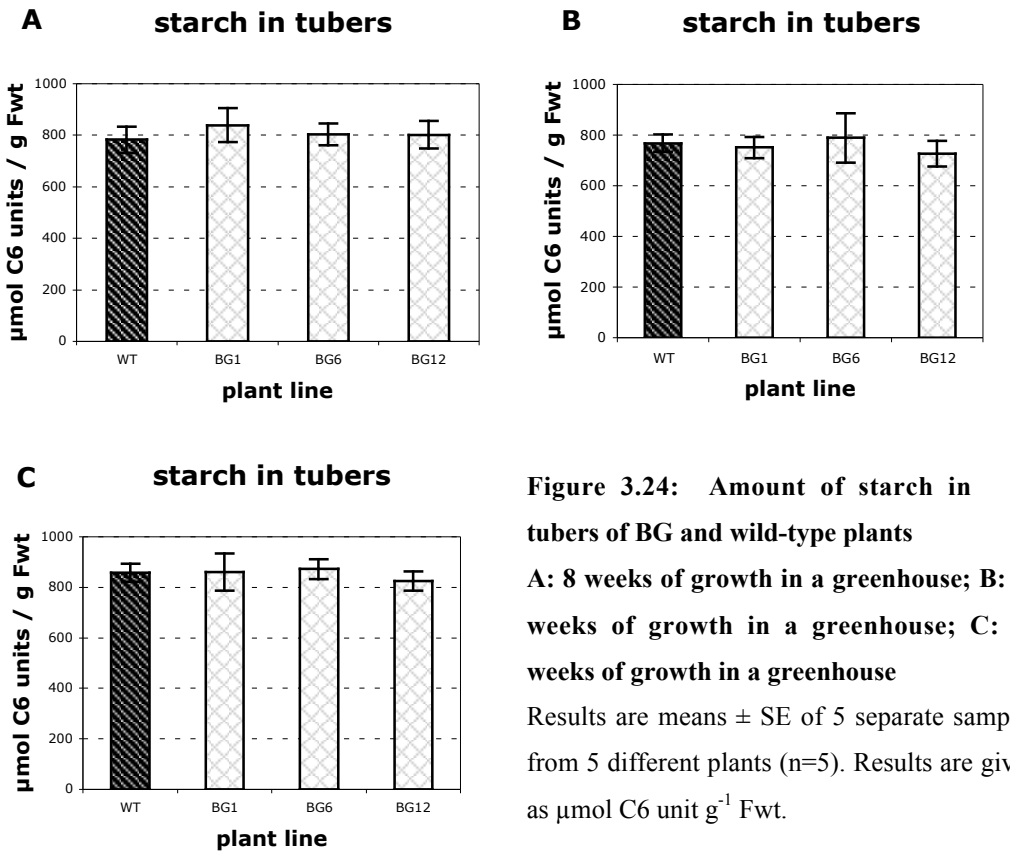


Figure 3.24: Amount of starch in tubers of BG and wild-type plants
A: 8 weeks of growth in a greenhouse; B: 10 weeks of growth in a greenhouse; C: 12 weeks of growth in a greenhouse
Results are means ± SE of 5 separate samples from 5 different plants (n=5). Results are given as µmol C6 unit g^{-1} Fwt.

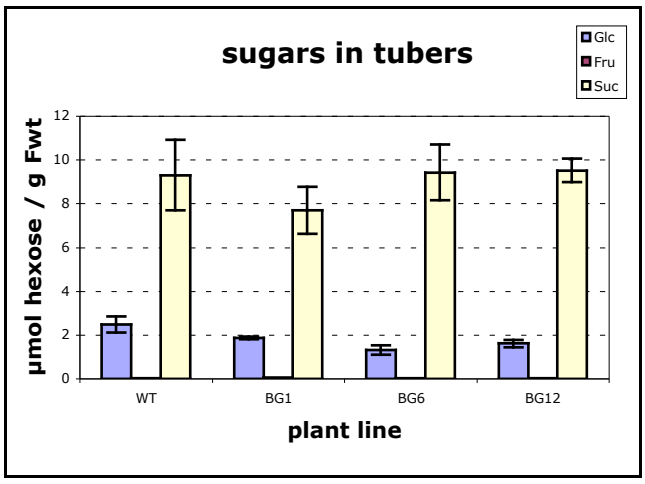


Figure 3.25: Soluble sugar contents in BG and wild-type tubers
Results represent one of four independent measurements. Results are means ± SE of 5 individual samples from 5 plants of each line (n=5). Values are given in µmol hexose g^{-1} Fwt.

3.5.6. Analysis of phosphorylated intermediates in tubers of BG plants

It has been shown that there were no marked differences in the levels of Glc6P and Fru6P in EW tubers compared with the wild type (Figure 3.17). To analyze this in BG tubers, samples were taken after 3 months of growth in a greenhouse (2.2.13.4). The results were similar in two batches. There was no big difference in Fru6P between BG and wild-type plants, whereas two of three BG lines examined (lines 1 and 12) exhibited a slight reduction of the Glc6P content compared with the wild type (Figure 3.26).

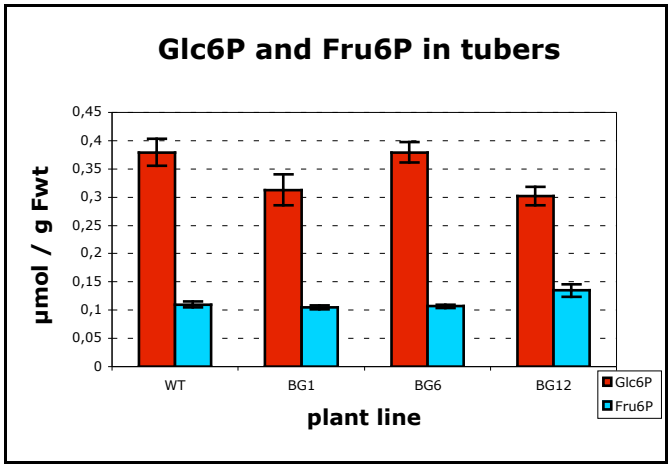


Figure 3.26: Phosphorylated intermediates in tubers of BG and wild-type plants

One of two measurements is presented. The results are means ± SE of five independent samples from five individual plants of one line (n=5). Values are given in µmol g^{-1} Fwt.

Taken together, BG plants did not exhibit increased tuber starch and yield compared with wild-type plants. Therefore, GPT and NTT were considered to be overexpressed in one plant simutaneously.

3.6. Overexpression of pea GPT in JT62 plants

JT62 plants contained more tuber starch than wild-type plants, while tuber yield was decreased in JT62 compared with wild-type plants (Tjaden et al., 1998a). GPT transports glucose-6-phosphate into amyloplasts of potato tubers, overexpressing pea GPT in JT62 plants may lead to an increase of tuber yield in the resulting double transformants.

3.6.1. Overexpression of pea GPT using the CaMV 35S promoter in JT62 plants (SA62SG)

Plants overexpressing pea GPT under control of the CaMV 35S promoter in JT62 plants were named SA62SG.

3.6.1.1. Analysis of pea GPT expression levels in SA62SG plants

30 hygromycin resistant independently transformed plants were regenerated from tissue-culture and transferred to the greenhouse. Expression levels were determined by PCR and Northern blot analysis. By use of gene-specific pea GPT primers, it was possible to amplify a PCR product of the expected size (about 1,6 kb) on SA62SG genomic DNA but not on DNA from wild-type and JT62 plants (data not shown). The accumulation of pea GPT mRNA from transformed and untransformed plants was monitored by Northern blot analysis. SA62SG plants exhibited different accumulation of pea GPT mRNA in both leaves and tubers. In contrast to this, no pea GPT mRNA accumulated in leaf or tuber tissue from wild-type and JT62 plants. Lines 7, 17 and 26 were used for subsequent analyses (Figure 3.27 and 3.28).

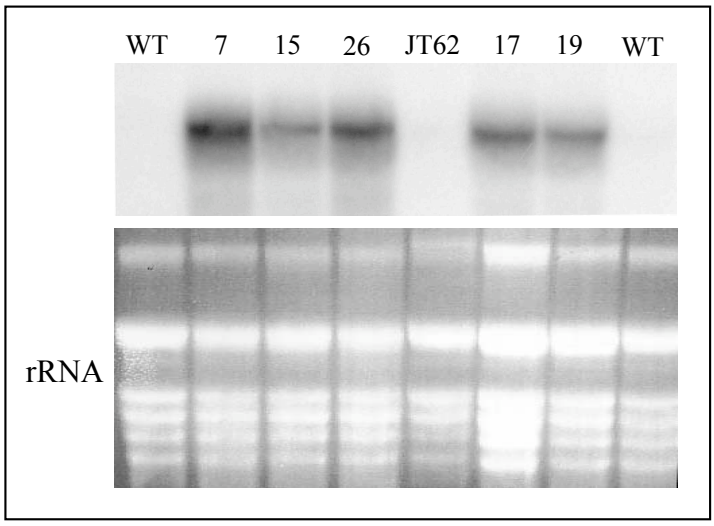


Figure 3.27: Northern blot analysis of potato leaves of SA62SG and control plants

40 µg denatured total RNA was loaded in each lane, EtBr staining indicated equal RNA loading. Probe: 1,6 kb fragment of the pea GPT. WT: wild type; JT62: AtNTT overexpressor line; 7, 15, 26, 17, 19: SA62SG lines.

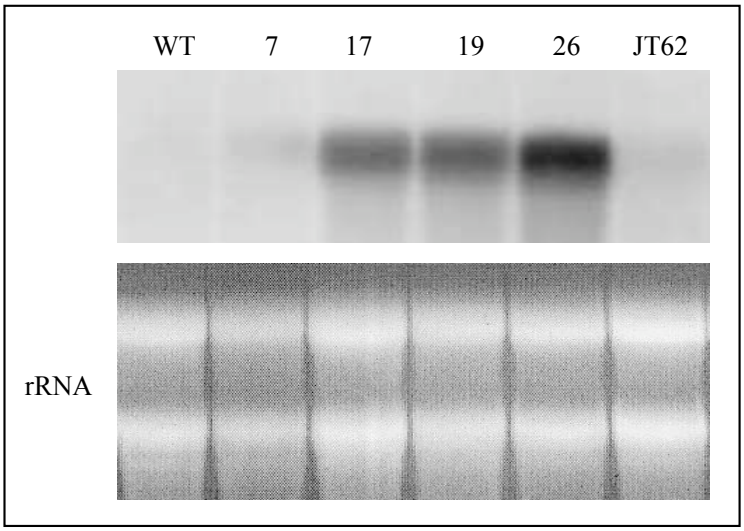


Figure 3.28: Northern blot analysis of potato tubers of SA62SG and control plants

40 µg denatured total RNA was loaded in each lane, EtBr staining indicated equal RNA loading. Probe: 1,6 kb fragment of pea GPT. WT: wild type; JT62: AtNTT overexpressor line; 7, 26, 17, 19: SA62SG lines.

3.6.1.2. General characteristics of SA62SG plants

JT62 plants were created by Tjaden et al. (1998a). The author did not describe any abnormal phenotype of JT62 aerial parts. However, we observed JT62 and SA62SG were smaller than wild-type plants, while some of SA62SG plants were slightly bigger than JT62 plants when grown in a greenhouse. In addition, younger leaves of JT62 and SA62SG plants were yellowish and crimple (Figure 3.29 and 3.30).

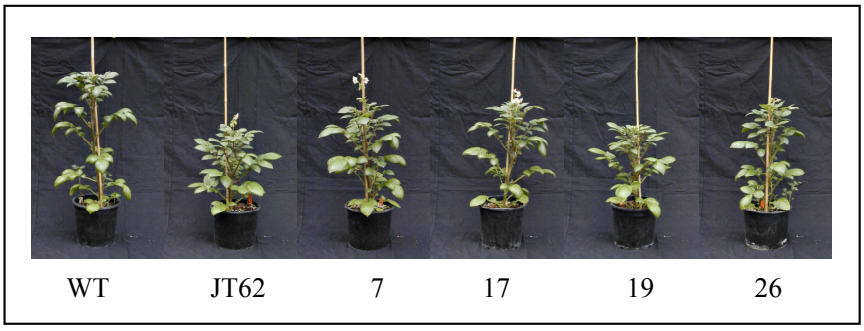


Figure 3.29: Phenotype of SA62SG and control plants

The plants were grown for 8 weeks under the same conditions in a greenhouse. WT: wild type; JT62: AtNTT overexpressor line; 7, 17, 19, 26: SA62SG lines.

Figure 3.30: Phenotype of wild-type and JT62 leaves

The plants were grown under the same conditions in a greenhouse for 8 weeks. Left: wild-type plants; Right: JT62-AtNTT overexpressor line.

3.6.1.3. Analysis of SA62SG tuber yield

To measure the tuber fresh weight, we harvested tubers after 12 weeks of growth in a greenhouse. Tuber yield is shown in Figure 3.31. In two experiments, tuber fresh weight of SA62SG lines was increased compared with that of JT62 plants, whereas only one of these three lines (line 17) contained slightly more tuber fresh weight than wild-type.

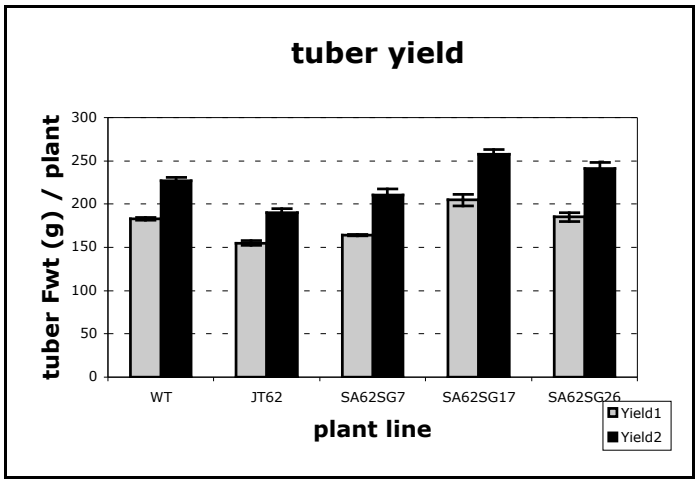


Figure 3.31: Tuber yield of two independently grown batches of SA62SG and control plants

The data given are means ± SE of four plants of each line of every batch (n=4). Grey and black columns represent two different batches. Results are given as tuber fresh weight (g) per plant.

3.6.1.4. Analysis of starch and soluble sugar contents in leaves and tubers of SA62SG plants

Starch and soluble sugar contents were measured from leaves (10 weeks of growth) and tubers (12 weeks of growth) (2.2.13.1, 2.2.13.2 & 2.2.13.3). The results were similar in two independent experiments. In leaves, the amount of starch was increased in SA62SG plants compared with the wild type, while the starch content was increased in two of three of the selected lines (lines 17 and 26) compared with JT62 plants. Tubers of SA62SG lines contained a slightly increased starch content compared to wild-type plants, and the similar tuber starch content as JT62 plants (Figure 3.32 A, B). On the other hand, no clear change was observed in glucose of leaves in SA62SG examined, while two of three SA62SG lines examined contained decreased glucose of tubers compared with control plants (wild type and JT62 plants). Sucrose was increased in leaves of two of three SA62SG lines examined (lines17 and 26), whereas a decreased sucrose amount of these two lines was observed in tubers (Figure 3.33 A, B). Result of one of two experiments is shown.

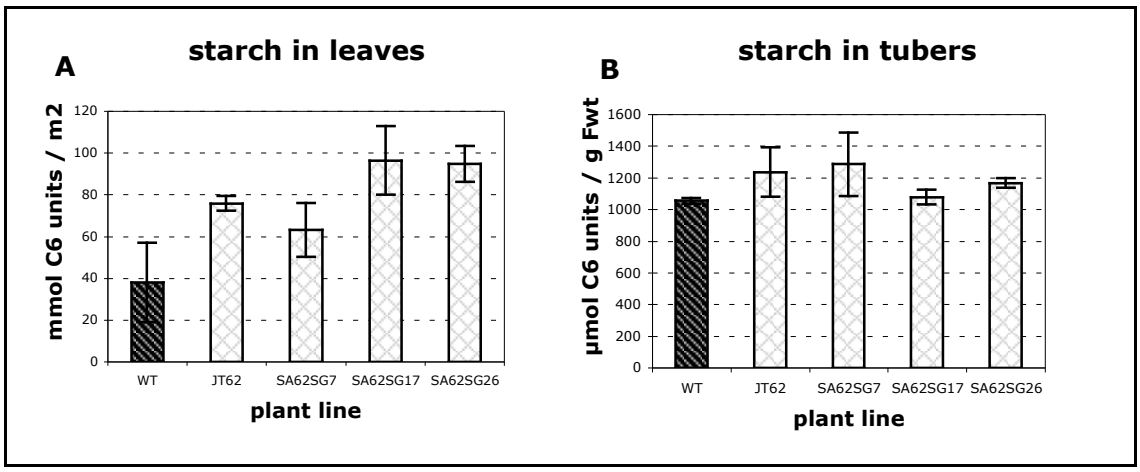


Figure 3.32: Amount of starch in leaves and tubers of SA62SG and control plants

A: starch content of leaves; B: starch content of tubers

Results are means ± SE, n=4 independent samples from different plants. In each time, three leaf discs (A) (r=0,4 cm) and tuber parts (B) were taken and analyzed. Values are given in mmol C6 units m^{-2} (A) and µmol C6 units g^{-1} Fwt (B).

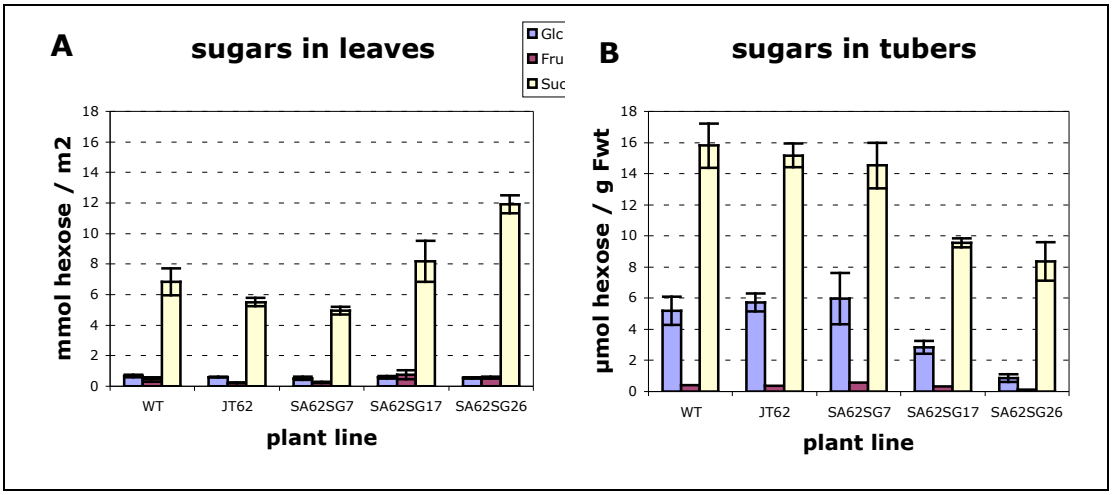


Figure 3.33: Measurement of soluble sugar contents in leaves and tubers of SA62SG and control plants

A: soluble sugar contents of leaves

Results are means ± SE, n=4 independent samples from different plants. At each time, three leaf discs were taken and analyzed. Values are given in mmol hexose unit m^{-2}.

B: soluble sugar contents of tubers

Results are means ± SE, n=4 independent tuber parts of different plants. At each time, one tuber parts was taken and analyzed. Values are given in µmol hexose unit g^{-1} Fwt.

3.6.2. Overexpression of pea GPT using patatin promoter B33 in JT62 plants (SA62BG)

Since SA62SG tubers did not contain an increased starch content and yield compared wild-type plants, the patatin promoter B33 was used to overexpress pea GPT in JT62 plants because it strongly drives gene expression in tubers. The plants were named SA62BG.

3.6.2.1. Analysis of pea GPT expression levels in SA62BG plants

27 hygromycin resistant independent transgenic plants were regenerated in tissue-culture and transferred to the greenhouse. Expression levels were detected in these plants by PCR and Northern blots. Pea GPT specific primers allowed to amplify a PCR fragment of the expected size using genomic DNA of SA62BG leaves as template but not from wild-type and JT62 plants (data not shown). To determine the transcript amount of pea GPT, a Northern blot analysis was performed. No transcripts specific for the pea GPT could be detected in wild-type and JT62 tubers, while expression of different strength was found in SA62BG tubers in Northern blot experiments. Lines 6, 12 and 20 were used for further studies (Figure 3.34).

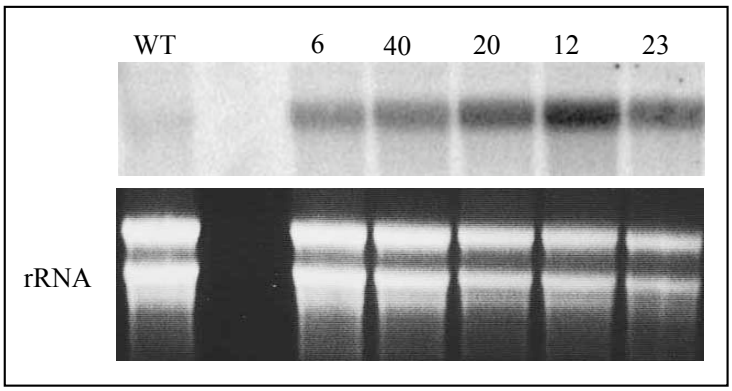


Figure 3.34: Northern blot analysis of potato tubers of SA62BG and wild-type plants

40 µg denatured total RNA was loaded in each lane, EtBr staining showed equal RNA loading. Probe: 1,6 kb fragment of pea GPT. WT: wild-type; 6, 12, 20, 23, 40: SA62BG lines.

3.6.2.2. General characteristics of SA62BG plants

Greenhouse grown SA62BG plants were found to be smaller than wild-type plants, while they were of similar size than JT62 plants. Yellowish and crimple leaves also appeared in younger leaves of SA62BG, as they do in JT62 plants (Figure 3.35 and 3.30).

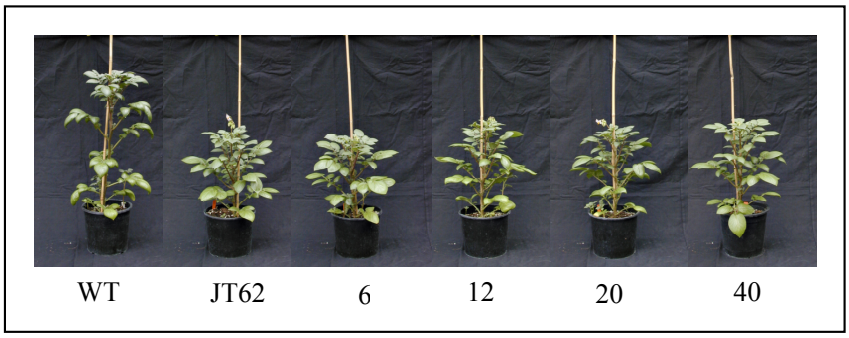


Figure 3.35: Phenotype of SA62BG and control plants

Plants were grown for 8weeks under the same conditions in a greenhouse. WT: wild-type; JT62: AtNTT overexpressor line; 6, 12, 20, 40: SA62BG lines.

3.6.2.3. Analysis of SA62BG tuber yield

Tubers were harvested after three months of growth in a greenhouse. In all SA62BG lines examined, there was less tuber fresh weight than in wild-type plants, but no significant difference was observed compared with JT62 plants in two different batches (Figure 3.36).

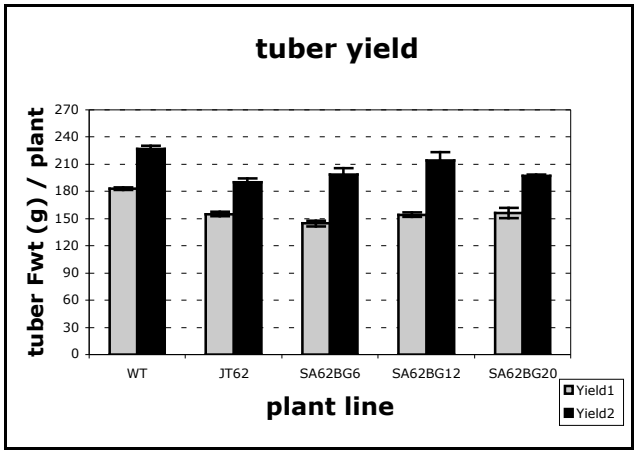


Figure 3.36: Tuber yield of two independently grown batches of SA62BG and control plants
The data given are means ± SE of 4 plants from each line of every batch (n=4). Grey and black columns represent two different batches. Results are given as tuber fresh weight (g) per plant.

3.6.2.4. Analysis of starch and soluble sugar contents in tubers of SA62BG plants

Starch and soluble sugar contents were measured in SA62BG tubers (2.2.13.1 &2.2.13.2). The results were similar in two batches (Figure 3.37). Tubers from line 6 contained a slightly increased starch content, while tubers from lines 12 and 20 of SA62BG showed no significant change compared to the wild type. In contrast, there was no significant difference in the starch content between SA62BG 6 and 20 and genetic background JT62 plants, whereas a slight reduction of the starch content was observed in line 12 of SA62BG compared with JT62 plants (Figure 3.37). Furthermore, there was no significant change in the level of sucrose, whereas glucose was decreased in tubers of SA62BG lines examined compared with control plants (Figure 3.38).

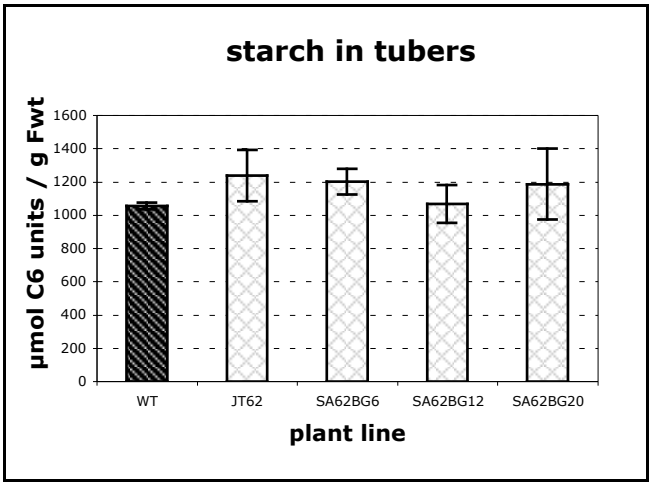


Figure 3.37: Amount of starch in tubers of SA62BG and control plants

Results are means ± SE n=4 independent tuber parts from different plants. Values are given in µmolC6 unit g^{-1} Fwt.

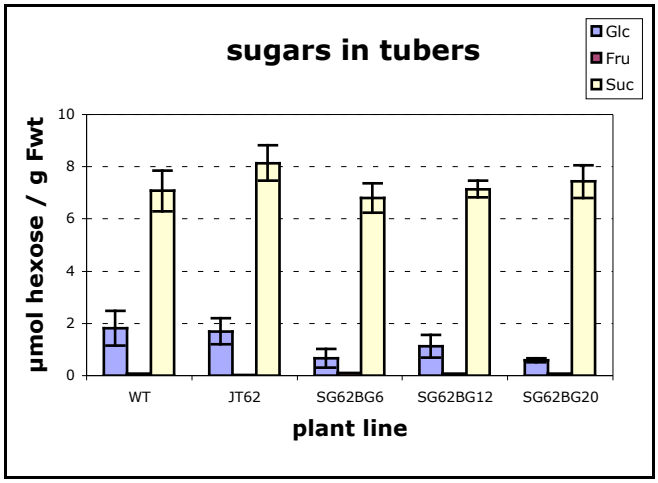


Figure 3.38: Soluble sugar contents in tubers of SA62BG and control plants

Results are means ± SE of 4 independent tuber parts of individual plants (n=4). Values are given in µmol hexose g^{-1} Fwt.

Together, neither SA62SG nor SA62BG plants did contain more tuber starch and yield than wild-type plants. It was therefore aimed to overexpress GPT and NTT simultaneously under the patatin promoter B33.

3.7. Overexpression of pea GPT and AtNTT together using the patatin promoter B33

Patatin promoter B33 mainly directs gene expression in potato tubers. However, the plants that overexpressed only the pea GPT or the AtNTT under the patatin promoter B33 did not exhibit significant changes in both starch content and yield of tubers compared with wild-type plants. In order to increase starch content and yield of tubers, pea GPT and AtNTT were overexpressed together under control of the patatin promoter B33. Two ways were used to create these transgenic plants. First, pea GPT and AtNTT were overexpressed in wild-type plants, and these plants were named BGBAM. Second, AtNTT was overexpressed in BG1 plants, which have been found to have increased pea GPT expression and GPT activity in tubers compared with wild-type. These plants were named BG1BA.

3.7.1. Overexpression of pea GPT and AtNTT in wild-type plants (BGBAM)

3.7.1.1. Analysis of pea GPT and AtNTT expression levels in BGBAM plants

Two B33-pBin19 vectors, that contained either pea GPT or AtNTT were transformed into *Agrobacterium tumefaciens* GV2260 by electroporation, respectively (2.2.4.2.2). The resulting Agrobacterium strains were mixed and used to transform potato plants (2.2.4.3). 40 kanamycin and hygromycin resistant independently transformed plants were regenerated from tissue-culture and transferred to the greenhouse. Gene-specific primers for pea GPT and AtNTT allowed to amplify the expected PCR products (1,6 kb and 1,9 kb), respectively, on genomic DNA of BGBAM leaves but not on that of the wild type (data not shown). Transcript amounts were determined in potato tubers. Different expression levels of pea GPT and AtNTT were found in BGBAM plants, whereas no transcripts specific for pea GPT and AtNTT could be detected in wild-type plants in Northern blots (Figure 3.39). Lines 16, 21 and 27 were used for further experiments.

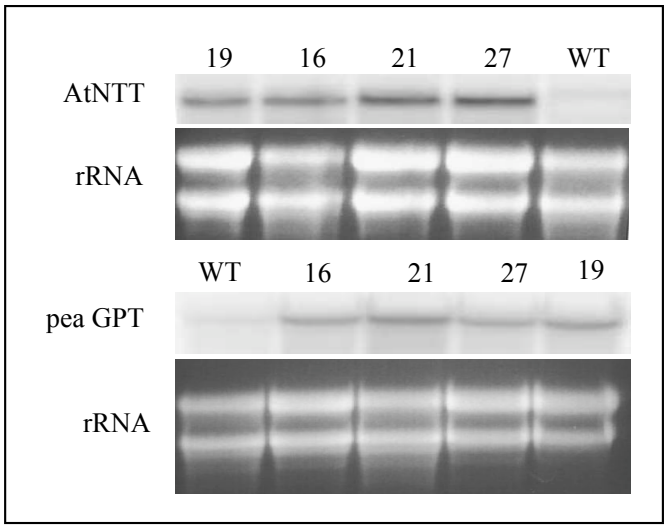


Figure 3.39: Northern blot analysis of potato tubers of BGBAM and wild-type plants
40 µg total RNA was loaded in each lane, EtBr staining indicated equal RNA loading. Probes: 1,6 kb pea GPT fragment and 1,9 kb AtNTT fragment. WT: wild type; 16, 19, 21, 27: BGBAM lines.

3.7.1.2. Analysis of BGBAM tuber yield

To measure the fresh weight of BGBAM tubers, plants were harvested after 8, 10 and 12 weeks of growth. No big change of tuber fresh weight was observed between BGBAM and wild-type plants of different age (Figure 3.40).

3.7.1.3. Analysis of starch and soluble sugar contents in tubers of BABGM plants

Although there was no pronounced change in tuber yield between BABGM and wild-type plants, the amount of starch and soluble sugars of tubers were measured at 8, 10 and 12 weeks of growth in a greenhouse (2.2.13.1 & 2.2.13.2). There were no consistent differences in tuber starch content, neither in tubers of different age, nor between BGBAM and wild-type plants (Figure 3.41). In addition, no strong changes in soluble sugar contents were observed (Figure 3.42).

3.7.1.4. Determination of phosphorylated intermediates in tubers of BABGM plants

The above results showed two of three BA (lines 8 and 12) and BG lines (lines 1 and 12) exhibited decreased Glc6P compared with the wild type, respectively. In contrast, tubers of two of three BGBAM lines revealed an increase in Glc6P, whereas there

was no marked change in Fru6P from BGBAM lines examined compared with the wild-type (Figure 3.43).

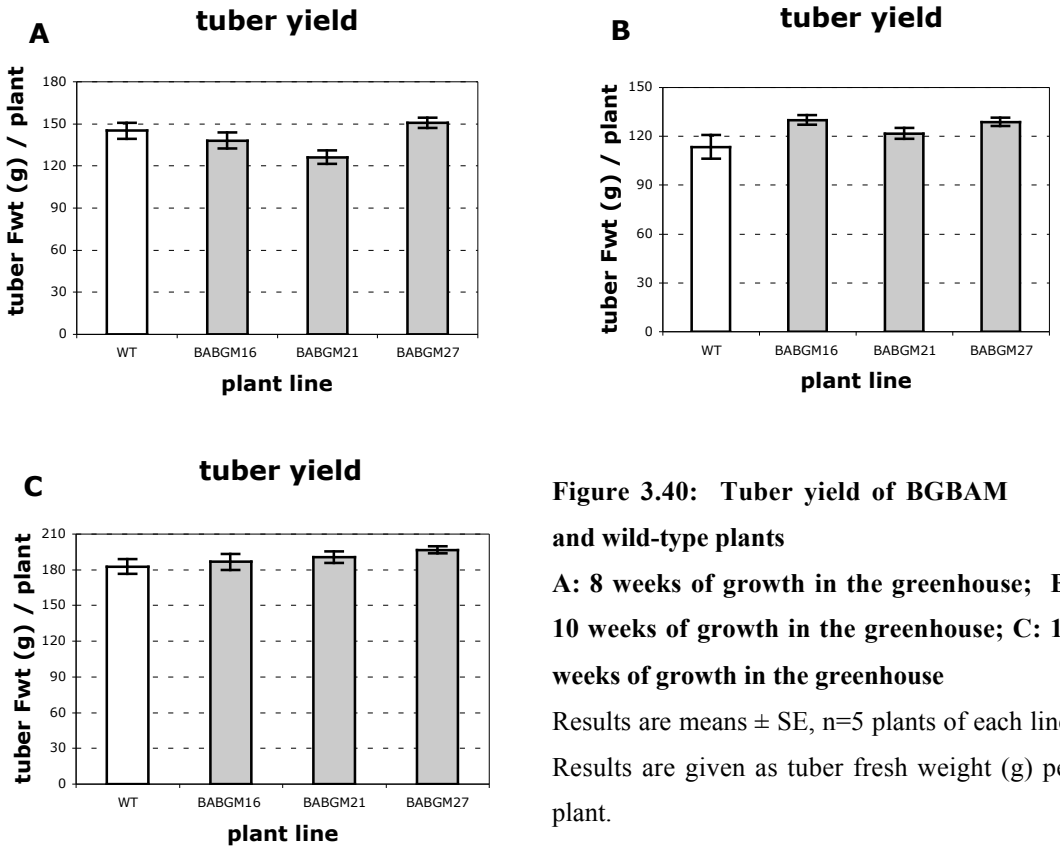


Figure 3.40: Tuber yield of BGBAM and wild-type plants

A: 8 weeks of growth in the greenhouse; B: 10 weeks of growth in the greenhouse; C: 12 weeks of growth in the greenhouse

Results are means ± SE, n=5 plants of each line. Results are given as tuber fresh weight (g) per plant.

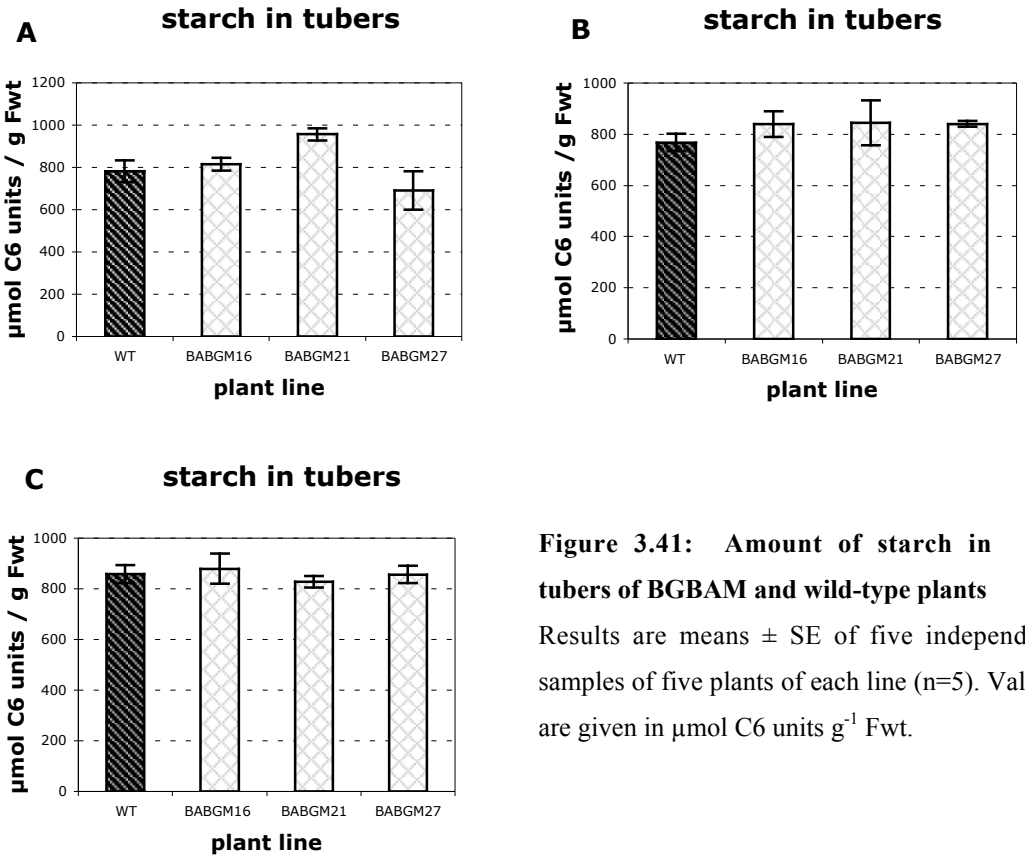


Figure 3.41: Amount of starch in tubers of BGBAM and wild-type plants
Results are means ± SE of five independent samples of five plants of each line (n=5). Values are given in µmol C6 units g^{-1} Fwt.

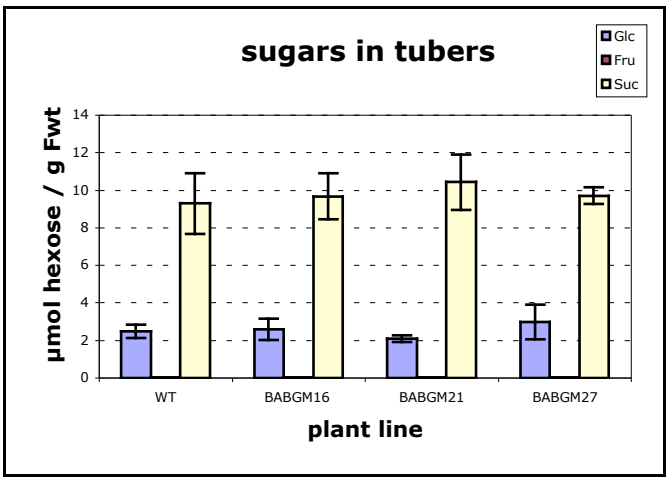


Figure 3.42: Soluble sugars in tubers of BGBAM and wild-type plants
One of three measurements is presented. Results are means ± SE of five independent samples of five plants of each line (n=5). Values are given in µmol hexose g^{-1} Fwt.

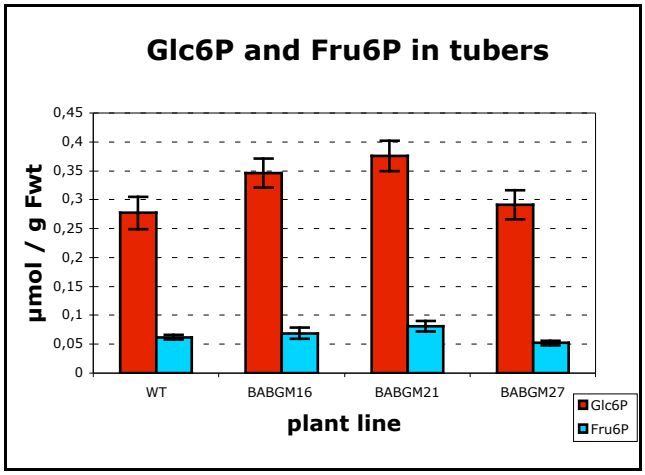


Figure 3.43: Phosphorylated intermediates in tubers of BGBAM and wild-type plants

Results are means ± SE of five independent samples of five plants of each line (n=5). Values are given in µmol g^{-1}Fwt.

3.7.2. Overexpression of the AtNTT in BG1 plants (BG1BA)

3.7.2.1. Analysis of AtNTT expression levels in BG1BA plants

B33-pBin19 containing AtNTT was transformed to *Agrobacterium tumefaciens*, the resulting *Agrobacterium* was used to transform BG1 plants (2.2.4.3). 36 kanamycin resistant individually transformed plants were regenerated in tissue-culture and transferred to the greenhouse. A fragment of expected size (about 1,9 kb) was amplified by PCR on BG1BA genomic DNA but not on DNA from wild-type leaves (data not shown). The accumulation of AtNTT mRNA in various lines of BG1BA plants was determined by Northern blot analysis. Several lines of BG1BA plants exhibited strong accumulation of AtNTT mRNA in tubers. In contrast to this, no AtNTT mRNA accumulated in tuber tissue from wild-type plants. Lines 24, 31 and 32 were used for subsequent analyses (Figure 3.44).

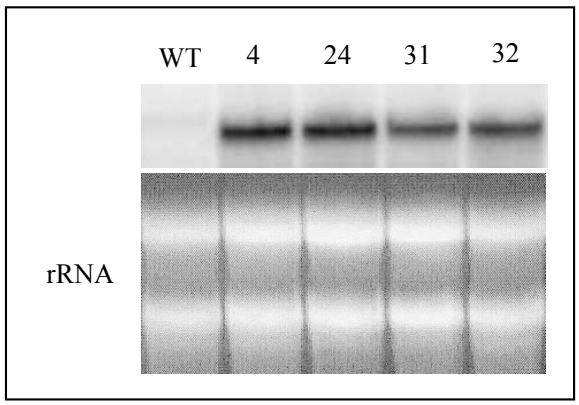


Figure 3.44: Northern blot analysis of tubers of BG1BA and wild-type plants

40 µg denatured total RNA was loaded in each lane, EtBr staining showed equal RNA loading. Probe: 1,9 kb AtNTT; WT: wild-type; 4, 24, 31, 32: BG1BA lines.

3.7.2.2. Analysis of activities of plastidic GPT and NTT in tubers of BG1BA plants

Since both pea GPT and AtNTT mRNA were detected in BG1BA plants, it is important to determine the activities of the plastidic glucose-6-phosphate/phosphate transloctor and the ATP/ADP translocator in these plants. To quantify the activity of GPT, proteins from tubers were reconstituted in proteoliposomes (2.2.14.1). Proteoliposomes were pre-loaded with phosphate, Glc6P and 3-PGA, respectively. BG1BA lines exhibited significantly increased GPT activities compared with the wild-type (Figure 3.45). To quantify the activity of the plastidic ATP/ADP translocator in BG1BA tubers, membrane proteins of enriched tuber membranes were reconstituted in proteolipsomes. Mitochondrial AAC activity is minimized by the addition of bongkrekic acid. This compound is known as an extremely specific inhibitor of adenylate transporter in animal and plant mitrochondia (Stubbs, 1998), but hardly affects the plastidic adenylate transporter (Schuenemann et al., 1993). Proteoliposomes were pre-loaded with ADP. BG1BA plants exhibited increased ATP uptake compared with wild-type plants in proteoliposomes containing membrane proteins where mitchondrial AAC activity was inhibited. Without inhibition of the AAC, the ATP uptake was about 10-fold higher, i.e. mitochondrial ATP transport activity is higher than plastid ATP transport activity. However, the relative ATP transport mediated by the plastidic NTT increased more than the total relative ATP transport in transgenic plants compared with the wild type (Figure 3.46 A and B).

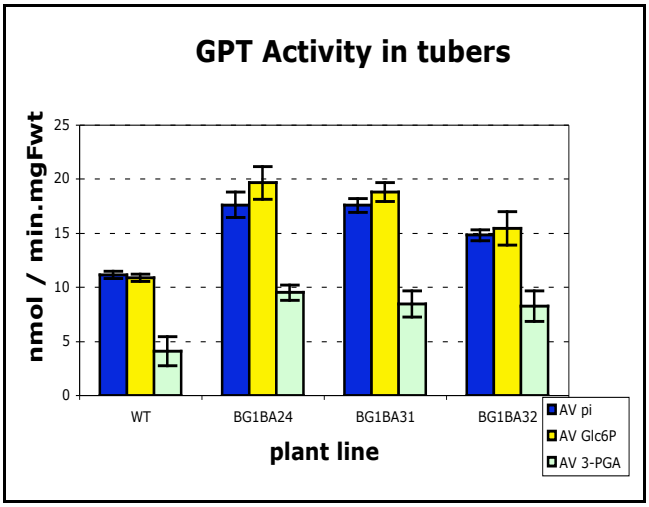


Figure 3.45: GPT activity in tubers of BG1BA and wild-type plants

All values are means ± SE of measurements made on three extracts from three different plants (n=3). Results are given as nmol $min^{-1}mg^{-1}$Fwt.

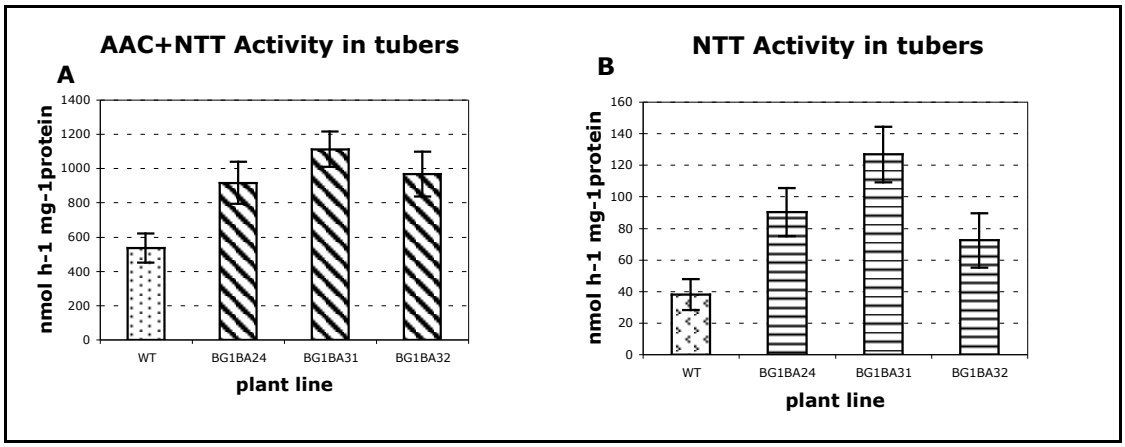


Figure 3.46: NTT activity in tubers of BG1BA and wild-type plants

A: AAC and NTT activity in tubers; B: NTT activity in amyloplasts of tubers

All values are means ± SE of measurements made on three extracts from three plants (n=3). Results are given as nmol $h^{-1}mg^{-1}$protein.

3.7.2.3. Analysis of BG1BA tuber yield

BG1BA plants were harvested after growth of 8, 10 and 12 weeks to measure the fresh weight of tubers. There was increased tuber yield in BG1BA plants compared with the wild type at every harvest except for BG1BA32 at 12 weeks (Figure 3.47).

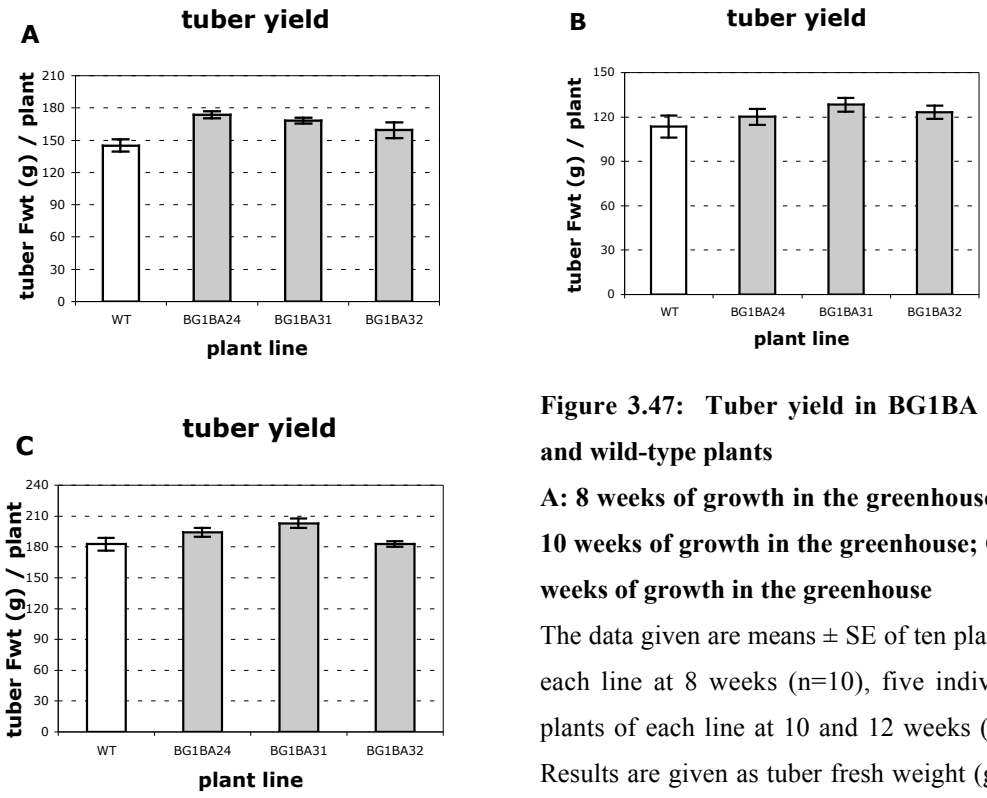


Figure 3.47: Tuber yield in BG1BA and wild-type plants

A: 8 weeks of growth in the greenhouse; B: 10 weeks of growth in the greenhouse; C: 12 weeks of growth in the greenhouse

The data given are means ± SE of ten plants of each line at 8 weeks (n=10), five individual plants of each line at 10 and 12 weeks (n=5). Results are given as tuber fresh weight (g) per plant.

3.7.2.4. Analysis of starch and soluble sugar contents of BG1BA tubers

Increased tuber yield was observed in BG1BA plants compared with the wild type. It was interesting to determine starch and soluble sugar contents of tubers in these plants after 8, 10 and 12 weeks of growth in the greenhouse (2.2.13.1 & 2.2.13.2). In plants of different age, tubers of BG1BA plants contained increased starch content compared with control plants (Figure 3.48). In order to assess the starch content in tubers of each plant, it was calculated as follows: amount of starch per gram multiplied with the tuber weight of the corresponding plant. The results indicated notably increased starch content per plant in BG1BA tubers compared with control plants (Figure 3.49). On the other hand, soluble sugar contents were not significantly changed in the transgenic plants of different age. Figure 3.50 (10 weeks) indicated that glucose was not significantly changed in BG1BA tubers, whereas one of three BG1BA lines examined exhibited increased sucrose amounts compared with control plants.

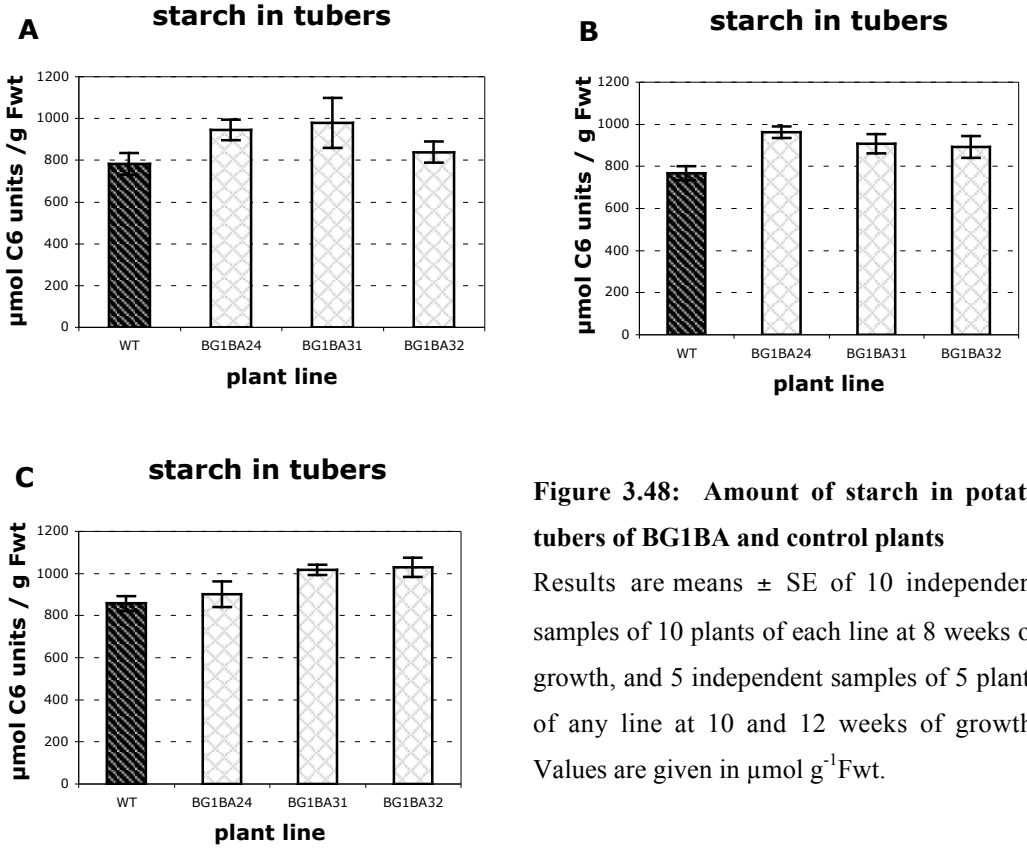
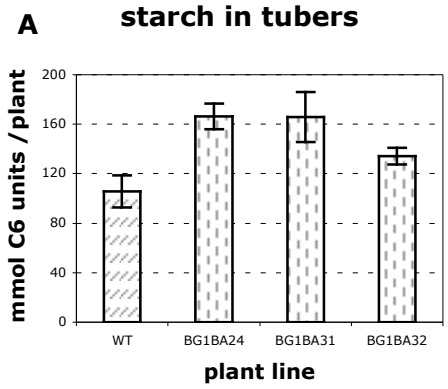


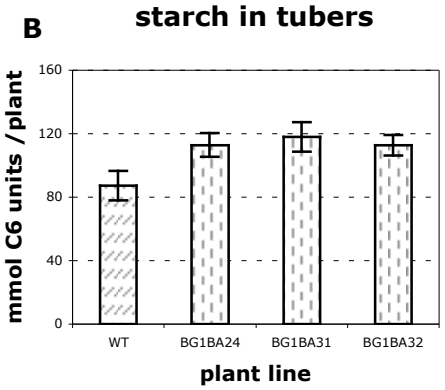
Figure 3.48: Amount of starch in potato tubers of BG1BA and control plants

Results are means ± SE of 10 independent samples of 10 plants of each line at 8 weeks of growth, and 5 independent samples of 5 plants of any line at 10 and 12 weeks of growth. Values are given in µmol g^{-1}Fwt.

3.7.2.5. Determination of phosphorylated intermediates in tubers of BG1BA plants

Two of three BGBAM lines examined exhibited increased Glc6P amounts compared with wild-type plants. Glc6P and Fru6P were also determined in BG1BA tubers when plants were grown in a greenhouse for 10 weeks. Tubers of BG1BA lines exhibited a reduction of Glc6P, while no strong change in Fru6P was observed compared with the wild type (Figure 3.51).





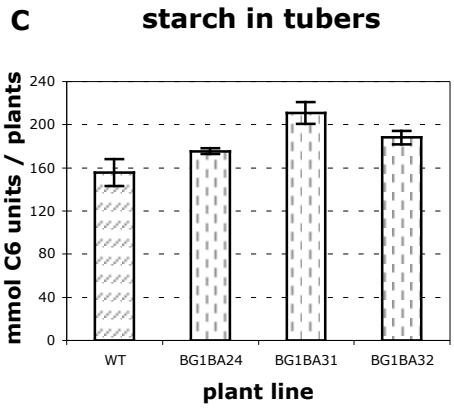


Figure 3.49: Amount of starch in tubers per plant in BG1BA and control plants

A: 8 weeks of growth in the greenhouse; B: 10 weeks of growth in the greenhouse; C: 12 weeks of growth in the greenhouse.

Results are means ± SE of 10 independent samples from 10 plants of each line at 8 weeks (n=10), and 5 independent samples of 5 individual plants of each line at 10 and 12 weeks (n=5). Values are given in mmol C6 units plant^{-1}.

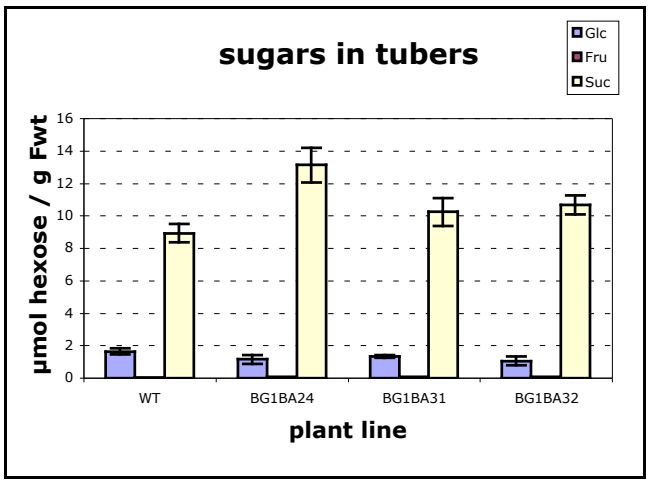


Figure 3.50: Soluble sugar amount in tubers of BG1BA and control plants

Results are means ± SE of 5 independent samples of 5 plants of each line at 10 weeks (n=5). Values are given in µmol g^{-1}Fwt.

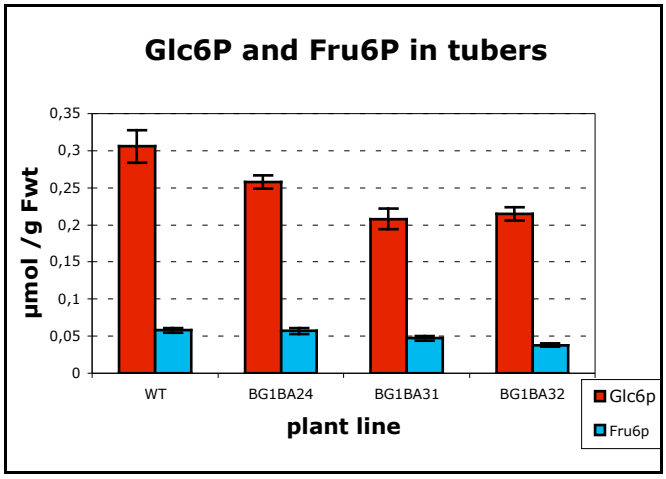


Figure 3.51: Phosphorylated intermediates of tubers in BG1BA and control plants

Results are means ± SE of 5 plants of each line at 10 weeks (n=5). Values are given in µmol g^{-1} Fwt.

3.7.2.6. Photosynthesis measurements of BG1BA plants

Photosynthetic parameters were measured for BG1BA plants after 10 weeks of growth in the greenhouse. Four plants of BG1BA31 and wild-type were selected for the experiment (2.2.16). There were no big differences in the rates of CO_2 assimilation and photosynthetic electron transport between BG1BA and wild-type plants (Figure 3.52 and 3.53).

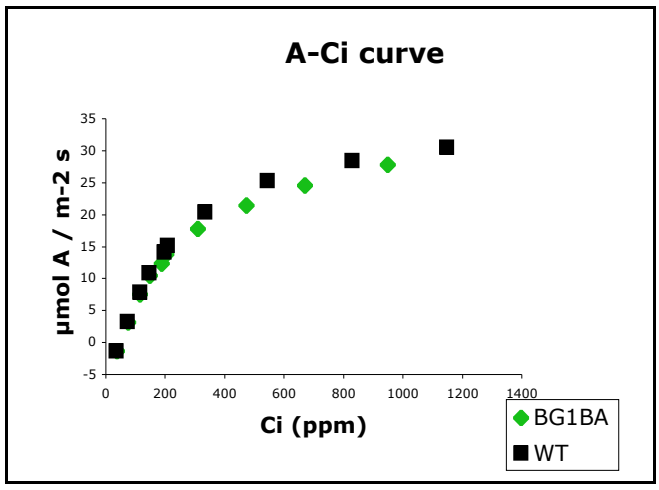


Figure 3.52: Assimilation-Ci curve in BG1BA and wild-type plants

The PFD was 827 µmol m^{-2} s^{-1}; A: assimilation; Values are given in µmol m^{-2} s^{-1}.

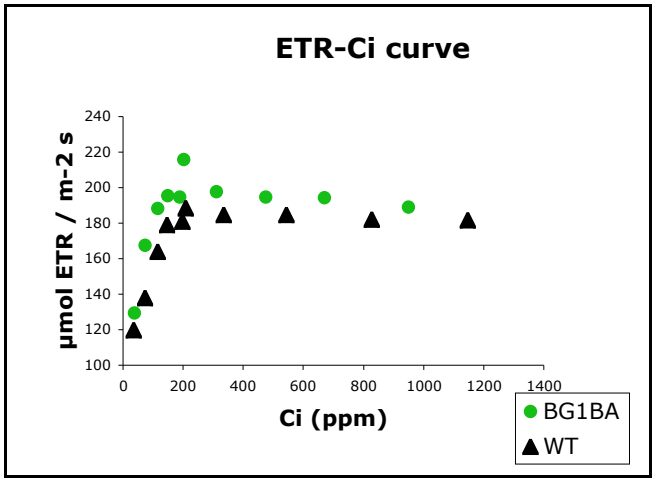


Figure 3.53: Electron transport-Ci curve in BG1BA and wild-type plants
The PFD was 827 µmol m^{-2} s^{-1}; ETR: electron transport; Values are given in µmol m^{-2} s^{-1}.

Together, BG1BA plants exhibited increased tuber starch and yield compared with wild-type plants. GPT and NTT can therefore co-limit starch biosynthesis in amyloplasts of potato tubers.

3.8. Overexpression of pea GPT and AtNTT under control of a bidirectionalized B33 patatin promoter

Since a polar promoter was first bidirectionalized in *Arabidopsis* (Xie et al., 2001), and patatin promoter B33 is mainly active in potato tubers, a bidirectionalized patatin promoter B33 was constructed to analyze if it is possible to direct the expression of two genes by one promoter in potato also.

3.8.1. Construction of a vector containing a bidirectionalized patatin promoter B33

A bidirectionalized CaMV 35S promoter worked in *Arabidopsis thaliana* (Xie et al., 2001). Patatin promoter B33 is mainly active in tubers. The bidirectionalized patatin promoter B33 was designed in studies. To prepare this construct, the B33-pBin19 was used as the basic vector. Another promoter (35S minimal promoter), a terminator (CaMV terminator) and a multiple cloning site (MCS) were introduced into this vector. In order to create a MCS between the 35S minimal promoter and the CaMV terminator, restriction enzyme sites were introduced between the 35S minimal promoter and the CaMV terminator. The promoter-MCS-terminator cassette was built

in a pBluescriptII (SK^-) vector.

The 35S minimal promoter was amplified from the plasmid pKannibal with primers containing SacI (primer 1) and StuI, SpeI (primer 2) restriction enzyme sites. The PCR product of the 35S minimal promoter was cloned into the pGEM T-easy vector, and then was cloned to the SacI/SpeI site of the pBluescriptII (SK^-) vector by digestion with SacI/SpeI. Furthermore, the CaMV terminator was amplified from the plasmid pCambia 1391XC with primers containing SpeI, AgeI (primer 3) and EcoRI (primer 4) restriction enzyme sites. The PCR product of the CaMV terminator was first cloned into the pGEM T-easy vector, and then into the SpeI/EcoRI sites of the pBluescriptII (SK^-) vector with digestion of the pGEM/CaMV plasmid by SpeI/EcoRI restriction enzymes. The 35S minimal promoter with a StuI site was then cloned into the plasmid containing the CaMV terminator with AgeI site by digestion with SacI/SpeI. The MCS (StuI, SpeI, AgeI) was created in the meanwhile. The pBluescriptII (SK^-) vector containing the 35S minimal promoter, MCS and CaMV terminator served as template to amplify the whole cassette with the primers (5/6) containing EcoRI sites. The PCR product of the 35S minimal promoter-MCS-CaMV terminator cassette was cloned into TOPO TA vector, and then cloned to the B33-pBin19 vector by digestion with EcoRI in the right direction (the 35S minimal promoter was connected to the B33 patatin promoter) giving the bidirectionalized B33-pBin19 vector (Figure 3.54). In this process, all PCR amplifications were performed using the proof reading Pfu DNA polymerase to avoid errors by PCR, and the 35S minimal promoter and CaMV terminator were sequenced in order to check for the advent of errors that may have arisen due to the PCR.

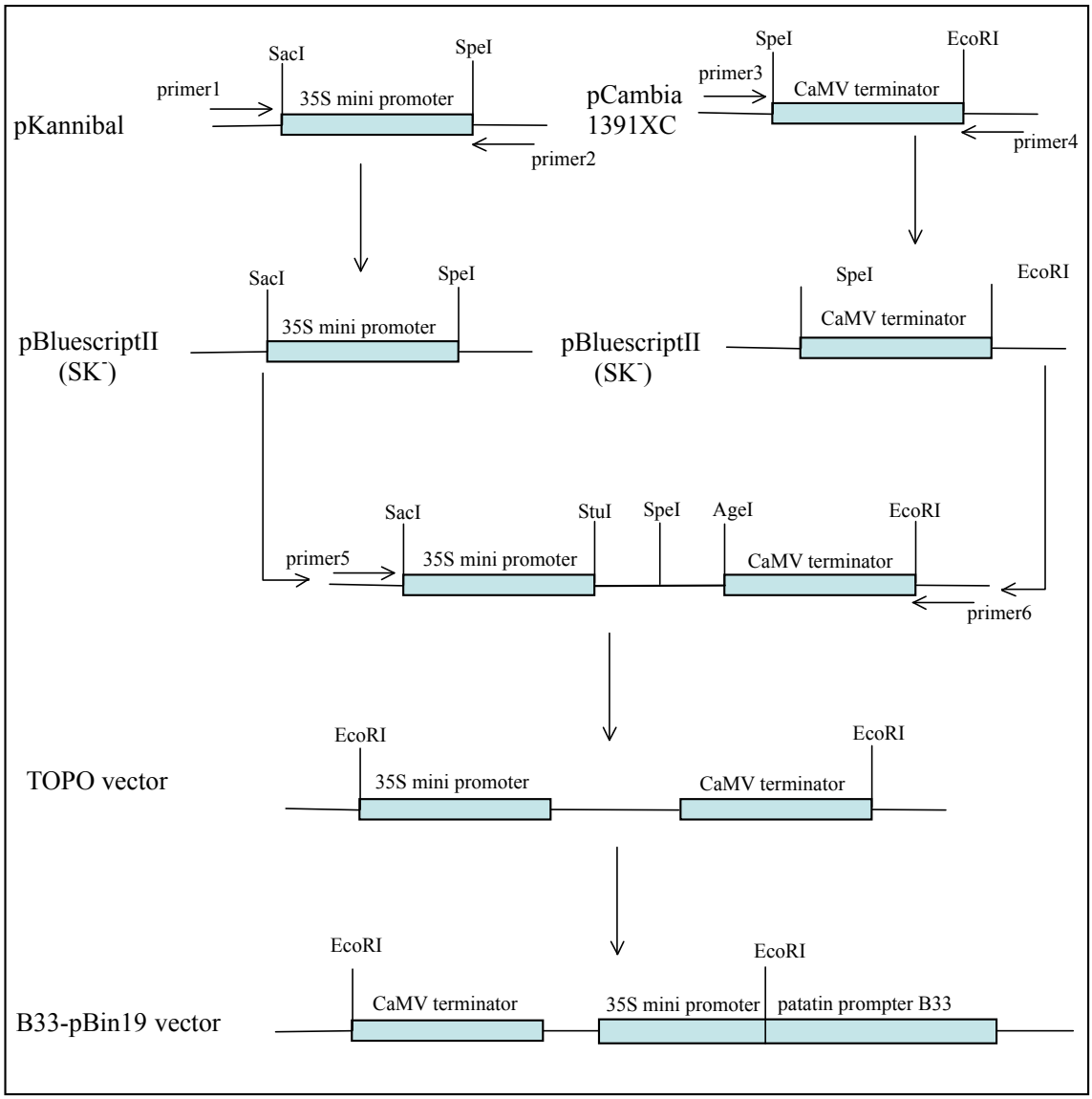


Figure 3.54: Construction of the bidirectionalized B33 patatin promoter

Primers 1/2: 35Smini promoter primers; Primers 3/4: CaMV terminator primers; Primers 5/6: 35Smini –CaMV cassette primers.

3.8.2. Cloning GUS and GFP into the bidirectionalized B33-pBin19 binary vector

In order to check if both parts of the bidirectionalized patatin promoter B33 are functional in potato plants, the reporter genes GUS and GFP were cloned into the bidirectionalized B33-pBin19 vector in two different directions, respectively. GUS and GFP fragments were amplified from the plasmid pCambia-1302 using primers containing BamHI (primer 7)/SalI (primer 8), and SpeI (primer 9)/AgeI (primer 10) restriction enzyme sites; GUS and GFP were subsequently cloned to TOPO TA vector independently, and were sequenced. GUS and GFP were excised from

corresponding TOPO/GUS and/or TOPO/GFP plasmids by digestion with either BamHI/SalI or SpeI/AgeI sites and were further cloned into the BamHI/SalI and SpeI/AgeI sites of the bidirectionlized B33-pBin19 vector, respectively. In construct A of the bidirectionalized B33-pBin19 vector, GUS was under control of the patatin promoter B33, and GFP was under control of the 35S minimal promoter. Moreover, another pair of complementary primers was designed for the amplification of GUS and GFP. Primers (11,12) containing SpeI/AgeI restriction enzyme sites were used to amplify GUS; primers (13, 14) containing BamHI/SalI restriction enzyme sites were designed to amplify GFP. Construct B was cloned in the above mentioned way. GFP was driven by patatin promoter B33, and GUS was expressed under control of the 35S minimal promoter in the bidirectionalized B33-pBin19 vector (Figure 3.55). Both of them were transformed to *Agrobacterium tumefaciens* strain GV2260. The resulting Agrobacterium strains were used to transform potato plants.

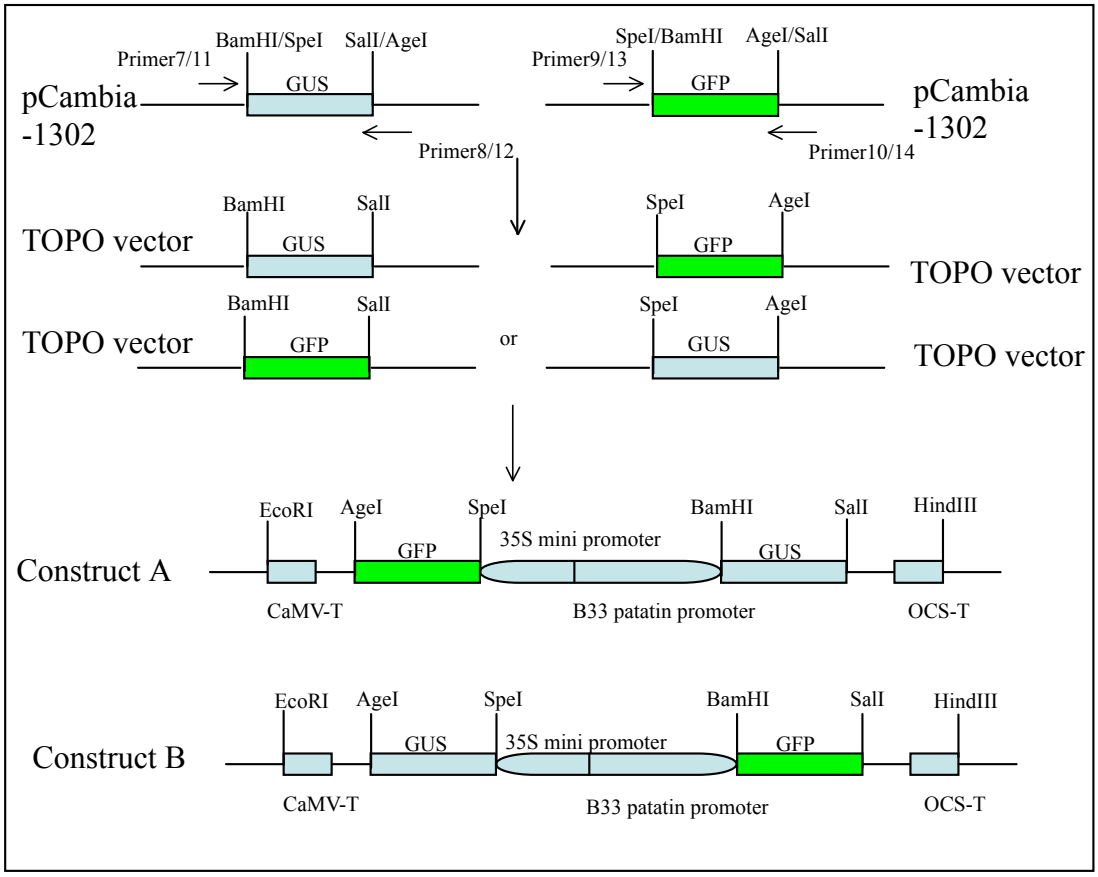


Figure 3.55: Construction of GUS / GFP into the bidirectionalized B33-pBin19 binary vector
Primers 7, 8, 9, 10 were used in construct A; primers 7/8: GUS primers; primers 9/10: GFP primers; primers 11, 12, 13, 14 were used in construct B; primers 11/12: GUS primers; primers 13/14: GFP primers.

3.8.3. Determination of GUS and GFP expression levels

The kanamycin resistant transformed plants harbouring construct A and B were regenerated from tissue-culture and transferred to the greenhouse. In plants carrying construct A, a histochemical analysis of transformed and untransformed potato tubers was carried out (2.2.17). The results revealed that signals of GUS staining were observed in 7 of 15 independently transformed plants, and no signals of GUS staining could be observed in untransformed tubers (Figure 3.56). In addition, no green fluorescence was detected in either transformed or untransformed tubers. GFP mRNA accumulation from tubers was below the detection limit in Northern blots. However, GFP specific primers 9/10 allowed to amplify a PCR product of the expected size on cDNA prepared from transformed tubers (Figure 3.57). On the other hand, a histochemical analysis of plant containing constract B did not yield a signal of GUS staining. In Northern blot experiments, GFP-specific transcripts could be detected, whereas GUS-specific transcripts were hardly detectable (Figure 3.58). However, a PCR product of 600 bp GUS on cDNA prepared from transformed tubers was amplified using GUS specific primers 11/11' (Figure 3.59). Taken together, the bidirectionalized patatin promoter B33 was not strong enough to drive strong gene expression in potato tubers.

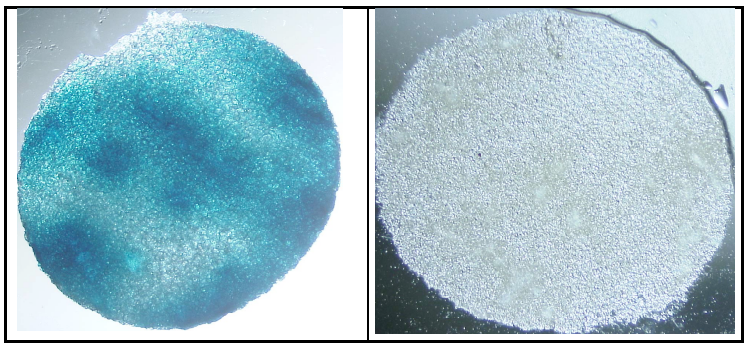


Figure 3.56: GUS staining of transformed and untransformed potato tubers

Left: a tuber cross-slice of transformed plants with construct A; Right: a tuber cross-slice of untransformed plants.

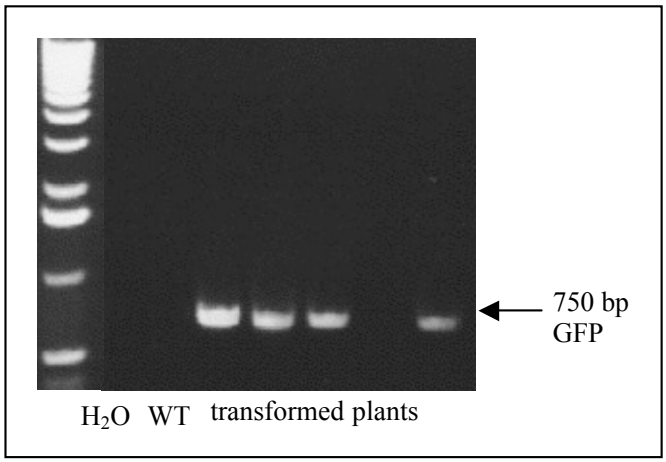


Figure 3.57: RT-PCR of tuber cDNA with construct A

5 µg total RNA was used for the reverse transcription reaction; PCR was carried out with 2,5 µl of 50 µl cDNA using GFP primers7/8.

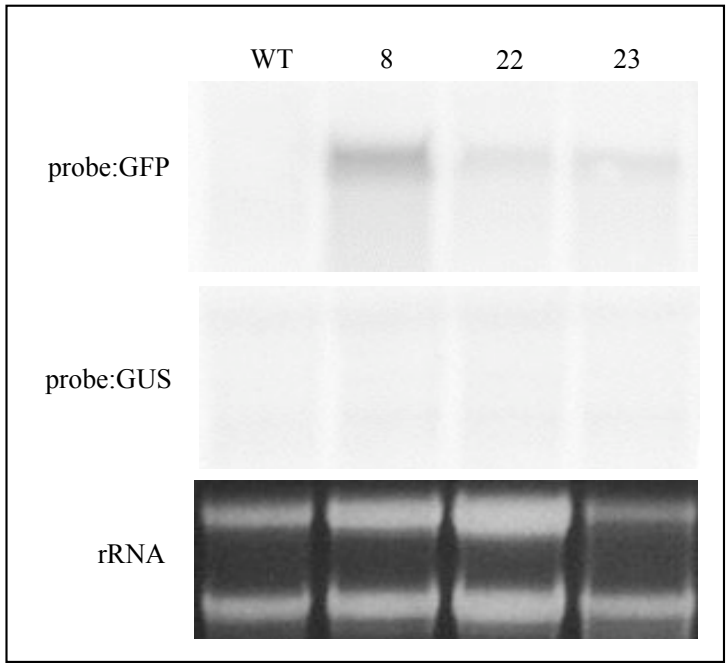


Figure 3.58: Northern blot analysis of tubers harbouring construct B

40 µg total RNA was load in each lane, EtBr staining showed equal RNA loading. Probe: 750 bp GFP fragment; WT: wild type ; 8, 22, 23: transformed plants.

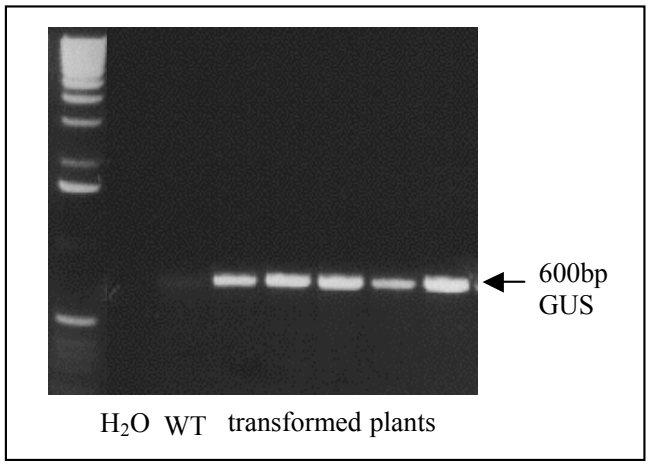


Figure 3.59: RT-PCR of GUS in tubers with construct B

5 µg total RNA was used for the reverse transcription reaction; PCR was carried out with 2,5 µl of 50 µl cDNA using GUS primers 11/11'.

3.8.4. Cloning of pea GPT and AtNTT into the bidirectionalized B33–pBin19 binary vector

A 1,6 kb pea GPT fragment was amplified from a B33-pBin19 vector containing pea GPT using primers 15 and 16. The PCR product was purified from the gel and cloned into the TOPO TA vector. The TOPO/pea GPT plasmid was digested with SpeI and AgeI restriction enzymes and subsequently cloned into the SpeI/AgeI sites of the bidirectionalized B33-pBin19 vector. A 1,9 kb AtNTT fragment was digested with BamHI and SalI restriction enzymes from TOPO/AtNTT plasmid, and cloned into these two sites of the bidirectionalized B33-pBin19 binary vector. In this construct, the pea GPT gene was under control of the 35S minimal promoter, the AtNTT gene was under control of the patatin promoter B33 (Figure 3.60).

3.8.5. Analysis of pea GPT and AtNTT expression levels

The bidirectionalized B33-pBin19 binary vector containing AtNTT/pea GPT was transferred to *Agrobacterium tumefaciens* GV2260 by electroporation (2.2.4.2.2). The resulting Agrobacterium strain was used to transform wild-type potato plants (2.2.4.3). 39 kanamycin resistant individual plants were regenerated from tissue-culture and transferred to a greenhouse. By use of pea GPT and AtNTT specific primers, the expected PCR products were able to amplify on genomic DNA of transformed leaves but not on genomic DNA of wild-type (data not shown). To determine the transcript levels of pea GPT (13/14) and AtNTT (NTT1/NTT2),

Northern blots were performed with total RNA from transformed and untransformed tubers. The results showed no transcripts specific for pea GPT and/or AtNTT in wild-type plants. Different AtNTT expression levels were found in transformed tubers, whereas faint signals were detected in all transformed plants in Northern blots hybridized with a pea GPT specific probe (Figure 3.61 A). In order to know if there was really pea GPT expression in transformed tubers, an RT-PCR was performed. An expected PCR product on cDNA prepared from transformed tubers was observed (Figure 3.61 B).

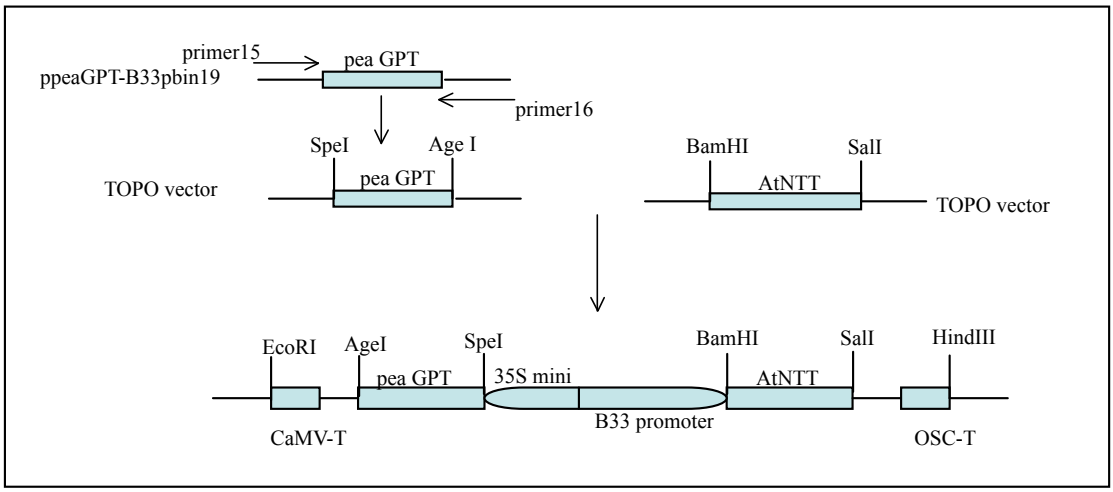


Figure 3.60: Construction of AtNTT/pea GPT in the bidirectionalized B33-pBin19 vector
Primers 15/16: pea GPT primers.

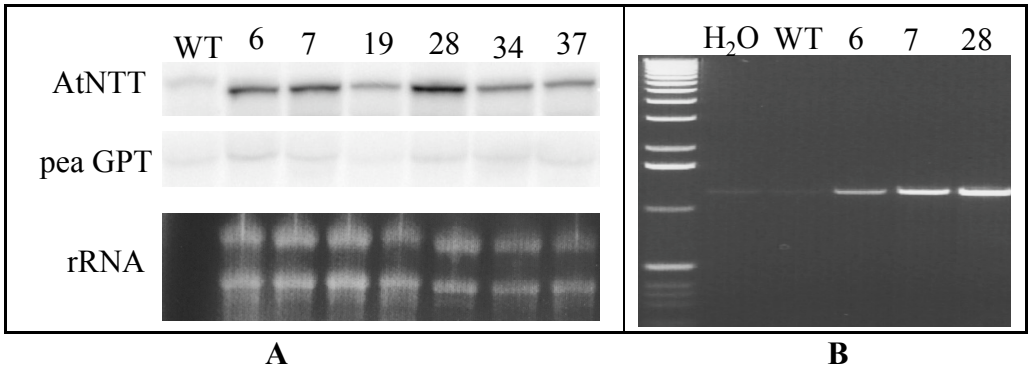


Figure 3.61: Expression analysis of potato tubers containing the construct of pea GPT/ AtNTT in the bidirectionalized B33-pBin19 binary vector
A: Northern blot analysis of tubers; B: RT-PCR analysis of tubers
40 µg total RNA was loaded in each lane, and EtBr staining indicated equal RNA loading. 5 µg total RNA was used for reverse transcription reaction, PCR was carried out with 2,5 µl of 50 µl cDNA using pea GPT primers 15/16. Probes: 1,6 kb pea GPT and 1,9 kb AtNTT fragments. WT: wild type; 6, 7, 19, 28, 34, 37: transformed plants.

Since the bidirectionalized patatin promoter B33 was not strong enough to efficiently drive gene expression in potato tubers, transgenic plants harbouring the bidirectionalized B33-pBin19 vector containing pea GPT and AtNTT were not analyzed further at the physiological level.

4. DISCUSSION

4.1. Potato transformation

Potato is the fourth most important crop in the world behind the three cereals maize, rice and wheat (FAO production yearbook, 1999). Furthermore, it is also a model system since this species is relatively easy to transform with chimeric genes (Rocha-Sosa et al., 1989) and because tubers of potato represent typical starch-storing storage sinks that accumulate high levels of starch in their amyloplasts (Mueller-Roeber et al., 1992; Frommer and Sonnewald, 1995). Potato transformation is highly unpredictable with respect to the integration and expression of transgenes and the frequency of somaclonal variation among transgenic lines. It is therefore necessary to select and regenerate numerous independently transformed potato lines to recover several lines with appropriate expression of the transgene and physiological characteristics (Conner and Christey, 1994). GPT and NTT play important roles in starch synthesis in amyloplasts of potato tubers. Several transgenic potato plants overexpressing pea GPT and AtNTT have been created. Kanamycin and/or hygromycin resistant transformed plants were regenerated from tissue culture and transferred to a greenhouse. Transgenic lines were screened from 30-40 independently transformed plants by PCR and Northern blot analysis. However, the integration of transgenes into the potato genome may take place at one or more sites in a complete, truncated or rearranged way, but single copies are preferred (Lindesy et al., 1993; Conner et al., 1993), although sometimes more than one copy was detected in some transgenic potato plants (Ludewig, pers. comm.). In addition, potato plants are always propagated from tubers with genetically stable perpetuity (Shepard et al., 1980). Therefore, it is not necessary to monitor the copy number of chimeric genes in potato plants.

4.2. Comparison of the three promoters used in transformants

Promoters have been cloned and widely used for biotechnological applications in plants. They are normally unidirectional, directing only one gene that has been fused at its 3' end. The CaMV 35S promoter provides a model plant nuclear promoter system (Odell et al., 1985). Although the CaMV 35S promoter showed no tissue-specificity of expression, the 'full-length' 35S promoter is more active in leaves than in tubers in potato plants (Jefferson et al., 1990). This is consistent with the result that there was more mRNA accumulation of pea GPT in leaves than in tubers when pea

GPT was overexpressed under control of the CaMV 35S promoter (EW plants) (Figure 3.8).

Patatin appears to serve as a storage protein in potato tubers. Although the formation of storage organs (tuberization) by potato plants is always associated with a high level of patatin expression in tubers, this does not mean that this is strictly dependent on tuber-specific factors since patatin synthesis has also been observed in other organs under a variety of conditions (Rocha-Sosa et al., 1989). Racusen (1983) reported that patatin accounted for 40% of the total soluble protein in stems and 7% of the total soluble protein in leaves of the cultivar Kennebec grown under field conditions. The B33 patatin class I gene carries a promoter that is mainly active in tubers. However, the mRNA of chimeric genes under control of the patatin promoter B33 can be accumulated in other organs in some growth condition (Wenzler et al., 1989; Gerrits et al., 2001), e.g. cultivating potato plants expressing a transgene under the control of this promoter in small pots led to the accumulation of large amounts of chimeric gene mRNA in leaves and stems because they became severely root-bound before tuberization (Wenzler et al., 1989). The investigation of BA plants that overexpressed AtNTT under control of the patatin promoter B33 is consistent with these results. Transcripts of AtNTT were detectable also in leaves of these plants (BA plants, data not shown). This is probably because these plants were grown in pots. However, the amount of RNA in leaves was much less compared with tubers as shown by Northern blot analysis.

Although promoters are generally unidirectional, bidirectional promoters are desirable to minimize or avoid transcriptional gene silencing caused by the repeated use of a single promoter when multiple genes are introduced into plants. Xie et al. (2001) bidirectionalized polar promoters, which were shown to function in *Arabidopsis*. Since pea GPT and AtNTT were expected to be introduced into potato tubers simultaneously, a bidirectionalized patatin promoter B33 was designed. Because the analysis of promoter-reporter gene fusions is one of the most widely used techniques for identifying sequences that control the temporal and spatial regulation of cloned genes (Taylor, 1997), GUS and GFP reporter genes were cloned behind and directed by the bidirectionalized patatin promoter B33. In potato tubers, there were no detectable GUS staining or green fluorescent signals when either GUS or GFP was under control of the 35S minimal promoter part of the bidirectionalized patatin promoter B33. However, transcripts of both genes could be detected in tubers by RT-

PCR (Figures 3.57, 3.59). This indicates that the 35S minimal promoter was not strong enough to mediate strong gene expression in tubers. On the other hand, no green fluorescence could be observed, while signals of GUS staining were found in corresponding transformed tubers when GFP or GUS were directed by the patatin promoter B33 part of the bidirectionalized patatin promoter B33. Transcripts of GFP could be detected in these tubers by Northern blot analysis although the signal was weak. To be able to detect GUS gene expression, more than 100 molecules need to be present in the cell, whereas for a GFP signal more than 10 000 molecules are needed (Koehler, 1998). However, in principle GFP can be used as a reporter in amyloplasts of tubers. Hibberd et al. (1998) reported that green fluorescence was detectable in amyloplasts of potato tubers following microprojectile bombardment when the *gfp* coding region was under control of the tobacco chloroplast ribosomal RNA (rrn) promoter, indicating that the rates of transcription and translation were sufficient for GFP to be detected. This is because that there is a large difference in overexpressing a gene under control of a nuclear promoter compared with a plastidic promoter. In the latter case, much more protein can be yielded than in the former case (Syab and Maliga, 1993). Overall, the bidirectionalized patatin promoter B33 was not qualified for driving gene expression in two directions simultaneously in potato tubers.

4.3. Comparison of transgenic potato plants with different plastidic transporters involved in starch synthesis

4.3.1. Comparison of potato plants overexpressing the AtNTT under control of different promoters

The site of starch synthesis is the plastids, either photosynthetic or non-photosynthetic ones. It is generally accepted that autotrophic and heterotrophic plastids possess an ATP-translocator protein (Heldt, 1969). In chloroplasts, the role of this protein is unknown; in amyloplasts or chromoplasts, it catalyzes the import of ATP (Kleinig and Liedvogel, 1980; Neuhaus et al., 1993a). Starch is synthesized by two different metabolic pathways in heterotrophic storage tissues. In cereals such as maize or barley, the endosperm contains a cytosolic AGPase (Denyer et al., 1996; Thorbjørnsen et al., 1996) that synthesizes ADPG which is subsequently imported into the amyloplasts (Moehlmann et al., 1997). In dicotyledonous plants, AGPase is exclusively located in the amyloplastic stroma (Emes and Neuhaus, 1997) making it

necessary to import ATP in addition to hexose phosphates for ADPG synthesis. In potato tubers, ATP is synthesized in the mitochondria, subsequently transported into the cytosol, and from there into the amyloplast via an ATP/ADP translocator (Heldt, 1969; Schuenemann et al., 1993; Kampfenkel et al., 1995; Tjaden et al., 1998a). The endogenous potato ATP/ADP translocator (Kampfenkel et al., 1995; Moehlmann et al., 1998) mRNA accumulated most strongly in tuber tissues suggesting that the provision of ATP for amyloplasts during starch synthesis is important for the starch content (Tjaden et al., 1998a). Tjaden et al. (1998a) reported that transgenic potato plants overexpressing AtNTT under control of the CaMV 35S promoter exhibited a significant increase of starch content in one (JT62 line: 36% above wild type) of three lines examined, and tuber yield was decreased in all these three lines compared with the wild type, while starch biosynthesis was inhibited in all antisense lines with decreased StNTT translocator activities. Moreover, amylose correlated with altered starch levels. The amylose content in antisense lines with low starch was decreased, whereas it was increased in sense lines (Tjaden et al., 1998a). However, only JT62 tubers contained significantly more starch than wild-type plants, this may be due to the integration site of the chimeric gene in the potato genome. The previous results indicated that the plastidic ATP/ADP transloctor limits starch biosynthesis *in vivo* (Tjaden et al., 1998a), but it may be not the only factor for controlling starch synthesis in amyloplasts of potato tubers. However, JT62 plants were observed to be smaller than wild-type plants, and younger leaves were crimple and yellowish under some growth condition (Figures 3.29, 3.30). This may lead to a reduced source capacity, e.g. less photoassimilates can be transported to the sinks. In order to avoid the leaf phenotype and increase tuber yield, transgenic plants overexpressing AtNTT under control of the patatin promoter B33 (BA plants, see 3.3) were created because this promoter is much more active in tubers than in other organs. In these plants, there were no observable phenotypical changes in leaves. The introduction of a chrimeric gene containing the coding sequence of the plastidic ATP/ADP translocator led to the formation of the appropriate mRNA in heterotrophic tissues (Figure 3.4). However, BA plants did not exhibit significant changes in tuber yield and starch content compared with wild-type plants (Figures 3.5, 3.6 A-C). The reasons could be that (I) ATP is not the sole limiting factor for starch synthesis in amyloplasts, (II) the patatin promoter B33 is not as strong as the CaMV 35S promoter although it is mainly active in tubers.

Conversion of Suc to starch is ATP dependent. Soluble sugar contents were analyzed in BA tubers. The results showed that glucose and sucrose were not significantly changed in BA compared with wild-type plants. These results are in agreement with the conclusion drawn from plants overexpressing the AtNTT under control of the CaMV 35S promoter. Geigenberger et al. (2001) showed that supply and initial mobilization of Suc does not restrict starch synthesis in AtNTT sense tubers. However, soluble sugars were increased markedly in AtNTT antisense tubers compared with the wild-type (Geigenberger et al., 2001).

The altered plastidic NTT translocator activity might also induce changes in the concentration of other metabolites known to be involved in starch synthesis. There were significantly decreased levels of Glc1P, Glc6P and Fru6P in antisense plants with decreased NTT translocator activity, while sense plants with increased NTT translocator activity did not exhibit differences in hexose phosphate levels compared with wild-type plants (Tjaden et al., 1998a; Geigenberger et al., 2001). In BA plants, no significant change in Fru6P was observed, while BA8 and BA12 contained decreased Glc6P levels compared with wild-type plants (Figure 3.7). Since BA plants did not exhibit increased tuber starch and yield compared with wild-type plants, they were not analyzed further.

4.3.2. Comparison of potato plants overexpressing pea GPT under control of different promoters

Starch biosynthesis takes place exclusively in plastids that are the sole location of starch synthases and starch-branching enzyme (Preiss, 1997). In leaves, starch is synthesized transiently in the light. In potato tubers, starch is synthesized and stored in amyloplasts. Starch synthesis within the plastids is completely dependent on the import of carbon from the cytosol, where it is initially delivered as sucrose from the phloem. There have been various reports that hexose phosphates, free sugars (Gerrits et al., 2001) and ADPG (Moehlmann et al., 1997) can be imported from the cytosol. The form in which carbon enters amyloplasts appears to be species-dependent (Naeem et al., 1997). In the developing endosperm of wheat and in soybean suspension cultures, Glc1P can support starch synthesis (Coates and ap Rees, 1994; Tetlow et al., 1994; Tyson and ap Rees, 1988). Glc6P is shown to be the preferred substrate for starch synthesis and not Glc1P in cauliflower bud amyloplasts (Neuhaus et al., 1993b), pea cotyledons (Hill and Smith, 1991), pea roots (Bowsher et al., 1989, 1992;

Borchert et al., 1989,1993), pea leaf guard cells (Overlach et al., 1993) and developing pea and rapeseed embryos (Hill and Smith, 1991; Kang and Rawsthorne, 1994). Analysis of starchless mutant lines that are deficient in the plastidic PGM (catalyzing the interconversion of Glc6P and Glc1P) further confirmed that Glc6P is the sole precursor for starch synthesis in most plants (Harrison et al., 2000; Kofler et al., 2000). In potato tubers, Glc6P is imported into amyloplasts (Mueller-Roeber et al., 1992; Schott et al., 1995).

Since GPT is thought to be important in controlling the supply of carbon precursors for starch synthesis in potato tubers, the pea GPT was overexpressed under control of the CaMV 35S promoter (EW plants, see 3.4) and patatin promoter B33 (BG plants, see 3.5), respectively. The introduction of the pea GPT led to the formation of the appropriate mRNA in green and/or heterotrophic tissues (Figures 3.8 A, B, 3.20). Under normal growth conditions, there are two endogenous GPTs (StGPT1 and StGPT2) in potato plants. When plants are treated with pathogen, the third one (StGPT3) can be detected (Ludewig, pers. comm.). In EW plants, there were no significant effects on either StGPT1 or StGPT2 mRNA accumulation (Figure 3.9), where a decreased transcript level of StGPT1 and StGPT2 was found in three of five BG lines examined compared with wild-type plants (Figure 3.21). The reason is unclear. However, pea GPT integrated in the potato genome may not have impact on the RNA level, but on e.g. the protein level.

The mode of GPT action is a 1:1 exchange of Glc6P mainly with inorganic phosphate and trioseP or an exchange of trioseP with inorganic phosphate. 3-PGA is a poorer substrate of the GPT (Kammerer et al., 1998). However, 3-PGA can be transported by the GPT under physiological conditions when the concentration of 3-PGA in the tissue is at least one order of magnitude higher than that of trioseP (Liu and Shannon, 1981). GPT proceeds via a ping-pong type of reaction mechanism, i.e. the first substrate is transported across the membrane before the second substrate can be bound and transported (Flügge, 1992). In proteolipsomes, an increased uptake of Glc6P, Pi or 3-PGA was detected in BG plants (Figure 3.22), i.e. the newly introduced pea GPT mRNA obviously led to an increased activity of plastidic GPT protein in tubers. This observation demonstrated that the heterologously synthesized transporter protein from pea is functionally integrated into the plastid envelope.

GPT belongs to the nucleotide sugar transporter (NST) / TPT superfamily. The nuclear-encoded plastidic proteins acquire an N-terminal extension (the transit

peptide) that directs the attached protein to the plastids (Knappe et al., 2003). Although GPT expression is mainly restricted to heterotrophic tissues (Kammerer et al., 1998), GPT can be localized in chloroplast when it is overexpressed under control of the CaMV 35S promoter. It is worth to analyze the diurnal variations in starch and soluble sugar contents of EW leaves. Starch is synthesized in leaves during the day from photosynthetically fixed carbon and is mobilized at night. It is believed that leaf starch synthesis is regulated by changes in the levels of phosphorylated metabolites and P_i that are generated when the rate of photosynthesis increases or when rising levels of sugars lead to feedback regulation of sucrose synthesis (Hendriks et al., 2003). The results indicate that EW leaves contained more starch than wild-type leaves during the daycourse (Figure 3.11). In chloroplasts, Glc6P is converted from Fru6P produced by the Calvin cycle, and Glc6P is a precursor for transitory starch synthesis. In EW leaves, there are two ways to form Glc6P. One part of Glc6P is formed from Fru6P; another part of Glc6P could be imported from the cytosol by the overexpressed pea GPT. Increased Glc6P content probably led to an increased starch content in EW leaves compared with wild-type leaves. The result of the $^{14}CO_2$ partitioning experiment indicates that more carbon was directed into starch biosynthesis in EW leaves (Figure 3.14). Photosynthetic carbon metabolism is a branched pathway leading to either sucrose or starch. In the simplest case, a decreased flux through one branch will be accompanied by a compensating increase in the flux through the other branch, and photosynthesis would be unaffected (Neuhaus et al., 1990). EW plants did not exhibit a different biomass of the aerial part (data not shown) and tubers compared with wild-type plants. There were also no differences in the rates of CO_2 assimilation and photosynthetic electron transport in saturated CO_2 between EW and wild-type plants (Figures 3.18, 3.19). The results reveal that an increase in starch synthesis is accompanied by a compensating decrease of sucrose synthesis from morning to 2:00 pm in EW leaves compared with wild-type plants (Figures 3.11, 3.12). This may result in an unchanged photosynthesis rate.

During photosynthesis, trioseP and 3-PGA are exported to the cytosol where they are converted to end products, including sucrose (Edwards and Walker, 1983). Some of the photosynthate is retained in the chloroplast to synthesize starch. Leaf starch represents a transient store, which is remobilized during the night to support leaf metabolism, and continued synthesis and export of sucrose (Geiger and Servaites, 1994; Caspar et al., 1986; Schulze et al., 1991; Geiger et al., 1995; Sun et al., 2002).

Sucrose is unloaded into storage tissues. From 2:00 pm to the evening, decreased levels of sucrose was observed in wild-type leaves (Figure 3.12 C). This may be because the rate of sucrose transported from source leaves to sink tissues is faster than the rate of sucrose synthesis. In contrast, increased sucrose was observed in EW leaves from 6:00 pm to the evening compared with wild-type plants (Figure 3.12 C). Since transitory starch is degraded in the dark, starch degraded and exported into cytoplasm probably led to increased sucrose synthesis in EW leaves compared with wild-type leaves. EW leaves contained increased Glc6P, Fru6P and Glc1P amounts compared with wild-type leaves, particularly during the dark period (Figures 3.13 A, B, C). The explanation could be, first, during the light, Glc6P was imported to chloroplasts as a precursor for starch biosynthesis, thereby the conversion of Fru6P to Glc6P was probably inhibited. Second, starch was degraded during the dark and products (maltose and glucose) were exported from chloroplasts to the cytosol. Increased starch was observed in EW leaves compared with wild-type leaves. In the cytosol, the exported starch degradation products finally fed up to the hexose phosphate pool. Therefore, increased Fru6P, Glc6P and Glc1P levels were detected in EW leaves. Moreover, in leaf tissue, 3-PGA is the primary product of photosynthesis, and is known to be exported from the chloroplasts via the TPT (Heldt and Rapley, 1970). Increased 3-PGA levels were observed at around 2:00 pm, while the level of trioseP was decreased at all time points in EW leaves compared with wild-type leaves. These probably can be explained as follows: Since Glc6P can be imported into the chloroplasts from the cytosol in EW leaves for transitory starch synthesis, conversion of Fru6P to Glc6P in plastids was circumvented. 3-PGA and/or trioseP were also accumulated since Fru6P was formed from 3-PGA and/or trioseP by several steps in the Calvin cycle. TPT has the same affinity towards trioseP and 3-PGA, whereas GPT has a lower affinity towards 3-PGA (Kammerer et al., 1998). More trioseP could be exported from the chloroplast to the cytosol than 3-PGA by the GPT. In the cytosol, trioseP was used to produce Glc6P in the sucrose biosynthesis pathway and Glc6P was continuously imported into chloroplast by the GPT. As a result, less trioseP and more 3-PGA were detected in EW leaves compared with wild-type plants.

In EW and BG plants, there were no significant changes in tuber starch content and yield compared with wild-type plants. However, RNAi plants with decreased StGPT1 and StGPT2 expression exhibited notably decreased tuber starch and yield (Ludewig, unpublished data). These results indicate that *in vivo* the plastidic GPT can limit starch

biosynthesis in potato tubers. Taken together, Glc6P is not the sole limiting factor for starch synthesis in amyloplasts and increasing only the Glc6P content in plastids by overexpressing the pea GPT does not lead to increased tuber starch and yield.

4.3.3. Comparison of potato plants overexpressing pea GPT under control of different promoters in JT62 plants

As mentioned above, JT62 plants contain more tuber starch and give less yield than wild-type plants. Since GPT also can affect starch biosynthesis in amyloplasts, pea GPT was overexpressed under control of the CaMV 35S promoter (SA62SG plants, see 3.6.1) and patatin promoter B33 (SA62BG plants, see 3.6.2) in JT62 plants, respectively. The introduction of pea GPT led to the formation of the appropriate mRNA in green and/or heterotrophic tissues (Figures 3.27, 3.28, 3.34). However, pea GPT mRNA levels were abundant in SA62SG7 leaves, whereas scarce pea GPT transcript level was detected in SA62SG7 tubers. The reason for this is unknown. JT62 plants exhibit yellowish and crimple younger leaves and are smaller than wild-type plants (Figure 3.30). SA62SG and SA62BG plants had the same phenotype as JT62, suggesting that overexpression of another starch limiting factor (GPT) in JT62 plants could not help to escape the leaf phenotype. Yellowish and crimple leaves of JT62, SA62SG and SA62BG might lead to plants smaller than wild-type plants. The reason may be that abnormal leaves cause less total photoassimilate amounts. However, SA62SG were slightly bigger than JT62 plants (Figure 3.29), while SA62BG were of similar size than JT62 plants (Figure 3.35). During development, crimple and yellowish leaves disappeared earlier in SA62SG than in SA62BG plants. This might be caused by different promoters used in these plants. CaMV 35S promoter shows no tissue-specific expression, whereas patatin promoter B33 is mainly active in tubers. Since CaMV 35S promoter can drive gene expression in leaves, this may be the reason that the leaf phenotype disappeared earlier in SA62SG than in SA62BG plants. In SA62SG plants, in which the pea GPT was overexpressed in leaves under control of the CaMV 35S promoter, more Glc6P was imported into chloroplasts. Taken together, with increased ATP, Glc6P can be used for transitory starch synthesis, and increased ADP and Pi are produced in this process. In SA62BG and JT62 plants, increased ATP could not match increased Glc6P in chloroplasts for starch biosynthesis. The situation of ADP and Pi were changed in SA62SG compared with SA62BG and JT62 plants.

Altered plastidic transporter activities might induce changes in the content of starch and tuber yield as well as other metabolites known to be involved in starch synthesis. In SA62SG leaves (e.g. line 17, 26), the starch content was increased; glucose was not changed, whereas sucrose was increased compared with control leaves (Figures 3.32 A, 3.33 A). These results are in agreement with the results of EW leaves, suggesting that the impact was caused by GPT in SA62SG leaves. On the other hand, glucose and sucrose were decreased in SA62SG tubers (line 17, 26) compared with control plants (wild-type and JT62 plants) (Figure 3.33 B). Glucose was decreased in SA62BG tubers compared with control plants. SA62BG tubers contained less sucrose compared with JT62 plants, while they contained the same amount of sucrose as wild-type plants (Figure 3.38). In the apoplast and cytosol of tubers, sucrose is converted to glucose and fructose in a reaction catalyzed by invertase. In addition, in the cytosol of tubers, sucrose is also converted to fructose and UDPG in a reaction catalyzed by SuSy. Less sucrose led to less glucose. Furthermore, the amount of starch was higher (1000-1200 µmol C6 units / g Fwt) in these experiments than in the other experiments (700-1000 µmol C6 units / g Fwt). This is probably because the tubers were drier when the plants were harvested in these batches. It is necessary that the transgenic plants have to be compared with the wild type in every batch. However, starch content was slightly increased in SA62SG tubers compared with wild-type plants, while they contained similar amounts of starch as JT62 plants (Figure 3.32 B). The tuber yield was increased compared with JT62 plants and a similar tuber yield was detected as in wild-type plants (Figure 3.31). In contrast, SA62BG tubers contained a slightly increased starch content compared with wild-type plants; a slightly reduced amount of starch was found compared with JT62 plants (Figure 3.37), while there was less tuber yield in SA62BG tubers than in wild-type plants, and no significant difference was observed compared with JT62 plants (Figure 3.36). These results suggest, first, that reduced tuber sucrose levels do not significantly affect tuber starch content. This is in agreement with the conclusion from potato plants overexpressing a yeast invertase (Sonnewald et al., 1997; Gerrits et al., 2001; Hajirezaei et al., 2003). Second, there was a more notable impact on leaves than on tubers when the pea GPT was expressed under control of the CaMV 35S promoter (EW and SA62SG plants). If Glc6P and ATP are not adequately balanced, tuber starch and yield probably might not be changed as expected.

4.3.4. Comparison of potato plants overexpressing pea GPT and AtNTT under control of the patatin promoter B33 and other transgenic plants with increased tuber starch contents

Increasing the starch content as well as tuber yield has been a major goal for many years, both classical plant breeding and biotechnological approaches were taken extensively over the last few decades (Martin and Smith, 1995; Smith et al., 1997). Some modulation of starch biosynthesis can be achieved through metabolic control of flux through the pathway. ADPG is the first committed precursor for starch biosynthesis in higher plants (Dickinson and Preiss, 1969; Lin et al., 1988a,b). ADPG is formed from immediate substrates (Glc1P and ATP) by the action of the enzyme AGPase. Suc is degraded in the cytosol via SuSy, UGPase and PGM to form Glc6P, which is imported into the plastid via GPT and converted back to Glc1P by the plastidic PGM. ATP is imported from the cytosol via the NTT. Up to now, there are no successful reports of increasing starch biosynthesis in heterotrophic storage organs by increasing the level of ADP-glucose via increasing the level of its immediate precursors in the pathway of starch synthesis (Sonnewald et al., 1997; Trethewey et al., 1998; Trethewey et al., 2001). This probably is because Glc1P and ATP were not increased in balance. Since plants with downregulated NTT or GPT resulted in reduced tuber starch and yield, NTT and GPT play important roles in starch synthesis. Previous work has shown that overexpressing GPT or NTT solely (EW, BG and BA plants) did not exhibit increase tuber starch and yield. Therefore, GPT and NTT were overexpressed together under control of the patatin promoter B33 (BGBAM and BG1BA plants, see 3.7.1 and 3.7.2).

The introduction of pea GPT and AtNTT led to the formation of the corresponding mRNAs in BGBAM tubers (Figure 3.39), whereas the insertion of an AtNTT caused the formation of the appropriate transcript level in BG1BA tubers (Figure 3.44). In proteoliposomes (containing membrane proteins including that of amyloplasts), an increased ATP uptake was detectable in BG1BA compared with wild-type tubers (Figure 3.46 B), suggesting the newly introduced AtNTT gene obviously induced increased amounts of plastidic NTT in tuber amyloplasts. However, in proteoliposomes containing mitochondrial and amyloplast membrane proteins from BG1BA tubers, there is up to 10-fold increased ATP uptake than in those with inhibited mitochondrial AAC activity (Figure 3.46 A). This result indicates that the AAC is more active than the plastidic NTT in potato tubers. Since BG1 was a

background in BG1BA plants, there is no significant difference in GPT activity in BG1BA tubers compared with BG1 plants (Figures 3.45, 3.22). Taken together, these results demonstrated that the heterologously synthesized transporter proteins are functionally integrated into the plastid envelope.

Significantly increased tuber starch content and yield was observed in BG1BA plants (Figures 3.47, 3.48, 3,49), whereas BGBAM plants did not exhibit increased tuber yield and starch (Figures 3.40, 3.41) compared with wild-type plants. One explanation could be that the simultaneous integration of an AtNTT and pea GPT in the wild–type genome could not cause increased starch and yield of tubers (BGBAM) because both integration events must have an impact on the corresponding activities. On the other hand, the introduction of the AtNTT into BG1 plants with increased GPT activity efficiently led to increased starch and tuber yield of BG1BA plants compared with wild-type plants. Both pea GPT and AtNTT were overexpressed under control of the patatin promoter B33, thereby, Glc6P and ATP may be increased in plastids in balance. Glc1P was increased by conversion of Glc6P in amyloplasts. It is tempting to speculate that increased Glc1P and ATP levels result in an increased level of ADPG. Therefore, the increase in starch can essentially be explained as a direct consequence of the increase in ADPG. This assumption was confirmed by previous work. Transgenic potato plants with downregulated activity of the plastidial isoform of adenylate kinase resulted in an increased starch content and tuber yield, accompanied by an increased ADPG level (Regierer et al., 2002). Glc1P and ATP are converted into ADP-glucose by AGPase and the rate of starch accumulation was modified by regulating AGPase activity (Geigenberger et al., 2001). Previous studies document that overexpression of an AGPase displaying modified allosteric properties also led to substantial increases in starch content (Giroux et al., 1996). In case of potato, reduction of AGPase activity led to a reduction in starch accumulation, and an increase in sucrose accumulation (Lee et al., 1999; Mueller-Roeber et al., 1992). However, the AGPase activity was not significantly altered in BG1BA compared to wild-type tubers *in vitro* (data not shown). Moreover, there was an increased tuber starch content, which was accompanied by an increased ADPG level and AGPase activity in growing potato tubers with increased PPase activity compared with wild-type plants (Geigenberger et al., 1998).

In BG1BA tubers, there is no significant difference in soluble sugars, with only the sucrose level of BG1BA24 being markedly increased, and there are also no big

changes in soluble sugars of BGBAM tubers compared with wild-type plants. These results indicate that sucrose does not restrict the rate of starch synthesis in double transformants with pea GPT and AtNTT. Glc6P and Fru6P were decreased in BG1BA tubers. Similar changes in the content of phosphorylated intermediates occurred in transgenic tubers with increased expression of PPase (Geigenberger et al., 1998).

The observation of photosynthetic parameters revealed that there is no pronounced difference in BG1BA plants compared with wild-type plants. However, the sum of the biomass of the aerial part (data not shown) and tubers of BG1BA plants was increased compared with that of wild-type plants. Since only one attached leaf of a plant was measured for photosynthetic parameters at one period of plant growth (10 weeks), this probably led to some deviation in results. The photosynthesis probably is increased in some developmental stages of BG1BA plants compared with wild-type plants, or is only increased very little and sums up to a larger biomass within 10 weeks.

Taken together, NTT and GPT co-limit starch synthesis in amyloplasts of potato tubers when available amounts of Glc6P and ATP are in balance.

5. SUMMARY

Potato is one of the most important crops in the world. Starch is the most important carbohydrate used for food and feed purposes and represents the major resource for our diet. Potato tubers represent typical starch-storing storage sinks that accumulate high levels of starch in the amyloplasts. For starch synthesis in potato tubers, amyloplasts depend on the import of carbon and energy. Carbon enters the amyloplasts in form of glucose-6-phosphate via the GPT, and energy is imported as ATP via the NTT. The impact of increasing Glc6P and ATP import into amyloplasts of potato tubers on starch synthesis as well as tuber yield was analyzed. Overexpressing GPT or NTT solely under the control of either CaMV 35S promoter or patatin promoter B33 (EW, BG and BA plants) did not lead to an increase of starch of tubers as well as yield. However, GPT overexpression under control of the CaMV 35S promoter (EW plants) resulted in an increase of leaf starch content. Overexpression of the GPT under control of the CaMV 35S promoter or of the patatin promoter B33 in NTT overexpressor (JT62) plants also did not show increased tuber starch and yield. However, starch amount in tubers and tuber yield were increased when GPT and NTT were overexpressed together under control of the patatin promoter B33. Heterologously synthesized transporter proteins from pea and Arabidopsis were functionally integrated into the plastid envelope of potato tubers. These results suggest that GPT and NTT co-limit starch synthesis in potato tubers. To obtain an increased starch content and tuber yield, increased carbon and energy import into amyloplasts should be in balance.

A bidirectionalized patatin promoter B33 was constructed. This construct, however, was not suitable to drive gene expression in two directions simultaneously in potato tubers.

6. ABBREVIATIONS

AAC	Adenylate carrier (mitochondrial)
ADP	Adenosine diphosphate
ADPG	ADP-glucose
AGPase	ADP-glucose pyrophosphorylase
ATP	Adenosine triphosphate
Amp	Ampicillin
A. tumefaciens	*Agrobacterium tumefaciens*
BAP	6-benzyl aminopurine
bp	base pair(s)
BSA	Bovine Serum Albumin
CaMV 35S promoter	Cauliflower mosaic virus 35S promoter
cDNA	Complementary deoxyribonucleic acid
Ci	Curie or CO_2 concentration
mCi	milliCurie
µCi	microCurie
dATP	Deoxyadenosinetriphosphate
dCTP	Deoxycytosinetriphosphate
dGPT	Deoxyguanosinetriphospfate
dTTP	Deoxythymidinetriphosphate
DEPC	Diethlypyrocarbonate
DHAP	Dihydroxyacetone-3-phosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DPE1	Disproportionating enzyme
DTT	Dithioththreitol
E.coli	*Escherichia coli*
EDTA	Ethylenediamine tetraacetic acid
EtBr	Ethidium bromide
EtOH	Ethanol
Fru6P	Fructose-6-phosphate
Fru1,6 bP	Fructose-1,6-bisphosphate
Fwt	Fresh weight
g	gram
mg	milligram
µg	microgram
ng	nanogram
GA3	Gibberellic acid
GAP	Glyceraldehyde-3-phosphate
Glc6P	Glucose-6-phosphate
Glc1P	Glucose-1-phosphate
GM	Genetic modification
G6PDH	Glucose-6-phoaphate-dehydrogenase
GPT	Glucose-6-phosphate/phosphate translocator
GUS	β-glucuronidase
GFP	Green fluorescence protein
GT	Glucose transporter
h	hour(s)

HEPES	4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid
HK	Hexokinase
IPTG	Isopropylthio-β-o-galactopyranoside
kb	kilobase(s)
kD	kilodalton
kV	kilovolt
l	Liter
ml	milliliter
µl	microliter
M	Molar
mM	millimolar
µM	micromolar
pM	picomolar
mmol	millmol
µmol	micromol
MEX1	Maltose transporter
min	minute
MOPS	3-(N-morpholino)-propanesulfonic acid
mRNA	messenger Ribonucleic acid
NAA	α-Naphethalene acetic acid
NAD(H)	Nicotinamide adenine dinucleotide
NADP(H)	Nicotinamide adenine dinucleotide phosphate
NST	Nucleotide sugar transporter
NTT	ATP/ADP translocator (plastidic)
MES	2-(N-morpholino)ethane sulfonic acid
OD	Optical density
Oppp	Oxidative pentose phosphate pathway
PAM	Pulse-amplitude-modulation
PEG	Polyethylene glycol
PFD	Photo flux density
3-PGA	3-phosphorygelicerate
PGI	Glucose phosphate isomerase
PGK	Phosphoglycerate kinase
PGM	Phosphoglucomutase
PMSF	Phenylmethanesulfonyl fluoride
PPase	Pyrophosphatase
PPi	Pyrophosphate
ppm	parts per million
PPT	Phosphoenolpyruvate/phosphate translocator
PVP	Polyvinylpyrrolidone
RNA	Ribonucleic acid
rpm	rounds per minute
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
Rubiso	Ribulose-1,5-bisphosphate carboxylase/oxygenase
sec or s	second
SAP	Shrimp alkaline phosphatase
SDS	Sodium dodecyl sulfate
SPS	Sucrose-phosphate synthase
Suc	Sucrose

SuSy	Sucrose synthase
SUT	Sucrose transporter
TE	Tris-HCl / EDTA
TCA	Trichloro-acetic acid
TIM	Triose phosphate isomerase
TPT	Triose-phosphate/phosphate translocator
TrioseP	Triose phosphate
U	Unit(s)
UDP	Uridine diphosphate
UDPG	UDP-glucose
UGPase	UDP-glucose pyrophosphorylase
UTP	Uridine triphosphate
UV	Ultravioletray
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
X-gluc	β-bromo-4-chloro-3-indolyl-β-D-gluronic acid cyclohexyl ammonium salt
XPT	Xylulose 5-phosphate/phosphate translocator
Ω	ohm

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ABSTRACT

Potato is one of the most important crops in the world. Starch is the most important carbohydrate used for food and feed purposes and represents the major resource for our diet. Potato tubers represent typical starch-storing storage sinks that accumulate high levels of starch in the amyloplasts. For starch synthesis in potato tubers, amyloplasts depend on the import of carbon and energy. Carbon enters the amyloplasts in form of glucose-6-phosphate via the GPT, and energy is imported as ATP via the NTT. The impact of increasing Glc6P and ATP import into amyloplasts of potato tubers on starch synthesis as well as tuber yield was analyzed. Overexpressing GPT or NTT solely under the control of either CaMV 35S promoter or patatin promoter B33 (EW, BG and BA plants) did not lead to an increase of starch of tubers as well as yield. However, GPT overexpression under control of the CaMV 35S promoter (EW plants) resulted in an increase of leaf starch content. Overexpression of the GPT under control of the CaMV 35S promoter or of the patatin promoter B33 in NTT overexpressor (JT62) plants also did not show increased tuber starch and yield. However, starch amount in tubers and tuber yield were increased when GPT and NTT were overexpressed together under control of the patatin promoter B33. Heterologously synthesized transporter proteins from pea and Arabidopsis were functionally integrated into the plastid envelope of potato tubers. These results suggest that GPT and NTT co-limit starch synthesis in potato tubers. To obtain an increased starch content and tuber yield, increased carbon and energy import into amyloplasts should be in balance.

A bidirectionalized patatin promoter B33 was constructed. This construct, however, was not suitable to drive gene expression in two directions simultaneously in potato tubers.

ZUSAMMENFASSUNG

Die Kartoffel gehört zu den wichtigsten Kulturpflanzen der Welt. Das Kohlenhydrat Stärke stellt eine wesentliche Ressource für die menschliche und tierische Ernährung dar. Kartoffelknollen sind typische Speicher-sink-Organe, die große Mengen Stärke in Amyloplasten akkumulieren. Um Stärke synthetisieren zu können, müssen Amyloplasten Kohlenstoffgerüste und Energie importieren. Kohlenstoff wird dabei in Form von Glukose-6-Phosphat über den GPT, Energie in Form von ATP über den NTT aufgenommen. Der Einfluss eines erhöhten Glukose-6-Phosphat- und ATP- Imports in Kartoffelknollen- Amyloplasten auf die Stärkesynthese und den Knollenertrag wurde analysiert. Die alleinige Überexpression von

GPT oder NTT unter Kontrolle des 35S CaMV- bzw. B33 Patatin-Promotors führte nicht zur Erhöhung der Stärkemenge in Knollen, genauso wenig ergab sich eine Ertragserhöhung. Die Überexpression des GPT unter Kontrolle des 35S CaMV Promotors resultierte in einer Erhöhung des Blattstärkegehalts. Die Überexpression des GPT unter Kontrolle des 35S CaMV- oder des B33 Patatin Promotors im Hintergrund von NTT überexprimierenden (JT62) Pflanzen ergab weder eine erhöhte Knollenstärkemenge noch einen erhöhten Ertrag. Die Stärkemenge in Knollen und der Knollenertrag waren erhöht, wenn GPT und NTT zusammen unter Kontrolle des B33 Patatin Promotors überexprimiert wurden. Heterolog synthetisierte Transportproteine aus Erbse und Arabidopsis wurden also funktionell in die innere Plastidenmembran von Kartoffelknollen integriert. Die Ergebnisse legen nahe, dass GPT und NTT gemeinsam die Stärkesynthese in Kartoffelknollen limitieren. Um einen erhöhten Stärkegehalt und Knollenertrag zu erhalten, muss folglich der Kohlenstoff- und Energieimport in die Amyloplasten ausbalanciert sein.

Weiterhin wurde im Rahmen der Arbeiten ein bidirektionalisierter B33 Patatin Promotor hergestellt. Dieses Konstrukt war aber in Kartoffelknollen nicht dazu geeignet, eine nennenswerte Genexpression in beide Richtungen gleichzeitig zu vermitteln.

Erklärung:

Ich versichere, daß ich die von mir vorgelegte Dissertation eigenständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder Sinn nach entnommen sind, jeweils kenntlich gemacht habe und daß sie weder einer anderen Fakultät oder Universität zur Prüfung vorgelegen hat, noch in Teilen publiziert worden ist und daß ich eine solche Veröffentlichung vor Abschluß des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordnung der Universität zu Köln sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. UI. Flügge betreut worden.

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