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Production of the biodegradable polymer cyanophycin in transgenic *Nicotiana tabacum* and *Solanum tuberosum* plants

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Summary

The aim of this thesis was to establish the production of the biopolymer cyanophycin in tobacco and potatoes. Both production systems were optimized to achieve maximal amounts of cyanophycin by minimal impairments of the plant growth. The study was part of the network project "Production of biological degradable polymers in transgenic potato tubers" (phase I and II).

A first milestone was achieved by accumulation of cyanophycin in the cytoplasm of tobacco and potato by constitutive expression of the cyanophycin synthetase from *Thermosynechococcus elongatus* BP-1 in both crops. The polymer formed up to 1.1% of the plant dry weight (dw) in tobacco leaves and 0.2% dw of potato leaves and tubers. Neither tobacco nor potatoes with the highest cyanophycin content were fertile or could be used for further propagation.

As a next step the cyanophycin production was improved in transgenic tobacco plants by targeting the cyanophycin synthetase to the plastids. In order to realize this, several different translocation pathway signal sequences for import into the chloroplasts were fused to the coding region of the cyanophycin synthetase gene from *T. elongatus* BP-1. Only one of these signal peptides led to cyanophycin production in plastids. In these plants, the polymer content was significantly increased up to 6.8% dw. Although all transgenic plants tested were fertile, the progeny of the highest cyanophycin-producing event showed reduced seed production compared to control plants.

The tuber-specific production of the polymer in potato could facilitate cyanophycin accumulation. The expression of the cyanophycin synthetase under control of the tuber-specific class 1 promoter (B33) led to increased polymer accumulation in cytoplasm up to 2% dw, however resulting in small and deformed tubers. The dislocation of cyanophycin production only in amyloplasts resulted in higher cyanophycin content up to 7.5% dw and also normal tuber weight and size and sometimes tubers exhibited brown sunken spots. After a storage period of 32 weeks, the cyanophycin content in potato tubers was stable, but the stress symptoms increased up to 31.8%. However all tubers were able to germinate.

A Introduction

The finite of the fossil energy providers oil, natural gas and carbon represents one of the major future problems. Since additionally, there are expectations that until 2030 the worldwide energy requirement will have increased up to 60% (Qaim 2006), there is an urgent need to identify alternative renewable energy sources based on wind, water or solar forces. The plants use of solar energy via photosynthesis is extremely efficient; hence plants as renewable resources are surely an option. Nevertheless, an economic use still depends on governmental subsidies; in addition it seems to be unavoidable to intensify the agricultural land use in order to satisfy the energy demand, which might lead to a reduction of the food supply (Thrän et al. 2005). One of the main possibilities to increase harvest is plant breeding leading to higher biomass production or to the formation of new and special ingredients of economic interest like biopolymers. The combination of both to create a double use plant might increase the outcome for the farmer, reduce the amount of arable land used for non food purpose and in addition reduce CO_2 emission (Hühns and Broer, in press). Naturally occurring polymers are e.g. polysaccharides, polyamides and polyesters produced by bacteria or plants, with a broad range of application (Table 1).

Table 1: Industrial usable polymers and their corresponding application area.

Polymer group	polymer	Area of application
polysaccharides	cellulose	constituent of paper and cardboards; textiles made from cotton, linen and other fibres, in packaging industry
	starch	as food additive; papermaking; thickener; glue; in packaging material
polyamides	spider silk	Strong and elastic fibres; used for textile manufacture
	polyglutamate	substitute for polyacrylates; water softener; thickener in cosmetics; treatment of leather; super absorber in hydrogels
	polylysine	super absorber in hydrogels; addition in animal feed
	polyaspartate	dispersant; water softener
polyester	polyhydroxybutyrate	packaging material; utilisation in compound materials

However all these groups have different drawbacks. Cellulose isolated from wood for the paper industry has some limitations, because costly and environmentally damaging processes are used to extract the lignin in order to obtain pure cellulose fibres. Therefore paper industry is very interested in trees with lower lignin content or with modified lignin that can be easily separated from the cellulose (Baucher et al. 2003). The usage of natural starch is limited due to its composition of amylose and amylopectin, components with different characteristics and separate uses in industrial processes. Generally, only the thickening properties of amylopectin are required, while the amylose component is undesirable in many products and can additionally interfere with certain processes (Pickardt and de Kathen 2004). Unfortunately, the chemical modification or separation of amylopectin and amylose is associated with increased consumption of water and energy. Naturally produced polyamids like spider silk can not be obtained in large quantities from spiders, and the most commonly used polyamides like nylons, aramids and sodium polyaspartate have never been detected in nature (taken from Hühns and Broer in press).

The major problem for commercial production and application of natural polyesters like polyhydroxyalkanoates (PHA) in consumer products is the high costs of bacterial fermentation, making it 5-10 times more expensive then the petroleum-derived polymers like polyethylene and polypropylene (Poirier 2002).

Envisioning these drawbacks and on the other hand the huge potential plants have to perform cheap biomass, new production technologies are required to improve the competitiveness of plant made biopolymers. Gene technology provides us with the tools to add new facilities to the plants metabolic pathways, which should lead to the production of either high quality or even new polymers in plants, possibly as a by-product to traditional materials like starch, oil or sucrose (Hühns and Broer in press).

Polyhydroxybutyrate and polyaspartate are such biologically degradable polymers.. Polyhydroxybutyrate (PHB) is a thermoplastic with properties similar to polyethylene and polypropylene and has a broad range of application in production of plastic bags, fibers and films. The polymer naturally occurs in *Ralstonia eutropha* where three enzymes are necessary for PHB synthesis. The first enzyme, β-ketothiolase, catalyzes the reversible condensation of two acetyl-CoA moieties to form acetoacetyl-CoA. The acetoacetyl-CoA reductase in turn reduces acetoacetyl-CoA to R-3-hydroxybutyryl-CoA, which is subsequently polymerized through the PHA synthase to form PHB. As an alternative to petrochemicals, PHA production was established in plants, first in *Arabidopsis thaliana* by the expression of the PHB synthase in the cytoplasm leading to max. 0.1% dw PHB present in the cytoplasm, nucleus or vacuoles (Poirier et al. 1992). However, the plants showed strong growth retardation and reduced seed production. PHB synthesis in the cytoplasm of tobacco (0.01% dw) (Nakashita et al. 1999), cotton (0.3% dw) (John and Keller 1996) and

oilseed rape (0.1% dw) (Poirier and Gruys 2001) showed similar plant damage. The deleterious effects of PHB production in the cytoplasm of plants might be caused by the diversion of acetyl-CoA and acetoacetyl-CoA away from the endogenous flavonoid and isoprenoid pathways, which are responsible for the synthesis of a range of plant hormones and sterols (van Beilen and Poirier 2008). Due to their high metabolic flow of acetyl-CoA, chloroplasts might provide a more suitable production platform although the β-ketothiolase is not present. Therefore the required enzymes -including the β-ketothiolase- were targeted to the plastids using signal sequences for plastid import. The highest PHB accumulation was observed in Arabidopsis with a maximum of 14% dw in leaves without significant effects on plant growth but visible leaf chlorosis (Nawrath et al. 1994). In seeds of oil seed rape up to 8% dw PHB accumulation was detected in leucoplasts after the transfer of all three genes (Houmiel et al. 1999), a strategy leading to even 30-40% dw in leaves of *A. thaliana*. Nevertheless, in contrast to the intact canola seeds, these plants were heavily reduced in growth and did not produce any seeds. Slightly reduced amounts were detected in corn leaves (6% dw) (Poirier, Gruys 2001), sugar cane leaves (2% dw) (Petrasovits et al. 2007) and sugar beet hairy roots (5% dw) (Menzel et al. 2003), whereas expression of the PHB pathway in plastids of alfalfa and tobacco leads to only low amounts (<0.5% dw) (Arai et al. 2001; Saruul et al. 2002). Since nuclear encoded proteins are expressed to a lesser extends then those encoded by plastidic genes, it was supposed, that the direct expression of the PHB pathway in the plastid genome might increase the PHB yield without increasing the deleterious effects. Nevertheless, in tobacco this strategy only leads to relatively low amounts up to 1.7% dw, accompanied by reduced growth and male sterility (Lössl et al. 2003) (taken from Hühns and Broer in press).

Other polymers are polyaminoacids, such as poly-γ-glutamate, poly-α-aspartate and poly-ε-lysine, which have a wide range of applications, for example as dispersants, thickeners or additives to hydrogel (Chang and Swift 1999; Oppermann-Sanio et al. 1999; Oppermann-Sanio and Steinbüchel 2002; Lössl et al. 2003). Polyaspartate is a soluble, non-toxic and biodegradable polycarboxylate (Tabata et al. 2000) that could replace the non-biodegradable polyacrylates in many industrial, agricultural, and medical applications (Schwamborn 1998; Zotz et al. 2001; Joentgen et al. 2001; Oppermann-Sanio and Steinbüchel 2002). The worldwide requirement of polycarboxylates is about 265000 tons a year (Eissen et al. 2002). Up to now no polyaspartate producing organism has been identified, so the polymer is chemically synthesized (Schwamborn 1996) by three to four manufacturers with a capacity of 12500 tons a year. Synthetic polyaspartate is already on the market, for example Baypure™ DS100 as substitute for polyacrylates and phosphates in washing powder. However, polyaspartate can also be obtained from cyanophycin (multi-$_L$-arginyl-poly-$_L$-aspartic acid). Cyanophycin is a cyanobacterial reserve polymer composed of

a poly-α-aspartic acid backbone with arginine residues linked via their α-amino group to the β-carboxyl group of each aspartate residue (Simon 1976; Simon and Weathers 1976; Simon 1987) and was discovered in 1887 by Borzi. Cyanophycin is synthesized by most cyanobacteria as a temporary nitrogen reserve material during the transition of the cells from the exponential phase to the stationary phase (Mackerras et al. 1990).
Mild hydrolysis of cyanophycin (Joentgen et al. 2001) results in homo- and copolymers of polyaspartate and L-arginine. The basic amino acid $_{L}$-Arginine has been suggested to be a regulator of some immunological and physiological processes, e.g. being an immune system stimulator (Cen et al. 1999; Taheri et al. 2001; de Jonge et al. 2002; Nieves, Jr. and Langkamp-Henken 2002; Tapiero et al. 2002; Yeramian et al. 2006; Li et al. 2007; Popovic et al. 2007), as growth inductor (Roth et al. 1995; Lenis et al. 1999; Wu et al. 2007) or as tumour cell growth inhibitor (Amber et al. 1988; Flynn et al. 2002; Caso et al. 2004). Alternatively, aspartate and arginine from cyanophycin could serve as a starting point for the synthesis of a range of chemicals (Scott et al. 2007) (Figure 1). Arginine can be converted to 1,4-butanediamine, which can be used for the synthesis of nylon-4,6. Aspartate is converted in several chemicals like 2-amino-1,4-butanediol, 3-aminotetrahydrofuran (analogs of high-volume chemicals used in the polymer industry), fumaric acid (used for polyester resins) and acrylamide (used as thickener, manufacture of dyes or papermaking).

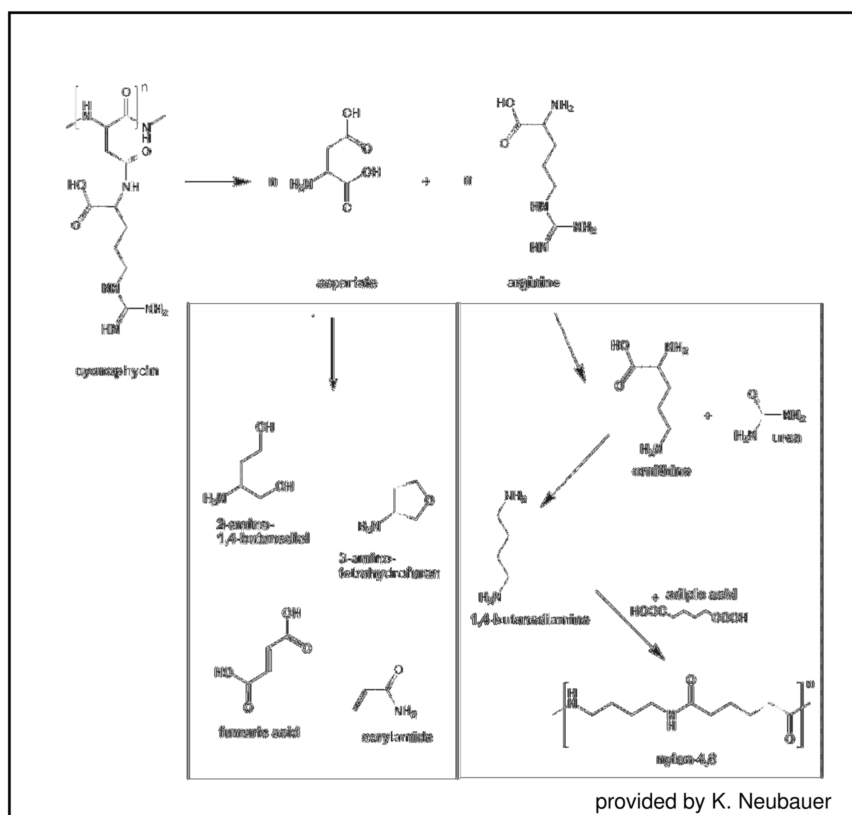

Figure 1: Potential products derived from the polymer cyanophycin.

Cyanophycin is synthesized via non-ribosomal polypeptide synthesis in many cyanobacteria (Simon 1987) and some other non-photosynthetic bacteria (Krehenbrink et al. 2002; Ziegler et al. 2002) (Figure 2).

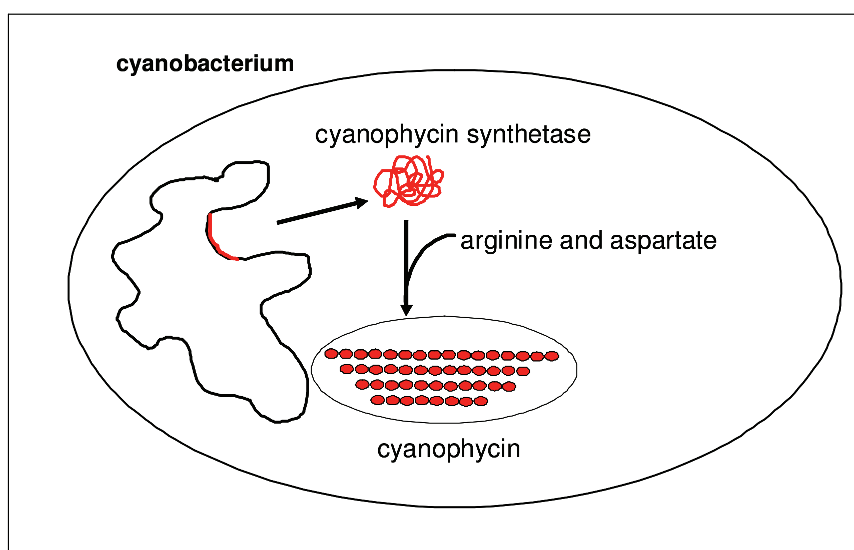

Figure 2: Mechanism of non-ribosomal polypeptide synthesis of cyanophycin in cyanobacteria.

For cyanophycin synthesis, only one enzyme, the cyanophycin synthetase encoded by *cph*A, is necessary to catalyze the ATP-dependent elongation of a cyanophycin primer by the consecutive addition of L-aspartic acid and L- arginine (Ziegler et al. 2002). In cyanobacteria, the polymer is variable in length (25-125kDa), water-insoluble, and stored in membraneless granules (Allen 1984; Simon 1987).
The production of cyanophycin in cyanobacteria for large scale production is not suitable, because of low polymer content and slow growth resulting in only low cell densities (Mooibroek et al. 2007). *E. coli* is one of the most commonly used bacterial hosts for the production of recombinant proteins (Lee 1996). However production of cyanophycin in this bacteria is not possible, because of instability of recombinant *E. coli* strains like DH1 and DH5α caused by loss of the plasmid during fermentation (Mooibroek et al. 2007). In other bacteria like *Corynebacterium glutamicum*, *Ralstonia eutropha* and *Pseudomonas putida* comparable high amounts of the polymer were produced (up to 50% of cell dry weight) in a much shorter period of time (1-2 days) as compared to cyanobacteria (about 4 weeks) (Aboulmagd et al. 2001). Due to material costs and fermentation costs of bacteria plants are advantageous compared to bacteria for high scale production of cyanophycin. A further advantage of cyanophycin accumulation in plants is the possibility to produce the polymer as a by-product to existing industrial production ways, e.g. starch extraction from potato tubers (Figure 3) to increase the value added of the specific cultivar.

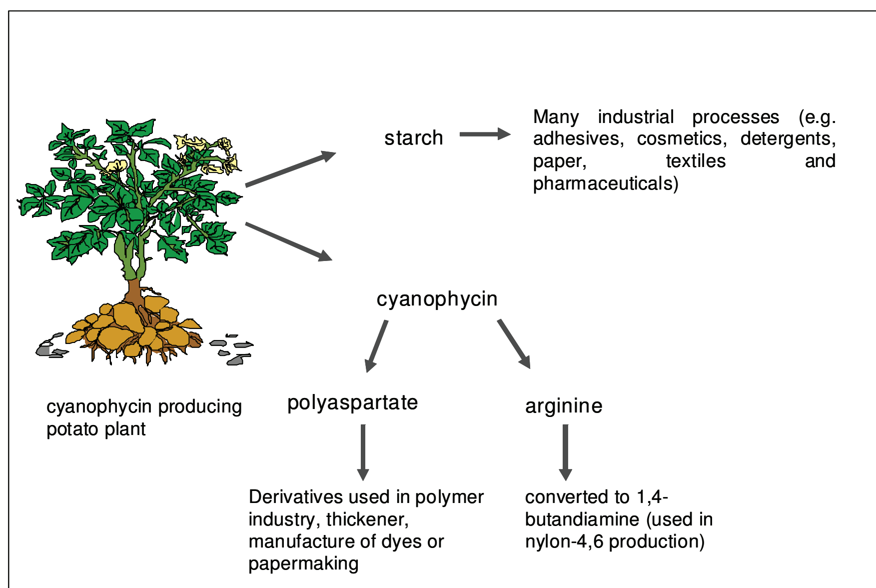

Figure 3: Enhancement of usability of potato plants through cyanophycin accumulation and their corresponding derivatives.

Potato is the fourth most important food crop in the world following wheat, rice and maize. However it is also important as renewable resource for non-food applications, so 40% of isolated starch is used for adhesives and lubricants in paper- and corrugated board manufacture and also for packages and building materials. The production of new ingredients like polymers in potato tubers ought to increase the usability of the whole plant.

A1 Aim of this work

As stated above, no polyaspartate producing organism has been identified up to now. The production of the alternative cyanophycin in bacteria is restricted due to slow growth of the bacteria, low content of the polymer and fermentation and material costs. The production of cyanophycin in plants might therefore be a useful alternative.
The aim of this work was to establish cyanophycin production in different plant species. Tobacco was chosen as model organism because it is easily transformable, cultivable and quick to investigate. The potato plant was chosen as target plant for cyanophycin accumulation as a by-product of starch.
In this study the following aspects were investigated:

Does the expression of the cyanobacterial genes coding for the cyanophycin synthetase lead to polymer accumulation in different plant species?

Is it possible to enhance the cyanophycin production by relocation of the cyanophycin synthesis to plastids?

Is it possible to increase cyanophycin accumulation by tuber specific expression of the cyanophycin synthetase?

The study was part of the network project “Production of biological degradable polymers in transgenic potato tubers (phase I and II) funded by the German federal ministry for food, agriculture and consumer protection (BMELV) coordinated by the University of Rostock. The close collaboration with the Humboldt University of Berlin, University of Bielefeld, University of Tuebingen, BioMath GmbH as well as the NORIKA GmbH and biovativ GmbH was the necessary basis for the success of the study.

B Results

Most of the results are presented in three peer reviewed publications and one invited textbook chapter

B 1 Neumann, K., Stephan, D.P., Ziegler, K., Hühns, M., Broer, I., Lockau, W., and Pistorius, E.K. (2005) "Production of cyanophycin, a suitable source for the biodegradable polymer polyaspartate, in transgenic plants." Plant Biotechnology Journal 3: 249-258.

B 2 Hühns, M. Neumann, K.; Hausmann, T.; Ziegler, K.; Klemke, F.; Kahmann, U.; Staiger, D.; Lockau, W.; Pistorius, E.K.; Broer, I. (2008) "Plastid targeting strategies for cyanophycin synthetase to achieve high-level polymer accumulation in *Nicotiana tabacum*." *Plant Biotechnol. J.* 6:321-336

B 3 Hühns, M.; Neumann, K.; Hausmann, T.; Klemke, F.; Lockau, W.; Kahmann, U.; Kopertekh, L.; Staiger, D.; Pistorius, E.K.; Reuther, J.; Waldvogel, E.; Wohlleben, W.; Effmert, M.; Junghans, H.; Neubauer, K., Kragl, U.; Schmidt, K.; Schmidtke, J.; Broer, I. (2009) „Tuber specific *cph*A expression to enhance cyanophycin production in potatoes." *Plant Biotechnol. J.* 7(9): 883-898

B 4 Hühns, M.; Broer, I. Characters of transgenic plants and their Application in Plant production: Biopolymers.
In: Biotechnology in Agriculture and Forestry Vol. X. Genetic Modification of Plants-Agriculture, Horticulture and Forestry. (eds.: F.Kempken and C. Jung); Springer, Heidelberg, in press

B 1

Neumann, K., Stephan, D.P., Ziegler, K., Hühns, M., Broer, I., Lockau, W., and Pistorius, E.K. (2005) “Production of cyanophycin, a suitable source for the biodegradable polymer polyaspartate, in transgenic plants.” Plant Biotechnology Journal 3: 249-258.

Summary:

The production of biodegradable polymers in transgenic plants in order to replace petrochemical compounds is an important challenge for plant biotechnology. Polyaspartate, a biodegradable substitute for polycarboxylates, is the backbone of the cyanobacterial storage material cyanophycin. Cyanophycin, a copolymer of L-aspartic acid and L-arginine, is produced via non-ribosomal polypeptide biosynthesis by the enzyme cyanophycin synthetase. A gene from *Thermosynechococcus elongatus* BP-1 encoding cyanophycin synthetase has been expressed constitutively in tobacco and in potato. The presence of the transgene-encoded mRNA correlated with changes in leaf morphology and decelerated growth. Such transgenic plants were found to produce up to 1,1% per dry weight of a polymer with cyanophycin-like properties. Aggregated material, able to bind a specific cyanophycin antibody, was detected in the cytoplasm and the nucleus of the transgenic plants.

B 2

Hühns, M. Neumann, K.; Hausmann, T.; Ziegler, K.; Klemke, F.; Kahmann, U.; Staiger, D.; Lockau, W.; Pistorius, E.K.; Broer, I. (2008) "Plastid targeting strategies for cyanophycin synthetase to achieve high-level polymer accumulation in *Nicotiana tabacum*." *Plant Biotechnol. J.* 6:321-336

Summary:

The production of biodegradable polymers in transgenic plants is an important challenge in plant biotechnology; nevertheless it is often accompanied by reduced plant fitness. In order to diminish the phenotypic abnormalities caused by cytosolic production of the biodegradable polymer cyanophycin, and to increase polymer accumulation four translocation pathway signal sequences for import into chloroplasts were individually fused to the coding region of the cyanophycin synthetase gene (*cph*A_{Te}) of *Thermosynechococcus elongatus* BP-1 resulting in the constructs named pRieske-*cph*A_{Te}, pCP24-*cph*A_{Te}, pFNR-*cph*A_{Te} and pPsbY-*cph*A_{Te}. These constructs were expressed in *Nicotiana tabacum* var. Petit Havanna SRI under the control of the constitutive CaMV35S promoter. Three of the four constructs led to polymer production. However, only the construct pPsbY-*cph*A_{Te} led to cyanophycin accumulation exclusively in chloroplasts. In plants transformed with the pCP24-*cph*A_{Te} and pFNR-*cph*A_{Te} construct water-soluble and insoluble forms of cyanophycin were only located in the cytoplasm, which resulted in phenotypic changes similar to those observed in plants, transformed with constructs lacking a targeting sequence. The plants transformed with pPsbY-*cph*A_{Te} produced predominantly the water insoluble form of cyanophycin. The polymer accumulated to up to 1.7% of dry matter in primary (T0) transformants. Specific T2 plants produced 6.8% of dry weight as cyanophycin, which is more than five-fold higher than the previously published value. Although all lines tested were fertile, the progeny of the highest cyanophycin producing line had reduced seed production compared to control plants.

B 3

Hühns, M.; Neumann, K.; Hausmann, T.; Klemke, F.; Lockau, W.; Kahmann, U.; Kopertekh, L.; Staiger, D.; Pistorius, E.K.; Reuther, J.; Waldvogel, E.; Wohlleben, W.; Effmert, M.; Junghans, H.; Neubauer, K., Kragl, U.; Schmidt, K.; Schmidtke, J.; Broer, I. (2009) „Tuber specific *cph*A expression to enhance cyanophycin production in potatoes.“ *Plant Biotechnol. J.* 7(9): 883-898

Summary:

The production of biodegradable polymers that can be used to substitute petrochemical compounds in commercial products in transgenic plants is an important challenge for plant biotechnology. Nevertheless it is often accompanied by reduced plant fitness. In order to decrease the phenotypic abnormalities of the sprout and to increase polymer production, we restricted cyanophycin accumulation to the potato tubers by using the cyanophycin synthetase gene (*cph*A_{Te}) from *Thermosynechococcus elongatus* BP-1, which is under the control of the tuber-specific class 1 promoter (B33). Tuber specific cytosolic (pB33-*cph*A_{Te}) as well as tuber specific plastidic (pB33-PsbY-*cph*A_{Te}) expression resulted in significant polymer accumulation solely in the tubers. In plants transformed with pB33-*cph*A_{Te} both cyanophycin synthetase and cyanophycin were detected in the cytoplasm leading to an increase up to 2.3% cyanophycin of dry weight and resulting in small and deformed tubers. In B33-PsbY-*cph*A_{Te} tubers, cyanophycin synthetase and cyanophycin were exclusively found in amyloplasts leading to a cyanophycin accumulation up to 7.5% of dry weight. These tubers were normal in size, some clones showed reduced tuber yield and sometimes exhibited brown sunken staining starting at tubers navel. During a storage period over of 32 weeks of one selected clone, the cyanophycin content was stable in B33-PsbY-*cph*A_{Te} tubers but the stress symptoms increased. However all tubers were able to germinate. Nitrogen fertilisation in the greenhouse led not to an increased cyanophycin yield, slightly reduced protein content, decreased starch content, and changes in the amounts of bound and free arginine and aspartate, as compared to control tubers were observed.

B 4

Hühns, M.; Broer, I. (2010) Characters of transgenic plants and their Application in Plant production: Biopolymers.
In: Biotechnology in Agriculture and Forestry Vol. X. Genetic Modification of Plants-Agriculture, Horticulture and Forestry. (eds.: F.Kempken and C. Jung); Springer, Heidelberg,

Biopolymers are intended to serve as substitutes for fuel derived compounds. As their production in plants, driven by solar energy and photosynthesis, is CO_2 neutral and they are normally completely biodegradable, they support a sustainable use of renewable resources. Unfortunately, in agriculturally usable cultivars, most biopolymers with technical applications are produced in low amounts, unfavourable combinations or are even absent. Hence, gene technology has to be used to create plants with an optimal concentration of e.g. polysaccharides, poly-amino acids ore even polyhydroxyalkanoates. In order to reduce costs and the amount of farmland taken away from food production, the biopolymers should be a by product in plants already used for biomass, carbohydrate or fatty acid production. Several biopolymers are already produced in plants, sometimes in interesting amounts; nevertheless application is not in sigh, due to necessary optimization and, even more, regulatory frameworks.

C Unpublished results

All relevant methods used are described in Neumann et al., 2005; Hühns et al.; 2008 and Hühns et al., 2009. Methods, which were not previously described, are indicated in the text or figure description.

C1 Tuber specific expression of the cyanophycin synthetase using the pGBSS promoter

To restrict the cyanophycin synthetase to the potato tuber, the tuber-specific GBSS promoter from the granule bound starch synthetase (GBSS) from *Manihot esculenta* was fused to the coding region of the cyanophycin synthetase (*cph*A_{Te}). The fusion of the GBSS promoter with the *cph*A_{Te} coding region in the binary vector p35S-*cph*A_{Te} (Neumann et al. 2005), was not successful, possibly based on sequence incompatibility between the promoter and the present sequences of the binary vector. The fusion of the GBSS promoter with the *cph*A_{Te} coding region might be facilitated by integration of a non-coding sequence upstream the *cph*A_{Te} coding region. For this purpose two fragments of 50bp or 200bp respectively were amplified via polymerase chain reaction (PCR). Only the 200bp fragment was successfully integrated into p35S-*cph*A_{Te} via the restriction enzymes *Sfi*I/*Sma*I. The cloning scheme is shown in Figure 4.

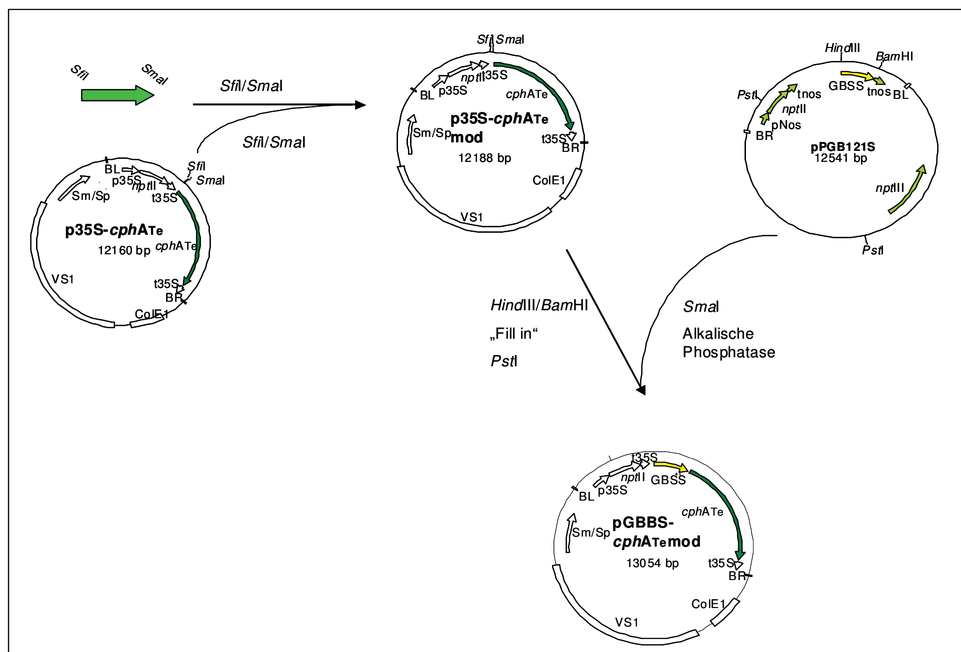

Figure 4: Cloning scheme for generating the plant transformation vector pGBSS-*cph*A_{Te}mod. GBSS: tuber-specific promoter of the granule bound-starch synthase; pnos/tnos: promoter/terminator of the nopaline synthetasegene from Agrobacterium tumefaciens; *npt*II: neomycine phosphotransferase gene II; BL, BR: border sequences of the T-DNA; nptIII: neomycine phosphotransferase gene III; cphATe: cyanophycin synthetase gene from *Thermosynechococcus elongatus* BP-1; Sm/Sp: bacterial expressed Streptocin/Spectinomycin resistance gene; p35S: cytoplasmic expressed 35S RNA promoter of the cauliflower mosaic virus; t35S: cauliflower mosaic virus-35S-terminator; ColE1, VS1: origin of replication for maintenance in *Escherichia coli* or Agrobacteria.

Subsequently the pGBSS promoter from pGB121S was integrated into the modified vector (Figure 4). For the generation of stabile transgenic plants via Agrobacterium-mediated transformation an integration of the pGBSS-*cph*A$_{Te}$mod vector into *Agrobacterium tumefaciens (A. tumefaciens*) strain LBA4404 or ATHV C58C1 is required. However, neither the frost-shock method nor electroporation led to an integration of the plasmid. To overcome this problem, the plasmid was transferred to *E. coli* strain S17.1 (Simon et al. 1983) and subsequently integrated into *A. tumefaciens* LBA4404 using the two-factor-mating procedure. Unfortunately the target gene was not detectable any more already after the first sub-cultivation step, whereas *npt*II was still present. Similar problems occurred after the fusion of the GBSS promoter with the *ctx*B coding region (Mikschofsky, H. 2006). Nevertheless, this phenomenon of instability in agrobacteria was not observed using other binary vectors carrying the *cph*A gene. Therefore, the PCR amplified GBSS promoter was exchanged with the CaMV35S promoter using the enzymes *Sfi*I and *Sma*I in the vector p35S-*cph*A$_{Te}$ forming vector pGBSS-*cph*A$_{Te}$ (Figure 5).

The following transformation of the pGBSS-*cph*A$_{Te}$ vector into *Agrobacterium tumefaciens* LBA4404 was now possible with the frost-shock method; the plasmid was stable for more then three years in agrobacteria.

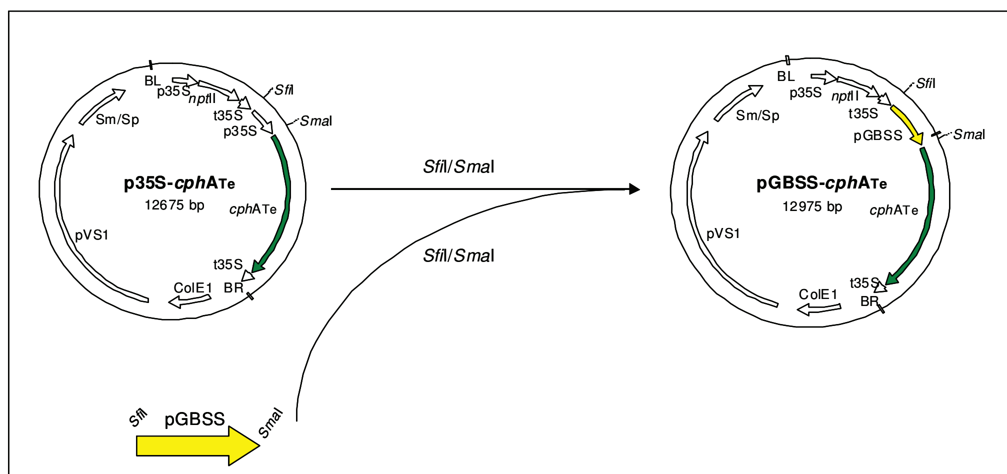

Figure 5: Cloning scheme for generating the plant transformation vector pGBSS-*cph*A$_{Te}$. GBSS: tuber-specific promoter of the granule bound-starch synthase; *npt*II: neomycine phosphotransferase gene II; BL, BR: border sequences of the T-DNA; nptIII: neomycine phosphotransferase gene III; *cph*A$_{Te}$: cyanophycin synthetase gene from *Thermosynechococcus elongatus* BP-1; Sm/Sp: bacterial expressed Streptocin/Spectinomycin resistance gene; p35S: cytoplasmic expressed 35S RNA promoter of the cauliflower mosaic virus; t35S: cauliflower mosaic virus-35S-terminator; ColE1, VS1: origin of replication for maintenance in *Escherichia coli* or Agrobacteria.

The construct described above was used for three independent transformation experiments in *Solanum tuberosum* cv. Albatros. The plants obtained were named GBSS-*cph*A$_{Te}$. A clone of the genetically unmodified cultivar Albatros, which was regenerated after treatment with the plasmid-free *Agrobacterium* strain, was used as near isogenic control.

Regeneration frequencies were evaluated in comparison to the control vector pLH9000, carrying only the *npt*II gene, (Table 2).

Table 2 Summary of the regeneration rate and characterization of regenerated transgenic plants. *Solanum tuberosum* transformed with the specific vector for tuber specific expression of the cyanophycin synthetase, in comparison to the control vector pLH9000.

construct	Shoot/ex-plantat (mean rate)	Regenerated transgenic plants	Plants with *cph*A_{Te} DNA	Phenotypical changes of leaves	of tubers	plants producing cyanophycin in tubers
pGBSS-*cph*A_{Te}	0.1	36	10/36	0/10	0/10	0/10
pLH9000	0.24	10				

The regeneration frequencies for plants transformed with pGBSS-*cph*A_{Te} were reduced compared to the vector control pLH9000. The presence of the cphA transgene in the potato plants was proven by PCR analysis of leaf DNA, which revealed that only 10 out of 36 tested plants carried the *cph*A_{Te} gene (Table 2, Figure 6).

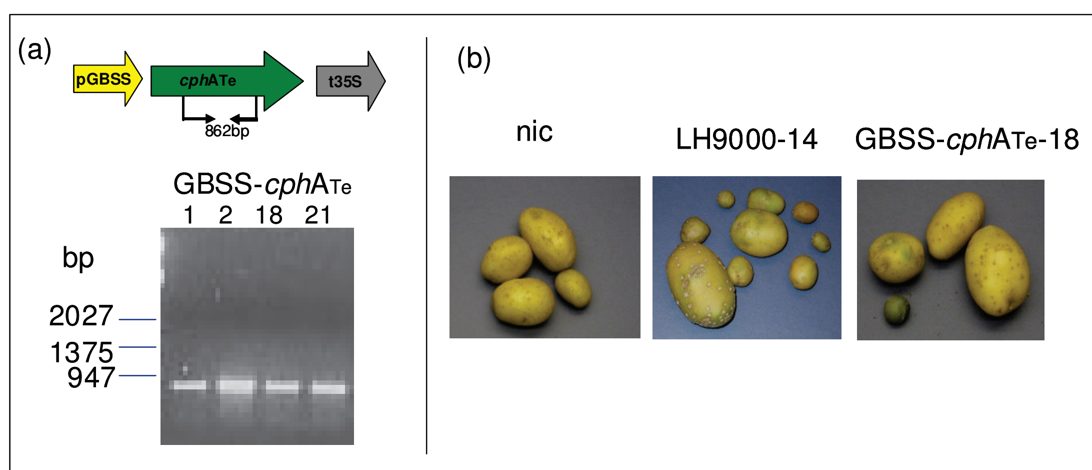

Figure 6 Detection of *cph*A_{Te} specific DNA in plant leaves and phenotype of transgenic tubers compared to controls. (a) *cph*A_{Te} gene and PCR of four transgenic GBSS-*cph*A_{Te} clones with specific *cph*A fw/rv primer; (b) phenotype of GBSS-*cph*A_{Te} tubers compared to controls. nic, near isogenic control.

The positively tested plants were grown in a greenhouse and analysed. The height of the sprout was quantitatively determined after three month of growth. No difference was detected in comparison to control plants of near isogenic control (nic) and vector control (LH9000),.

Also the tubers produced from the analyzed GBSS-*cph*A_{Te} plants were comparable in weight and size to the control tubers (Table 3, Figure 6).

Table 3 The tuber weights were determined in different cultivations, therefore data for control plants was provided for each independent cultivation and indicated above investigated events. nic, near isogenic control.

Plant	Transgenic clone	Number of tubers	Weight of tubers in g
LH9000	9	2	25.3
nic		5	21.6
GBSS-*cph*A_{Te}	1	5	23.6
	2	7	18.3
	3	4	23.4
	4	6	22.4
	5	3	25.6
	6	2	24.8
LH9000	14	9	71.24
nic		8	80.3
GBSS-*cph*A_{Te}	18	10	86.36
	21	5	66.19
	23	9	66.21
	24	4	61.54

Cyanophycin content of transgenic GBSS-*cph*A_{Te} tubers was measured in the working group of Prof. Lockau (Humboldt University Berlin). In none of the transgenic clones investigated cyanophycin was detected.

C2 Attempts to increase the amino acid pool via expression of different bacterial glutamine and aspartate synthase genes

As stated earlier the cytoplasmic production of cyanophycin in plants is accompanied by different stress symptoms such as variegated leaves, small and deformed tubers, reduced growth and early flower induction. It might be speculated that these visible changes were caused by biochemical changes in the cell. Especially influences of the nitrogen metabolism such as changes in the biosynthesis of aspartate and arginine, which are used directly for the formation of cyanophycin, are possible. This would in addition lead to a poor production of the polymer. To overcome such problems, the coincident expression of bacterial genes coding for enzymes involved in amino acid biosynthesis and the cyanophycin synthetase in plants might enhance the biosynthesis of amino acids. Thereby this approach could ensure the production of sufficient amounts of amino acids. Therefore Katrin Neumann fused seven different bacterial coding regions responsible for the synthesis of glutamine synthetase (*gln*A from *E. coli*, *gln*A from *Streptomyces coelicolor*, *gln*A4 from *S. coelicolor*, *gln*II from *S. coelicolor*), ornithine acetyltransferase (*arg*J from *S. coelicolor*, *arg*J from *Mycobacterium tuberculosis*) and *N*-acetylglutamate-5-semialdehyde-reductase (*arg*C from *S. coelicolor*) to the CaMV35S promoter and transferred them to tobacco. The transcription was verified by analysis of the total mRNA with Northern Blot analysis using transgene specific mRNA probes. However, the correct transcript size was only detected for three out of the seven genes (FKZ 98NR090 2003). Therefore, as part of my thesis, plants expression on of these

three genes (*gln*A, *gln*II and *arg*J) (Figure 7) were used in matings with tobacco plants carrying the 35S-*cph*A_{Te} construct.

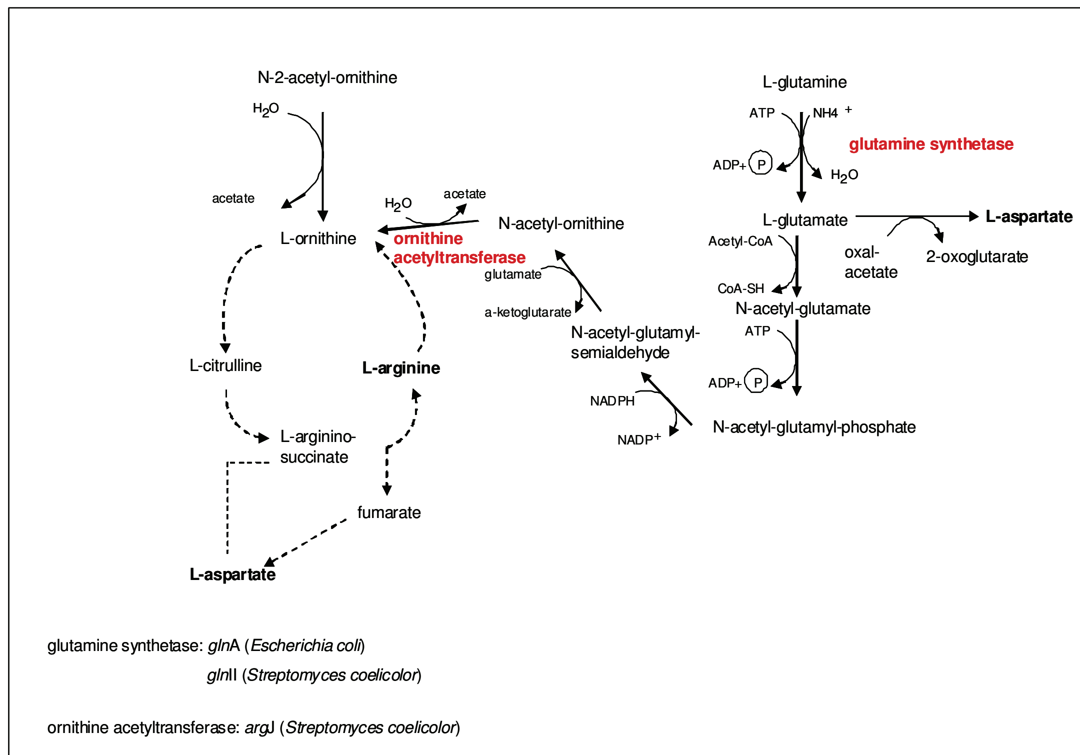

Figure 7 Biochemical pathways for arginine and aspartate synthesis. Overexpressed enzymes are marked in red.

The selection of the mating partners carrying the p35S-*cph*A_{Te} construct was based on their cyanophycin content (Table 4) and the level of phenotypical stress symptoms (Figure 8).

Table 4 Cyanophycin content of 35S-*cph*A_{Te} events chosen for crossing experiments.

Transgenic event	total cyanophycin content (mg/g dw)
14	0.7
31	0.4
39	2.6

The phenotypic changes of the transgenic mating partners were classified into two different categories, which were termed stressed and vigorously stressed plants. The first class, including event 14 and 39, displayed stress symptoms like reduced leaf blade, unfolded petals and spotted leave, but in spite of these phenotypic differences to the near isogenic control (nic) they seemed to be healthy. In contrast, the second class termed vigorously stressed and represented by event 31, was clearly much more stressed showing a crippled leaf laminar as well as strongly variant and reduced size. The stem was weak and thin and the plants could hardly stand in the soil. Only the flower developed normally.

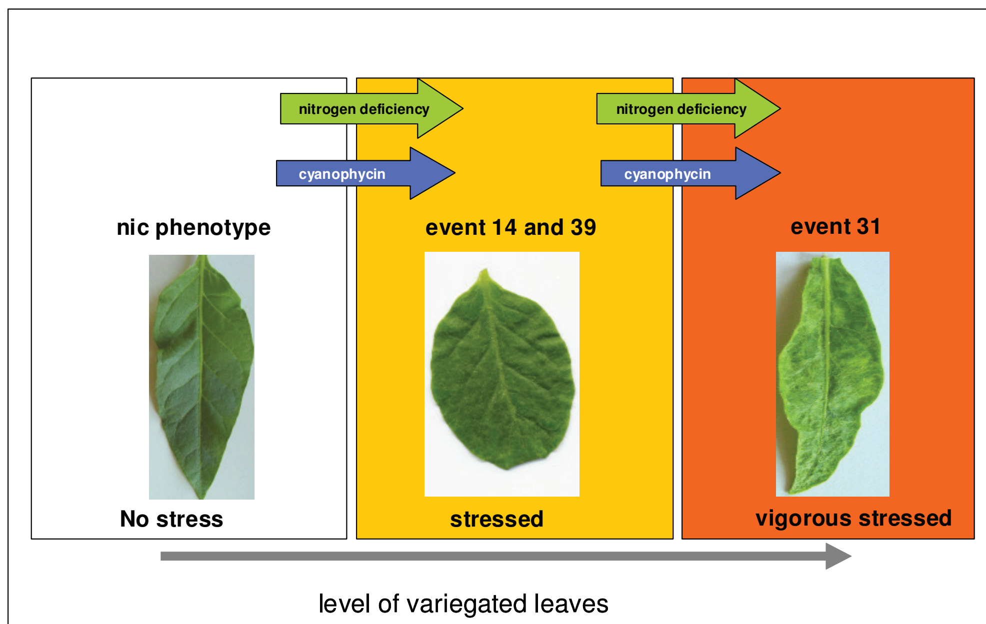

Figure 8 Selection of cyanophycin-producing mating partners in dependence on the intensity of leaf variegation. The reduction of these stress symptoms should be achieved by the enhancement of nitrogen supply.

Compared to the correlation to cyanophycin content observed for the other parameters the correlation to the phenotypic stresses symptoms was not obvious. Despite of the fact, that they belong to two different stress classes; events 14 and 31 had similar cyanophycin contents. Furthermore, the cyanophycin content of event 14 and 39 were much different although they belong to the same stress class.

In the mating experiments with plants carrying the genes involved in amino acid biosynthesis, the cyanophycin producing plants were used as pollen acceptor and also as pollen donor (Figure 9).

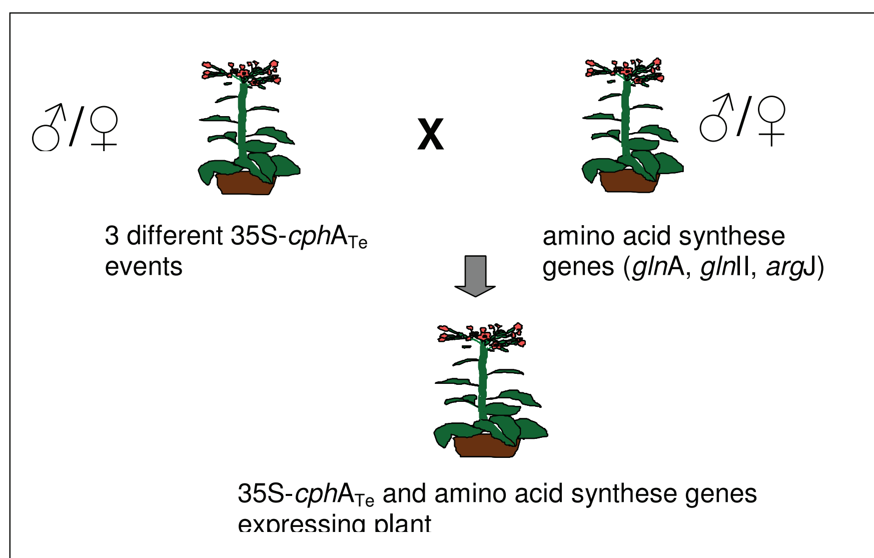

Figure 9 Scheme of crossing of 35S-*cphA*$_{Te}$ tobacco plants with genes involved in amino acid biosynthesis. All plants were used as pollen acceptor as well as pollen donor.

The progeny of the different crossings were further investigated. At first 150 seeds were determined and 20 descendants were analysed via sequence specific PCR of both genes. In Figure 10 all descendants are indicated, which contained transgenes, the *cph*A gene and the gene involved in amino acid biosynthesis. Thereby, one box represents one investigated transgenic plant and its corresponding phenotype.

Figure 10 Summary of the analysed crossing plants via PCR including the phenotype. nic, near isogenic control

Remarkably, in the progeny of event 31 only very few plants were detected carrying both transgenes (Figure 10). Furthermore, all descendants exhibited a normal, unstressed phenotype. Random samples from selected plants were analysed for cyanophycin content (working group of Prof. Lockau, Humboldt University Berlin), but no polymer was detected (Table 5).

The F1 offspring of event 14 and 39, which carried both transgenes (Figure 10), did not show a reduction of stress symptoms compared to T0 plants, rather the level of variegated leaves increased during plant cultivation. The cyanophycin content was also analysed from selected plants at the Humboldt University Berlin (Table 5), but no increase in cyanophycin content was detected.

Table 5 Cyanophycin content of various transgenic crossing progenies. n.d. not detected.

crossing	transgenic event	total cyanophycin content (mg/g dw)
35S-*cph*A_{Te}-14 x *gln*A	4	0.3
	5	0.4
	6	0.3
	20	0.1
*gln*A x 35S-*cph*A_{Te}-14	10	0.4
	13	0.08
	15	0.1
	18	0.09
35S-*cph*A_{Te}-14 x *gln*II	9	0.2
	13	0.2
*gln*II x 35S-*cph*A_{Te}-14	10	0.3
	11	0.3
	12	0.02
nic x 35S-*cph*A_{Te}-14	1	0.7
	17	1.3
	18	0.3
35S-*cph*A_{Te}-14 x nic	17	0.09
	18	0.2
35S-*cph*A_{Te}-39 x *gln*A	5	2.7
	12	2.2
*gln*A x 35S-*cph*A_{Te}-39	15	3.1
	16	2.9
	17	0.05
	18	2.2
*gln*A x 35S-*cph*A_{Te}-31	6	n.d.
	11	n.d.

C3 Attempts to increase the amino acid pool via external supply of amino acids

As stated in 4.2 the expression of bacterial genes involved in amino acid biosynthesis was not appropriate to increase the cyanophycin content in 35S-*cph*A_{Te} tobacco plants. In the cyanophycin producing PsbY-*cph*A_{Te} tobacco plants maximal polymer content up to 1.7% of dw in primary (T0) transformants was detected. It is not known whether during regeneration phase, regenerating transgenic PsbY-*cph*A_{Te} plants with high cyanophycin content were suppressed caused by the limited availability of amino acids in the regeneration media. This could in addition lead to reduced cyanophycin content. To overcome this problem, the external supply of casein hydrolyzate could ensure sufficient amounts of amino acids. Casein hydrolysate is a milk protein product composed of amino acids and other substances that can be incorporated in basal media to provide plant cells with a source of organic nitrogen (George 1993). Therefore, in literature no information about casein hydrolysate concentration for tobacco transformation was found. Therefore, three different casein hydrolysate concentrations (200mg/l to 1000mg/l) were used to ascertain their influence on the transformation rate of construct pPsbY-*cph*A_{Te} into tobacco. The regeneration rate of transgenic sprouts was analysed in three independent transformation experiments (Table 6).

Table 6 Summary of the regeneration rate of transgenic PsbY-*cph*A_{Te} plants transformed with casein hydrolysate in the regeneration media. *Nicotiana tabacum* transformed with plastidic expression of the cyanophycin synthetase, in comparison to the control vector pLH9000.

concentration of casein hydrolysate in mg/l	Shoot/ explantat (mean rate) of pLH9000	Shoot/ explantat (mean rate) of pPsbY-*cph*ATe
0	1.31	0.56
200	1.27	0.84
500	0.75	0.49
1000	0.88	0.55

Based on these data a positive influence on regeneration rate of plants transformed with the pPsbY-*cph*A_{Te} construct was observed with 200mg/l casein hydrolysate in the regeneration media. In contrast, higher concentrations of casein hydrolysate showed no influence and where comparable to regeneration frequencies without casein hydrolysate in the regeneration media. The supplementation with casein hydrolysate exhibited no increased regeneration frequencies and rather led to reduced rates of the control vector pLH9000, carrying only the *npt*II gene and the 35S promoter. The regenerated transgenic shoots of PsbY-*cph*A_{Te} had normal growth rates and phenotypes (data not shown). The presence of the *cph*A_{Te} transgene in tobacco plants was proven by PCR analysis of leaves. As indicated in Table 7, the majority of regenerated plants carried the *cph*A_{Te} gene. The positively tested plants were grown in a greenhouse and the cyanophycin content was analysed at the Humboldt University Berlin (working group of Prof. Lockau, Table 7).

Table 7 Summary of characterization and determination of cyanophycin content of transgenic PsbY-*cph*A_{Te} plants transformed with casein hydrolysate in the regeneration media.

Casein hydrolysate concentration in mg/l	Regenerated transgenic plants	Plants with *cph*A_{Te} DNA	Maximuml total content of cyanophycin in mg/g dry weight
0	8	6	1.45
200	21	12	1.44
500	15	11	1.17
1000	16	14	1.02

The cyanophycin content in the transgenic PsbY-*cph*A_{Te} events was not increased by supplement of casein hydrolysate to the regeneration media compared to regenerated plants without casein hydrolysate.

C4 Accumulation of cyanophycin in plastids of potato

In order to establish a plastidic generation of cyanophycin in potato, the gene coding for the cyanophycin synthetase of *T. elongatus* BP-1 was fused to the PsbY translocation pathway signal sequences for import into chloroplasts (Hühns et al. 2008). The transit peptide belongs to PsbY, a nuclear encoded integral protein of photosystem II, which is transported to the chloroplasts after translation (Gau et al. 1998). It has previously been shown to mediate the import of the cyanophycin synthetase (CphA) enzyme into tobacco chloroplasts

(Hühns et al. 2008) or potato amyloplasts (Hühns et al. 2009). The construct named pPsbY-*cph*A_{Te} also contain the *npt*II gene as selection marker (Herrera-Estrella et al. 1983) and was used for five transformation experiments in *Solanum tuberosum* cv. Albatros (Table 8). The plants obtained were named PsbY-*cph*A_{Te}.

Table 8 Summary of the regeneration rate and characterization of regenerated transgenic plants. *Solanum tuberosum* transformed with plastidic expression of the cyanophycin synthetase, in comparison to the control vector pLH9000.

construct	Shoot/ex-plantat (mean rate)	Regenerated transgenic plants	Plants expressing *cph*A_{Te} mRNA	Phenotypical changes of leaves	of tubers	plants producing cyanophycin in tubers
pLH9000	0.46	5	-	-	-	-
pPsbY-*cph*A_{Te}	0.27	14	9 of 14	0 of 9	0 of 9	9 of 9

Plants carrying the pPsbY-*cph*A_{Te} construct had reduced regeneration frequencies compared to the control vector pLH9000, carrying only the *npt*II gene and the p35S promoter. However, the transgenic shoots of PsbY-*cph*A_{Te} had normal growth rates and phenotypes (Figure 11A).

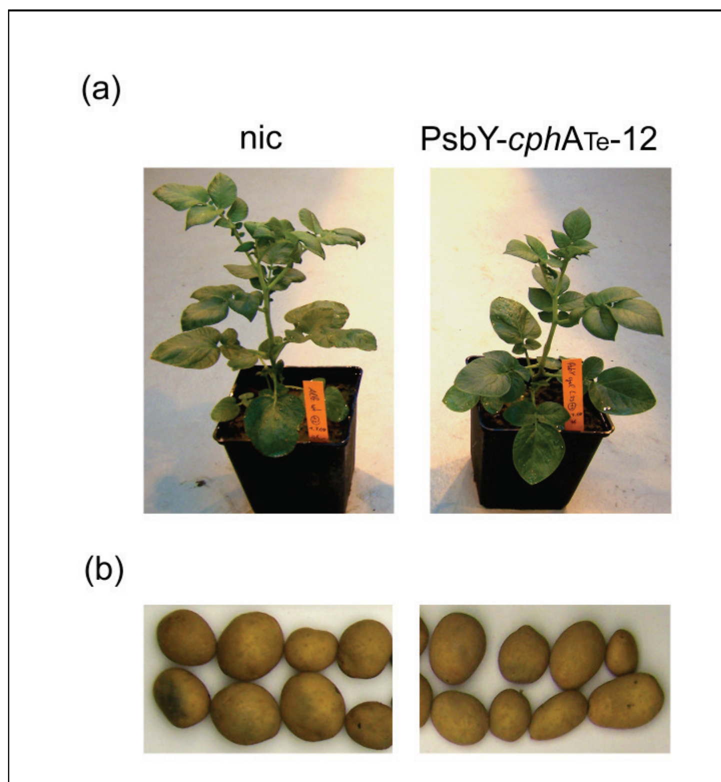

Figure 11 Phenotype of a transgenic PsbY-*cph*A_{Te} potato clone in comparison with the near isogenic control plant (nic). (a) The PsbY-*cph*A_{Te} event 12 was normal in growth in comparison to the near isogenic control. (b) Phenotype of produced tubers.

The presence of the *cph*A_{Te} transgene in potato plants was proven by PCR analysis of leaves. As indicated in Table 6, the majority of generated plants contained the *cph*A_{Te} gene.

The presence of the transgene specific, full length mRNA of *cph*A_{Te} was demonstrated by Northern blot analysis in 9 of 14 of the regenerated plant events (Table 8). No transgenic PsbY-*cph*A_{Te} potato events analyzed did show any phenotypic changes or growth retardation *in vitro* or in greenhouse cultivation compared to the near isogenic control and LH9000 plants. As summarized in Table 8 and Figure 11B, all PsbY-*cph*A_{Te} plants analyzed produced tubers of normal size and weight (Table 8, Figure 11 B).

The determination of the cyanophycin content in leaves and tubers of each of the PsbY-*cph*A_{Te} plants was measured twice at the Humboldt University Berlin (working group of Prof. Lockau, Table 9). The mean cyanophycin content in PsbY-*cph*A_{Te} leaves was 16.0 mg/g dry weight (dw), with a maximum amount of 37.0 mg/g dw in clone 12 (Table 9). In tubers the mean content was 5.6 mg/g dw, with a maximum amount of 9.3 mg/g dw in clone 23 (Table 9).

Table 9 Determination of cyanophycin content and composition (soluble and insoluble form) in tubers and phenotype of various transgenic potato tubers. n.d., not detected.

Plant	**Trans-genic event**	**Content of cyanophycin (mg/g dry weight) in leaves**			**Content of cyanophycin (mg/g dry weight) in tubers**			**Weight of tubers in g**
		Total	**soluble**	**insoluble**	**Total**	**soluble**	**insoluble**	
LH9000	53	-	-	-	-	-	-	69.69
PsbY-			21.9	1.3	7.4			
cphA_{Te}	9	23.2				7.2	0.2	59.89
	10	29.2	28.3	0.9	8.3	8.0	0.3	38.42
	12	37.0	35.3	1.7	9.8	9.3	0.5	47.04
	21	0.5	0.4	0.1	6.1	5.8	0.3	28.38
	23	10.3	9.5	0.8	9.3	8.9	0.4	51.93
	24	21.1	20.5	0.6	5.0	4.7	0.3	50.33
	25	0.2	0.2	n.d.	0.2	0.1	0.1	35.85
	31	11.5	10.8	0.7	2.4	2.3	0.1	9.09
	35	11.0	11.0	n.d.	2.1	2.0	0.1	21.54

The localization of cyanophycin plants was analysed at the University of Bielefeld (working group of Prof. Pistorius/ Prof. Staiger) by electron microscopy. As expected, cyanophycin aggregates were exclusively visible in chloroplasts (leaves) and amyloplasts (tubers) of the PsbY-*cph*A_{Te} plants (Figure 12 A, B). Like in tobacco (Hühns et al., 2008) and potato (Hühns et al., 2009) the aggregates were composed of cyanophycin strands, which differed in thickness and length, were arranged in a pile, and reacted with an antibody raised against cyanobacterial cyanophycin (Figure 12 C).

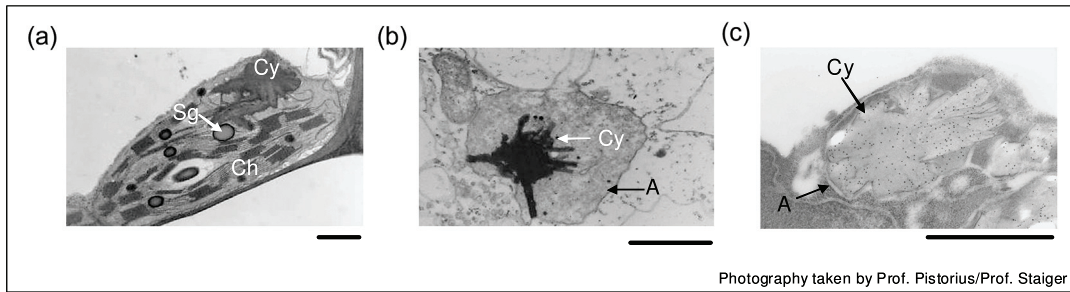

Figure 12 Electron microscopy of cyanophycin in leaves and tubers of PsbY-*cph*A_{Te} potato plants. (a) In leaves. (b) In tubers. (c) Immunocytochemical detection of cyanophycin by the anti-cyanophycin-antiserum and gold-coupled anti-rabbit immunoglobin G (IgG) antibody. Bars, 1µm. Cy, cyanophycin; Ch, chloroplast; Sg, starch grain; A, amyloplast.

To investigate the influence of T-DNA integration and copy number of the *cph*A gene in PsbY-*cph*A_{Te} clones on cyanophycin production, genomic DNA from leaves of PsbY-*cph*A_{Te} events 9, 10, 12, 23, 24, 31 and 35 were tested by Southern blot hybridization (Figure 13). DNA was digested with *Bcl*I and *Eco*RI, blotted and hybridized with the *cph*A probe. For PsbY-*cph*A_{Te} clone 9 and 12 two bands were observed for *Bcl*I- and *Eco*RI -digested DNA probed with the *cph*A sequence. For PsbY-*cph*A_{Te} events 9 and 12 two bands were observed for *Bcl*I- and *Eco*RI -digested DNA probed with the *cph*A sequence indicating two copies of the *cph*A gene. In contrast, a single band was observed for PsbY-*cph*A_{Te} event 10, 23, 24, 31 and 35, which indicates a single locus T-DNA insertion event (Figure 13).

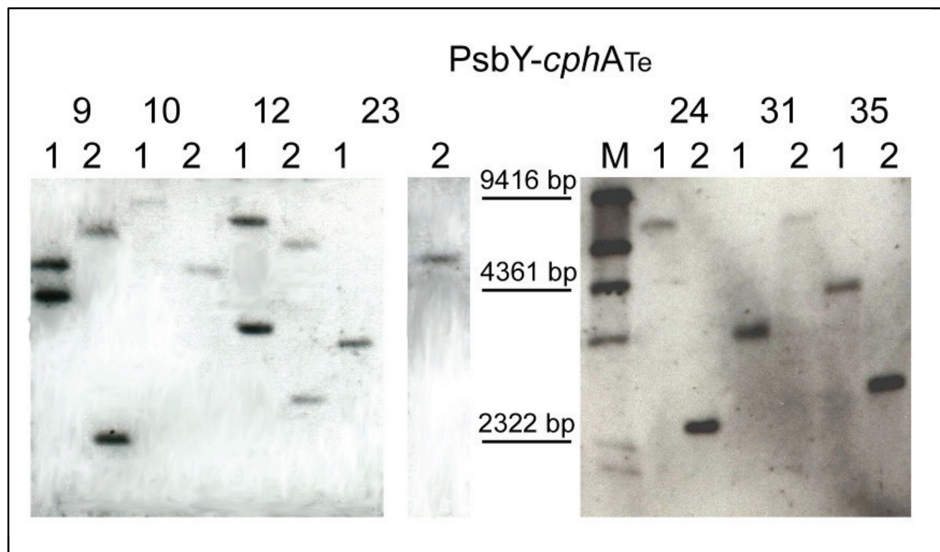

Figure 13 Analysis of *cph*A_{Te} transgene copy number. Southern blots showing the number of *cph*A_{Te} gene copies integrated in the genome of transgenic PsbY- *cph*A_{Te} potato plants; 50g of DNA digested with *Bcl*I or *Eco*RI was probed with the *cph*A_{Te} coding sequence. M, marker. The sizes of the fragments are indicated between the blots. DNA digested with *Bcl*I in lane 1 and with *Eco*RI in lane 2. Beyond transgenic events are indicated.

These results indicate that there is no correlation between cyanophycin content and copy number of the *cph*A gene.

C5 Analysis of the possible reasons for stress symptoms in B33-PsbY-*cph*A_{Te} potato tubers

The transgenic B33-PsbY-*cph*A_{Te} potato tubers exhibited stress symptoms, resulting in brown spots starting from the navel of the tubers (Hühns et al. 2009). The symptoms were observed in the whole tuber, the tissue was macerated which led in conclusion to the death of the tubers. The brown spots resembled symptoms observed in bacterial (*Erwinia atroseptica*) or fungal (*Phytopthora infestans*) infections. In order to find out whether they are caused by these infectious agents, potato tubers of B33-PsbY-*cph*A_{Te} event 38 and controls were sliced and incubated at room temperature for five days (Figure 14).

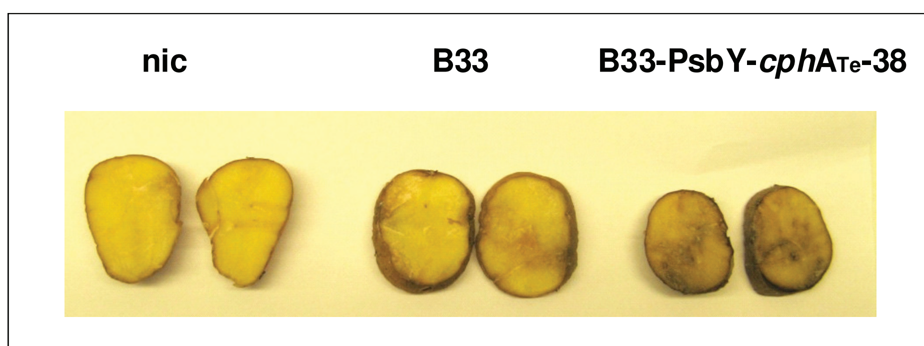

Figure 14 Sliced potato tubers to investigate potential affection of pathogens. Potato tubers were surface sterilized and sliced. The potato slices were incubated on wet filter paper in Petri dishes for five days by room temperature. Nic, near isogenic control; B33 control vector, expressing only the kanamycin resistance gene.

Nevertheless, neither the control tubers, nor the transgenic B33-PsbY-*cph*A_{Te} event 38 potato tubers showed any bacterial or fungal infection.

The brownish discoloration of the tuber tissue indicated a peroxidase mediated polymerization of phenolic compounds which is also observed after wounding.

To investigate this possibility, tuber slices of B33-PsbY-*cph*A_{Te} event 38 and the near isogenic control were placed between two nitrocellulose membranes in an electrical field which allowed the separation of cell wall bound and soluble extracellular plant peroxidases (Figure 15).

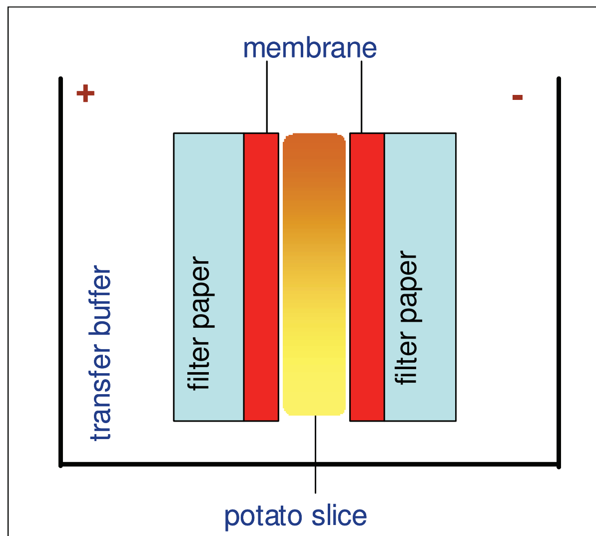

Figure 15 Experimental setup for investigations of bound and free plant peroxidases. The potato tuber was cut and a slice was incubated in water for ten minutes, later it was prepared between two nitrocellulose membrane filter. The transfer buffer (25mM Tris) allowed the separation of membrane bound and free peroxidases in an electrical field. The optical detection was performed by DAB-Cl_2.

The cell wall bound peroxidases moved to the cathode and the soluble peroxidases to the anode. The optical detection was performed with the DAB-Cl_2 staining of the nitrocellulose membrane (Figure 16).

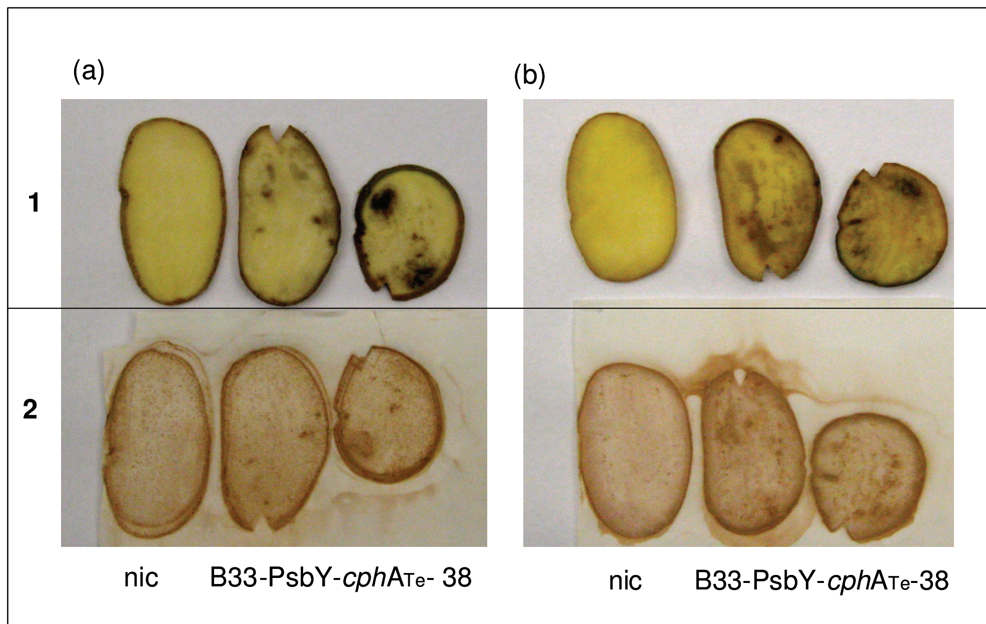

Figure 16 Optical detection of DAB-Cl_2 dyed potato slices for 30 seconds. (a) Free peroxidases from the anode. (b) Membrane bound peroxidases from the cathode. The peroxidase activity was detected by a brownish staining of the nitrocellulose membrane. nic: near isogenic control. 1, potato slices; 2, nitrocellulose membrane.

The highest cell wall bound and soluble extracellular peroxidase activities were detected congruent to the brown coloration of the potato slices. The brown spots of the potato tubers exhibited the strongest brown staining, beside the potato peel. The results indicated an increased plant peroxidase activity in brown spots of cyanophycin producing potatoes, possibly caused by defence of the plant.

D Discussion

The results, presented in this thesis clearly demonstrate that the ability for cyanophycin production can be transferred from the cyanobacterium *Thermosynechococcus elongatus* BP-1 to plants by inserting the responsible bacterial gene into the plant genome (Neumann et al., 2005). Furthermore, we showed tissue as well as organelle specific formation of the polymer when specific target sequences were introduced (Hühns et al. 2008; Hühns et al. 2009). Both, potato and tobacco, showed under greenhouse conditions a maximal cyanophycin accumulation up to 7.5% dry weight (dw). It was demonstrated for the first time, that the production of this biopolymer for industrial demands is feasible in plants. The three main questions expressed in the goal of this thesis are answered and discussed in the publications attached. Here, the currently unpublished results will be discussed in the context of the information published.

D1 The stress symptoms observed in plants expressing the cyanophycin synthetase differ between cultivars and tissues

The results clearly indicate that the severity of stress symptoms positively correlates with the cyanophycin concentration. Nevertheless, the symptoms and their threshold differ between the cultivars and tissues; tobacco leaves tolerate higher cyanophycin concentrations compared to potato tubers and brown spots are only observed in tubers. The different reasons might be responsible for these divergent reactions.

Potential causes for the appearance of brown spots on the cyanophycin producing potato tubers were discussed in Hühns et al. 2009. However, the paper provides no data that support one of these hypotheses.

Although the brown spots on potatoes were comparable to symptoms caused by bacterial (*Erwinia atroseptica*) or fungal (*Phytopthora infestans*) pathogens that are typical for pathogen defence and reactions mediated by plant peroxidases, no infection could be detected. Nevertheless strong peroxidase activity could be demonstrated in the DAB-Cl_2 staining of the nitrocellulose membrane that displayed a mirror image of the discoloration found after cutting the potatoes into slices. Although this newly developed technique to visualize the peroxidase activity *in planta* needs to be improved, the data obtained clearly show that the peroxidase activity is strongly increased in all brown tissue areas indicating strong parallels to pathogen attack or the activated defence system of the plant. Biochemical tissue analysis verified the involvement of peroxidases which were found to be up to 20-fold increased compared to phenotypically “normal” tuber tissue (Unger, unpublished data). Due to the fact that peroxidases are involved in wound or pathogen defence the increase upon cyanophycin accumulation was not expected and is not understood. Further development of these brown spots leads to macerated tissue areas indicating a total destruction of cells.

Additionally the epidermal cell layer is opened for secondary infections of bacteria or fungi. The exact reason for this plant reaction is not known. In the worst case, cyanophycin accumulation in potato tubers could be recognized as foreign compound initializing the plant defence system and leading to the final stage of completely macerated potato tubers. This interesting interaction between cyanophycin and the plant defence system remains to be investigated further.
In tobacco several reasons might be casual for the stress symptoms; (i) the production of the polymer acts at the expense of amino acids and potentially causes a deficit of the cytoplasmic amino acids resources; (ii) the presence of the bulky cyanophycin aggregates in cytoplasm might interfere with the normal functions of this compartment (chapter D3); (iii) the fraction of the water soluble form of the polymer to the water-insoluble form (chapter D4); (iv) the polymer might compete for the available space. This last point is highly speculative and remains to be investigated in future experiments.
To find out the possible reason for stress symptoms the first item was analyzed further. Cyanophycin is an amino acid consuming process. Therefore the availability of amino acids could play a central role in cyanophycin producing plants. The increase of plant available amino acids could reduce stress symptoms and additionally increase cyanophycin content in transgenic plants. Internal and external supply of amino acids in cyanophycin producing plants should help to reduce stress symptoms of plants. The external supply of amino acids should be achieved via casein hydrolysate in the regeneration media and should provide sufficient amounts of amino acids at a very early stage of polymer accumulation and also for regeneration of transgenic plants. The theory that the negative correlation between cyanophycin accumulation in plants and regeneration frequencies might be caused by the deprivation of amino acids for polymer synthesis has already been published (Hühns et al. 2008, Hühns et al. 2009). It is now supported by the fact, that this effect could be compensated through 200mg/l casein hydrolysate supply in regeneration media. Similar reactions of casein hydrolysate additive to regeneration media were observed in other plant species like almond (Ainsley et al. 2000) or rice (Rafiq et al. 2005)and led to increased callus and shoot regeneration. Interestingly higher casein hydrolysate concentrations led to reduction of regeneration frequencies compared to those without casein hydrolysate additive. However the supply did not lead to an increase in cyanophycin content. This might be due to the fact, that the massive formation of cyanophycin takes place in later developmental stages of the plant life where no external amino acids were supplied. Unfortunately, the internal increase in amino acids, that was supposed to occur in plants expressing bacterial glutamine synthetase genes, was not successful- no improvement of the phenotype or increased polymer content of cyanophycin producing tobacco plants was observed. This was due to the fact, that although the transgenic *gln*A plants exhibited 50%

increased glutamine synthetase activity and a tenfold increase in glutamine content compared to the near isogenic control plant no effect in total amino acid content was detected. In transgenic *gln*II and *arg*J plants neither an increased glutamine nor arginine content was verified. (W. Wohlleben unpublished results). Since also plants carrying the *gln*A gene did not show any improvement in plant grows or cyanophycin production, it might be assumed that there might be also no increase in glutamine or arginine concentrations. The reasons for this are discussed in the next chapter.

D2 Effects of glutamine synthetase expression in cyanophycin producing plants

As indicated before, no positive effect on cyanophycin content or stressed phenotype was observed by overexpression of the bacterial *gln*A gene. In contrast, the overexpression of the bacterial *gln*A gene in rice resulted in an increased glutamine synthetase activity, total amino acid content and total nitrogen content (Cai et al. 2009). Possibly, there are differences in the recognition of the bacterial *gln*A gene in monocots like rice and dicots like tobacco. Several publications show that plant derived glutamine synthetase genes lead to an increased glutamine content and enhanced protein or biomass production in transgenic plants like tobacco (Lam et al. 1995; Migge et al. 2000; Habash et al. 2001; Oliveira et al. 2002), rice (Hoshida et al. 2000; Cai et al. 2009) or hybrid poplar (Gallardo et al. 1999). However, other studies also showed an overexpression of heterologous glutamine synthetase genes, but the increase of glutamine synthetase activity was not associated with enhanced protein or biomass production (Eckes et al. 1989; Hirel et al. 1992; Vincent et al. 1997; Limami et al. 1999). The reason for this discrepancy is not known.

In conclusion, the overexpression of plant derived glutamine synthetase genes in cyanophycin producing plants might be able to increase glutamine and protein content. However this approach should be tested in future experiments.

D3 Approaches for optimization of cyanophycin production in plants

D3.1 Improvement of the cyanophycin production by relocation the cyanophycin synthesis to plastids

Based on results of cytoplasmic cyanophycin accumulation, a new strategy for increased cyanophycin content was developed by targeting the cyanophycin synthetase into plastids. Thus, the storage of the polymer cyanophycin was restricted to the plastid as well. As consequence the stress symptoms in tobacco and potato were notedly reduced (Hühns et al. 2008). Supplementary to the discussion in the publication Hühns et al. 2009 further aspects have to be discussed why cyanophycin accumulation in plastids is more biocompatible than in cytoplasm.

It might be speculated that this phenomenon is due to the fact that plastids are descendants of cyanobacteria, the origin of the cyanophycin production (Borzi 1887). Like in cyanobacteria, all precursors for cyanophycin synthesis, especially the key enzymes for L-aspartate and L-arginine synthesis, are present in the chloroplast (Forchhammer and Demarsac 1994; Sugiyama et al. 2004; Slocum 2005; Chen et al. 2006). Apparently, the transgenic plants favour the cyanophycin accumulation in chloroplasts according to the amounts detected in leaves and tubers. Generally, in chloroplasts the reduction of nitrate for amino acid production is a very energy consuming process and is linked to the photosynthesis (Figure 17). In addition to the normal demand of the plant, the cyanophycin production displays a new consumer of metabolites like amino acids. An important benefit of the cyanophycin production in chloroplasts in comparison to cytoplasm is that the produced amino acids aspartate and arginine could be directly used for polymer accumulation (Figure 17).

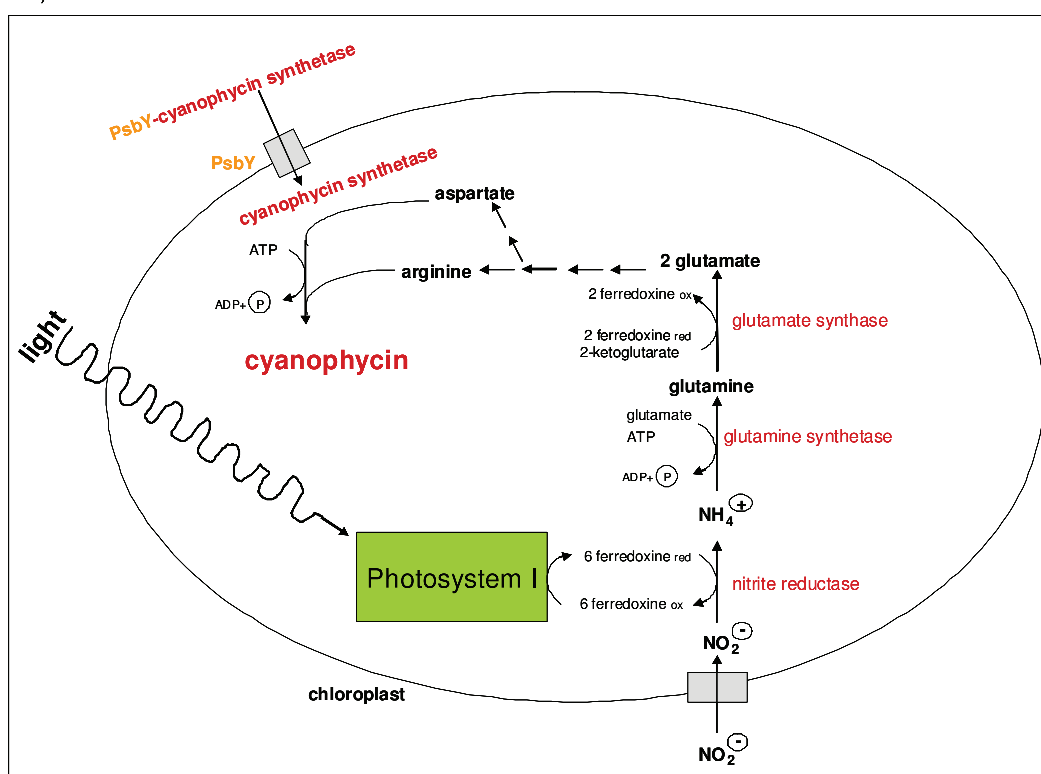

Figure 17 Scheme of pathways for synthesis of (i) arginine and aspartate from nitrite, (ii) cyanophycin. Scheme represents production of the amino acids arginine and aspartate, which could direct used for cyanophycin synthesis. The essential electrons for the nitrite reductase are allocated by photosystem I. red, reduced; ox, oxidised

D3.2 Optimization of cyanophycin production by tuber specific expression of the cyanophycin synthetase under control of the GBSS promoter

As an alternative to the tuber specific B33 promoter, the GBSS promoter of the granule-bound starch synthetase is often used for tuber specific transgene expression in potato plants (Kortstee et al. 1996; Shekhawat et al. 2007). The GBSS promoter exhibited a

stronger expression rate in tubers compared to the CaMV35S promoter or the B33 promoter. Similar to the B33 promoter, sugars like sucrose can induce the GBSS promoter recognition in leaves, but not to levels as high as it does for the patatin promoter (pB33). In different publications the GBSS promoter was used for the overexpression of transgenes in potato tubers (Vandersteege et al. 1992; Kortstee et al. 1996; Lopez et al. 2008). In order to improve the cyanophycin production further experiments were designed to integrate the GBSS promoter into the plant genome. All attempts to express the cyanophycin synthetase under control of the GBSS promoter in potato plants failed caused by instability of the binary vector in agrobacteria or the integrated gene was inactive in potato. The second reason was further investigated. Up to now the induction of the GBSS promoter with other sugars like glucose and fructose is known (Visser et al. 1991), but not to levels obtained with sucrose induction. Based on these facts, it might be possible that in regenerating transgenic cells cyanophycin production is induced by glucose which is normally contained in regeneration media. As a consequence high cyanophycin content could lead to toxic effects on cell. Furthermore only cells could regenerate which includes an inactive copy of the cyanophycin synthetase gene. That in turn represents transgenic plants without cyanophycin accumulation, although the transgene is present. This hypothesis is supported by the strong reduced regeneration rate of the GBSS-*cph*A_{Te} construct (Table 2).

D4 Different solubility and molecular masses of the cyanophycin polymer formed by transgenic plants and cyanobacteria and their influence on plant health

In contrast to cyanobacteria, tobacco and potato form a second polymer type, a water-soluble form of cyanophycin. The ratio of these two forms differed in the plants depending on the sub cellular formation of cyanophycin. In transgenic tobacco events producing cytoplasmic cyanophycin, the mean ratio of insoluble to water-soluble form was 1:0.6. In transgenic PsbY-*cph*A_{Te} events with cyanophycin production in plastids, only traces of the water-soluble form were detected.

Interestingly, all potato tubers carrying the B33-PsbY-*cph*A_{Te} construct, showed a direct correlation between the amount of water-soluble cyanophycin and the level of damage. No correlation of water-insoluble cyanophycin to phenotypical damages was found. In conclusion, the data indicated a correlation between the presence of the water-soluble polymer and the deleterious effects on phenotype of tobacco and potato. Amazingly, the chemical structure of the two forms of the polymer has been reported to be identical (Ziegler et al. 2002; Füser and Steinbüchel 2005). However, the soluble form of cyanophycin was also found in the recombinant *Saccharomyces cerevisiae* (Steinle et al. 2008) and occurred naturally in the non-cyanobacterial eubacterium *Desulfitobacterium hafniense* (Ziegler et al. 2002). It is still unclear, what causes the appearance of a soluble form of cyanophycin and

what is its difference to the insoluble cyanophycin produced in cyanobacteria. Hence it also remains to be investigated what might be the cause for the correlation between the concentration of soluble cyanophycin and damages observed in transgenic plants.
In addition to the unexpected appearance of water soluble cyanophycin in plants, also the molecular mass distribution of cyanophycin differs with 20-35 kDa formed in transgenic tobacco and potato plants to the cyanobacterial product. Nevertheless distributions similar to those in plants were observed for recombinant bacteria, *S. cerevisiae* and *in vitro* synthesized cyanophycin (Ziegler et al. 1998; Oppermann-Sanio et al. 1999; Aboulmagd et al. 2001; Frey et al. 2002 ;Steinle et al. 2008).
In contrast, in cyanobacteria the molecular masses were much higher ranging up to 130 kDa (Hai et al. 1999) compared to the plant cyanophycin. By now proper reasons are not known but it might be speculated that it is caused by (i) an abruption of synthesis after definite size of the polymer; (ii) the conditions for cyanophycin synthesis in cyanobacteria that differ in comparison to recombinant organism like bacteria, yeast and plant; (iii) the optimal temperature for synthesis; (iv) further unknown factors which are necessary for or influence synthesis. It might be assumed that in cyanobacteria not only the cyanophycin synthetase influences the polymer accumulation, but additional genes are present that allow polymer size up to 130kDa. These specific genes are not transferred to recombinant organism like bacteria, yeast and plant leading to polymer masses only up to 35kDa.

D5 Stability of cyanophycin in plants

The structure of cyanophycin aggregates changed during plant growth in tobacco leaves as well as in potato tubers but did not lead to any decrease in cyanophycin concentration. The changes occur when chloroplasts or amyloplasts mature to gerontoplasts or chromoplasts, respectively. According to the electron microscope studies carried out at the University of Bielefeld, the compact structure of cyanophycin aggregates changed to a loosely packed structure. When the inner plastid membrane looses its integrity the compact shape of the cyanophycin aggregates is lost as well. Hence it might be possible that the cyanophycin aggregates are tied to the membrane as a compact structure which is consequently lost during the aging of the inner membrane.
Another possibility is the biochemical degradation of the polymer cyanophycin which has up to now only be reported in cyanobacteria where it is catalyzed by a specialized enzyme called cyanophycinase (EC 3.4.15.6) (Allen 1984; Simon 1987). Similar enzymes have never been described in plants. Nevertheless, the changes might be correlated to changes in cyanophycin synthesis. This assumption is supported by the fact that no cyanophycin synthetase could be detected via immuno gold labelling in the loosely packed structure of cyanophycin, whereas the CphA enzyme was found in the compact structure. These

differences might indicate that the loosely packed structure is synthesized at first and thereby older than the compact structure.

It is not known, whether synthesis of cyanophycin occurs in gerontoplasts or chromoplasts. These organelles have degenerated membrane systems and reduced thylakoid systems (Tuquet and Newman 1980; Thomson and Platt-Aloia 1987 ;Matile 1992; Thomas 1997). Conceivably, the cyanophycin synthetase might not be able to integrate into the inner membrane of these maturated plastids via the PsbY transit peptide due to its changes during senescence (Springer 2007) and the protein import apparatus enables no protein translocation.

D6 *Commercial application of cyanophycin producing potato plants*

The profitability of a large scale production of cyanophycin in cyanobacteria is limited by a low polymer content slow growth of the bacteria (Mooibroek et al. 2007). Up to now, the production of cyanophycin in *E. coli* is not possible, since in recombinant *E. coli* strains like DH1 and DH5α the plasmid carrying the *cph*A gene is lost during fermentation (Mooibroek et al. 2007). In other bacteria like *Corynebacterium glutamicum*, *Ralstonia eutropha* and *Pseudomonas putida* high amounts of the polymer were produced in one to two days (up to 50% of cell dry weight). This is much faster compared to cyanobacteria, which need about four weeks (Aboulmagd et al. 2001). Nevertheless, the cultivation of bacteria needs the supply of substrate, energy, a sterile environment and disposal of waste material, which causes high costs and energy demand. Therefore, the production in plants, supported only by fertilizer and solar energy, might be a lot more attractive. In addition, the costs can be further reduced if cyanophycin is produced as a by-product in plants that are already cultivated to build other industrial products like starch. In this case, cyanophycin can be isolated from the left over and should therefore be a cheap alternative.

Cyanophycin formation in plants is a CO_2-neutral and environmentally safe production of a biopolymer as substitute for fossil hydrocarbon. Therefore potato was chosen as plant to establish the cyanophycin synthesis.

Potato farming splits into two branches – an industrial branch e.g. for industrial starch production and a food branch. The accumulation of cyanophycin in industrial potato tubers is of high interest to the potato starch industry as a by-product to starch. Production in these plants does not require any additional infrastructure or other special equipment. In an evaluation of economic efficiency within the interdisciplinary working group "Zukunftorientierte Nutzung ländlicher Räume-Landinnovation-" the benefit of cyanophycin producing potatoes were calculated (Serr et al. 2007). Assuming that 5% of dry weight after isolation represents cyanophycin the following calculation is possible (Table 10).

Table 10: Calculation of feasible benefit of polyaspartate and arginine from cyanophycin producing potato tubers.

	ratio/ton in kg	**price/kg in €**	**price/ton rest protein in €**
dry weight	250		
cyanophycin	12.5		
polyaspartate	6.25	3.50 €	ca. 22 €
arginine	6.25	89.00 €	ca. 556 €
costs of isolation of the polymer			100 €
reprocessing			200 €
benefit			278 €
benefit/hectare (with 300deci tons harvest)			**8340 €**
actual benefit/ hectare from starch production			5000 €

Investments for process engineering performing the cyanophycin isolation have to be detached from the benefit, because so far this item is not quantifiable at present. Additionally, the profit from the disposal of potato pulp after isolation of cyanophycin might increase the potential value of the harvested tubers. The prices for polyaspartate and arginine reflect the present market value. The published data show only a slightly reduced starch content of cyanophycin producing potato tubers (Hühns et al. 2009). Nevertheless the market value of cyanophycin versus starch is 1.6 fold higher. The reduction of starch content in advantage of cyanophycin accumulation could increase the market potential of the potato additionally due to the higher market value of cyanophycin. The exact maximum cyanophycin content in tubers is not predictable yet and more research is needed to answer open questions like stress symptoms of tubers, storage capacity of the tubers and metabolite stream to polymer synthesis.

D7 Prospects for enhanced cyanophycin accumulation in plants and further suitable plant systems for cyanophycin production

The tuber specific high cyanophycin accumulation in plastids of transgenic potato plants was accompanied by stress symptoms visible as shrunken tubers with brown spots. One of the possibilities which caused these symptoms was a reduced ability to produce essential proteins due to depletion of L-amino acids, most likely arginine and/ or aspartate. These tubers showed a reduced content of bound glutamate/glutamine and also the free glutamate pool was significantly reduced compared to the near isogenic control and vector control tubers. For cyanophycin synthesis this reduced glutamate pool might be a strong limiting factor, because arginine is synthesized via glutamate. As a next step it could be advantageous to optimize cyanophycin synthesis in plants by an increase in the amount of

amino acids that are available for essential proteins as well as for cyanophycin accumulation. In heterotrophic bacteria and in the cyanobacterium *Synechocystis* sp. PCC 6803 arginine was identified as a limiting factor for cyanophycin accumulation (Elbahloul et al. 2005; Maheswaran et al. 2006) because a strong positive influence of externally added arginine on cyanophycin production was observed. The key enzyme for arginine biosynthesis represents the N-acetyl glutamate kinase (NAGK), that acts as a signal protein, widely spread in bacteria, archae, and plants (Ninfa and Atkinson 2000; Arcondeguy et al. 2001; Forchhammer et al. 2004) and its activity is regulated by the PII protein. The enzyme acts as sensor in the nitrogen assimilation regulation and is located in the plastids of plants (Forchhammer and Demarsac 1994; Ninfa and Atkinson 2000; Sugiyama et al. 2004; Chen et al. 2006). Through the expression of the NAGK in cyanophycin producing plants, the increased arginine requirement could be covered, since cyanophycin accumulation and arginine biosynthesis occurred in the same compartment of the cell. The regulation of the bacterial NAGK could be carried out by the highly conserved PII protein of the plant.

The cyanophycin accumulation in transgenic plants may be restricted by the activity of the cyanophycin synthetase from *T. elongatus* BP1. The enzyme is thermally resistant and retains 80% activity after a 60-min incubation at 50℃ (Arai and Kino 2008). Furthermore the highest activity was detected at pH 9.0 in *in vitro* experiments (Arai and Kino 2008), although CphAs from *Synechocystis* sp. PCC6308 (Aboulmagd et al. 2001) and *A. cyclindrica* (Hai et al. 2002) showed the highest activity at pH 8.2. In plants the direction of the cyanophycin synthetase to chloroplast using the PsbY transit peptide led to polymer accumulation; however, the exact localization of the enzyme within chloroplasts is not known. The targeting sequence of PsbY belongs to the integral protein of photosystem II (PsbY), located in the thylakoid membrane, with its N terminus exposed to the lumen, and C terminus to the stroma (Gau et al. 1998). Chloroplast exhibited different pH values in the stroma space and the thylakoid space in the dark and by illumination, which range from 5.5 to 8 (Werdan et al. 1975). At these pH values the cyanophycin synthetase from *T. elongatus* BP-1 has not the optimal enzyme activity, possibly leading to less cyanophycin production. Furthermore in plants under normal cultivation conditions the optimal temperature for the cyanophycin synthetase is not reached. It might be speculated that under controlled and optimal temperature conditions, an increased cyanophycin production is possible. Since it might be assumed, that optimal temperature conditions for the enzyme might already harm the plant, plants with a higher temperature tolerance like tobacco might be preferable.

In tubers, nitrogen transport occurs through the amino acids glutamine and aspartic acid, their corresponding amides and also serine and alanine (Schilling 2000). Interestingly, asparagine was identified as a reaction partner by the production of acrylamide, which is formed in fried potato products. In toxicological animal feeding experiments acrylamide was

identified as carcinogen and genetically harmful. It might be speculated that cyanophycin production in potato tubers could reduce the formation of asparagine (Figure 18) and as a consequence the production of acrylamide by the reduction of the free aspartate content. Possibly, this effect is an interesting topic for future research and of high interest of potato breeders.

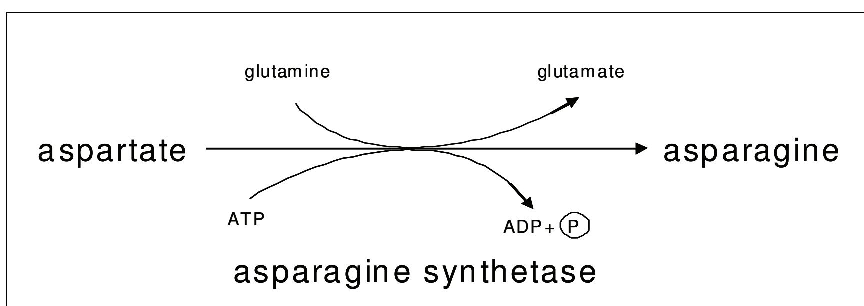

Figure 18 Biosynthesis of asparagine. ATP is used to activate aspartate, forming β-aspartyl-AMP. Glutamine donates an ammonium group which reacts with β-aspartyl-AMP to form asparagine and free AMP.

The relevance of tobacco plants changed from a model organism to a seriously discussed producer of cyanophycin. By now high contents of cyanophycin increased the commercial relevance as a by-product of pharmacological nicotine and other value adding components like organic acids, fats, waxes and vitamins. Furthermore, the cultivation of tobacco in Germany is threatened by cancellation of subsidies in 2010. The use of transgenic tobacco as a production system for economical interesting products enables farmers to use their available know how, existing resources, equipment and accoutrements. Up to now, cyanophycin accumulation was established in *Nicotiana tabacum* Petit Havanna SRI, a short stature plant, which is not interesting for high biomass yield. In preliminary experiments the cyanophycin synthetase from *T. elongatus* BP-1 was transformed in the tobacco species Badischer Geudertheimer and Virginia Golta, used for commercial smoking tobacco, which led to cyanophycin accumulation up to 4.9% dw (Hausmann, unpublished data). An advantage of tobacco versus potato as cyanophycin producer is given by the fact that tobacco is never involved in any step of the food chain. The accidentally appearance of cyanophycin containing plants in the food production chain is nearly impossible.
The use of additional cultivable and agricultural important plants opens the possibility to allow polymer accumulation besides existing production processes. Naturally potatoes store energy in form of carbohydrate polymers, which might explain phenotypically damages when the tubers accumulated economically interesting amounts of cyanophycin. It might be of benefit that for accumulation of nitrogen containing polymer like cyanophycin the use of a plant which naturally stores nitrogen could solve the problem. These include economically interesting plants like legumes. *Pisum sativum* would increase the biodiversity of crop rotation and also allows a threefold usage of plant: (i) isolation of cyanophycin from the

sprout; (ii) the rest of the sprout can be used as feed or for energetically purposes; (iii) the roots are used for green fertilization. For the farmers the market value of pea would increase, since an additional valuable component is produced without additional input. A further advantage is that green fertilization supports the building of humus, enrichment of the soil with nitrogen, as well as soil erosion and eluviations of nutrients counteracts.

E References

Aboulmagd, E., Voss, I., Oppermann-Sanio, F.B., and Steinbüchel, A. (2001) Heterologous expression of cyanophycin synthetase and cyanophycin synthesis in the industrial relevant bacteria Corynebacterium glutamicum and Ralstonia eutropha and in Pseudomonas putida. *Biomacromolecules* 2:1338-1342.

Ainsley, P.J., Collins, G.G., and Sedgley, M. (2000) Adventitious shoot regeneration from leaf explants of almond (Prunus dulcis Mill.). *In Vitro Cellular & Developmental Biology-Plant* 36:470-474.

Allen, M.M. (1984) Cyanobacterial Cell Inclusions. *Annual Review of Microbiology* 38:1-25.

Amber, I.J., Hibbs, J.B., Taintor, R.R., and Vavrin, Z. (1988) The L-Arginine dependent effector mechanism is induced in murine adenocarcinoma cells by culture supernatant from cyto-toxic activated macrophages. *Journal of Leukocyte Biology* 43:187-192.

Arai, T. and Kino, K. (2008) A cyanophycin synthetase from *Thermosynechococcus elongatus* BP-1 catalyzes primer-independent cyanophycin synthesis. *Applied Microbiology and Biotechnology* 81:69-78.

Arai, Y., Nakashita, H., Suzuki, Y., Kobayashi, K., Shimizu, T., Yasuda, M., Doi, Y., and Yamaguchi, I. (2001) Plastid targeting of polyhydroxybutyrate biosynthetic pathway in tobacco. *Plant Biotechnology* 18:289-293.

Arcondeguy, T., Jack, R., and Merrick, M. (2001) P-II signal transduction proteins, pivotal players in microbial nitrogen control. *Microbiology and Molecular Biology Reviews* 65:80-+.

Baucher, M., Halpin, C., Petit-Conil, M., and Boerjan, W. (2003) Lignin: Genetic engineering and impact on pulping. *Critical Reviews in Biochemistry and Molecular Biology* 38:305-350.

Borzi, A. (1887) Le communicazioni intracellulari delle Nostochinee. *Malpighia* 1:27-74.

Cai, H.M., Zhou, Y., Xiao, J.H., Li, X.H., Zhang, Q.F., and Lian, X.M. (2009) Overexpressed glutamine synthetase gene modifies nitrogen metabolism and abiotic stress responses in rice. *Plant Cell Reports* 28:527-537.

Caso,G., McNurlan,M.A., McMillan,N.D., Eremin,O., and Garlick,P.J. 2004. Tumour cell growth in culture: dependence on arginine. *Clinical Science* 107:371-379.

Cen, Y., Luo, X., and Liu ,X. (1999) Effect of arginine on deep partial thickness burn in rats. Hua Xi Yi Ke Da Xue Xue Bao. 30[2], 198-201. Ref Type: Generic

Chang, C.J. and Swift, G. (1999) Poly(aspartic acid) hydrogel. *Journal of Macromolecular Science-Pure and Applied Chemistry* A36:963-970.

Chen, Y.M., Ferrar, T.S., Lohmeier-Vogel, E.M., Morrice, N., Mizuno, Y., Berenger, B., Ng, K.K.S., Muench, D.G., and Moorhead, G.B.G. (2006) The PII signal transduction protein of *Arabidopsis thaliana* forms an arginine-regulated complex with plastid N-acetyl glutamate kinase.(vol 281, pg 5726, 2006). *Journal of Biological Chemistry* 281:24084.

de Jonge, W.J., Kwikkers, K.L., te Velde, A.A., van Deventer, S.J.H., Nolte, M.A., Mebius, R.E., Ruijter, J.M., Lamers, M.C., and Lamers, W.H. (2002) Arginine deficiency affects early B cell maturation and lymphoid organ development in transgenic mice. *Journal of Clinical Investigation* 110:1539-1548.

Eckes, P., Schmitt, P., Daub, W., and Wengenmayer ,F. (1989) Overproduction of alfalfa glutamine-synthetase in transgenic tobacco plants. *Molecular & General Genetics* 217:263-268.

Eissen, M., Metzger, J.O., Schmidt, E., and Schneidewind, U. (2002) 10 years after Rio - concepts on the contribution of chemistry to a sustainable development. *Angewandte Chemie-International Edition* 41:414-436.

Elbahloul, Y., Krehenbrink, M., Reichelt, R., and Steinbüchel, A. (2005) Physiological conditions conducive to high cyanophycin content in biomass of *Acinetobacter calcoaceticus* strain ADP1. *Applied and Environmental Microbiology* 71:858-866.

FKZ 98NR090 (2003) Abschlussbericht: Produktion von biologisch abbaubaren Polymeren in transgenen Kartoffelknollen. *im Rahmen des Forschungsverbundes Wissenschaft-Wirtschaft im BML-Förderschwerpunkt Nachwachsende Rohstoffe*.

Flynn, N.E., Meininger, C.J., Haynes, T.E., and Wu, G. (2002) Dossier: Free amino acids in human health and pathologies - The metabolic basis of arginine nutrition and pharmacotherapy. *Biomedicine & Pharmacotherapy* 56:427-438.

Forchhammer, K. and Demarsac, N.T. (1994) The PII protein in the cyanobacterium *Synechococcus* sp. strain PCC-7942 is modified by serine phosphorylation and signals the cellular N-status. *Journal of Bacteriology* 176:84-91.

Forchhammer, K., Irmler, A., Kloft, N., and Ruppert, U. (2004) P-II signalling in unicellular cyanobacteria: analysis of redox-signals and energy charge. *Physiologia Plantarum* 120:51-56.

Frey, K.M., Oppermann-Sanio, F.B., Schmidt, H., and Steinbüchel, A. (2002) Technical-scale production of cyanophycin with recombinant strains of *Escherichia coli*. *Applied and Environmental Microbiology* 68:3377-3384.

Füser, G. and Steinbüchel, A. (2005) Investigations on the solubility behavior of cyanophycin. Solubility of cyanophycin in solutions of simple inorganic salts. *Biomacromolecules* 6:1367-1374.

Gallardo, F., Fu, J.M., Canton, F.R., Garcia-Gutierrez, A., Canovas, F.M., and Kirby, E.G. (1999) Expression of a conifer glutamine synthetase gene in transgenic poplar. *Planta* 210:19-26.

Gau, A.E., Thole, H.H., Sokolenko, A., Altschmied, L., Herrmann, R.G., and Pistorius, E.K. (1998) PsbY, a novel manganese-binding, low-molecular-mass protein associated with photosystem II. *Molecular and General Genetics* 260:56-68.

George, F.F. (1993) Plant propagation by tissue culture. Part 1: The technology, 2nd edn., Exegetics ed pp 285-291.

Habash, D.Z., Massiah, A.J., Rong, H.L., Wallsgrove, R.M., and Leigh, R.A. (2001) The role of cytosolic glutamine synthetase in wheat. *Annals of Applied Biology* 138:83-89.

Hai, T., Oppermann-Sanio, F.B., and Steinbüchel, A. (1999) Purification and characterization of cyanophycin and cyanophycin synthetase from the thermophilic *Synechococcus* sp MA19. *Fems Microbiology Letters* 181:229-236.

Herrera-Estrella, L., Deblock, M., Messens, E., Hernalsteens, J.P., Vanmontagu, M., and Schell, J. (1983) Chimeric genes as dominant selectable markers in plant cells. *Embo Journal* 2:987-995.

Hirel, B., Marsolier, M.C., Hoarau, A., Hoarau, J., Brangeon, J., Schafer, R., and Verma, D.P.S. (1992) Forcing expression of a soybean root glutamine-synthetase gene in tobacco leaves induces a native gene encoding cytosolic enzyme. *Plant Molecular Biology* 20:207-218.

Hoshida, H., Tanaka, Y., Hibino, T., Hayashi, Y., Tanaka, A., Takabe, T., and Takabe, T. (2000) Enhanced tolerance to salt stress in transgenic rice that overexpresses chloroplast glutamine synthetase. *Plant Molecular Biology* 43:103-111.

Houmiel, K.L., Slater, S., Broyles, D., Casagrande, L., Colburn, S., Gonzalez, K., Mitsky, T.A., Reiser, S.E., Shah, D., Taylor, N.B., Tran, M., Valentin, H.E., and Gruys, K.J. (1999) Poly(beta-hydroxybutyrate) production in oilseed leukoplasts of *Brassica napus*. *Planta* 209:547-550.

Hühns, M. and Broer, I. (in press) Characters of transgenic plants and their application in plant production: Biopolymers. *In: Biotechnology in Agriculture and Forestry Vol. X Genetic Modification of Plants-Agriculture, Horticulture and Forestry (eds. F. Kempken and C. Jung) Springer, Heidelberg*.

Hühns, M., Neumann, K., Hausmann, T., Klemke, F., Lockau, W., Kahmann, U., Kopertekh, L., Staiger, D., Pistorius, E.K., Reuther, J., Waldvogel, E., Wohlleben, W., Effmert, M., Junghans, H., Neubauer, K., Kragl, U., Schmidt, K., Schmidtke,J ., and Broer, I. (2009) Tuber specific *cph*A expression to enhance cyanophycin production in potato. *Plant Biotechnology Journal*.

Hühns, M., Neumann, K., Hausmann, T., Ziegler, K., Klemke, F., Kahmann, U., Staiger, D., Lockau, W., Pistorius, E.K., and Broer, I. (2008) Plastid targeting strategies for cyanophycin synthetase to achieve high-level polymer accumulation in *Nicotiana tabacum*. *Plant Biotechnology Journal* 6:321-336.

Joentgen, W., Groth, T.S.A., Hai, T., and Oppermann, F.B. (200) Polyasparaginic acid homopolymers and copolymers, biotechnical production and use thereof. *US Patent* 6.

John, M.E. and Keller, G. (1996) Metabolic pathway engineering in cotton: Biosynthesis of polyhydroxybutyrate in fiber cells. *Proceedings of the National Academy of Sciences of the United States of America* 93:12768-12773.

Kortstee, A.J., Vermeesch, A.M.S., deVries, B.J., Jacobsen, E., and Visser, R.G.F. (1996) Expression of Escherichia coli branching enzyme in tubers of amylose-free transgenic potato leads to an increased branching degree of the amylopectin. *Plant Journal* 10:83-90.

Krehenbrink, M., Oppermann-Sanio, F.B., and Steinbüchel, A. (2002) Evaluation of non-cyanobacterial genome sequences for occurrence of genes encoding proteins homologous to cyanophycin synthetase and cloning of an active cyanophycin synthetase from *Acinetobacter* sp strain DSM 587. *Archives of Microbiology* 177:371-380.

Lam, H.M., Coschigano, K., Schultz, C., Melooliveira, R., Tjaden, G., Oliveira, I., Ngai, N., Hsieh, M.H., and Coruzzi, G. (1995) Use of arabidopsis mutants and genes to study amide amino acid biosynthesis. *Plant Cell* 7:887-898.

Lee, S.Y. (1996) High cell-density culture of *Escherichia coli*. *Trends in Biotechnology* 14:98-105.

Lenis, N.P., van Diepen, H.T.M., Bikker, P., Jongbloed, A.G., and van der Meulen, J. (1999) Effect of the ratio between essential and nonessential amino acids in the diet on utilization of nitrogen and amino acids by growing pigs. *Journal of Animal Science* 77:1777-1787.

Li, P., Yin, Y.L., Li, D., Kim, S.W., and Wu, G.Y. (2007) Amino acids and immune function. *British Journal of Nutrition* 98:237-252.

Limami, A., Phillipson, B., Ameziane, R., Pernollet, N., Jiang, Q.J., Poy, R., Deleens, E., Chaumont-Bonnet, M., Gresshoff, P.M., and Hirel, B. (1999) Does root glutamine synthetase control plant biomass production in *Lotus japonicus* L. *Planta* 209:495-502.

Lopez, A.B., Van Eck, J., Conlin, B.J., Paolillo, D.J., O'Neill, J., and Li ,L. (2008) Effect of the cauliflower or transgene on carotenoid accumulation and chromoplast formation in transgenic potato tubers. *Journal of Experimental Botany* 59:213-223.

Lössl, A., Eibl, C., Harloff, H.J., Jung, C., and Koop, H.U. (2003) Polyester synthesis in transplastomic tobacco (*Nicotiana tabacum* L.): significant contents of polyhydroxybutyrate are associated with growth reduction. *Plant Cell Reports* 21:891-899.

Mackerras, A.H., Dechazal, N.M., and Smith, G.D. (1990) Transient accumulations of cyanophycin in *Anabaena cylindrica* and *Synechocystis*-6308. *Journal of General Microbiology* 136:2057-2065.

Maheswaran, M., Ziegler, K., Lockau, W., Hagemann, M., and Forchhammer, K. (2006) P-II-regulated arginine synthesis controls accumulation of cyanophycin in *Synechocystis* sp strain PCC 6803. *Journal of Bacteriology* 188:2730-2734.

Matile, P. (1992) Chloroplast senescence. *In Baker NR, Thomas, H. , eds. Crop photosynthesis: spatial and temporal determinants Amsterdam: Elsevier*413-440.

Menzel, G., Harloff, H.J., and Jung, C. (2003) Expression of bacterial poly (3-hydroxybutyrate) synthesis genes in hairy roots of sugar beet (*Beta vulgaris* L.). *Applied Microbiology and Biotechnology* 60:571-576.

Migge, A., Carrayol, E., Hirel, B., and Becker, T.W. (2000) Leaf-specific overexpression of plastidic glutamine synthetase stimulates the growth of transgenic tobacco seedlings. *Planta* 210:252-260.

Mikschofsky, H. (2006) Charakterisierung des Produktionssystems Pflanze für die rekombinante Impfstofferzeugung. Logos Verlag Berlin, Ref Type: Thesis/Dissertation

Mooibroek, H., Oosterhuis, N., Giuseppin, M., Toonen, M., Franssen, H., Scott, E., Sanders, J., and Steinbüchel, A. (2007) Assessment of technological options and economical feasibility for cyanophycin biopolymer and high-value amino acid production. *Applied Microbiology and Biotechnology* 77:257-267.

Nakashita, H., Arai, Y., Yoshioka, K., Fukui, T., Doi, Y., Usami, R., Horikoshi, K., and Yamaguchi, I. (1999) Production of biodegradable polyester by a transgenic tobacco. *Bioscience Biotechnology and Biochemistry* 63:870-874.

Nawrath, C., Poirier, Y., and Somerville, C. (1994) Targeting of the polyhydroxybutyrate biosynthetic pathway to the plastids of *Arabidopsis thaliana* results in high levels of polymer accumulation. *Proceedings of the National Academy of Sciences of the United States of America* 91:12760-12764.

Nieves, C., Jr. and Langkamp-Henken, B. (2002) Arginine and immunity: a unique perspective. *Biomed. Pharmacother.* 56:471-482.

Ninfa, A.J. and Atkinson, M.R. (2000) PII signal transduction proteins. *Trends in Microbiology* 8:172-179.

Oliveira, I.C., Brears, T., Knight, T.J., Clark, A., and Coruzzi, G. (2002) Overexpression of cytosolic glutamate synthetase. Relation to nitrogen, light, and photorespiration. *Plant Physiology* 129:1170-1180.

Oppermann-Sanio, F.B., Hai, T., Aboulmagd, E., Hezayen, F.F., Jossek, S., and Steinbüchel, A. (1999) Biochemistry of polyamide metabolism. *Biochemical Principles and Mechanisms of Biosynthesis and Biodegradation of Polymers: Proceedings of the International Symposium (Steinbüchel, A. ed.)*185-193.

Oppermann-Sanio, F.B. and Steinbüchel, A. (2002) Occurrence, functions and biosynthesis of polyamides in microorganisms and biotechnological production. *Naturwissenschaften* 89:11-22.

Petrasovits, L.A., Purnell, M.P., Nielsen, L.K., and Brumbley, S.M. (2007) Production of polyhydroxybutyrate in sugarcane. *Plant Biotechnology Journal* 5:162-172.

Pickardt, T. and de Kathen, A. (2004) Gentechnisch veränderte Pflanzen mit neuen oder verbesserten Qualitäts-und Nutzungseigenschaften: Futtermittel- und rohstoffliefernde Nutzpflanzen, Pflanzen zur Bodensanierung und Zierpflanzen. *im Auftrag des Büros für Technikfolgenabschätzung* De Kathen &Pickardt BiotechConsult GbR.

Poirier, Y. and Gruys, K.J. (2001) Production of PHAs in transgenic plants. *In Biopolyesters (Doi, Y. and Steinbüchel, A. eds)*401-435.

Poirier, Y. (2002) Polyhydroxyalknoate synthesis in plants as a tool for biotechnology and basic studies of lipid metabolism. *Progress in Lipid Research* 41:131-155.

Poirier, Y., Dennis, D.E., Klomparens, K., and Somerville, C. (1992) Polyhydroxybutyrate, a biodegradable thermoplastic, produced in transgenic plants. *Science* 256:520-523.

Popovic, P.J., Zeh, H.J., and Ochoa, J.B. (2007) Arginine and immunity. *Journal of Nutrition* 137:1681S-1686S.

Qaim, M. (2006) The role of plant breeding for global food security. *Berichte Uber Landwirtschaft* 84:198-212.

Rafiq, M., Fatima, T., Husnain, T., Bashir ,K., and Riazuddin, S. (2005) Effect of different media on callus formation and regeneration of different genotypes of maize (*Zea mays* L.). *Plant Tissue culture* 15:57-65.

Roth, F.X., Fickler, J., and Kirchgessner, M. (1995) Effect of dietary arginine and glutamic acid supply on the N-balance of piglets . Communication on the importance of nonessential amino acids for protein retention. *Journal of Animal Physiology and Animal Nutrition-Zeitschrift fur Tierphysiologie Tierernahrung und Futtermittelkunde* 73:202-212.

Saruul, P., Srienc, F., Somers, D.A., and Samac, D.A. (2002) Production of a biodegradable plastic polymer, poly-beta-hydroxybutyrate, in transgenic alfalfa. *Crop Science* 42:919-927.

Schilling, G. (2000) Pflanzenernährung und Düngung. *Verlag Eugen Ulmer Stuttgart*146-170.

Schwamborn, M. (1996) Polyasparaginsäuren. *Nachr. Chem. Techn. Lab* 44:1167-1179.

Schwamborn, M. (1998) Chemical synthesis of polyaspartates: a biodegradable alternative to currently used polycarboxylate homo- and copolymers. *Polymer Degradation and Stability* 59:39-45.

Scott, E., Peter, F., and Sanders, J. (2007) Biomass in the manufacture of industrial products - the use of proteins and amino acids. *Applied Microbiology and Biotechnology* 75:751-762.

Serr, A., Pühler, A., and Broer, I. (2007) Der Beitrag von Pflanzen mit neuartigen Eigenschaften zur Inwertsetzung ländlicher Räume. *Materialien der Interdisziplinären Arbeitsgruppe "Zukunftsorientierte Nutzung ländlicher Räume" Nr. 19. Berlin: Berlin-Brandenburgische Akademie der Wissenschaften.*

Shekhawat, U.K.S., Ganapathi, T.R., Kumar, G.B.S., and Srinivas, L. (2007) Sucrose-inducible expression of hepatitis B surface antigen using potato granule-bound starch synthase promoter. *Plant Biotechnology Reports* 1:199-206.

Simon, R., Priefer, U., and Pühler, A. (1983) A broad host range mobilization system for in vivo genetic engineering - transposon mutagenesis in gram-negative bacteria. *Bio-Technology* 1:784-791.

Simon, R.D. (1976) Biosynthesis of multi-L-arginyl-poly(L-aspartic acid) in filamentous cyanobacterium *Anabaena cylindrica*. *Biochimica et Biophysica Acta* 422:407-418.

Simon, R.D. (1987) Inclusion bodies in the cyanobacteria: cyanophycin, polyphosphate, polyhedral bodies. *The cyanobacteria*199-225.

Simon, R.D. and Weathers, P. (1976) Determination of structure of novel polypeptide containing aspartic acid and arginine which is found in cyanobacteria. *Biochimica et Biophysica Acta* 420:165-176.

Slocum, R.D. (2005) Genes, enzymes and regulation of arginine biosynthesis in plants. *Plant Physiology and Biochemistry* 43:729-745.

Springer, A. (2007) Methyljasmonat induzierte Seneszenz in Chloroplasten aus Primärblättern der Gerste (*Hordeum vulgare* L.): Analyse der Ultrastruktur und des Proteinimports. urn:nbn:de:bvb:703-opus-3537. Ref Type: Thesis/Dissertation

Steinle, A., Oppermann-Sanio, F.B., Reichelt, R., and Steinbüchel, A. (2008) Synthesis and accumulation of cyanophycin in transgenic strains of *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology* 74:3410-3418.

Sugiyama, K., Hayakawa, T., Kudo, T., Ito, T., and Yamaya, T. (2004) Interaction of N-acetylglutamate kinase with a PII-like protein in rice. *Plant Cell Physiol* 45:1768-1778.

Tabata, K., Abe, H., and Doi, Y. (2000) Microbial degradation of poly(aspartic acid) by two isolated strains of *Pedobacter* sp. and *Sphingomonas* sp. *Biomacromolecules* 1:157-161.

Taheri, F., Ochoa, J.B., Faghiri, Z., Culotta, K., Park, H.J., Lan, M.S., Zea, A.H., and Ochoa, A.C. (2001) L-arginine regulates the expression of the T-cell receptor zeta chain (CD3 zeta) in Jurkat cells. *Clinical Cancer Research* 7:958S-965S.

Tapiero, H., Mathe, G., Couvreur, P., and Tew, K.D. (2002) I. Arginine. *Biomed. Pharmacother.* 56:439-445.

Thomas, H. (1997) Chlorophyll: a symptom and a regulator of plastid development. *New Phytologist* 136:163-181.

Thomson, W.W. and Platt-Aloia, K.A. (1987) Ultrastructure and senescence in plants. *In Thomson WW, Nothnagel EA, Huffaker, RC, eds. Plant senescence: its biochemistry and physiology Rockville, MD: American Society of Plant Physiologists*20-30.

Thrän, D., Weber, M., Scheuermann, A., Fröhlich, N., Zeddies, J., Henze, A., Thoroe, C., Schweinle, J., Fritsche, U.R., Jenseit, W., Rausch, L., and Schmidt, K. (2005) Sustainable strategies for biomass use in the european context. Analysis in the charged debate on national guidelines and the competition between sloid, liquid and gaseous biofuels. *Institute for Energy and Environment gGmbH (IE), im Auftrag des Bundesministeriums für Umwelt, Naturschutz und Reaktorsicherheit*.

Tuquet, C. and Newman, D.W. (1980) Aging and regreening in soybean cotyledons. 1 Ultrastructural changes in plastids and plastoglobuli. *Cytobios* 29:43-59.

Vandersteege, G., Nieboer, M., Swaving, J., and Tempelaar, M.J. (1992) Potato granule bound starch synthase promoter controlled gus expression - regulation of expression after transient and stable transformation. *Plant Molecular Biology* 20:19-30.

Vincent, R., Fraisier, V., Chaillou, S., Limami, M.A., Deleens, E., Phillipson, B., Douat, C., Boutin, J.P., and Hirel, B. (1997) Overexpression of a soybean gene encoding cytosolic glutamine synthetase in shoots of transgenic *Lotus corniculatus* L. plants triggers changes in ammonium assimilation and plant development. *Planta* 201:424-433.

Visser, R.G.F., Stolte, A., and Jacobsen, E. (1991) Expression of a chimeric granule bound starch synthase gus gene in transgenic potato plants. *Plant Molecular Biology* 17:691-699.

Werdan, K., Heldt, H.W., and Milovancev, M. (1975) Role of pH in regulation of carbon fixation in chloroplast stroma - studies on CO_2 fixation in light and dark. *Biochimica et Biophysica Acta* 396:276-292.

Wu, G.Y., Bazer, F.W., Davis, T.A., Jaeger, L.A., Johnson, G.A., Kim, S.W., Knabe, D.A., Meininger, C.J., Spencer, T.E., and Yin, Y.L. (2007) Important roles for the arginine family of amino acids in swine nutrition and production. *Livestock Science* 112:8-22.

Yeramian, A., Martin, L., Arpa ,L., Bertran, J., Soler, C., McLeod, C., Modolell, M., Palacin, M., Lloberas, J., and Celada, A. (2006) Macrophages require distinct arginine catabolism and transport systems for proliferation and for activation. *European Journal of Immunology* 36:1516-1526.

Ziegler, K., Deutzmann ,R., and Lockau, W. (2002) Cyanophycin synthetase-like enzymes of non-cyanobacterial eubacteria: Characterization of the polymer produced by a recombinant synthetase of *Desulfitobacterium hafniense*. *Zeitschrift fur Naturforschung C-A Journal of Biosciences* 57:522-529.

Ziegler, K., Diener, A., Herpin, C., Richter, R., Deutzmann, R., and Lockau, W. (1998) Molecular characterization of cyanophycin synthetase, the enzyme catalyzing the biosynthesis of the cyanobacterial reserve material multi-L-arginyl-poly-L-aspartate (cyanophycin). *European Journal of Biochemistry* 254:154-159.

Zotz ,R.J., Schenk, S., Kuhn, A., Schlunken, S., Krone, V., Bruns, W., Genth, S., and Schuler, G. (2001) Safety and efficacy of LK565 - a new polymer ultrasound contrast agent. *Zeitschrift fur Kardiologie* 90:419-426.

F Appendix

F1 List of abbreviations

A. tumefaciens	*Agrobacterium tumefaciens*
*arg*J	gene of the ornithine acetyltransferase from *S. coelicolor*
ATP	adenosinetriphosphate
B33	tuber-specific class1 promoter
CaMV	Cauliflower mosaic virus
CO_2	carbon dioxide
cphA_{Te}	coding region of the cyanophycin synthetase
CphA	cyanophycin synthetase
*ctx*B	coding region of the cholera toxin B
DAB-Cl_2	diamino benzidine chloride
DNA	desoxyribonucleotide acid
dw	dry weight
e.g.	for example
*gln*A	gene of the glutamine synthetase from *E. coli*
*gln*II	gene of the glutamine synthetase from *S. coelicolor*
g	gram
mg	milligram
mRNA	messenger ribonucleic acid
NAGK	N-acetyl glutamate kinase
nic	near isogenic control
*npt*II	coding of the neomycin phosphotransferase gene
kDa	kilo Dalton
PCR	polymerase chain reaction
pGBSS	granule bound starch synthetase
p35S	promoter of the 35S RNA gene from the cauliflower mosaic virus
%	percent
PHB	polyhydroxybutyrate
T-DNA	transfer DNA
t35S	terminator of the 35S RNA gene from the cauliflower mosaic virus

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F4 Erklärungen (in German)

F4.1 Anteilserklärung für Maja Hühns

Neumann, K., Stephan, D.P., Ziegler, K., Hühns, M., Broer, I., Lockau, W., and Pistorius,E.K. (2005) "Production of cyanophycin, a suitable source for the biodegradable polymer polyaspartate, in transgenic plants." Plant Biotechnology Journal 3: 249-258

- Anzucht und Analyse der Nachkommen der transgenen Tabakpflanzen hinsichtlich DNA-und RNA Nachweis
- Anzucht und Analyse der vegetativ vermehrten Kartoffelpflanzen hinsichtlich DNA- und RNA Nachweis
- Mitarbeit am Manuskripts

Hühns, M. Neumann, K.; Hausmann, T.; Ziegler, K.; Klemke, F.; Kahmann, U.; Staiger, D.; Lockau, W.; Pistorius, E.K.; Broer, I. (2008) "Plastid targeting strategies for cyanophycin synthetase to achieve high-level polymer accumulation in *Nicotiana tabacum*." *Plant Biotechnol. J.* 6:321-336

- Klonierung der Pflanzentransformationsvektoren
- Überführung der Pflanzentransformationsvektoren in *Agrobacterium tumefaciens*
- Transformation von *Nicotiana tabacum*
- Charakterisierung der resultierenden transgenen Pflanzen mittels PCR
- Phänotypische Charakterisierung
- Durchführung der Southern Analysen
- Durchführung der Northern Analysen
- Anzucht und Analyse der Nachkommen der transgenen Pflanzen
- Koordination der Partner für die Analysen hinsichtlich der Bestimmung des Cyanophycingehaltes und der elektronenmikroskopischen Aufnahmen
- erstellen des Manuskripts

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- Transformation von *Solanum tuberosum*
- Charakterisierung der resultierenden transgenen Pflanzen mittels PCR
- Phänotypische Charakterisierung
- Durchführung der Southern Analysen
- Durchführung der semiquantitativen RT-PCR
- Anzucht und Analyse der Nachkommen der transgenen Pflanzen
- Koordination der Partner für die Analysen hinsichtlich der Bestimmung des Cyanophycingehaltes, der elektronenmikroskopischen Aufnahmen, sowie der Bestimmung des Stickstoff- und Aminosäuregehaltes
- erstellen des Manuskripts

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- Literaturrecherche und erstellen des Manuskripts gemeinsam mit Frau Prof. Broer

F4.2. Erklärung zur Selbständigkeit

Ich versichere hiermit an Eides statt, dass ich die vorliegende Arbeit selbständig angefertigt und ohne fremde Hilfe verfasst habe, keine außer den von mir angegebenen Hilfsmitteln und Quellen dazu verwendet habe und die den benutzten Werken inhaltlich und wörtlich entnommenen Stellen als solche gekennzeichnet habe.

Maja Hühns   Rostock, den 15.01.2010

F4.3 Danksagung

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F4.4 Publikationsliste

Neumann, K.; Stephan, D.P.; Ziegler, K.; **Hühns, M.**; Broer, I.; Lockau, W.; and Pistorius, E.K. (2005) "Production of cyanophycin, a suitable source for the biodegradable polymer polyaspartate, in transgenic plants." Plant Biotechnology Journal 3: 249-258.

Hühns, M.; Neumann, K.; Hausmann, T.; Ziegler, K.; Klemke, F.; Kahmann, U.; Staiger, D.; Lockau, W.; Pistorius, E.K.; Broer, I. (2008) "Plastid targeting strategies for cyanophycin synthetase to achieve high-level polymer accumulation in *Nicotiana tabacum*." Plant Biotechnol. J. 6:321-336

Hühns, M.; Neumann, K.; Hausmann, T.; Klemke, F.; Lockau, W.; Kahmann, U.; Kopertekh, L.; Staiger, D.; Pistorius, E.K.; Reuther, J.; Waldvogel, E.; Wohlleben, W.; Effmert, M.; Junghans, H.; Neubauer, K., Kragl, U.; Schmidt, K.; Schmidtke, J.; Broer, I. (2009) „Tuber specific *cph*A expression to enhance cyanophycin production in potatoes." Plant Biotechnol. J. 7(9). :883-898

Hühns, M.; Broer, I. "Characters of transgenic plants and their Application in Plant production: Biopolymers."
In: Biotechnology in Agriculture and Forestry Vol. X. Genetic Modification of Plants-Agriculture, Horticulture and Forestry. (eds.: F.Kempken and C. Jung); Springer, Heidelberg, in press

F4.5 wissenschaftliche Vorträge

Regionale wissenschaftliche Konferenz „Pflanzenbiotechnologie“ IAPTC&B, Wien 22.03-24.03.06

Hühns, M.; Neumann, K.; Stephan, D.P.; Ziegler, W.; Lockau, W.; Pistorius, E.K.; Broer, I. „Bioplastik in transgenen Pflanzen: Cyanophycin als geeignete Quelle für Polyaspartat“

BBA Braunschweig, 04.04.06

Hühns, M.; Neumann, K.; Stephan, D.P.; Ziegler, W.; Lockau, W.; Pistorius, E.K.; Broer, I. “Bioplastic in transgenic plants: cyanophycin as a suitable resource for polyaspartate”

GVC/DECHEMA-Jahrestagungen 2006 mit 24. DECHEMA-Jahrestagung der Biotechnologen, Wiesbaden 26.09-28.09.06

Hühns, M.; Pistorius, E.K.; Stephan, D.P.; Ziegler, K.; Lockau, W..; Broer, I.: „Produktion von biologisch abbaubaren Polymeren in transgenen Pflanzen“

ProcessNet Jahrestagung 2009 mit 27. DECHEMA-Jahrestagung der Biotechnologen, Mannheim 08.09-10.09.09

Hühns, M.; Hausmann, T.; Klemke, F.; Lockau, W.; Kahmann, U.; Pistorius, E.K.; Schmidt, K.; Broer, I. „Produktion eines biologisch abbaubaren Polymers in transgenen Pflanzen“

Workshop der Gesellschaft für Pflanzenbiotechnologie e.V. 2. Zirkular der Arbeitskreise „somatische Embryogenese“ und „Gentechnik“, Rostock 01.10.-03.10.09

Hühns, M.; Hausmann, T.; Unger, C.; Broer, I. „ Produktion des biologisch abbaubaren Polymers Cyanophycin in Pflanzen“

F4.6 Lebenslauf

Persönliche Angaben

Name:	Maja Hühns
Geboren am:	09.03.1979 in Kühlungsborn

Studium

Seit März 2003	Beginn der experimentellen Arbeiten zur Dissertation an der Universität Rostock am der Argrar-und Umweltwissenschaftliche Fakultät, Fachbereich Agrobiotechnologie (Prof. Dr. Inge Broer). Thema der Dissertation: „Production of the biodegradable polymer cyanophycin in transgenic *Nicotiana tabacum* and *Solanum tuberosum* plants."
Seit April 2005	wissenschaftliche Mitarbeiterin am Fachbereich Agrobiotechnologie der Universität Rostock
Nov. 2003-März 2005	Landesgraduiertenstipendium des Landes Mecklenburg-Vorpommern, Universität Rostock
1997-2003	Biologiestudium an der Universität Rostock, Abschluss: Diplom; Thema der Diplomarbeit: „ Hemmung der Phosphinothricin-Resistenz durch spezifische RNA-Interferenz: Ein neuer Weg zum induzierten Zelltod in transgenen Pflanzen"