Universität Rostock

Traditio et Innovatio

Agrobiotechnology and Risk Assessment of Bio- and Gene Technology

Faculty of Agriculture and Environmental Science

Increasing the Arginine Content in Plants by the Coexpression of Cyanophycin and Cyanophycinase

Dissertation

submitted for the Degree of

Doctor of Agriculture (doctor agriculturae, Dr. agr.)

at the faculty of Agriculture and Environmental Science

of the University of Rostock

Rostock 2017

Submitted by

Daniel Helmut Ponndorf, M.Sc.

From Rostock

Born on 17.01.1985 in Reutlingen

urn:nbn:de:gbv:28-diss2017-0087-6

First Reviewer:

Prof. Dr. rer. nat. habil. Inge Broer
Chair of Agrobiotechnology and Risk Assessment for Bio- und Gene Technology
Faculty of Agricultural and Environmental Sciences
University of Rostock

Second Reviewer:

Prof. Dr. rer. nat. habil. Cornelia C. Metges
Institute of Nutritional Physiology “Oskar” Kellner”
Leibniz Institute for Farm Animal Biology (FBN) Dummerstorf

Third Reviewer

Prof. Dr. Rer. Nat. habil. Alexander Steinüchel
Institute for molecular microbiology and biotechnology
Westfälische Wilhelms-Universität Münster

Date of Submission: 31.01.2017

Date of Disputation: 20.06.2017

Universität Rostock

Traditio et Innovatio

Professur für Agrobiotechnologie und Begleitforschung zur Bio-und Gentechnologie

Der Agrar- und Umweltwissenschaftlichen Fakultät

Erhöhung des Arginingehaltes in Pflanzen durch die Koexpression von Cyanophycin und Cyanophycinase

Dissertation

zur Erlangung des akademischen Grades

Doktor der Agrarwissenschaften (doctor agriculturae, (Dr. agr.))

an der Agrar und Umweltwissenschaftlichen Fakultät

der Universität Rostock.

Rostock 2017

Vorgelegt von

Daniel Helmut Ponndorf, M.Sc.

aus Rostock

Geboren am 17.01.2017 in Reutlingen

Erster Gutachter:

Prof. Dr. rer. nat. habil. Inge Broer
Professur für Agrobiotechnologie und Begleitforschung für Bio- und Gentechnologie
Agrar- und Umweltwissenschaftliche Fakultät
Universität Rostock

Zweiter Gutachter:

Prof. Dr. rer. nat. habil. Cornelia C. Metges
Institut für Ernährungsphysiologie “Oskar” Kellner”
Leibniz Institut für Nutztierbiologie (FBN) Dummerstorf

Dritter Gutachter:

Prof. Dr. rer. nat. habil. Alexander Steinüchel
Institut für molekulare Mikrobiology und Biotechnologie
Westfälische Wilhelms-Universität Münster

Datum der Einreichung: 31.01.2017

Datum der Verteidigung: 20.06.2017

“Once you learn to quit, it becomes a habit”

Vince Lombardi

Für meine Eltern.

This work was funded by a scholar of the “Landesgraduiertenförderung” of the University of Rostock and conducted in cooperation with the working group Nutritional Physiology “Oscar Kellner” of the Leibniz Institute for Farm Animal Biology (FBN) in Dummerstorf/Germany, the department of Nutrition Physiology and Animal Nutrition of the University of Rostock, and the chair of nutritional sciences of the Martin-Luther-University Halle-Wittenberg. All the support and cooperation leading to the success of this work is highly appreciated.

Content

Summary

This work describes a new strategy for the supply of the semi-essential amino acid (AA) Arginine (Arg) to food and feed: The production of the Arg rich, bacterial storage compound cyanophycin (CGP) and its degrading enzyme cyanophycinase (CGPase), which hydrolyzes CGP to β-Asp-Arg dipeptides, in plants. These dipeptides can be administrated in two ways: The supplementation of food and feed with isolated CGP and CGPase, and the coexpression of both proteins in one plant and direct administration in the chow.

In a first step the CGP production in the chloroplasts of plants was optimized for a commercialized application by the use of the cultivars *Nicotiana tabacum* Badischer Geudertheimer (BG) and Virginia Golta (VG). So far, CGP was produced in *N. tabacum* Petit Havana SRI (PH) and *Solanum tuberosum* cultivars. BG produced 170 % more biomass than PH and in the T0 generation with up to 8.8 % more CGP in the dry weight compared to PH (1.74 %) and *S. tuberosum* (7.5 %) without the phenotypic changes as described for *S. tuberosum* where high CGP yields were associated with an aberrant tuber phenotype. Furthermore, the CGP content in BG plants was more homogenous between parents and descendants compared to VG. Consequently, BG is the most suitable cultivar for a commercialized CGP production. In a second step, we could show that plants are able to produce an active form of two CGPases, CPHB and CPHE, and that the coexpression of CGP and CGPase in one plant is possible. CPHE was produced in higher amounts and showed a higher activity than CPHB. While CPHE was stable in BG plants and degraded CGP after cell homogenization, no activity was detected for CPHB due to its instability in crude extract of BG plants and low expression level in the chloroplast.

Feeding studies with isolated CGP and CGPase in mice also revealed that CGP can be degraded in the gastro intestinal tract of mice (GIT), but unexpectedly no increase in the Arg concentration, but of β-Asp-Arg dipeptides was detected in the plasma. This indicates that these dipeptides can’t be hydrolyzed in the enterocytes of mice and are probably not bioavailable.

In conclusion, we showed that high amounts of CGP and CGPase can be produced for a commercial application and that the coexpression in plants and a degradation of CGP in crude plant extracts and in the GIT of mice is possible. Though more work is necessary according to the bioavailability of the β-Asp-Arg dipeptides, our strategies show the potential to replace the conventional Arg supplementation.

A Introduction

Amino acids (AA) are essential for the synthesis of proteins and are precursors of many low molecular substances with high physiological value. In the last century the effort to define the optimal content of AA in food of humans and livestock increased (Wu, 2009). This applies not only for essential AA, which can't be synthesized by the organism and have to be provided in food and feed, but also for non-essential and conditional AA, which are only essential for some species or during certain developmental stages and environmental conditions (Hou et al., 2015; Li et al., 2011; Rose and Rice, 1939). One example for a semi-essential and conditional AA is arginine (Arg), which is non-essential for adult mammals but is indispensable for young mammals and avian species (Rose and Rice, 1939).

Beside the synthesis of Arg in the organism, Arg is mainly released from food proteins by proteases and peptidases as short chain peptides or free Arg in the lumen of the gastrointestinal tract (GIT) (reviewed by Wu et al. (2016)). In mammals, 40 % of dietary Arg is utilized directly in the enterocytes and in bacteria in the lumen. The other 60 % are transported to the vascular circulation in two ways: Free Arg is transported from the lumen of the small intestine into the enterocytes via the cationic amino acid transporters of the $b^{0,+}$ system while Arg, which is bound in di- or tripeptides, is transported by PepT1 or PepT2 transporters (Klang et al., 2005; Rubio-Aliaga and Daniel, 2008). The uptake of di- and tripeptides is more efficient and furthermore, promotes the single AA uptake (Matthews and Adibi, 1976; Wenzel et al., 2001). After the transport of di- and tripeptides into the enterocyte, they are cleaved in the cytosol by peptidases and free Arg is transported into the vascular circulation and to the extra-intestinal tissue by y^+LAT1 and to a smaller extent by y+LAT2 transporters (reviewed by Bröer (2008)). Arg is then utilized during protein synthesis and as precursor for multiple low molecular substances such as creatine, nitric oxide, polyamines and other AA like proline (Pro), citrulline (Cit) and glutamic acid (Glu) (reviewed by Wu et al. (2016)).

Because the rate of utilization could be higher compared to the rate of synthesis and insufficient amounts of Arg might be provided by food or feed, Arg has to be supplemented to meet the optimal supply (Li et al., 2011; Wu, 2009; Wu et al., 2016). This is the case in conventional animal production, because commonly used soy and corn diets for poultry and pig nutrition are low in Arg (Li et al., 2011). The supplementation of this diets with Arg leads to beneficial effects like higher reproduction rates, or better development and health of young mammals (Mateo et al., 2007; Wu et al., 2010; Yang et al., 2016; Zeng et al., 2008). It increases muscle gain and meat quality (Ma et al., 2010; Ma et al., 2015; Tan et al., 2009) and a positive effect of Arg supplementation on human athletes performance is also

discussed (Hwang and Willoughby, 2015). Arg also improves health and immune response (reviewed by Hou et al. (2015)), while no adverse effect on health were found for rats and pigs (Hu et al., 2015; Yang et al., 2015).

The AA can be supplemented to the forage using Arg rich components like for example fishmeal (Li et al., 2011), chickpea seeds (Cortes-Giraldo et al., 2016) or free Arg synthesized by bacterial fermentation (Utagawa, 2004). These supplementation forms are in the case of fermentation more expensive (Merlin et al., 2014) and in the case of fishmeal not as sustainable as the direct increase of the content in plants (Hardy, 2010). Hence, it's of interest to increase Arg in plants to improve their nutritional value.

A1 Biofortification as strategy to increase the Arg content in plants

Increasing the nutritional value of plants by breeding or plant biotechnology is called biofortifcation and is described for vitamins and minerals (reviewed by De Steur et al. (2015) as well as for AA (reviewed by Galili et al. (2016)).

For the increase of the AA content in plants conventional breeding has mostly failed and if AA were increased, the crop yield decreased (Galili and Amir, 2013; Galili et al., 2016). Besides the decrease in crop yield, the failure of conventional breeding is caused by the feedback inhibition of AA synthesis on different levels and the lack of possibilities to avoid this regulation (reviewed by Galili et al. (2016)). In contrast to conventional breeding, four transgenic approaches have led to an increase of essential AA in different plant species and cultivars like *A. thaliana,* tobacco, potato, soybean, alfalfa, rice, barley, and canola.

One approach was the overexpression of different feedback insensitive regulatory enzymes of the respective AA synthetic pathway, which led to an increase in the pool of the AA phenylalanine and tryptophan (Trp) and their secondary metabolites in *Arabidopsis thaliana (Tzin et al., 2012)*, tomato (Tzin et al., 2013) and petunia (Oliva et al., 2015). This approach was also successful in multiple studies for Trp, threonine (Thr), methionine (Met) and lysine (Lys) in various plants as reviewed by Galili and Amir (2013) and Galili et al. (2016).

Another possibility is the suppression of seed storage proteins which contain low amounts of the AA of interest. One example is the RNAi mediated decrease of the Lys-poor, 19 kilo Dalton (kDa) seed storage protein α-zein. This strategy led to an increase of Lys and Met in the total AA concentration in maize seeds (Huang et al., 2004). A similar approach was used by Sikdar et al. (2016) who used RNAi to reduce the prolamins C-hordein and ω-gliadins in barley and showed an increase in the content of Pro, Lys, Thr, Met, leucine (Leu) and glutamine (Gln). Combining the expression of feedback insensitive enzymes and decreasing the zein content led to an even higher accumulation of Lys and other AA like Arg, Trp and histidine (His) in maize seeds (Frizzi et al., 2010; Huang et al., 2006; Huang et al., 2005).

Besides the suppression of storage proteins with a low content of AA, the down regulation of the catabolic enzymes for these AA is another possibility which might lead to a reduction of the respective catabolism and hence to an increase in AA. This was successfully shown in plants for Lys (Frizzi et al., 2008; Hournard et al., 2007; Reyes et al., 2009) and Met (Avraham and Amir, 2005). Additionally, the combination of the down regulation of the Lys catabolism and the expression of a bacterial feedback insensitive enzyme for Lys synthesis led to an 80 fold increase in Lys and 38 fold increase in Met content in *A. thaliana* (Zhu and Galili, 2003).

The overexpression of storage proteins which contain a high amount of the AA of interest is another strategy, which has been used to increase the AA content in plants. One approach was the design of synthetic proteins which contain high amounts of the desired AA and showed promising results in experiments in bacteria, but transferring these proteins to plants did not lead to much success, because of low protein production (Beauregard and Hefford, 2006; Galili and Amir, 2013). In a second strategy, the sequence of genes encoding storage proteins was fortified with additional codons for Lys and Met (De Clercq et al., 1990; Forsyth et al., 2005; Hoffman et al., 1988; Torrent et al., 1997). In addition, chimeric genes were designed to support an increase in the AA content of plants. For example a fusion of the Pro rich region of the gamma-zein from maize and phaseoline from bean (“zeoline”) or a fusion of the rice glutelin storage protein to the Lys rich protein from winged bean were designed (Mainieri et al., 2004; Sun and Liu, 2008). Additionally, Wong et al. (2015) overexpressed endogenous Lys rich histone proteins in rice seeds and measured an increase in Lys of up to 35 %. The use of storage proteins from other plants as heterologous source of AA was also investigated. Expression of the Sb401 protein from potato in maize for example increased the Lys content by 16.1-54.8 % and the total protein content up to 11.6-39.0 % (Yu et al., 2005). Comparable results were described by Liu et al. (2016) who expressed the Lys rich protein gene (*LRP*) of winged bean in the endosperm of rice and achieved an average increase in the Lys content of 34,76 % combined with an increase in all other AA compared to the controls. In line with this result, the expression of a Lys rich protein gene from cotton (*GhLRP*) led to up to 65 % more Lys in maize compared to the controls (Yue et al., 2014). Another storage protein which led to an increase in Met and Lys in different cultivars was 2S albumin from Brazil nut and Sunflower. In tobacco and canola the Met content increased to about 30-33 % (Altenbach et al., 1992; Altenbach et al., 1989) and up to 62 % in rice (Lee et al., 2003). Expressing the sunflower 2S albumin in lupine even increased the Met content up to 97 % (Molvig et al., 1997).

While all of these approaches led to promising results, they also had to deal with drawbacks like protein instability, interference with other endogenous proteins, unfavorable phenotypes

like lower seed biomass and reduced germination rates and in the case of S2 Albumin the high allergenic potential of the protein, which limits its biotechnological use (Angelovici et al. (2011), Liu et al. (2016), reviewed by Galili and Amir (2013)).

Despite these drawbacks all these strategies have the potential to avoid the feedback regulation which also regulates the Arg synthesis (Fig. A1). Arg is synthesized in the chloroplast via the cyclic pathway as reviewed by Slocum (2005) and Winter et al. (2015) in two steps: First Glu is utilized in multiple steps to form ornithine (Orn) in the cyclic pathway and secondly, Orn is catabolized to form Arg (Fig. A1). The feedback inhibition regulates the Arg synthesis on three levels. *N*-acetylglutamate synthase (NAGS), the first enzyme in the Arg synthesis, which acetylates Glu to *N*-acetylglutamate, is down regulated in presence of Arg (Sancho-Vaello et al., 2009; Winter et al., 2015). The second enzyme, *N*-Acetylglutamate kinase (NAGK) (Mckay and Shargool, 1981), which forms *N*-acetylglutamate-5-P, is also inhibited by Arg, but only under low nitrogen levels, while the enzyme activity is increased under high nitrogen availability, because the binding of PII proteins to NAGK decreases the enzymes sensitivity for Arg under these conditions (Burillo et al., 2004). The third step of the regulation of Arg synthesis is the inhibition of N^2 acetylornithine:glutamate acetyltransferase (NAOGAcT), which forms Orn by removing the acetyl group of N^2 acetylornithine and represents the cycle part of Arg synthesis, recycling the free acetyl group to Glu. (Shargool and Jain, 1985). This step might limit the Orn production and therefore the amount of substrate to form Arg (Slocum, 2005).

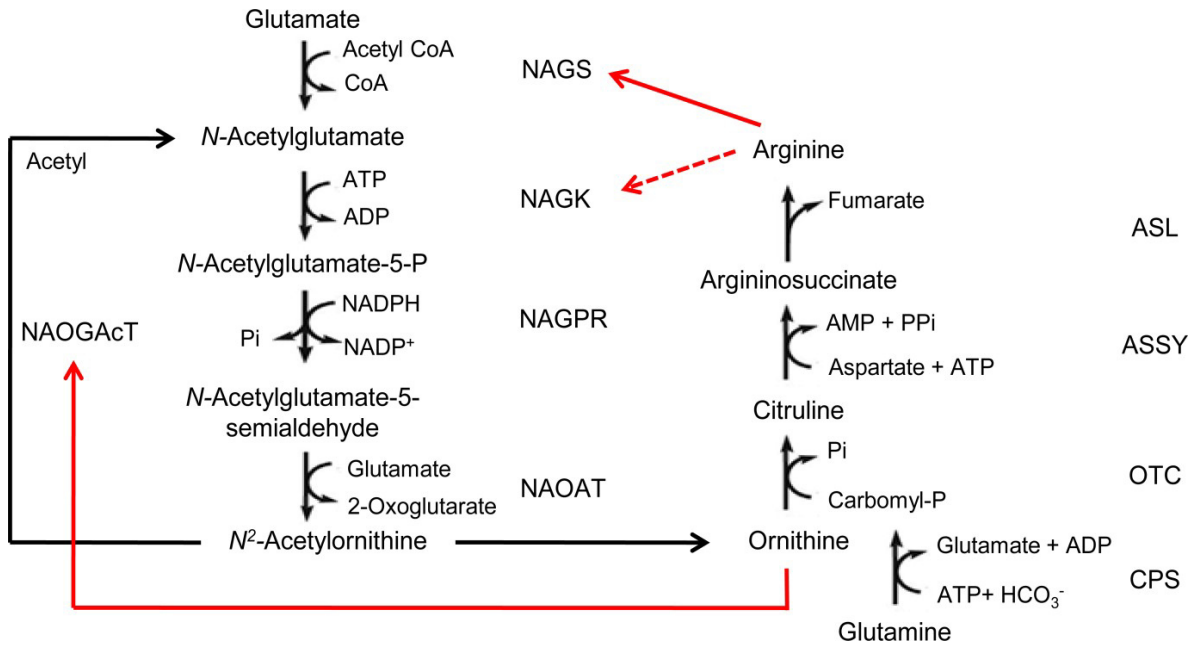


Fig A 1: Arginine synthesis and feedback regulation of respective enzymes in plants according to Slocum (2005) and Winter et al. (2015). Red arrows indicate feedback regulation steps. The red dashed arrow indicates feedback inhibition at low nitrogen availability. Acetyl CoA: Acetyl coenzyme A; ATP: Adenosine triphosphate; ADP: Adenosine diphosphate; AMP: adenosine monophosphate; $NADP^+$/NADPH: Nicotinamide adenine dinucleotide phosphate; P: Phosphate; PPi: Diphosphate; NAGS: *N*-Acetylglutamate synthase; NAGK: *N*-Acetylglutamate kinase; NAGPR: *N*-acetylglutamate -5-P reductase; NAOT: N^2-acetylornithine aminotransferase; NAOGAcT: N^2 acetylornithine:glutamate acetyltransferase; CPS: Carbomyl phosphate synthetase; OTC: Ornithine transcarbamylase; ASSY: Argininosuccinate synthase; ASL: Argininosuccinate lyase

Since the Arg synthesis is not yet fully understood in plants (Winter et al., 2015) and its feedback inhibition depends on different environmental conditions and is regulated on different levels, the most suitable strategy to increase the Arg content is the expression of stable storage proteins in which Arg is bound in high amounts. One candidate protein for this approach is the bacterial storage protein cyanophycin.

A2 Cyanophycin as an arginine source in plants

Cyanophycin (multi-L-arginyl-poly-L-aspartic acid) or cyanophycin granule polypeptide (CGP), is a nitrogen, carbon and energy storage protein which is synthesized during the transition of the cells from the exponential to the stationary phase by most of the cyanobacteria and also several non- photosynthetic bacteria (Allen et al., 1984; Mackerras et al., 1990; Simon, 1987; Simon and Weathers, 1976; Ziegler et al., 2002). It is built via non ribosomal biosynthesis by the enzyme cyanophycin synthetase (CPHA) (Ziegler et al., 1998) and consist of a L aspartic acid (Asp) backbone, which is linked via the β-carboxyl group to the α-amino group of -L-Arg residues (Simon and Weathers, 1976) (Fig. A2).

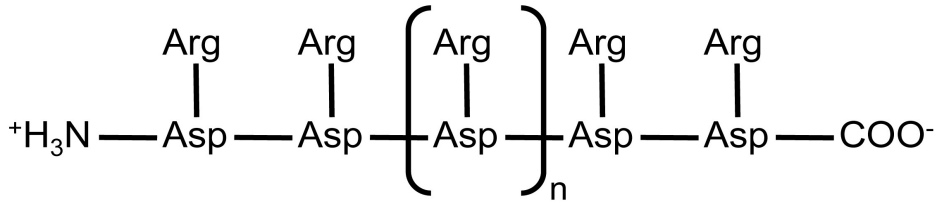


Fig A 2: Structure of cyanophycin granule polypeptide (CGP) (Simon and Weathers, 1976)

CGP is insoluble under physiological conditions but soluble at acidic or alkaline pH values (Simon et al., 1980). Besides its natural function it is of interest for industrial purposes, because it can be converted into Polyaspartate and L-Arg by mild hydrolysis (Joentgen et al., 2001). Polyaspartate can be used in various technical and medical processes to replace non-biodegradable polyacrylates (Joentgen et al., 2001; Oppermann-Sanio and Steinbuchel, 2002; Schwamborn, 1998), while L-Arg, could serve as a starting point for the synthesis of a broad range of chemicals (Mooibroek et al., 2007; Scott et al., 2007). Hence, in the past a lot of effort was taken to optimize the biotechnological production of CGP in several bacteria such as *Escherichia coli (E.coli)* (Abd-El-Karem et al., 2011; Du et al., 2013; Du et al., 2016; Hai et al., 2006; Hai et al., 2002; Krehenbrink et al., 2002; Ziegler et al., 2002; Ziegler et al., 1998), *Ralstonia eutropha* (Aboulmagd et al., 2001; Voss et al., 2004; Voss and Steinbuchel, 2006), *Pseudomonas putida* (Aboulmagd et al., 2001; Voss et al., 2004), *Corynebacterium glutamicum*.(Aboulmagd et al., 2001) and *Sinorhizobium meliloti* (Abd-El-Karem et al., 2011). Furthermore, also eukaryotic expression systems like *Saccoromyces cerivisiae* (Steinle et al., 2009) and *Pichia pastoris* (Steinle et al., 2010) were used. Both eukaryotic organisms accumulated CGP to about 15 % of the cell dry matter (CDM) (Steinle et al., 2009; Steinle et al., 2010), while much higher expression levels of CGP where achieved in *Ralstonia eutropha* (40 % of CDM) (Voss and Steinbuchel, 2006), *Sinorhizobium meliloti* (40 % of CDM) (Abd-El-Karem et al., 2011) and up to 43,4 % in *Pseudomonas Putida* (Wiefel et al., 2011).

Interestingly, the recombinant CGP varies in properties like composition and solubility from the natural occurring CGP. In the recombinant CGP producing strains Lys was partially incorporated in the side chains instead of Arg (Krehenbrink et al., 2002; Steinle et al., 2009; Steinle et al., 2010; Ziegler et al., 1998). Furthermore, also Cit (Steinle et al., 2009; Wiefel et al., 2011) and Orn (Steinle et al., 2009) were detected in CGP. Besides these structural changes, recombinant strains also produced soluble CGP additionally to the insoluble polypeptide (Fuser and Steinbuchel, 2005; Steinle et al., 2010; Wiefel et al., 2011; Ziegler et al., 2002). Frommeyer and Steinbuchel (2013) and Wiefel and Steinbuchel (2014) found that the Lys content in the polypeptide determines the solubility of CGP, but the cultivation conditions and the temperature during CGP isolation might also have an influence (Du et al.,

2016; Wiefel and Steinbuchel, 2014). Furthermore, the production of CGP in different plant species revealed that the Lys content was very low in plants but soluble CGP was still detected, indicating a possible influence of the CGP producing organism on the proportion of soluble CGP (Nausch et al., 2016).

For CGP production in plants the CPHA encoding gene (*cph*A_{Te}) from *Thermosynechococcus elongatus* BP-1 was expressed in the cytosol and chloroplast of *Nicotiana tabacum* cv. Petit Havanna SRI (PH) and *Solanum tuberosum* cv. Desirée (STD) and Albatross (STA) (Hühns et al., 2009; Hühns et al., 2008; Neubauer et al., 2012; Neumann et al., 2005). Constitutive expression in the cytosol of PH led to a CGP accumulation of about 1.1 % of the dry weight (DW). Arg was partially replaced by Lys in a ratio of 1:1.05:0.1 (Asp-Arg-Lys) and the insoluble and soluble fraction were measured in a ratio of 1:0.6 (Neumann et al., 2005). In STD the expression of *cph*A_{TE} led to only up to 0.24 % total CGP (0.19 % insoluble) of the DW in leaves in a ratio of insoluble to soluble CGP of 1:0.26. The CGP content in tubers was below the detection level, but was shown by electron microscopy (Neumann et al., 2005). In both cultivars the expression of CGP led to an aberrant phenotype, like limited growth rate, variegated leaves which were thicker compared to the control plants and reduced number and size of the grana stacks in the chloroplasts (Neumann et al., 2005). These stress symptoms might be caused by the interference of CGP with the endogenous metabolism (Nausch et al., 2016; Neumann et al., 2005). Targeting the CGP production to the chloroplasts of PH and STA led to a normal phenotype of the plants and an increased CGP production (Hühns et al., 2008; Neubauer et al., 2012). In primary transformants of PH a CGP content of up to 1.74 % DW was measured and in descendants of the T2 generation an elite plant even produced up to 6.8 % CGP of the DW. In these plants the fraction of soluble CGP in the total CGP pool was much lower than described before (ratio of 1:0.003) (Hühns et al., 2008). In STA the mean CGP content increased to 1.6% DW in the leaves and 0.56 % DW in the tubers while the ratio of insoluble CGP to the soluble CGP decreased to about 1:0.05 compared to plastidic expression in STA (Neubauer et al., 2012). Hühns et al. (2009) showed that the tuber specific expression of CGP can increase the accumulation in STA to about 1.35 % DW in the cytosol of the tubers, while no CGP was found in the leaves. The respective tubers were smaller and deformed compared to the control. As described before, targeting of the CGP production to the plastids, led to an increase in CGP content to up to 7.5 % DW (mean 3.76 % DW). These tubers did not differ in the size, but showed brown staining (Hühns et al., 2009). Hühns et al. (2009) also analyzed the pool of bound and free Arg, Asp, Lys and Glu in hydrolyzed soluble cell protein, including the soluble CGP but not the insoluble CGP, which was the major fraction of total CGP. It was shown that the amount of bound Arg and Lys was lower in higher CGP

producing tubers compared to the control plants which might result from its possible incorporation into the insoluble polymer. Additionally, the pool of free Glu, which is a precursor of Arg, was lower in the transgenic plants compared to the controls, while this did not hold true for free Arg. (Hühns et al., 2009). During plant growth and long term storage of plant material CGP reveals to be very stable and does not seem to be metabolized or degraded in plants (Hühns et al., 2009). Hence CGP is a potent storage peptide for bound Arg in plants. This stability is most likely due to the fact that the degradation of CGP is solely catalyzed by a group of enzymes called cyanophycinase (CGPase), which have not been found in plants yet.

CGPases occur in two classes: First, the intracellular CGPase from cyanobacteria, called CPHB, which was described in cell extracts of *Anabena 7120* by Gupta and Carr (1981), *Aphanocapsa 6308* (Allen et al., 1984) and *Synechocystis sp.* PCC 6803 (Richter et al., 1999). The overexpression and analysis of CPHB in *E.coli* revealed that it is a serine type exopeptidase with a dimeric structure (Law et al., 2009; Richter et al., 1999) and its binding is highly specific for the substrate features of the β-linked aspartyl peptides (Law et al., 2009). The second CGPase class is called CPHE, which is an extracellular Cyanopyhcinase expressed by non cyanobacteria (Obst et al., 2002) and which was also found in the colon of mammals, birds and fish (Sallam and Steinbuchel, 2009). Both CGPases catalyze the same reaction, the degradation of CGP to β-Asp-Arg dipeptides (Gupta and Carr, 1981; Richter et al., 1999) (Fig. A3).

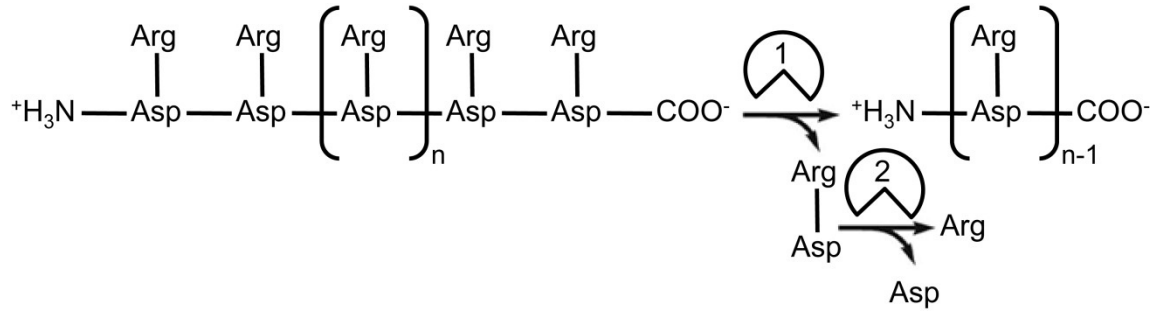


Fig A 3: Degradation of cyanophycin (CGP) by the enzyme cyanophycinase (1) to β-Asp-Arg dipeptides and hydrolysis to single amino acids by either isoaspartyl dipeptidase, isoaspartyl aminopeptidase or plant type arginases (2) according to Simon and Weathers (1976), Gupta and Carr (1981) and Hejazi et al. (2002)

The release of β-Asp-Arg dipeptides with high nutritional value as supplement for food and feed in nutrition and therapy is an additional use of CGP to the above mentioned applications and was already investigated in bacterial cell cultures (Sallam et al., 2009; Sallam and Steinbuchel, 2010; Santos et al., 2012). However, cell cultures may have different disadvantages compared to plants, depending on the produced protein, and might be less suitable for large-scale synthesis, because they are limited in terms of productivity, scalability

and are less cost effective (Ma et al., 2003; Merlin et al., 2014; Sabalza et al., 2014). Hence, the use of plants as source for β-Asp-Arg dipeptides could be a sustainable alternative to cell cultures by the expression of CGP and CGPase in plants and supplementation of both isolated components to the feed. Recently the expression of active CPHE was described in *Nicotiana benthamiana* (Nausch and Broer, 2016).

The production of CGP and CGPase in one plant is a more interesting approach and might result in a cheap and sustainable supply of Arg to livestock. Therefore, CGPase has to be coexpressed in CGP producing plants. To achieve this goal, two strategies are possible. First: CGPase expression in the chloroplast where CGP is present (Hühns et al., 2009; Hühns et al., 2008), which might result in the release and accumulation of β-Asp-Asg dipeptides during plant growth. Hereby, it has to be considered that β-Asp-Arg dipeptides might be further hydrolyzed to the single amino acids Arg and Asp by peptidases (Fuser and Steinbuchel, 2007; Richter et al., 1999) (Fig. A3), which could result in the feedback inhibition of the Arg synthesis. However, a β-aspartyl dipeptidase from *Arabidopsis thaliana* did not cleave the β-Asp-Arg dipeptides (Hejazi et al., 2002), but unwanted cleavage of the dipeptides in plants can't be ruled out. The second strategy might be the cytosolic expression of CGPase, resulting in a spatial separation of enzyme and substrate. The CGP degradation is then restricted to the homogenization of the plant cell during the food uptake and the β-Asp-Arg dipeptides are available in the gastro intestinal tract (GIT). Following these two approaches it might be possible to increase the Arg content in plants and replace Arg supplementation in the diet of mammals.

A3 Aims of this work and hypotheses

Because of its high stability and its Arg content, CGP is a suitable candidate protein to increase the pool of bound Arg in plants, avoiding the feedback inhibition of Arg synthesis and provide β-Asp-Arg peptides with high nutritional value. The aim of this work was to increase the CGP content in the plant for a commercial production and enable its timely degradation in order to allow substantial Arg supply in feed by the delivery of β-Asp-Arg dipeptdes.

The production of CGP was already shown in potato and tobacco plants where tobacco showed a higher CGP yield (Hühns et al., 2009; Hühns et al., 2008; Neubauer et al., 2012; Neumann et al., 2005). Anyhow, the production in PH is not applicable for the production of high amounts of CGP, because its biomass production is low. Hence, the CGP production in plants with higher biomass yield is of interest. Two possible cultivars which were bred for commercial tobacco production and high biomass yield are *N. tabacum* cv. Badischer Geudertheimer (BG) and *N. tabacum* cv. Virginia Golta (VG). Due to the higher biomass we

assume that the expression of CPHA leads to a higher CGP yield in BG and VG compared to PH. Hence, CPHA should be expressed in named plants via direct transformation as well as by breeding.

In contrast to CGP, CGPase expression is not established in plants. Recently the successful expression of CHPE was shown in *N. benthamina* (Nausch and Broer, 2016), but CPHB was never expressed in plants. We use the MagniCON® transient expression system (Icon Genetics GmbH, Halle/Saale, Germany) to analyze the expression of a *cph*B gene from *Thermosynechococcus elongatus* BP-1 in *N. benthamiana* for fast evaluation of the gene expression and protein behavior in plants. In a second step we analyzed the expression of CPHB in CGP producing BG plants to determine whether the enzyme is able to degrade CGP *in planta* and in crude plant extracts. Since CPHE was also produced successfully in plants, we compared both enzymes according their ability to degrade CGP in the same experiment to identify which enzyme is more suitable for this project.

After successful CGPase production, the enzyme should be used for feeding studies in mice, which should give us insights about the ability of CGPase to hydrolyze CGP in the GIT of mammals and the bioavailability of β-Asp-Arg dipeptides.

On the basis of our assumptions I formulate the following hypotheses:

1. The expression of cyanophycin synthetase (CPHA) in *N. tabacum* cv. Badischer Geudertheimer and cv. Virginia Golta leads to a higher production of cyanophycin compared to *Solanum tuberosum* and *N. tabacum* Petit Havanna SRI.
2. Plants are able to produce an active form of the intracellular cyanophycinase CPHB.
3. The coexpression of cynaophycin and cyanophycinase is possible and cyanophycin can be degraded *in planta* in the chloroplast as well as after cell homogenization in crude plant extracts.
4. Feeding of cyanophycin and cyanophycinase leads to the release of β-Asp-Arg dipeptides in the gastro intestinal tract of mammals which increases the arginine concentration in the plasma.

Finally, the results will be summarized to discuss the main thesis of this work:

“The coexpression of cyanophycin and cyanophycinase in plants can replace the conventional Arg supplementation in the diets of mammals.”

B Results

The results of this work are published in three peer reviewed journal articles.

B1 Tobacco as platform for a commercial production of cyanophycin.

Henrik Nausch, Tina Hausmann, **Daniel Ponndorf**, Maja Hühns, Sandra Hoedtke, Petra Wolf, Annette Zeyner, Inge Broer (2016): Tobacco as platform for a commercial production of cyanophycin. New Biotechnology 33, 842-851.

Author's contribution: I performed Southern blot analysis, determination of biomass production and CGP quantification of *N. tabacum* var Badischer Geudertheimer plants (Event 116, 128, 125, 137, 157, 176).

New Biotechnology 33 (2016) 842–851

Contents lists available at ScienceDirect

New Biotechnology

journal homepage: www.elsevier.com/locate/nbt

Full length Article

Tobacco as platform for a commercial production of cyanophycin

Henrik Nausch[a,*], Tina Hausmann[a], Daniel Ponndorf[a], Maja Hühns[a], Sandra Hoedtke[b], Petra Wolf[b], Annette Zeyner[c], Inge Broer[a]

[a] University of Rostock, Faculty of Agricultural and Environmental Sciences, Department of Agrobiotechnology and Risk Assessment for Bio- und Gene Technology, Justus-von-Liebig Weg 8, Mecklenburg-Western Pomerania, 18059, Rostock, Germany
[b] University of Rostock, Faculty of Agricultural and Environmental Sciences, Department of Nutrition Physiology and Animal Nutrition, Justus-von-Liebig-Weg 6b, Mecklenburg-Western Pomerania, 18059, Rostock, Germany
[c] Martin-Luther-University Halle-Wittenberg, Institute for Agricultural and Nutritional Sciences, Chair of Animal Nutrition, Theodor-Lieser-Str. 11, 06120, Halle (Saale), Germany

ARTICLE INFO

Article history:
Received 15 May 2016
Received in revised form 4 August 2016
Accepted 4 August 2016
Available online 5 August 2016

Keywords:
Cyanophycin
Nicotiana tabacum cv. Petit Havana SR1
Badischer Geudertheimer
Virginia Golta
Commercial production

ABSTRACT

Cyanophycin (CP) is a proteinogenic polymer that can be substituted for petroleum in the production of plastic compounds and can also serve as a source of valuable dietary supplements. However, because there is no economically feasible system for large-scale industrial production, its application is limited. In order to develop a low-input system, CP-synthesis was established in the two commercial *Nicotiana tabacum* (*N. tabacum*) cultivars 'Badischer Geudertheimer' (BG) and 'Virginia Golta' (VG), by introducing the cyanophycin-synthetase gene from *Thermosynecchococcus elongatus* BP-1 ($CphA_{Te}$) either via crossbreeding with transgenic *N. tabacum* cv. Petit Havana SR1 (PH) T2 individual 51-3-2 or by agrobacterium-mediated transformation. Both in F1 hybrids (max. 9.4% CP/DW) and T0 transformants (max. 8.8% CP/DW), a substantial increase in CP content was achieved in leaf tissue, compared to a maximum of 1.7% CP/DW in PH T0 transformants of Hühns et al. (2008). In BG CP, yields were homogenous and there was no substantial difference in the variation of the CP content between primary transformants (T0), clones of T0 individuals, T1 siblings and F1 siblings of hybrids. Therefore, BG meets the requirements for establishing a master seed bank for continuous and reliable CP–production. In addition, it was shown that the polymer is not only stable *in planta* but also during silage, which simplifies storage of the harvest prior to isolation of CP.

1. Introduction

Currently, plastic polymers as well as cosmetics and dietary supplements are predominantly produced from fossil resources [1,2]. However, the use of petrochemicals is under debate from two aspects: (i) it is one of the main contributors to climate change, and (ii) fossil resources are limited and will become scarcer in the future [3,4]. Renewable biomaterials derived from plants can substitute petroleum based materials [5] and plant made polymers such as rubber, starch, cellulose or lignin are already used extensively [6]. The range of biomaterials made by plants can be expanded by the addition of genes encoding enzymes that convert endogenous plant metabolites into polymers that do not occur naturally in plants [6].

Cyanophycin (CP) is one such attractive polymer with the potential to replace polyacrylates and polyamide-based plastics [1,7]. In addition, it can be used for the production of food and feed supplements of high nutritional value [8–10]. It occurs naturally in cyanobacteria, where it serves as transient storage for nitrogen, carbon and energy. The CP-polymer is composed of mainly arginine-aspartate dipeptides and is synthesised by a single enzyme, cyanophycin-synthetase (CphA), via non-ribosomal protein biosynthesis [11]. Since the branched conformation of CP differs substantially from the tertiary structure of proteins, CP cannot be degraded by common proteases. Its degradation is restricted to a special class of cyanophycinases, occurring exclusively in bacteria, [2,12].

Previously, CP has been produced in prokaryotic cell cultures and yeast by introducing the *cphA*-gene from various bacteria, yielding up to 28% of dry weight (DW) [13,14]. However, cell

Abbreviations: PH, Petit Havana SR1; BG, Badischer Geudertheimer; VG, Virginia Golta; NIC, near isogenic control; CP, cyanophyin; $CphA_{Te}$, cyanophycin-synthetase from *Thermosynecchococcus elongatus* BP-1; FW/DW, fresh/dry weight.

* Corresponding author.
E-mail address: henrik.nausch@uni-rostock.de (H. Nausch).

http://dx.doi.org/10.1016/j.nbt.2016.08.001
1871-6784/

cultures are unsuitable for large-scale synthesis, since they are limited in terms of scalability, productivity and cost [15–17]. Therefore, CP was produced in plants such as tobacco and potato [18–20]. Yields of up to 6.8% of DW in tobacco leaves and 7.5% of DW in potato tubers could be achieved by targeting the CP-synthetase from *Thermosynecchococcus elongatus* BP-1 ($CphA_{Te}$) to the plastids [18,19]. Tobacco as non-food crop is a promising platform and is already used for the production of therapeutic proteins [21,22]. In addition to its suitability for producing high-value compounds, tobacco also fits the needs of industrial large-scale production of low-value materials [23]. Tobacco can be grown without special demands on soil, fertilisation and environment, and it rapidly forms high amounts of leaf biomass [24–26]. Tobacco is commonly harvested manually in order to keep the leaves intact [27]; however, since biomass and CP-content (not leaf quality) are critical for CP-production, cultivation of CP-producing tobacco can be mechanised using the existing agricultural infrastructure, such as for corn or canola. Mechanised harvesting is already practised by some tobacco farmers in Europe, yielding up to 3.5 t leaf DW per year and ha [24,25]. In addition, plants might be allowed to regrow after harvesting the shoot, potentially yielding multiple crops per season. It has been estimated that up to 140–165 t leaf fresh weight (FW) per season could be generated by this practice, which is equivalent to 16–32 t leaf DW [27–29].

However, up to now, cyanophycin-producing tobacco lines have been based on the cultivar Petit Havana SR1 (PH) [19,20]. This cultivar is not eligible for the industrial production of CP since it has not been bred for high leaf biomass yields. For commercial CP production, tobacco breeders have developed two cultivars that yield high biomass and that are more resistant to changing weather conditions and abiotic stress: 'Badischer Geudertheimer' (BG) and 'Virginia Golta' (VG) [24,25]. Consequently, the production of CP has been established in these two cultivars via crossing with the PH T2 plant 51-3-2 with 6.8% CP per leaf DW, leading to a CP accumulation of up to 9.4% CP/DW or via direct transformation, yielding up to 8.8% CP/DW in T0 transformants. BG with a homogenous CP-accumulation in the T1 offspring seems to be suitable for generation of a master-seed bank. Since CP is stable during ensilage of plant tissue, CP-containing tobacco can be stored at ambient conditions after harvest.

2. Material and methods

2.1. Plasmid construction, plant material and transformation

For constitutive, plastidic expression of $CphA_{Te}$, the plasmid pPsbY-*cphA*$_{Te}$ was used [30]. Leaf specific expression was achieved by substituting the 35S promoter with the C1 promoter from *Beta vulgaris* [31], amplified from the plasmid pC1-TL with the primer *Spe*I-C1-fw (5′-TACGACTAGTAGCTTGAGGATCAACATT-3′) and *Sma*I-C1-rv (5′-TACGCCCGGGGGTATATTTTGGTTTCAAC-3′; restriction sites underlined). The resulting vector pC1-PsbY-$CphA_{Te}$ (Fig. S1a) was verified by sequencing (GATC Biotech AG, Konstanz, Germany). Transformation of tobacco plants with *Agrobacterium tumefaciens* strains LBA4404, C58C1 and AGL1 was carried out as described [32]. Transgene integration was confirmed by PCR using cphA-fw (5′-GTGCCGGCCCATGTGATTGG-3′) and cphA-rv (5′-AGCCAGGAGCGGCATTGACC-3′) for the coding region of *cphA*$_{Te}$ and C1-fw (5′-CACCTGTCAACCACTAGATGGATAGC-3′) in combination with cphA-rv for the C1-promoter.

2.2. Kanamycin germination assay

At least 150 seeds from self-fertilised transgenic plants were germinated on LS-medium containing 100 μg/mL kanamycin as described in [19]. Germination frequency was analysed by germinating 50 seeds on LS-medium without Kanamycin. Taking selection-free germination as 100%, the germination rate on antibiotic-containing medium was calculated.

2.3. Greenhouse cultivation

Transgenic individuals were transferred from tissue culture 4 weeks after the last subculture, directly into 5 L pots containing peat soil (Stender AG, Schermberg, Germany). Plants were fertilised once a week using 0.2% Hakaphos Blue (Hermann Meyer KG, Rellingen Germany). Leaf samples were taken 6 and 12 weeks after potting.

2.4. CP quantification

Cyanophycin analysis was conducted as described in [19] with modifications. In 2 mL reaction tubes 30–35 mg of freeze dried tobacco leaf material was homogenised with ceramic pills using a Precellys 24 homogenisator (VWR International GmbH, Erlangen, Germany). Soluble proteins were extracted with 1 mL 50 mM Tris (pH8) for 30 min in a shaker. After a centrifugation step, the pellet resuspended in 1 mL of 0.1 M HCl. After another centrifugation step, 800 μL of the supernatant was used for CP analysis. Tubes with 1 to 10 μL of sample were filled up with 0.1 M HCl to a final volume of 800 μL. After addition of 200 μL of 5× RotiQuant Bradford reagent (Carl Roth GmbH+ Co. KG, Karlsruhe, Germany), samples were measured at 595 nm. A calibration curve was prepared with purified CP from potato tubers, extracted after the method of [33] and range of 1–5 μg/mL CP. OD values of leaf samples from transgenic plants that where transformed with the control vector pLH9000 were subtracted from OD values of samples of transgenic plants.

2.5. SDS-PAGE and coomassie-staining

HCl extracts were prepared from freeze-dried leaf material as for the CP quantification. The extracts were freeze-dried, separated in a 12% SDS-PAGE, as described [34], and stained with Coomassie Brilliant Blue R250. CP, serving as positive control, was isolated from potato tubers as described above.

2.6. Ensilage of tobacco plants

At the age of 12 weeks, whole tobacco plants of BG hybrids, BG near-isogenic controls (NICs), VG hybrids and VG NICs were harvested at a DW of 16.2, 16.8, 20.4 and 21.1% FW and chopped with a shredder (GE 210, Viking, Dieburg, Germany) to a particle size of 50 mm. Approximately 400 g was ensiled in vacuum-sealed polyethylene bags according to [35]. Silage treatments were made in quintuplicates either as control without (CON) or with the addition of a lactic acid bacteria (LAB) inoculant containing *Lactobacillus plantarum* (DSM 8862 and 8866, application rate 3×10^5 cfu/g). Samples were stored at ambient temperature (20 °C) for 49 days.

At opening day, silages were homogenised and an aliquot was taken for freeze-drying. Afterwards, samples were milled and the absolute DW of the lyophilised samples was determined by oven drying at 105 °C for 3 h, whereas silage DW was corrected for the loss of volatiles according to [36]. Another 50 g of silage was weighed into a beaker (600 mL), mixed with 200 mL deionised water and stored overnight at 4 °C. Silage extracts were filtered and the pH value was measured. Lactic acid was determined by HPLC (Aminex HPX-87H, Biorad, Hercules, USA) as described in [35]. Fermentation losses were calculated by the difference in sample weight at day 0 and the respective weight at the opening day.

2.7. Southern blot analysis

Southern blot analysis was conducted according to [19]. Chromosomal DNA was digested with *Bcl*I and *Eco*RI. The probe was amplified from the plasmid pPsbY-CphA$_{Te}$ using the primer pair cphA-fw and −rv.

2.8. Statistical methods

Statistical analysis was performed with SPSS, using either the non-parametric (Mann-Whitney-*U* or unpaired *T*-test) or univariate ANOVA (including the post-hoc Bonferroni, Duncan and Tukey test). A *P*-value $P \leq 0.05$ (two-sided) was considered significant. The variability of different events and siblings was characterised by the relative coefficient of variation (CV, %):

$$V_r[\%] = \frac{1}{\sqrt{n}} \frac{s}{y} .100$$

3. Results

3.1. Biomass production of N. tabacum cv. 'Badischer Geudertheimer' and 'Virginia Golta' in the greenhouse

The biomass of BG and VG was evaluated by two independent greenhouse trials in parallel with PH. The leaf biomass was determined after 6 and 12 weeks. For all three cultivars, biomass yields were reproducible between the two trials (data not shown). At both time points PH yielded significantly less biomass compared to the other commercial cultivars (Table S2). After 6 weeks, BG and VG were similar in their FW, with approximately 170% more biomass taking PH as reference. After 12 weeks, VG yielded significantly more leaf material than BG, with 251.0% (VG) while PH and BG nearly had no increase in yield (Fig. 1). In addition, PH started flowering at 6 weeks whereas both BG and VG started at 12 weeks. The ratio between leaf FW and DW did not differ significantly between cultivars and harvest time points (Table S2).

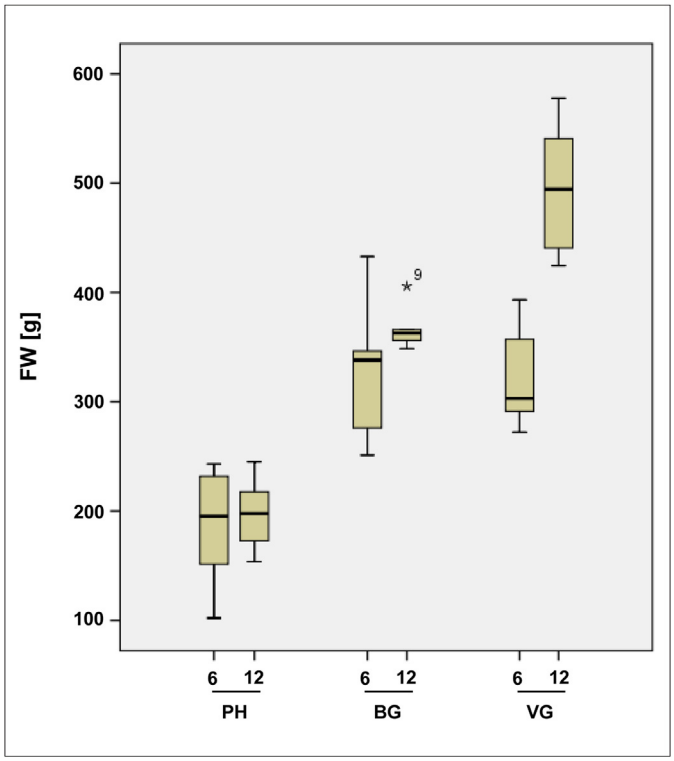


Fig. 1. FW of the *N. tabacum* cultivars, harvested after 6 and 12 weeks cultivation in the greenhouse. Asterisk: extreme value, identified by SPSS analysis.

3.2. CP production in N. tabacum cv. 'Badischer Geudertheimer' and 'Virginia Golta'

Recombinant CP synthesis can be achieved by two approaches: (i) the direct transformation with a *Psby-cphA$_{Te}$*-encoding construct, or (ii) by crossing both cultivars with transgenic PH plants. Both attempts were conducted and compared in terms of CP yield and plant biomass.

3.3. Crossbreeding of N. tabacum cv. 'Badischer Geudertheimer' and 'Virginia Golta' plants with the transgenic Petit Havana SR1 elite event

The PH T2 plant 51-3-2 [19], which has proven to be the highest CP producer with 6.8% DW, served either as pollen donor (NIC X 51-3-2) or pollen recipient (51-3-2 X NIC) in the crossings. The hybrids with the highest CP yield were backcrossed.

51-3-2 served both as pollen donor and pollen recipient for BG and VG hybrids. Independently of the crossing scheme, the mean CP content of the F1 offspring was not significantly higher compared to the 51-3-2 PH parent, measured in this study in parallel (Fig. 2, Table S3). However, in the case of BG, in some individual F1 hybrids a significantly higher CP content was found, yielding up to 9.4% CP/DW (Table S3). F1 hybrids with 51-3-2 as the pollen recipient had a significantly higher CP yield than F1 plants from 51-3-2 as pollen donor, independent of the cultivar. Hence only the former were used for backcrossing.

The mean CP content in F2 hybrids was similar to that of the F1 generation and even reduced in the case of BG (Fig. 2; Table S3). Only for VG, individual F2 hybrids had increased CP levels, yielding

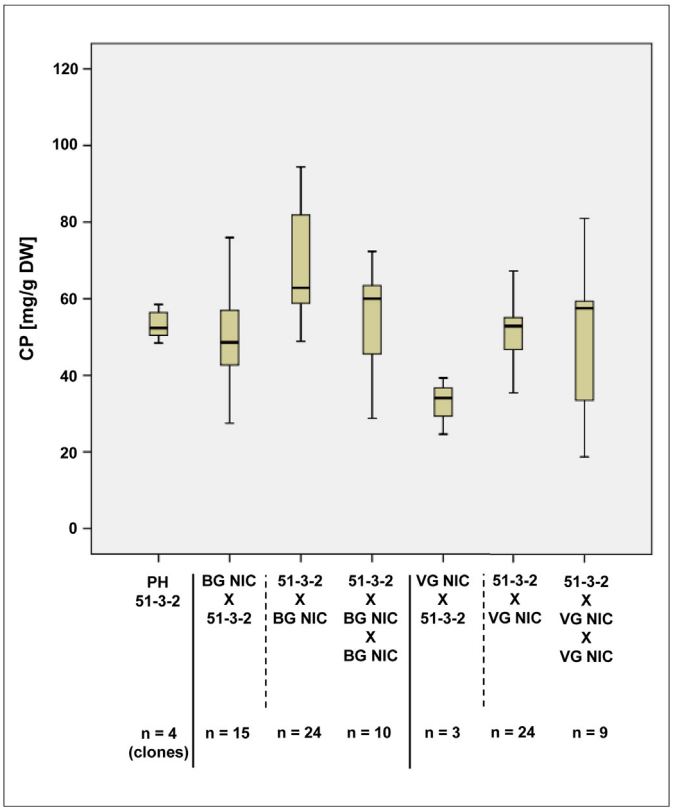


Fig. 2. Average CP content in hybrids of BG and VG plants crossed with the PH elite event 51-3-2. Crossing scheme mother (pollen recipient) X father (pollen donor). Samples were taken from 12 week old plants, grown in the greenhouse.

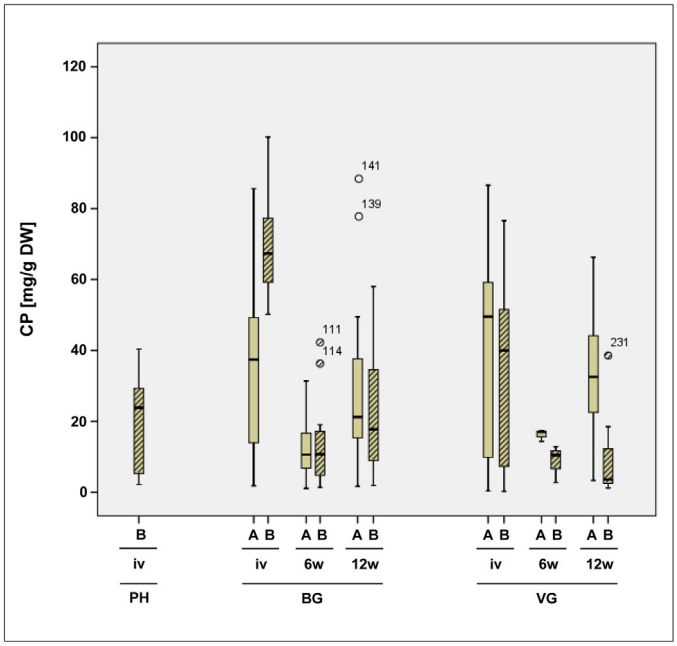


Fig. 3. CP content in leaves of T0 transformants of the *N. tabacum* cultivars *in vitro* and in the greenhouse. iv: *in vitro*; 6w/12w: 6 and 12 weeks of cultivation in the greenhouse; (A) plants, transformed with the constitutive expression construct pPsbY-CphA$_{Te}$; (B) plants transformed with the leaf-specific expression construct pC1-PsbY-CphA$_{Te}$ (dashed bars); Circle: outlier, identified by SPSS analysis.

up to 8.1% CP/DW (Table S3). BG hybrids in general showed the highest homogeneity in variance that was only less pronounced in clones of PH 51-3-2 (Table S4). The mean FW of F2 BG and VG hybrids averaged 76–84% of the NIC after 12 weeks (data not shown).

3.4. CP content in T0 events, transformed with pPsbY-CphA$_{Te}$ and pC1-Psby-CphA$_{Te}$

Transgenic BG and VG events with either constitutive or leaf-specific plastidic CP synthesis were generated, using the vectors pPsbY-CphA$_{Te}$ and pC1-PsbY-CphA$_{Te}$. (Fig. S1a) and three different *A. tumefuciens strains*: LBA4404, C58C1 and AGL1. The vector pC1-PsbY-CphA$_{Te}$. was also introduced into PH. Transformation with C58C1 and AGL1 yielded more shoots per explant compared to LBA4404, independent of the construct (Table S1b). VG could not be transformed using LBA4404. Transgene integration was verified by PCR (data not shown).

CP content in transgenic BG and VG T0 plants was measured in leaf samples of *in vitro* cultured individuals and after cultivation in the greenhouse for 6 and 12 weeks (Fig. 3). CP accumulation was confirmed in the events with the highest CP accumulation *in vitro* by separation in a Coomassie-stained SDS-PAGE (Fig. 4). In the case of PH transformed with pPsbY-CphA$_{Te}$, samples were taken from the T2 plant 51-3-2. Comparing BG and VG plants that expressed the CP-synthetase under the control of the constitutive 35S promoter, the mean CP content *in vitro* was nearly equal, 3.4% and 3.9% CP/DW (Table S5). Plants in the greenhouse did not reach this CP yield, even when grown to 12 weeks (Table S5). Although mean CP content was quite similar in BG and VG, top producers differed significantly with 8.8% and 6.6% CP/DW respectively.

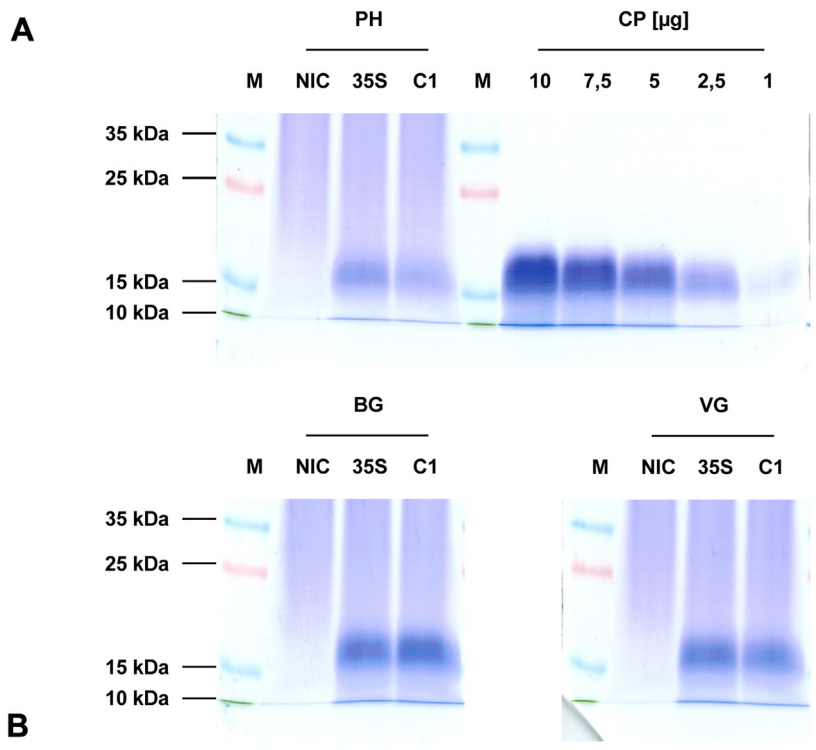
Similar to the analysis for PH in [19], the CV value was determined for the 7 events of BG and VG with the highest CP content. The homogeneity of variances proved to be comparable in both cultivars, with a relative variation coefficient of BG 5.26 and VG 4.99 (Table S6).

The C1 promoter was significantly more efficient in BG both compared to the C1 in VG and the 35S promoter in BG and VG under *in vitro* conditions (Fig. 3). In the greenhouse, CP accumulation levels were similar between 35S and C1 in BG but lower in VG (Table S5). Comparing the variation in CP content in BG, VG and PH with the C1 construct, the variance was significantly more homogenous in BG compared to VG and PH (Table S7). Independently of the construct and cultivar, there was no statistically significant increase in CP content between 6- and 12-week old transgenic BG and VG plants (Fig. 3). CP content was also determined in single leaves of four 12-week old BG individuals, carrying the constitutive pPsbY-CphA$_{Te}$ construct at one time point (Fig. 5). The levels of CP in all leaves were similar independent of the age and did not differ significantly.

3.5. CP-content in T1 descendants of N. tabacum *cv. 'Badischer Geudertheimer'*

Increased yields of recombinant proteins and polymers through conventional breeding have already been reported [37–39]. In addition, establishing a master seed bank of transgenic events to enable consistent yield is a prerequisite for commercial production. Since in the greenhouse the leaf-specific C1 construct did not perform any better than its 35S counterpart and because VG did not show any significantly higher CP content than BG under the same conditions, breeding attempts were only made for the crop/construct-combination BG + pPsbY-CphA$_{Te}$. The T1 generation of six events was generated by self-pollination (Table 1). Seeds from two events (BG 128 and 176) had considerably reduced germination rates on the selection-free medium (Table 1). In addition, for two events of BG (116 and 125) germination rates were 100% on selection-free medium but were considerably reduced on selective medium. Adverse effects on the germination rate correlated neither to the amount of CP in the parents nor to the copy numbers, which were determined by Southern blot (Table 1; Fig. S8).

Most of the T1 plants showed very slight reductions in CP level compared to T0 plants, but this trend was not significant (Fig. 6).



	35s		C1	
	event	CP [mg / g DW]	event	CP [mg / g DW]
PH	51-3-2	66.42	167	40.35
BG	409	85.60	51	100.15
VG	383	86.59	98	67.57

Fig. 4. Coomassie-stained SDS-PAGE of HCl-extracts from leaves of the *N. tabacum* cultivars from *in vitro* culture. (A) 35S: transgenic events, transformed with pPsbY-CphA$_{Te}$; C1: transgenic events, transformed with pC1-PsbY-CphA$_{Te}$; leaf samples were taken 8 weeks from *in vitro* plants after the last sub-culturing; M: PageRuler Plus Prestained Protein Ladder Mix (ThermoScientfic; Darmstadt/Germany): numbers: purified CP in µg. (B) events, analysed in the SDS-PAGE.

However, individual T1 descendants had considerably higher CP content compared to the parent as well. For example the T1 plant 128-28 yielded 3.5% CP/DW compared to 2.5% in the parent 128 (Table S9). In addition, the relative variation coefficient was more homogenous in the T1 generation compared to the T0 parents (Table S10).

3.6. Comparison of the variation of CP-content in clones and siblings of the transgenic tobacco cultivars N. tabacum *cv. 'Badischer Geudertheimer'*

Besides the influence of the host genome, CP-content might be impacted by environmental factors. In order to differentiate between both factors, the variation in the CP amount among clones of BG was determined and compared to the variation among siblings/descendants of T0 events (Fig. 7). Samples were taken from 12-week old plants cultivated in the greenhouse. The variation between clones was equal to or slightly lower than that among siblings, with a CV-value of 12.97 in clones and 12.36 (±3.13) as mean of 6 lines of siblings (Table S11), which was also true when comparing the 7 siblings with the highest CP content (Table S12). However, only clones from one individual were investigated.

3.7. Evaluation of the fermentation quality of silages of N. tabacum *cv. 'Badischer Geudertheimer' and 'Virginia Golta'*

Efficient storage of VG and BG would enable decoupling of biomass production and processing and thereby significantly reduce the costs of CP production under industrial conditions [40,41]. Neither cultivar has been used for the production of tobacco silage before and they differ substantially in key parameters that are essential for fermentation quality, such as sugar content [24,25]. Hence, ensilability was determined using 12-week-old F2 hybrids BG 25 and VG 24, as well as using 12-weeks-old BG and VG NICs. Experiments were conducted in two

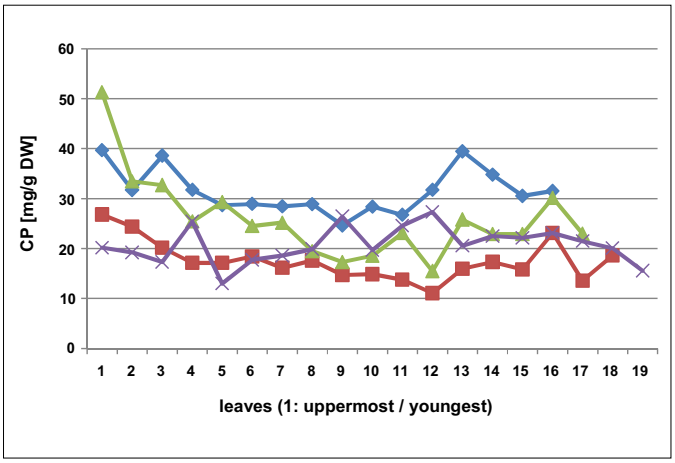


Fig. 5. CP content in leaves of different development stages. Lines represent CP content in individual plants of BG transformed with expression cassette pPsbY-CphA$_{Te}$. Samples were taken from 12 week old plants, grown in the greenhouse.

independent trials in 2010 and 2011. At harvest, CP content in BG 25 and VG 24 hybrids averaged 13.38 and 13.44 mg CP/g DW (2010) and 24.60 and 21.96 mg CP/g DW (2011), respectively. Representative data for the trial in 2011 are shown in Table 2.

The most important variable to establish anaerobically stable silages is the pH value, as undesired microorganisms are hampered in an acidic silage environment. The pH value in silage extracts of both cultivars after 49 days of ensilage ranged between 3.44 and 3.87 (Table 2). Silages inoculated with LAB were always lower in pH than control silages without LAB addition ($P < 0.05$), although differences between treatments were only marginal in the CP producing BG 25 and VG 24. Corresponding to the low pH, in all hybrids and NICs considerably higher content of lactic acid between 10.4 and 15.7% of DW were measured, indicating that lactic acid as the strongest organic acid in silages was responsible for the pH decrease. In BG and VG NICs, the addition of LAB positively influenced the lactic acid production ($P < 0.05$). However, treatments with LAB unexpectedly showed lower lactic acid levels in BG 25 ($P < 0.05$) and VG 24 ($P > 0.05$) hybrids than non-inoculated silages. Lactic acid was the primary acid in all silages and made up between 85 and 97% of the total acid content.

Fermentation losses expressed as a proportion of the initial silage weight before ensiling were between 1.14 and 2.34%. Coefficients of variation were considerable and ranged from 15 to 70% among all hybrid and NIC treatments. No clear influence of either cultivar or variant could be detected. Silages without addition of LAB in BG 25 and NICs tended to have higher losses ($P > 0.05$), whereas only in VG NICs were significant differences detected ($P < 0.05$). In VG 24 fermentation, losses were even higher in the LAB-treated silages. However, due to high standard deviations of the treatment means, this is not considered significant.

Table 1
Germination rates of T0 events of the transgenic BG, used for breeding.

N. tabacum cultivar	T0 events			germination rate of seeds from T0 events	
	plant	CP content [mg/g DW]	transgene copy number	−Km (n = 50) [%]	+Km (n = 150) [%]
BG	116	22.93	2	118.9	51.5
	125	10.72	>2	102.5	68.2
	128	25.05	>2	78.9	81.1
	137	7.88	>2	118.7	98.7
	157	11.64	1	104.6	94.4
	176	16.66	1	81.0	90.4

BG: *N. tabacum* cv. Badischer Geudertheimer; Km: Kanamycin.

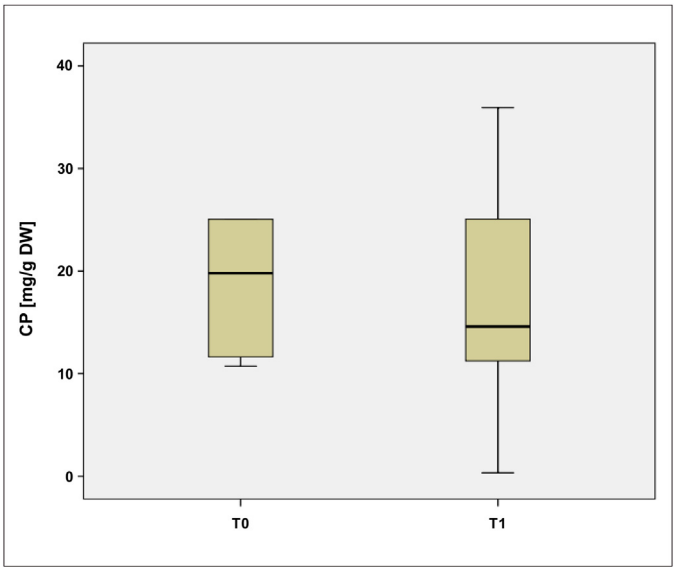


Fig. 6. Comparison of the mean CP content in T0 elite events of BG and in their T1 descendants. CP-content was measured after 12 weeks of cultivation in the greenhouse.

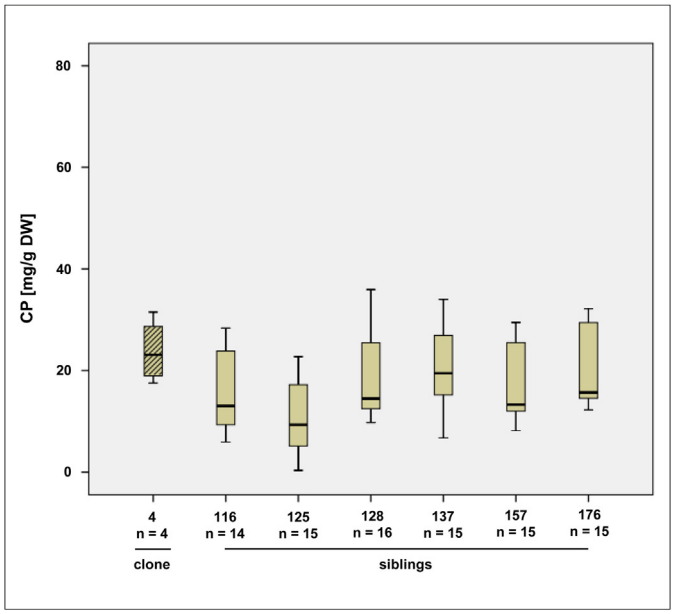


Fig. 7. Comparison of the variation of CP-content in clones and siblings of the transgenic BG. CP-content was measured after 12 weeks of cultivation in the greenhouse.

3.8. Stability of CP N. tabacum *cv. 'Badischer Geudertheimer' and 'Virginia Golta' during ensilage*

In order to allow efficient storage of CP-containing plant material, CP has to be stable during ensilage. Hence, at the beginning and end of the fermentation of BG and VG tobacco material, samples were taken and analysed for their CP content. In the two trials, no significant degradation of CP was observed independently of the treatment (Fig. 8a and b). CP integrity was verified by isolation of CP from samples and subsequent separation in a SDS-PAGE (Fig. 8c). The occasional slightly increasing trend was probably due to the general degradation of biomass. Additionally, at the beginning of the experiment only one sample was taken and this cannot be considered significant.

4. Discussion

4.1. Transformation might be more suitable than crossing to introduce CP production into 'Badischer Geudertheimer' and 'Virginia Golta'

Both by crossing and transformation CP production was successfully introduced into the BG and VG genetic backgrounds. Comparing both methods, CP content in F1 and T0 top-producers were comparable (Tables S3 and S5). Comparing the cultivars, CP yields in BG elite plants were substantially higher and more homogenous in their variation (Tables S4, S6 and S7). Hence, both methods seem to be equal, and BG seems to be more advantageous for CP production. However, F1 hybrids yielded substantially less biomass compared to the T0 transformants. Backcrossing increased the biomass in the F2 generation but reduced the CP content, limiting the overall CP yield in hybrids. On the other hand transformants were not affected in their biomass yield. Although the mean CP content was not increased in the T1 generation of BG (Fig. 6; Table S9), breeding generated T1 individuals with significantly higher CP content compared to the T0 parent, indicating that the CP yield in transformants could be increased by selective breeding. Similarly, [19] showed that although the mean CP content in the PH T1 generation (1.0% CP/DW) was lower compared to the T0 transformants (1.3% CP/DW), there were individual plants with a substantially higher accumulation and a 4.0-fold increase from the T0 to the T2 generation could be achieved. The increase by conventional breeding which has been observed for other recombinant proteins and polymers as well [37–39], indicates that this is a general phenomenon and not related to a specific recombinant compound. Since the mean CP content was higher both in the T0 and the T1 generations of BG compared to PH (Fig. 6), it might be assumed that BG has a higher potential to increase the CP content by this approach.

However, direct transformation has two inherent disadvantages. First, a suitable transformation protocol had to be established for both cultivars. As the results of this study clearly demonstrate, transformation efficiencies of *A. tumefaciens* strains can differ not only between plant species but even between cultivars. As reviewed in [42,43], this might be attributed to different alleles of the plant genes involved in the infection process. Secondly, due to the random transgene integration, transformants need to be analysed not only for their CP content, but also for their transgene copy number, integration locus and pleiotropic off-target effects. Hence, both approaches have their advantages and disadvantages, but direct transformation might have the potential to yield more CP due to a higher accumulation level.

In general CP content in transformants could not be increased by the use of a leaf-specific promoter. Although it increased the yield 2-fold in BG compared to 35S under *in vitro* conditions, this could not be reproduced in the greenhouse and was also not shown for VG and PH (Table S5; [19]). In addition, the CV values of BG transformants were similar for both constructs, but three times higher in VG and PH for the leaf-specific construct (Tables S6 and S7; [19]). Thus, the positive effects observed by [18] when using a tuber-specific promoter in potato could not be mirrored with the leaf-specific C1 promoter in tobacco.

4.2. CP content is higher and more homogenous in transformants of 'Badischer Geudertheimer' compared to 'Petit Havana SR1'

Due to the higher biomass yield of BG compared to PH (Table S2), this cultivar led to a higher absolute CP production when CP concentration is equal. Interestingly, when *cphA*$_{Te}$ gene

Table 2
Fermentation parameters of BG and VG hybrids (BG 25, VG 24) and NICs (BG_NIC, VG_NIC), ensiled either without (CON) or with addition of a lactic acid bacteria inoculant (LAB) after 49 days of storage duration (n = 5).

	BG_25		BG_NIC		VG_24		VG_NIC	
Parameter	CON	LAB	CON	LAB	CON	LAB	CON	LAB
pH	$3.47^{aB} \pm 0.02$	$3.44^{bD} \pm 0.01$	$3.87^{aA} \pm 0.16$	$3.57^{bA} \pm 0.01$	$3.48^{aB} \pm 0.01$	$3.46^{bC} \pm 0.00$	$3.63^{aA} \pm 0.04$	$3.49^{bB} \pm 0.01$
Lactic acid (% DW)	$15.7^{aA} \pm 0.37$	$14.9^{bA} \pm 0.47$	$10.5^{bC} \pm 1.86$	$14.9^{aA} \pm 0.60$	$14.2^{B} \pm 0.36$	$13.8^{B} \pm 0.41$	$10.4^{bC} \pm 0.92$	$12.6^{aC} \pm 0.33$
Fermentation losses[1]	$1.84^{AB} \pm 1.28$	1.23 ± 0.44	$1.39^{AB} \pm 0.37$	1.29 ± 0.70	$1.14^{B} \pm 0.27$	1.30 ± 0.63	$2.34^{aA} \pm 0.34$	$1.25^{b} \pm 0.22$

DW – dry weight.
[a,b]Means with different lower case letters within the same parameter and plant material differ significantly between treatments ($p < 0.05$).
[A,B,C,D]Means with different capital letters within the same parameter and treatment differ significantly between plant materials ($p < 0.05$).
[1] Given as% of the initial weight before ensiling.

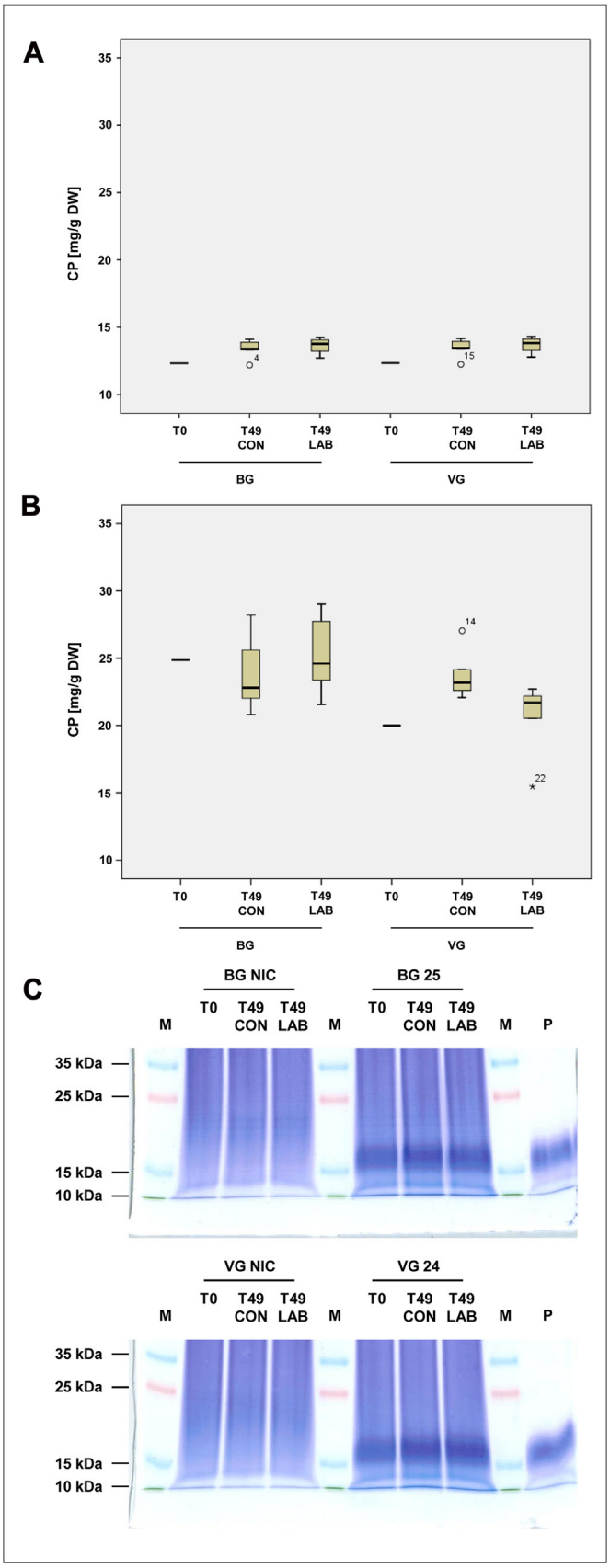


Fig. 8. CP content in tobacco leaf material before and after ensilage. BG 25 and VG 24: transgenic hybrids events of BG and VG; T0/T49: CP content in samples before (T0; n = 1) and after ensilage for 49 days (T49) either without (CON; n = 5) or with addition of a lactic acid bacteria inoculant (LAB; n = 5). Two independent trials were

expression is driven by the constitutive 35S promoter, CP content was also increased by higher accumulation levels in comparison to the T0 transformants of PH described in [19]. Under in *in vitro* culture, yields were three times higher in BG (Table S5). Top-producers of the constitutive construct produced up to 8.5% CP/DW in BG compared to 1.7% in the PH elite T0 event 51 and even higher than the T2 elite event 51-3-2 with 6.8%. Not only in the T0 but also in the T1 generation, the mean CP content in BG (Fig. 6; Table S9) was significantly higher compared to the T1 of PH of [19]. In addition, the CP content proved to be significantly more homogenous in BG both in the T0 and T1 generation (Tables S6, S7 and S10, [19]). The same homogenous variation was found in BG, but not in PH, when the CP content between clones from one individual was compared to siblings, descended from the same individual (Fig. 7, Tables S11 and S12, [19]). Hence yield and homogeneity seem to be influenced by the genetic background of the plant, favoring BG. In contrast to our results, [44] could not observe any significant difference between *Nicotiana* cultivars when analyzing the accumulation level of four recombinant proteins. Unfortunately, PH, BG and VG were not among them. Moreover, the four recombinant proteins were directly encoded by the transgene, whereas in case of CP not the polymer but the corresponding synthetase is encoded by the transgene. It cannot be excluded that the non-ribosomal protein biosynthesis of CP might be differentially affected by the host metabolism compared to the regular biosynthesis of proteins.

4.3. BG and VG are suitable for ensilage and fermentation quality is not affected by CP

A key requirement for successfully fermented silage is a sufficient decrease in the pH through lactic acid fermentation within a short time after the silo is closed and, furthermore, that this pH remain consistently low to maintain anaerobic stability of the silage [45]. In all evaluated silages of both cultivars the critical pH value was far below the necessary values [46]. However, there is no information about the pH decline over the course of time as only one storage duration was analysed. Nevertheless, high lactic acid levels and the comparatively low content of undesired products from secondary fermentations (data not shown) suggest that even in non-inoculated silages lactic acid fermentation was spontaneous and probably mostly completed at an early stage of ensilage. Unless silage fermentation is not entirely homolactic, losses are likely to take place [47] and in accordance with silages made from forage crops [48,49]. Concerning the application of a bacterial inoculant to enhance fermentation, it can be assumed that fermentation stimulants are not mandatory for successful ensilage of tobacco.

4.4. CP is stable during ensilage of BG and VG

During ensilage, in all samples CP content was not decrease and actually showed a slight (though insignificant) increase. The data clearly demonstrated that there were no bacteria present during the ensilage process that are able to degrade CP. The relative increase can be explained by biomass losses which commonly occur during fermentation [47–49]. This is in line with the data of [18,19], where no CP degradation was observed either in senescing tobacco leaves or in potato tubers, which were stored for 32 weeks. In potato, the relative amount of CP also increased, explained by loss of biomass without reduction of the total CP content [18]. Thus, tobacco biomass can be stored in a practical and cost-efficient manner until processing, reducing overall production costs.

5. Conclusion

In this study, conditions necessary for the industrial large-scale production of CP in tobacco have been established. Based on the cultivars BG and VG, which produced high amounts of leaf biomass, events were generated yielding up to 8.8% CP per leaf DW in T0 transformants. Due to the homogenous CP-accumulation in BG, this cultivar seems to be suitable for generating a uniform master seed bank, making CP production reliable. The stability of CP in leaf tissue and the ability to ensilage BG allows the decoupling of biomass production and processing, which enables the adaptation of automated crop management practices such as those used for corn or canola, benefiting overall economics. In addition the protocol for CP isolation from tobacco leaf tissue developed by [33] might support industrial large-scale purification from tobacco. Ultimately, the real value of this system has to be proven in field trials since CP production could significantly differ under field conditions.

Acknowledgement

We would like to express the warmest of thanks to Alex Rajewski for proof-reading and the helpful suggestions.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.nbt.2016.08.001.

References

[1] Börnke F, Broer I. Tailoring plant metabolism for the production of novel polymers and platform chemicals. Curr Opin Plant Biol 2010;13:353–61.
[2] Sallam A, Steinbüchel A. Biotechnological production of cyanophycin dipeptides. Patent US 8980845 B2. 2011.
[3] Bardi U. Plundering the planet – the 33rd report to the club of Rome. White River Junction, USA: Chelsea Green Publishing Co.; 2013.
[4] Randers J. 2052 – A Global Forecast for the Next Forty Years. White River Junction, USA: Chelsea Green Publishing Co.; 2012.
[5] van Beilen JB, Poirier Y. Plants as factories for bioplastics and other novel biomaterials. In: Altman A, Hasegawa PM, editors. *Plant biotechnology and agriculture*. London, UK; 2012. 481–94.
[6] Snell KD, Singh V, Brumbley SM. Production of novel biopolymers in plants: recent technological advances and future prospects. Curr Opin Biotechnol 2015;32:68–75.
[7] Mooibroek H, Oosterhuis N, Giuseppin M, Toonen M, Franssen H, Scott E, et al. Assessment of technological options and economical feasibility for cyanophycin biopolymer and high-value amino acid production. Appl Microbiol Biotechnol 2007;77:257–67.
[8] Sallam A, Steinbuchel A. Cyanophycin-degrading bacteria in digestive tracts of mammals, birds and fish and consequences for possible applications of cyanophycin and its dipeptides in nutrition and therapy. J Appl Microbiol 2009;107:474–84.
[9] Sallam A, Steinbuchel A. Dipeptides in nutrition and therapy: cyanophycin-derived dipeptides as natural alternatives and their biotechnological production. Appl Microbiol Biotechnol 2010;87:815–28.
[10] Santos S, Torcato I, Castanho MARB. Biomedical applications of dipeptides and tripeptides. Pept Sci 2012;98:288–93.
[11] Ziegler K, Diener A, Herpin C, Richter R, Deutzmann R, Lockau W. Molecular characterization of cyanophycin synthetase, the enzyme catalyzing the biosynthesis of the cyanobacterial reserve material multi- L-arginyl-poly- L-aspartate (cyanophycin). Eur J Biochem 1998;54:154–9.
[12] Law AM, Lai SWS, Tavares J, Kimber MS. The Structural Basis of β-Peptide-Specific Cleavage by the Serine Protease Cyanophycinase. J Mol Biol 2009;392:393–404.
[13] Elbahloul Y, Frey K, Sanders J, Steinbüchel A. Protamylasse, a Residual Compound of Industrial Starch Production, Provides a Suitable Medium for Large-Scale Cyanophycin ProductionApplied and Environmental Microbiology. Appl Environ Microbiol 2005;71:7759–67.
[14] Frommeyer M, Wiefel L, Steinbüchel A. Features of the biotechnologically relevant polyamide family cyanophycins and their biosynthesis in prokaryotes and eukaryotes. Crit Rev Biotechnol 2014;0:1–12.
[15] Ma JKC, Drake PMW, Christou P. The production of recombinant pharmaceutical proteins in plants. Nat Rev Genet 2003;4:794–805.
[16] Merlin M, Gecchele E, Capaldi S, Pezzotti M, Avesani L. Comparative evaluation of recombinant protein production in different biofactories: the green perspective. BioMed Res Int 2014.
[17] Sabalza M, Christou P, Capell T. Recombinant plant-derived pharmaceutical proteins: current technical and economic bottlenecks. Biotechnol Lett 2014;36:2367–79.
[18] Hühns M, Neumann K, Hausmann T, Klemke F, Lockau W, Kahmann U, et al. Tuber-specific cphA expression to enhance cyanophycin production in potatoes. Plant Biotechnol J 2009;7:883–98.
[19] Hühns M, Neumann K, Hausmann T, Ziegler K, Klemke F, Kahmann U, et al. Plastid targeting strategies for cyanophycin synthetase to achieve high-level polymer accumulation in Nicotiana tabacum. Plant Biotechnol J 2008;6:321–36.
[20] Neumann K, Stephan DP, Ziegler K, Hühns M, Broer I, Lockau W, Pistorius EK. Production of cyanophycin, a suitable source for the biodegradable polymer polyaspartate, in transgenic plants. Plant Biotechnol J 2005;3:249–58.
[21] Matoba N, Davis K, Palmer K. Recombinant Protein Expression in Nicotiana. In: Birchler JA, editor. *Plant chromosome engineering – methods and protocols*. New York; 2011. 199–219.
[22] Tremblay R, Wang D, Jevnikar AM, Ma S. Tobacco, a highly efficient green bioreactor for production of therapeutic proteins. Biotechnol Adv 2010;28:214–21.
[23] Tusé D, Tu T, McDonald KA. Manufacturing Economics of Plant-Made Biologics: Case Studies in Therapeutic and Industrial Enzymes. BioMed Res Int 2014;2014:256135.
[24] Billenkamp N. Cultivar description Burley] Sortenbeschreibung Burley 2010. Karlsruhe, Germany: Agricultural Technology Centre Augustenberg; 2010.
[25] Billenkamp N. Cultivar description Virgin] Sortenbeschreibung Virgin 2010. Karlsruhe, Germany: Agricultural Technology Centre Augustenberg; 2010.
[26] Billenkamp N. Guidepost for tobacco growing] Wegweiser durch die Tabakanzucht 2013. Germany: Agricultural Technology Centre Augustenberg; 2013.
[27] van Beilen JB, Möller R, Toonen M, Salentijn E, Clayton D. Industrial crop platforms for the production of chemicals and biopolymers. Berks: CPL Press; 2007.
[28] Long RC. Edible tobacco protein. Crops Soils Mag 1984;36:13–5.
[29] Wildman SG. Tobacco, a potential food crop. Crops Soil Mag 1979;31:7–9.
[30] Hausmann L, Töpfer R. [Development of plasmid vectors]. Entwicklung von Plasmid-Vektoren. In: Brauer D, Röbbelen G, Töpfer R, editors. [Lectures for plant breeding: tailored bioengineering of canola cultivars]. *Vorträge für Pflanzenzüchtung: Bioengineering für Rapssorten nach Maß*. Quedlinburg: Germany; 1999. 153–71.
[31] Hehl R, Kloos D, Stahl DJ. [Tissue-specific promoters from sugar beet]. *Gewebespezifische Promotoren aus der Zuckerrübe*. Patent WO 2002040687 A9. 2003.
[32] Wohlleben W, Arnold W, Broer I, Hillemann D, Strauch E, Punier A. Nucleotide sequence of the phosphinothricin N-acetyltransferase gene from *Streptomyces viridochromogenes* Tü494 and its expression in *Nicotiana tabacum*. Gene 1988;70:25–37.
[33] Neubauer K, Hühns M, Hausmann T, Klemke F, Lockau W, Kahmann U, et al. Isolation of cyanophycin from tobacco and potato plants with constitutive plastidic cphATe gene expression. J Biotechnol 2012;158:50–8.
[34] Nausch H, Mikschofsky H, Koslowski R, Meyer U, Broer I, Huckauf J. High-level transient expression of ER-targeted human interleukin 6 in *Nicotiana benthamiana*. PLoS One 2012;7:e48938.
[35] Hoedtke S, Zeyner A. Comparative evaluation of laboratory-scale silages using standard glass jar silages or vacuum-packed model silages. J Sci Food Agric 2011;91:841–9.
[36] Weißbach F, Strubelt C. [Correction of the dry matter content of grass silage as substrate for biogas plants] Die Korrektur des Trockensubstanzgehaltes von Grassilagen als Substrat für Biogasanlagen. Agric Technol Landtechnik 2008;63:210–1.
[37] de Jaeger GD, Scheffer S, Jacobs A, Zambre M, Zobell O, Goossens A, et al. Boosting heterologous protein production in transgenic dicotyledonous seeds using Phaseolus vulgaris regulatory sequences. Nat Biotechnol 2002;20:1265–8.
[38] Malik MR, Yang W, Patterson N, Tang J, Wellinghoff RL, Preuss ML, et al. Production of high levels of poly-3-hydroxybutyrate in plastids of Camelina sativa seeds. Plant Biotechnol J 2015;13:675–88.
[39] Mikschofsky H, Schirrmeier H, Keil GM, Lange B, Polowick PL, Keller W, Broer I. Pea-derived vaccines demonstrate high immunogenicity and protection in rabbits against rabbit haemorrhagic disease virus. Plant Biotechnol J 2009;7:537–49.
[40] Boothe J, Nykiforuk C, Shen Y, Zaplachinski S, Szarka S, Kuhlman P, et al. Seed-based expression systems for plant molecular farming. Plant Biotechnol J 2010;8:588–606.
[41] Kermode AR. Seed expression systems for molecular farming. In: Wang A, Ma S, editors. Molecular Farming in Plants: Recent Advances and Future Prospects. Germany: Heidelberg; 2012. p. 89–123.
[42] Gelvin SB. Agrobacterium-mediated plant transformation: the biology behind the gene-jockeying tool. Microbiol Mol Biol Rev 2003;67:16–37.

conducted in (A) 2010 and (B) 2011. Asterisk: extreme value, identified by SPSS analysis; Circle: outlier, identified by SPSS analysis; (C) Coomassie-stained SDS-PAGE of HCl-extracts from leave samples of 2011. M: PageRuler Plus Prestained Protein Ladder Mix (ThermoScientfic; Darmstadt/Germany): P: 5 μg of purified CP.

[43] Nester EW. Agrobacterium: nature's genetic engineer. Front Plant Sci 2015;5.
[44] Conley AJ, Zhu H, Le LC, Jevnikar AM, Lee BH, Brandle JE, et al. Recombinant protein production in a variety of Nicotiana hosts: a comparative analysis. Plant Biotechnol J 2011;9:434–44.
[45] McDonald P, Henderson AR, Heron SJE. The biochemistry of silage. 2nd ed. UK: Kingston; 1991.
[46] Weißbach F, Schmidt L, Hein E. Method for anticipation of the run of fermentation silage making, based on the chemical composition of green fodder. in: Iglovikov VG, Movsisiants AP, editors. *Proceedings of the XIIth international grassland congress*. (Jun 11–20 1974, Moscow, Russia): 1974; 663–73.
[47] Savoi P, Jofriet JC. Silage storage silage science and technology. Agronomy 2003;42:405–67.
[48] Filya I. The effect of lactobacillus buchneri and lactobacillus plantarum on the fermentation, aerobic stability, and ruminal degradability of low dry matter corn and sorghum silages. J Dairy Sci 2003;86:3575–81.
[49] Weinberg ZG, Szakacs G, Ashbell G, Hen Y. The effect of Lactobacillus buchneri and L. plantarum, applied at ensiling, on the ensiling fermentation and aerobic stability of wheat and sorghum silages. J Ind Microbiol Biotechnol 1999;23:218–22.

Supporting Information to B1:

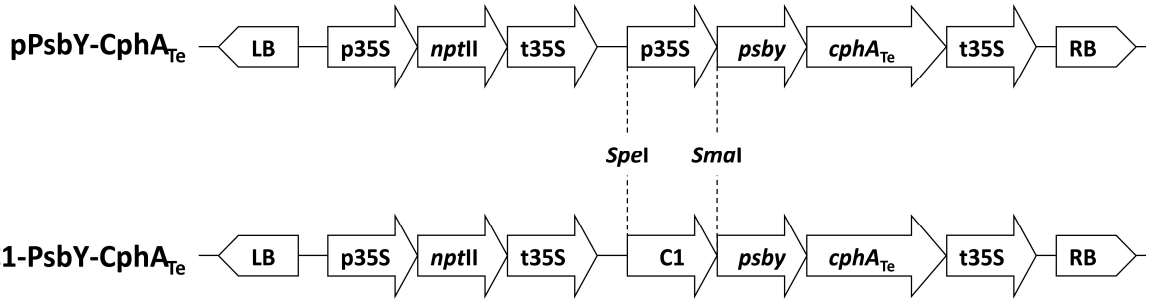


Fig B1-S 1 a: Map of the nuclear transformation vectors for constitutive and leaf-specific, plastidic expression of the cyanophycin synthetase. *cphA*$_{Te}$: cyanobacterial coding region of the CP-synthetase from *T. elongatus* BP-1, *psby*: peptide of the integral protein of photosystem II, p35S: constitutive cauliflower mosaic virus (CaMV) 35S promoter; t35S: CaMV terminator, C1: leaf-specific C1 promoter from *Beta vulgaris*, *npt*II: coding region of neomycin phosphotransferase gene, LB and RB, left and right borders of *A. tumefaciens* binary vector; *Spe*I and *Sma*I: cleavage sites for restriction enzymes

Table B1-S 1 b : Regeneration frequencies after transformation of the *N. tabacum* cultivars.

N. tabacum cultivar	construct	shoots/explant		
		LBA4404	AGL1	C58C1
PH	pLH9000	1.05 (± 0.32)	1.43 (± 0.41)	1.54 (± 0.35)
	pPsbY-cphA$_{Te}$	0.75 (± 0.35)	n.d.	n.d.
	pC1-PsbY-cphA$_{Te}$	0.54(± 0.26)	1.79(± 0.29)	n.d.
BG	pLH9000	0.88(± 0.35)	1.34(± 0.36)	1.35(± 0.35)
	pPsbY-cphA$_{Te}$	0.80(± 0.38)	1.73(± 0.35)	1.34(± 0.27)
	pC1-PsbY-cphA$_{Te}$	1.07(± 0.37)	n.d.	n.d.
VG	pLH9000	0	1.50(± 0.35)	1.44 (± 0.30)
	pPsbY-cphA$_{Te}$	0	1.52(± 0.37)	1.55(± 0.25)
	pC1-PsbY-cphA$_{Te}$	0	1.43(± 0.33)	n.d.

PH: *N. tabacum* cv. Petit Havana SR1; BG: *N. tabacum* cv. Badischer Geudertheimer; VG: *N. tabacum* cv. Virginia Golta; LBA4404, AGL1 and C58C1: *A. tumefaciens* strains; CphA$_{Te}$: cyanophycin synthetase from *T. elongatus BP-1*; pPsbY-CphA$_{Te}$: constitutive plastidic expression of CphA$_{Te}$; pC1-PsbY-CphA$_{Te}$: leaf-specifc plastidic expression of CphA$_{Te}$; pLH9000: empty vector; n.d. = not done. Shoots were counted at 6 weeks in 3 independent transformations. Numbers: mean of at least three independent transformations, standard deviation in parentheses.

Table B1-S 2: Correlation between fresh and dry weight of leaves of the *N. tabacum* cultivars PH, BG and VG, harvested after 6 and 12 weeks of cultivation in the greenhouse (n = 6).

	6 weeks			12 weeks		
	DW [g]	stdev	DW / FW [%]	DW [g]	stdev	DW / FW [%]
PH	26.03	6.80	14.22	23.25	1.83	12.04
BG	39.91	6.05	12.37	52.37	5.20	14.36
VG	44.37	4.99	14.06	83.63	8.35	17.26

Stdev: standard deviation, FW: fresh weight,

Table B1-S 3: Mean and highest CP content in *N. tabacum* hybrids of BG and VG crossed with the PH elite event 51-3-2

CP content [mg CP / g DW]							
	51-3-2	BG			VG		
		NIC x 51-3-2	51-3-2 x NIC	51-3-2 x NIC x NIC	NIC x 51-3-2	51-3-2 x NIC	51-3-2 x NIC x NIC
mean	53.08	49.70	67.98	55.05	32.65	50.61	50.13
stdev	3.86	11.97	13.38	13.69	7.45	7.45	22.11
max	58.49	75.94	94.40	72.34	39.30	67.24	80.98
no. of events	4	15	24	10	3	24	9

Table B1-S 4: Relative variation coefficientof the seven *N. tabacum* hybrids of BG and VG, crossed with the PH elite event 51-3-2, with the highest CP content

plants / crossing	generation	mean CP [mg CP / g DW]	stdev	n	variance	variation coefficient	relative variation coefficient
51-3-2	clones	53.08	3.86	4	14.87	0.07	3.63
BG NIC x 51-3-2	F1	59.51	8.13	7	66.05	0.14	5.16
53-3-2 x BG NIC	F1	85.92	4.40	7	19.33	0.05	1.93
53-3-2 x BG NIC x BG NIC	F2	62.82	4.77	7	22.79	0.08	2.87
VG NIC X 51-3-2	F1	32.65	7.45	3	55.57	0.23	13.18
51-3-2 x VG NIC	F1	58.87	4.10	7	16.82	0.07	2.63
51-3-2 x VG NIC x VG NIC	F2	57.49	18.89	7	356.93	0.33	12.42

Table B1-S 5 Mean CP-content in T0 transformants of of the *N. tabacum* cultivars PH, BG and VG, expressing PsbY-CphA$_{Te}$ either under the control of the 35S or the leaf-specific C1-promoter, measured in vitro and in the greenhouse (6 and 12 weeks), respectively

		CP [mg / g DW]							
		35S				C1			
		mean	stdev	highest	n	mean	stdev	highest	n
PH	*in vitro*					20,79	14.8	40.35	6
BG	*in vitro*	34.11	21.31	85.60	44	68.58	13.52	100.15	17
	6 weeks	11.62	7.36	31.39	26	14.06	12.61	42.25	13
	12 weeks	27.78	21.14	88.45	26	21.83	16.23	58.00	17
VG	*in vitro*	39.76	26.44	86.59	30	33.05	26.54	76.57	11
	6 weeks	16.22	1.67	17.38	3	8.68	5.28	12.84	3
	12 weeks	32.50	15.45	66.26	25	9.50	11.17	38.56	11

Table B1-S 6: Relative variation coefficient of the seven T0 eventsof the *N. tabacum* cultivars BG and VG, containing pPsbY-CphA$_{Te}$, with the highest CP content *in vitro*

	n	mean CP [mg / g DW]	stdev	variance	variation coefficient	Relative Variation Coefficient
BG	7	70.99	9.88	97.69	0.14	5.26
VG	7	69.22	9.14	83.55	0.13	4.99

Table B1-S 7: Relative variation coefficient of the seven T0 eventsof the *N. tabacum* cultivars PH, BG and VG, containing pC1-PsbY-CphA$_{Te}$, with the highest CP content *in vitro*

	n	mean CP [mg / g DW]	stdev	variance	variation coefficient	Relative Variation Coefficient
PH	6	20.79	14.80	219.04	0.71	29.07
BG	7	81.49	9.54	91.05	0.12	4.43
VG	7	49.67	16.47	271.34	0.33	12.53

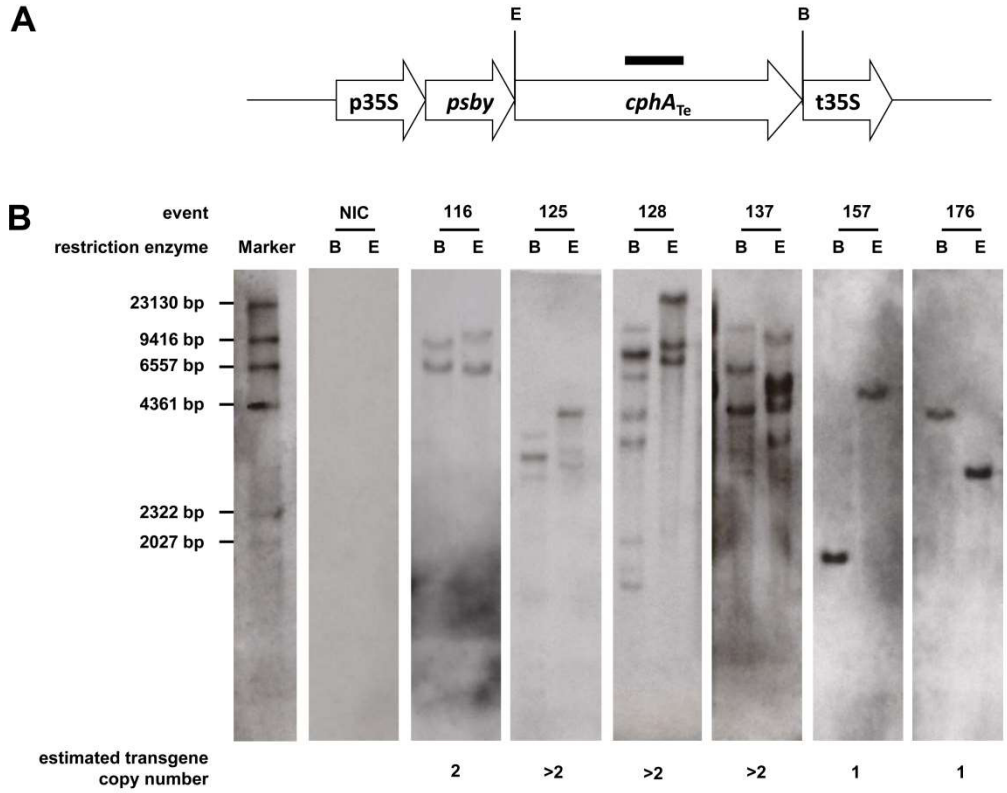


Fig B1-S 8: Southern blot analysis of BG T0 events. (A) transgene: black bar: *cph*A$_{Te}$ hybridization probe; B / E: restriction enzymes sites of *Bcl*I and *Eco*RI; *cphA*$_{Te}$: cyanobacterial coding region of the CP-synthetase from *T. elongatus* BP-1, *psby*: peptide of the integral protein of photosystem II, p35S: constitutive cauliflower mosaic virus (CaMV) 35S promoter; t35S: CaMV terminator. (B) Chromosomal DNA digested with *Bcl*I and *Eco*RI and hybridized with the *cph*A$_{Te}$ probe. Marker: DIG-labeled DNA Molecular Weight Marker II (Roche); NIC: near-isogenic control

Table B1-S 9: Mean and highest CP content in T0 transformants of the *N. tabacum* cultivar BG, containing pPsbY-CphA$_{Te}$, used for generation of the T1 generation

N. tabacum cultivar	T0		T1				
	event	CP [mg / g DW]	mean CP [mg / g DW]	stdev	n	max CP [mg / g DW]	Event
BG	116	22.93	15.14	7.59	14	28.36	116-30
	125	10.72	10.86	7.67	15	22.76	125-27
	128	25.05	18.45	8.30	16	35.95	128-28
	137	25.05	20.62	8.32	15	33.51	137-5
	157	11.64	17.47	7.44	15	29.49	157-10
	176	16.66	20.71	7.91	15	32.19	176-1
BG [mean]		17.82 (± 6.97)	17.24	8.40	90	35.95	128-28

Table B1-S 10 Relative variation coefficient of CP content in T0 transformants of the *N. tabacum* cultivar BG, containing pPsbY-CphA$_{Te}$, used for generation of the T1 generation

N. tabacum cultivar	generation	n	mean CP [mg / g DW]	stdev	variance	variation coefficient	Relative Variation Coefficient
BG	T0	6	17.82	6.97	48.62	0.39	15.97
	T1	88	17.38	8.44	71.25	0.49	5.18

Table B1-S 11: Relative variation coefficient of the CP content of clones and siblings of the *N. tabacum* cultivarBG.

			n	mean CP [mg / g DW]	stdev	variance	variation coefficient	Relative Variation Coefficient
BG	4	clones	4	23.83	6.18	38.19	0.26	12.97
	116	siblings	14	15.14	7.59	57.62	0.50	13.40
	128	siblings	16	18.45	8.30	68.95	0.45	11.25
	125	siblings	15	10.86	7.67	58.90	0.71	18.25
	137	siblings	15	20.62	8.32	69.22	0.40	10.42
	157	siblings	15	17.47	7.44	55.33	0.43	10.99
	176	siblings	15	20.71	7.91	62.64	0.38	9.87
	mean	siblings	90	21.24	8.40	34.64	0.25	11.50 (± 2.48)
	mean	lines	6	23.98	5.87	35.08	0.25	12.36 (±3.13)

Table B1-S 12: Relative variation coefficient of the CP content of clones and seven siblings with the highest CP content of lines of the *N. tabacum* cultivar BG.

			n	mean CP [mg / g DW]	stdev	variance	variation coefficient	Relative Variation Coefficient
BG	4	clones	4	23.83	6.18	38.19	0.26	12.97
	116	siblings	7	20.70	6.89	47.48	0.33	12.58
	128	siblings	7	26.00	7.02	49.24	0.27	10.20
	125	siblings	7	17.51	5.28	27.91	0.30	11.41
	137	siblings	7	27.56	5.42	29.40	0.20	7.44
	157	siblings	7	24.28	4.89	23.91	0.20	7.61
	176	siblings	7	27.97	5.43	29.46	0.19	7.34
	mean	siblings	42	21.11	8.44	31.14	0.25	9.49 (± 2.11)
	mean	lines	6	23.82	8.39	32.82	0.24	9.06 (± 2.13)

Chapter B1 and the supporting information was reprinted from Publication: New Biotechnology, 33, Henrik Nausch, Tina Hausmann, **Daniel Ponndorf**, Maja Hühns, Sandra Hoedtke, Petra Wolf, Annette Zeyner, Inge Broer 842-851, © 2016 Published by Elsevier B.V., with permission from Elsevier.

Additional results to B1:

Additional to the presented results and supporting information the total Arg and Asp content was measured in BG and VG plants to determine whether CGP production in plants increases the pool of both AA. CGP production leads to an increase in Arg content of about 4 times compared to the control, while the Asp pool nearly was doubled. In BG the Arg pool increased from 0.4 % DW in the control to 1.71 % DW, while Asp was increased from 1.06 to 2.05 % DW. This was comparable for VG where Arg was increased from 0.36 to 1.29 % DW and Asp from 0.78 to 1.59 % DW.

These experiments were conducted by S. Hoedtke of the department of Nutrition Physiology and Animal Nutrition of the University of Rostock and presented by Nausch (2016).

B2: Stable production of cyanophycinase in *Nicotiana benthamiana* and its functionality to hydrolyze cyanophycin in the murine intestine.

Daniel Ponndorf, Sven Ehmke, Benjamin Walliser, Kerstin Thoss, Christoph Unger, Solvig Görs, Gürbüz Daş, Cornelia C. Metges, Inge Broer, Henrik Nausch (2017): Stable production of cyanophycinase in Nicotiana benthamiana and its functionality to hydrolyse cyanophycin in the murine intestine. Plant Biotechnology Journal 15-5, 605-613

Author's contribution: I designed all of the used constructs, except the empty infiltration vectors and *gfp*-*cph*B. I cloned the constructs *cph*B-b, *cph*B-s, *cph*B-sA and *cph*B-s-c and performed Western blot analysis of infiltrated plants together with S.E. except for *gfp*-*cph*B. Northern blot analysis was conducted by K.T. according to my instructions. I prepared the material for the production of anti-CPHB antibodies as well as for the feeding studies in mice and assisted during both experiments and interpreted the plant experiments and wrote the plant sections of the article.

Plant Biotechnology Journal

Plant Biotechnology Journal (2017) **15**, pp. 605–613 doi: 10.1111/pbi.12658

Stable production of cyanophycinase in *Nicotiana benthamiana* and its functionality to hydrolyse cyanophycin in the murine intestine

Daniel Ponndorf[1], Sven Ehmke[1,†], Benjamin Walliser[1], Kerstin Thoss[1], Christoph Unger[1], Solvig Görs[2], Gürbüz Daş[2], Cornelia C. Metges[2,*], Inge Broer[1] and Henrik Nausch[1]

[1]*Faculty of Agricultural and Environmental Sciences, Department of Agrobiotechnology and Risk Assessment for Bio- and Gene Technology, University of Rostock, Rostock, Germany*

[2]*Leibniz Institute for Farm Animal Biology (FBN), Institute of Nutritional Physiology 'Oskar Kellner', Dummerstorf, Germany*

Received 8 July 2016;
revised 5 October 2016;
accepted 30 October 2016.
**Correspondence* (Tel +49-38208-68650; fax +49-38208-68-693; email metges@fbn-dummerstorf.de)
† Present Address: Paraxel International GmbH, Klinikum am Westend, Haus 18, SpandauerDamm 130, 14050, Berlin, Germany.

Keywords: arginine, cyanophycin, cyanophycinase, dipeptide, digestion, *Nicotiana benthamiana*, protein stability.

Summary

Food supplementation with the conditionally essential amino acid arginine (Arg) has been shown to have nutritional benefits. Degradation of cyanophycin (CGP), a peptide polymer used for nitrogen storage by cyanobacteria, requires cyanophycinase (CGPase) and results in the release of β-aspartic acid (Asp)-Arg dipeptides. The simultaneous production of CGP and CGPase in plants could be a convenient source of Arg dipeptides. Different variants of the *cph*B coding region from *Thermosynechococcus elongatus* BP-1 were transiently expressed in *Nicotiana benthamiana* plants. Translation and enzyme stability were optimized to produce high amounts of active CGPase. Protein stability was increased by the translational fusion of CGPase to the green fluorescent protein (GFP) or to the transit peptide of the small subunit of RuBisCO for peptide production in the chloroplasts. Studies in mice showed that plant-expressed CGP fed in combination with plant-made CGPase was hydrolysed in the intestine, and high levels of ß-Asp-Arg dipeptides were found in plasma, demonstrating dipeptide absorption. However, the lack of an increase in Asp and Arg or its metabolite ornithine in plasma suggests that Arg from CGP was not bioavailable in this mouse group. Intestinal degradation of CGP by CGPase led to low intestinal CGP content 4 h after consumption, but after ingestion of CGP alone, high CGP concentrations remained in the large intestine; this indicated that intact CGP was transported from the small to the large intestine and that CGP was resistant to colonic microbes.

Introduction

Arginine (Arg) is an indispensable amino acid (AA) for young mammals and birds (Wu *et al.*, 2004, 2009). In addition to its function as a building block of proteins, Arg plays important roles in regulating gene expression, cell signalling, vascular development, reproduction and immunity (Bazer *et al.*, 2012; Wang *et al.*, 2012; Wu, 2014). Furthermore, Arg has nutritional benefits for athletes and the elderly or immune-compromised patients, but because its concentration is relatively low in food proteins, it has been used as a supplement for therapy and as an additive in food (Sallam and Steinbuchel, 2010). Supplemental free Arg is commonly produced by fermentation (Utagawa, 2004). The oral application of Arg-containing dipeptides may increase the uptake of Arg in the small intestine compared to Arg monomers (Matthews and Adibi, 1976; Wenzel *et al.*, 2001). Currently, dipeptides are synthesized by enzymatic, chemical and combined methods (Yagasaki and Hashimoto, 2008). Overexpression of the polypeptide cyanophycin (CGP) followed by cyanophycinase (CGPase)-mediated degradation results in β-aspartic acid (Asp)-Arg dipeptides, which could produce Arg (Sallam and Steinbuchel, 2009b, 2010; Sallam *et al.*, 2009). Cyanophycin is synthesized by cyanobacteria and several nonphotosynthetic bacteria via nonribosomal biosynthesis by the enzyme cyanophycin synthetase (CPHA) (Allen *et al.*, 1984; Simon, 1987; Simon and Weathers, 1976; Ziegler *et al.*, 1998, 2002) and consists of an L-Asp backbone linked to L-Arg residues (Simon and Weathers, 1976). The expression of the CPHA-encoding gene from *Thermosynechococcus elongatus* BP-1 enables high and stable accumulation of CGP in tobacco and potato plastids (Hühns *et al.*, 2008, 2009; Neumann *et al.*, 2005). However, to the best of our knowledge, there are no reports on feeding CGP to animals and assessing its potential to produce Arg. CGP is highly stable and resistant to proteases (Simon and Weathers, 1976), except CGPase (Gupta and Carr, 1981). The cyanophycinase CPHB was described in cell extracts of *Anabaena 7120* (Gupta and Carr, 1981), *Aphanocapsa 6308* (Allen *et al.*, 1984) and *Synechocystis* sp. PCC 6803 (Richter *et al.*, 1999). Overexpression in *E.coli* and analysis of CPHB revealed that it is a serine (Ser)-type exopeptidase with a dimeric structure (Law *et al.*, 2009; Richter *et al.*, 1999), and its binding is highly specific for β-linked aspartyl peptides. If CGP and CGPase can be co-expressed in food plants, β-Asp-Arg dipeptides could become a source of dietary Arg. The degradation of CGP resulting in the release of β-Asp-Arg dipeptides might be achieved following two strategies: (i) accumulation of CGP in the plastid via separation of CGP (plastid) and CGPase (cytosol) leading to dipeptide formation after extraction when both components are joined and (ii) accumulation of the dipeptides in plastids by targeting cyanophycin synthetase (CPHA) and cyanophycinase (CPHB) to the chloroplast allowing dipeptide production during plant growth.

We used a transient expression system in *N. benthamiana* to determine whether plants can produce an active and stable form of CGPase that degrades CGP and whether the enzyme can be translocated to the chloroplast. In the second step, we formulated food pellets containing both, plant-made CGP and plant-made CGPase to investigate whether CGP is hydrolysed by CGPase and whether Arg from CGP is bioavailable in a mouse model.

Results

Cytosolic production and stabilization of CGPase in plants

A 5' truncated coding region of the *cph*$B_{tlr2169}$ gene (*cph*B-b) (Prof. Dr. Wolfgang Lockau (W.L.) Humbold Univesity Berlin) and two codon-optimized versions, *cph*B-s, designed to improve the efficiency of translation (Perlak *et al.*, 1991; Sharp and Li, 1987) and *cph*B-sA, where the sequence GCT TCCTCC encoding for Alanin (Ala)-Ser-Ser (Fig. 1), was added to improve efficiency of translation and protein stability (Sawant *et al.*, 2001) were transiently expressed in *N. benthamiana*. Total soluble protein (TSP) was assessed for the presence of CGPase using Western blot analysis (Fig. 2a). Infiltration with *cph*B-b did not result in detection of CGPase in 50 μg TSP, but faint, not always reproducible, signals were visible at the expected size of approximately 29 kDa in 100 μg TSP. Expression of *cph*B-s and analysis of 50 μg TSP showed faint CGPase signals, while expression of *cph*B-sA resulted in more pronounced signals. Bands were detected not only at 29 kDa but also at 60, 130 and 200 kDa. These bands were not observed in the empty vector control and their size corresponds to potential di- and trimers and higher aggregates of CPHB-SA. Additionally, we were not able to detect CGPase without the addition of protease inhibitors, suggesting its instability in the crude plant extract. Greater stability of recombinant proteins can be achieved by fusion of the protein to a stable fusion partner at the *N*- or *C*-terminus. One promising fusion partner is GFP which was previously used successfully to improve protein stability (Piron *et al.*, 2014). Because our constructs carry a 6*Histag at the *C*-terminus for enzyme purification, we used *N*-terminal fusions. The fusion of *cph*B-s to the green fluorescent protein (GFP) coding region, resulting in *gfp-cph*B-s, led to an increase in protein yield as shown in Figure 2b. An additional band was visible at approximately 100 kDa, corresponding to the calculated size of a GFP::CPHB-S dimer.

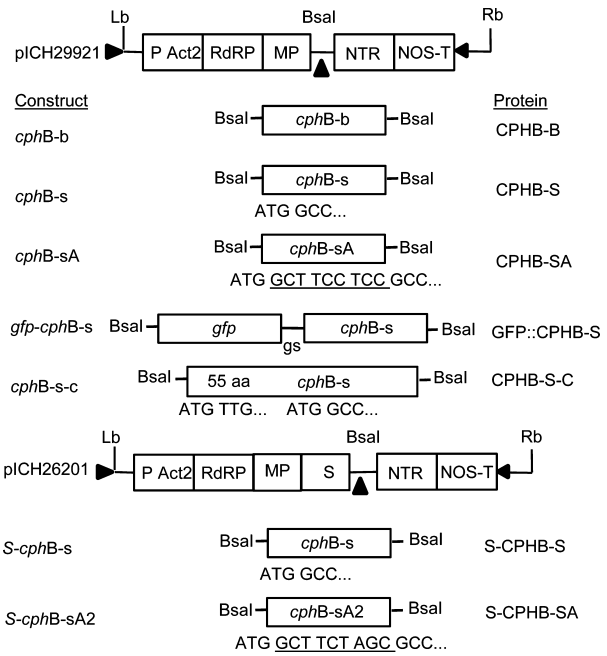


Figure 1 Plasmid constructs and corresponding labels of the respective protein variants: TMV-based viral vectors (Marillonnet *et al.*, 2005): pICH29912: empty vector, cphB-b: bacterial coding region of the cphB gene, cphB-s: cphB coding region adapted to the codon usage of *N. benthamiana*, cphB-sA: cphB-s with the addition of the amino acids Ala-Ser-Ser (A) (underlined sequence), gfp-cphB-s: fusion of gfp (sequence of the green fluorescent protein from pICH18711 (Marillonnet *et al.*, 2005)) and cphB-s, gs: linker, cphB-s-c: complete codon-optimized coding region of cphB as described in the database; pICH26201: containing a consensus sequence of the transit peptide of small subunit of RuBisCO (S) from dicotyl plants (Klimyuk *et al.*, 2004), cphB-sA2: cphB-s with the addition of the altered sequence of amino acids Ala-Ser-Ser (underlined sequence). LB and RB: left and right T-DNA borders; P Act2: Arabidopsis actin 2 promoter; RdRP: RNA-dependent RNA polymerase; MP: movement protein; NTR: 3' untranslated region of TMV; NOS-T: nos terminator.

Because *N*-terminal fusions stabilized the protein, we analysed whether the complete CGPase protein (CPHB-S-C) described in the database is more stable compared to the truncated version. Therefore, we used a codon-optimized version of the complete sequence. After infiltration with *cph*B-s-c, three bands were detected in 50 μg TSP at approximately 27, 29 and 35 kDa. The 35-kDa protein corresponds to the calculated size of the complete protein. The smaller bands are potential degradation products, while the band at 29 kDa is the size of the truncated protein.

Subcellular targeting of CPHB to the chloroplast

To determine whether CPHB-S and CPHB-SA can be targeted to the chloroplast *cph*B-s was fused to the plastid leader peptide of the small subunit of RuBisCO (S) (Klimyuk *et al.*, 2004) resulting in S-*cph*B-s. Due to sequence incompatibility between S and the sequence GCT TCC TCC of Ala-Ser-Ser (A) in *cph*B-sA, it was necessary to adapt the sequence to GCC ATT GGA (A2) prior to the fusion to S, resulting in S-*cph*B-sA2. Western blot analysis of 25 μg TSP showed that S-*cph*B-s produced substantially more CGPase than S-*cph*B-sA2 (Figs 1 and 2b) and also showed a higher yield compared to *gpf-cph*B-s (Fig. 2b). To determine a possible effect of A2 on protein folding, we conducted *in silico* analysis of S-CPHB-S and S-CPHB-A2 and found different potential α-helices between AA 4-25, caused by the integration of Ala-Ser-Ser (Fig. S1). This might have an effect on protein stability or folding of the transit peptide. In addition to the expected band at 29 kDa, a band at approximately 35 kDa was detected, corresponding to the unprocessed proteins (Fig. 2b) for S-*cph*B-s and S-*cph*B-sA2. The same bands were present in isolated chloroplasts (data not shown).

Putative S-CPHB-S dimers of 70 kDa were also visible when analysing higher protein concentrations (data not shown). Expression of S-*chp*B-s led to the greatest amount of enzyme detected compared to all other constructs. To determine whether differences between the constructs might be caused by different RNA patterns, we conducted Northern blot analysis.

RNA analysis of different CGPase variants

Northern blot assays were performed to compare the RNA steady-state levels of the different constructs (Fig. 3a). For all

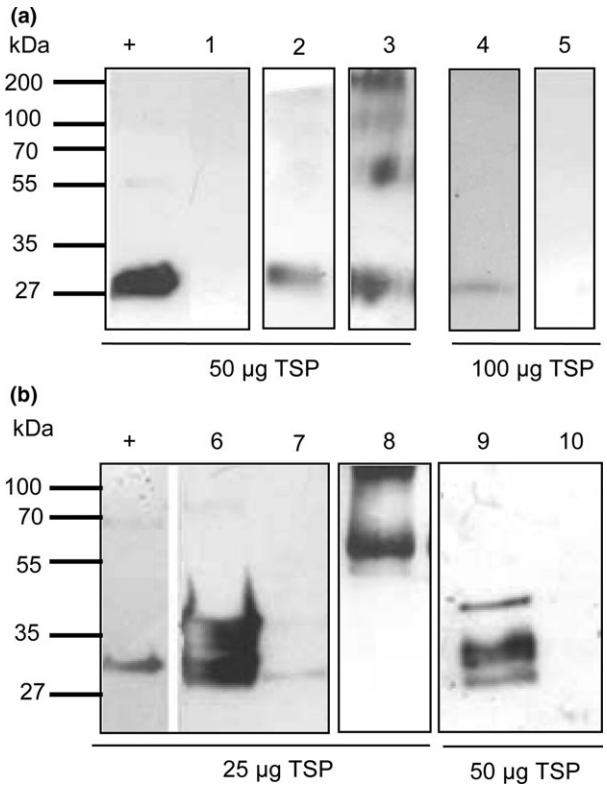
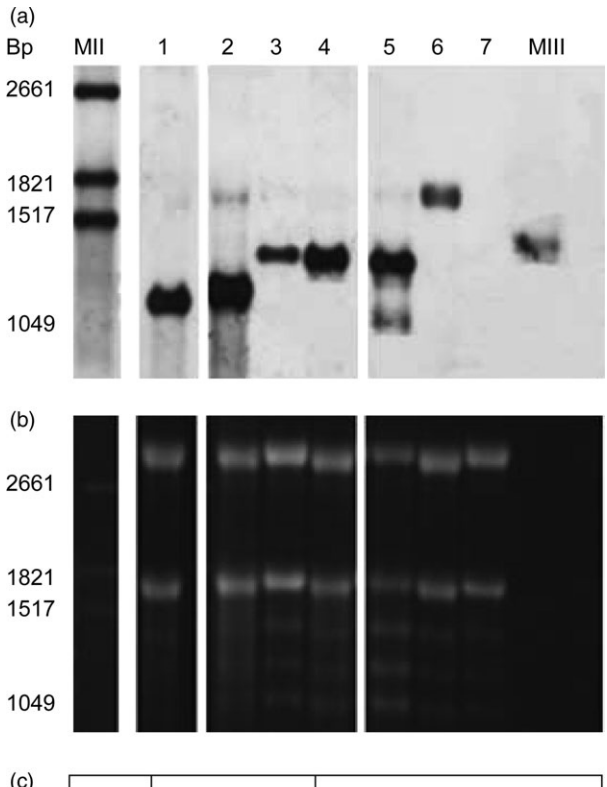


Figure 2 (a) Western blot analysis of 50 and 100 µg total soluble protein isolated from *N. benthamiana* leaves harvested 7 days post infiltration from one plant (dpi): + = Cyanophycinase-positive control isolated from *E. coli*; 1: CPHB-B; 2: CPHB-S; 3:CPHB-SA, 4: CPHB-B; 5: pICH29912 = empty vector control. b: 25 and 50 µg TSP harvested 7 dpi from one plant:+ = Cyanophycinase-positive control isolated from *E. coli* 6:S-CPHB-S, 7:S-CPHB-SA2; 8: GFP::CPHB-S; 9: CPHB-S-C; 10: pICH29912 = empty vector control. Plants were harvested 7 dpi.



(c)

Lane	Construct	Approximate RNA size
1	*cph*B-s	1100
2	*cph*B-sA	1100
3	S-*cpH*B-s	1300
4	S-*cph*B-sA2	1300
5	*cph*B-s-c	1300
6	*gfp-cph*B-s	1800

Figure 3 Northern blot (a) and RNA gel loading control (b) of 3 µg RNA isolated from *N. benthamiana* leaves. All samples showed the approximate expected size bands (c) but different signal strength. The loading control (b) shows that samples were loaded equally. MII: RNA marker II with a base pair (bp) range of 1516-6948 bp, MIII: RNA marker with a bp range of 310-1517 bp. 1: *cph*B-s, 2: *cph*B-sA, 3: S-*cph*B-s, 4: S-*cph*B-sA2, 5: *cph*B-s-c, 6: gfp-*cph*B-s, 7: empty vector control.

constructs, RNAs corresponding to the calculated size (Fig. 3c) were observed. For *cph*B-sA, an additional band at approximately 1700 bp was detected. This fragment was weakly detected for *cph*B-s-c, which also had a third band at approximately 1.000 bp. While the loading control showed equal amounts of total RNA (Fig. 3b), the strongest signal was observed with *cph*B-sA. *Cph*B-s, S-*cph*B-sA2, *cph*B-s-c and *gpf-cph*B-s had similar signals. The weakest signals were found for S-*cph*B-s. This indicates a possible positive influence of A on transcript stability.

Activity of *N*-terminal-modified CGPase in crude plant extracts

The activity of the plant-produced, modified enzymes was determined by adding 100 or 200 µg purified CGP to 600 µg TSP isolated from *N. benthamiana* plants, which were infiltrated with the respective vectors. One reaction was stopped immediately (T0), while the other sample was incubated at room temperature (RT) overnight (T1). Both CGP samples were degraded with proteins from plants infiltrated with S-*cph*B-s (Fig. 4:1a) and *gfp-cph*B-s (Fig. 4:2a). Extracts from plants infiltrated with *cph*B-s-c (Fig. 4:1b) led to a nearly complete substrate reduction in the 100 µg CGP sample, while the 200 µg sample of CGP was only partially reduced. No degradation was found after incubation in plant material infiltrated with the GFP-expressing control vector pICH18711 (Marillonnet *et al.*, 2005) (Fig. 4:2b) or with all other constructs used in this work (not shown).

Feeding CGP- and CGPase-containing pellets to mice results in the absorption of ß-Asp-Arg dipeptides

Feeding studies were performed using plant-made CGP and plant-made CGPase (S-CPHB-S) to investigate the activity of CGPase in the intestine and the bioavailability of the CGP constituent Arg and Asp. The mice were fed protein-free pellets supplemented with CGP, CGP+CGPase, Asp+Arg or none of these (CON). The pellet mass ingested was comparable between groups ($P > 0.6$) (Table S1). Intake of CGP was similar in mice fed pellets containing CGP or CGP+CGPase (Table S1). Intakes of Arg and Asp in the CGP, CGP+CGPase and Asp+Arg mouse groups were comparable, but intakes were zero in the CON group, as expected. Plasma Asp, ß-Asp-Arg, Arg and ornithine Orn

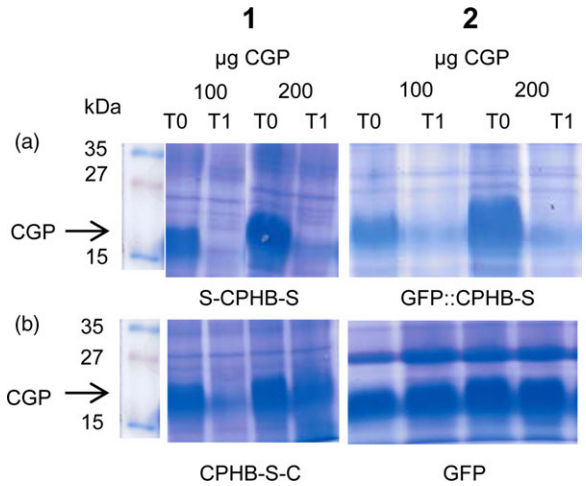


Figure 4 Enzyme activity test in 600 µg TSP crude plant extract. One hundred or 200 µg of cyanophycin was added, and the enzyme reaction was stopped by trichloroacetic acid (TCA) precipitation immediately (T0) or after 12h incubation at room temperature (T1). Total protein was precipitated with TCA and dissolved in 300 µL SDS sample buffer. Then, 30 µL of the sample was loaded on a 12% SDS gel and stained with Coomassie Brilliant Blue for 20 min; kDa: kilodalton; CGP: cyanophycin; 1a: S-CPHB-S, 2a: GFP:: CPHB-S, 1b: CPHB-S-C, 2b: GFP (green fluorescent protein expressed with vector pICH18711 (Marillonnet *et al.*, 2005)) was used as a control.

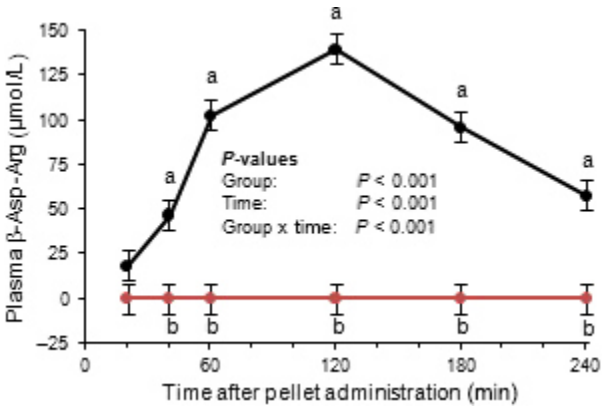


Figure 5 Course of ß-Asp-Arg concentrations in mouse plasma after administration of a pellet with cyanophycin only (red) and cyanophycin co-applied with cyanophycinase (black). LSMEANS ± SE (n = 6/group). Values with different letters (a, b) at the same time points differ between groups (Tukey, $P < 0.05$).

concentrations were affected by group, time after administration (with the exception of Orn), and group × time interaction (Table S1). In the plasma of the CGP+CGPase group, we found a relatively large peak, which was shown to be the ß-Asp-Arg dipeptide (Figs S2 and 5). This substance did not appear in the plasma of the CGP, Asp+Arg and CON mice, respectively (Fig. 5).

Arg from ß-Asp-Arg dipeptides is not bioavailable in mice

Free Arg concentrations in the plasma of Asp+Arg mice peaked 20–40 min after pellet intake and decreased thereafter to reach basal levels between 60 and 120 min ($P < 0.05$; Fig. 6a). In contrast, in the CON, CGP and CGP+CGPase groups, the courses of free plasma Arg, Asp (data not shown) and Orn, the product of Arg conversion, were similar and showed no increase (Fig. 6a,b). The course of plasma Orn concentrations in Asp+Arg mice followed the concentration curve of plasma Arg, although the maximal concentration of Orn was approximately twice that of Arg, and baseline was reached again at 180 min (Table S1; Fig. 6b). The pharmacokinetics of the plasma ß-Asp-Arg dipeptide in the CGP+CGPase group differed from those of the free plasma Arg in the group fed the pellets with free Asp and Arg (Figs 5, 6). The mean T_{max} and C_{max} of plasma Arg were 0.5 h and 172 µM, respectively, whereas plasma ß-Asp-Arg peaked with a T_{max} and C_{max} of 1.7 h and 224 µM, respectively ($P < 0.001$ and $P = 0.115$, for T_{max} and C_{max}, respectively). The plasma area under the curve (AUC) was greater for plasma ß-Asp-Arg than for free plasma Arg, 527 vs. 282 µM × h ($P = 0.003$), respectively, whereas plasma clearance (CL) for ß-Asp-Arg was lower than that for plasma Arg, 7.6 vs. 11.2 L/h ($P = 0.095$), respectively. Among the other proteinogenic AA, only plasma concentrations of glutamic acid (Glu), Ala, isoleucine (Ile) and lysine (Lys) showed a group effect ($P < 0.05$) with higher levels of Glu and Ala in the Asp+Arg group, and higher levels of Lys and Ile in the CON group which received the protein-free pellet (data not shown).

Group, intestinal location and group × location interaction affected the residual intestinal CGP content 4 h after pellet intake ($P < 0.05$). Co-administration of CGP+CGPase resulted in a low residual CGP content in the small and large intestine (0.8 and 1.3 µg/mg of dry matter (DM); $P > 0.1$), while in mice fed the pellets with CGP only, relatively high contents of residual CGP were detected in the large intestine (33.4 µg/mg of DM), with smaller amounts of CGP (8 µg/mg) in the small intestine ($P < 0.05$).

Discussion

CGPase production in plants

In the present work, we showed for the first time the expression of cyanophycinase in plants and its cyanophycin degrading activity in the gastrointestinal tract (GIT) of mice. We used a high-yield MagnICON® transient expression system (Marillonnet *et al.*, 2004) to determine whether plants can produce an active form of the enzyme cyanophycinase. In contrast to other researchers, who used similar vectors and described a high yield of recombinant protein of approximately 7% (Nausch *et al.*, 2012a), 10% (Webster *et al.*, 2009) and even 80% of the TSP (Gleba *et al.*, 2005), we observed low levels of detectable protein. Because the expected RNA patterns were detected, possible reasons for the low protein yield may be instability of the RNA, low efficiency of translation and protein instability (reviewed by Egelkrout *et al.* (2012) and Ullrich *et al.* (2015)). Improvements in RNA stability and translation can be achieved by adapting the coding region to the codon usage of plants (Barahimipour *et al.*, 2015; Perlak *et al.*, 1991; Sharp and Li, 1987) and integrating the sequence GCT TCC TCC, which encodes Ala-Ser-Ser (A), downstream of the initial start codon (Sawant *et al.*, 2001). Accordingly, both steps led to an increase in protein production, but the addition of protease inhibitors was necessary to detect the protein. As already assumed by Sawant *et al.* (2001) the results of this work indicate that the insertion of A led to an increased amount of RNA and also to an increase in protein prior to extraction, while the sensitivity to proteases was not changed.

In contrast to the aforementioned beneficial effect of the insertion of A, the combination of the RuBisCO transit peptide (S)

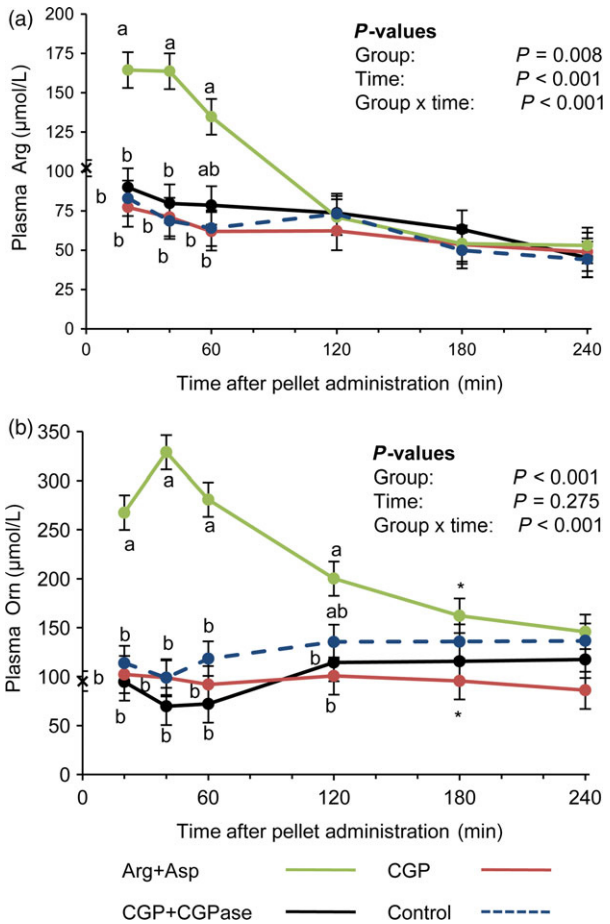


Figure 6 Course of Arg (a) and Orn (b) concentrations in mouse plasma after administration of a pellet with free Asp+Arg (green), cyanophycin only (red), cyanophycin co-ingested with cyanophycinase (black) and control pellet without supplement (dashed blue). LSMEANS ± SE (n = 6/group). Values with different letters (a, b) at the same time points differ between groups (Tukey, $P < 0.05$). *Values sharing the sign at the same time point tend to differ (Tukey, $P < 0.10$). x: The sign on the y-axis indicates the overall average basal plasma concentration across the four groups.

and A2 in the variant S-CPHB-A2 substantially reduced protein accumulation compared to S-CPHB-S and RNA amounts were not increased, indicating that transcription was not responsible for the difference in protein accumulation. Hence, it appears that the difference is caused by different translational efficiencies or different protein stability *in planta*. *In silico* analysis of the protein indicates an influence in the secondary structure of the transit peptide which might result in aberrant folding and protein degradation. In general, protein targeting to the chloroplast led to high enzyme production. The presence of high amounts of processed protein indicates the successful import of the protein to the chloroplast. In addition, this may indicate that the native protein is protected in the chloroplast because the same protein is unstable in the cytosol. One reason may be the absence of cytosol-specific proteases in the chloroplast, as previously described for recombinant proteins (Benchabane *et al.*, 2008; Pillay *et al.*, 2014).

In addition to the processed protein, the unprocessed 35 – kDa protein was detected in the chloroplast fraction. Gils *et al.* (2005), who used the same transit peptide with altered cleavage sites (valine-cysteine-Arg and proline-Ser-Arg instead of valine-glutamine-cysteine in our studies), made a similar observation and suggested that it was due to partially incorrect processing of the target protein. The unprocessed form may either be present in the cytosol or attached to the chloroplast membrane. Assuming that it is located in the cytosol, the *N*-terminal addition of the signal peptide may protect the protein from degradation as observed for the *N*-terminal addition of GFP or the *N*-terminal region of the complete protein.

While the fusion to GFP and S led to an increase in protein accumulation, it was less pronounced for the original *N*-terminus. The reduced stability of the complete protein was also indicated by the additional bands observed for CPHB-S-C, possibly representing degradation products. The *N*-terminal modification may result in a decreased sensitivity to proteases, as suggested for GFP (Moreau *et al.*, 2010; Piron *et al.*, 2014), ubiquitin (Hondred *et al.*, 1999; Jang *et al.*, 2012) and elastin-like proteins (Floss *et al.*, 2008; Patel *et al.*, 2007). Nevertheless, we cannot exclude the possibility that the increased levels of GFP::CPHB-S and S-CPHB-S are due to altered translational efficiency.

The increase in protein yield was mirrored by the activity of the protein. S-CPHB-S and GFP::CPHB-S, which had the highest levels of CGPase, showed similar activities. Both enzyme variants decreased the CGP content for both amounts of substrate tested. CPHB-S-C, which was produced at lower levels, led to almost complete degradation of 100 µg CGP but only slightly reduced 200 µg CGP. No activity was detected for all other constructs. This indicates that the degradation was independent of the *N*-terminal modifications but depended on the amount of protein. This is consistent with the observations that the fusion to GFP did not impair biological function of an antigen (Piron *et al.*, 2014) and phytochrome B (Yamaguchi *et al.*, 1999).

Absorption of CGP-derived ß-Asp-Arg dipeptides in mice

We report in this study for the first time that co-application of CGP and CGPase, both isolated from plants, results in the enzymatic breakdown of CGP into dipeptides in the murine GIT, as shown by the increase in β-Asp-Arg concentrations in plasma. Upon luminal cleavage of CGP into β-Asp-Arg dipeptides by CGPase in the GIT, ß-Asp-Arg is apparently absorbed by peptide transporters (Klang *et al.*, 2005; Rubio-Aliaga and Daniel, 2008). Although the T_{max} of β-Asp-Arg dipeptide occurred at 1.7 h after intake, increased plasma concentration of the constituent Arg or Asp was not detected. ß-Asp-Arg may be partly degraded by peptidases in the intestinal epithelium, liberating Asp and Arg. However, because Arg is degraded by arginase to form Orn, urea and, to a lesser degree, nitric oxide and polyamine in the GIT, detectable amounts of Arg do not enter the systemic circulation (Wu *et al.*, 2009). As we did not observe an increase in plasma Orn concentrations in the CGP+CGPase group, we conclude that Arg cannot be liberated from β-Asp-Arg dipeptides. This is likely because β-Asp-Arg dipeptides contain an unusual bond between the C1 amino group of Arg and the C4 carboxy group of Asp. This rare phenomenon was also described for β-Ala-(Met)-His and Gly-Gly (Matthews and Adibi, 1976). Hence, the lack of an Arg and Orn increase in the plasma indicates that mice do not possess a suitable peptidase to degrade ß-Asp-Arg dipeptides. It has been shown that isoaspartyl dipeptidases have different activities in different species (Hejazi *et al.*, 2002). Consequently, β-Asp-Arg accumulates and is eventually transferred to the blood. The increased plasma ß-Asp-Arg concentrations from 40 to 240 min

after pellet consumption in the CGP+CGPase group may be explained by two reasons: CGPase was stable at the pH in the small intestine and thus was active for 120 min after administration, after which plasma ß-Asp-Arg concentrations started to decline. Alternatively, upon entering the enterocytes, ß-Asp-Arg dipeptides accumulated but were only slowly released to the circulation and subsequently detoxified and excreted via urine, which we did not analyse.

Furthermore, the higher Glu and Ala plasma concentrations observed in the Asp+Arg group indicates the interconversion of Arg (via Orn) and Asp to Glu, whereas Asp is also biochemically related to Ala. The higher plasma levels of Lys and Ile in the CON group fed the protein-free pellet suggest cellular proteolysis and AA efflux to the plasma as a consequence of the lack of a suitable AA pattern necessary for protein synthesis.

The AUC in the Asp+Arg compared to the CGP+CGPase groups suggests that although equimolar amounts of Arg were consumed, a large portion of the free Arg was degraded in the small intestinal tissues to form Orn, as shown by the substantial increase in plasma Orn concentrations. Others have shown that supplemental Arg is rapidly catabolized to Orn by arginase present in the hepatocytes and also in plasma (Wu *et al.*, 2009). Judged by the timing of the increase in plasma ß-Asp-Arg concentration compared to free Arg (1.7 vs. 0.5 h), we hypothesized that the major site of CGP degradation is the small intestine and not the stomach. This is further supported by the comparatively low residual CGP contents in the small intestine when mice were co-administered CGP and CGPase, which additionally indicates that CGP was not completely degraded by CGPase within 4 h. When mice were fed pellets with CGP only, CGP levels were high in the large intestine, suggesting that CGP was resistant to colonic fermentation, although bacteria expressing CGPase have been reported in the caecum microbiota of rabbits, sheep and carp (Sallam and Steinbuchel, 2009a).

In conclusion, we showed that plants are able to produce high amounts of active CGPase. Differences in enzyme activity were caused by different CGPase accumulation, indicating that the successful CGP degradation by CGPase depends on the enzyme amount. The greatest accumulation was observed for S-CPHB-S and GFP::CPHB-S; therefore, these two variants are suitable for further investigations related to the production of CGP in plants. The results obtained in the mouse study suggest that plant-derived CGPase, when co-ingested with CGP, is active in the mammalian intestine and hydrolyses CGP to form ß-Asp-Arg dipeptides, which can be absorbed. However, Arg from these dipeptides is not bioavailable owing to the lack of a suitable dipeptidase. This problem might be solved by the co-expression of a suitable dipeptidase in combination with CGP and CGPase in plants.

Experimental procedures

Construction of transient plant expression vectors

For transient expression, we used the MagnICON® vectors pICH29912 (cytosolic expression) (Marillonnet *et al.*, 2005) and pICH26201 for chloroplast-targeted expression (Fig. 1), which were kindly provided by Nomad Bioscience (Halle/Saale, Germany). The *cph*B coding fragments were integrated using the *Bsa*I cloning site. Vector pet22b*cph*B, carrying a 55 AA *N*-terminal truncated coding region of the *cph*$B_{tlr2169}$ gene from *Thermosynechococcus elongatus* BP-1 (UniProt Accession No. P0C8P3) with a *C*-terminal 6xHistag, was provided by Prof. Dr. Wolfgang Lockau (Institute of Biology-Plant Biochemistry of the Humboldt-University of Berlin, Germany). This sequence, called *cph*B-b, was adapted to the codon usage of *N. tabacum* (Eurofins MWG Operon, Ebersberg, Germany), resulting in *cph*B-s. The GCT TCC TCC sequence encoding Ala-Ser-Ser (A) was integrated downstream of the start codon to improve the efficiency of translation (Sawant *et al.*, 2001), using primer *Bsa*I-*cph*B-sA-fw (Table S2), resulting in *cph*B-sA. *N*-terminal modifications were added to the coding regions by cloning the corresponding PCR fragments. The sequence of green fluorescent protein (GFP) was amplified from vector pICH18711 (Marillonnet *et al.*, 2005), flanked by *Bsa*I (5′) and *Bam*HI (3′) and subcloned into pJet (CloneJET PCR cloning kit, Thermo Scientific, Bonn, Germany). *Cph*B-s was also flanked by *Bsa*I (3′) and *Bam*HI (5′) and integrated into pJet. Subsequently, *gfp* was fused to *cph*B-s using the *Bam*HI sites. To create *cph*B-s-c, the unmodified coding region as described in the database, a synthetic sequence corresponding to the first 70 AA of the full-length *cph*B sequence (*cph*B-s-c-pI), was adapted to the codon usage of *Nicotiana tabacum* (Eurofins MWG Operon, Ebersberg, Germany) and cloned into vector pEXA2-*cph*B-s-pI. The sequence coding for AA 71-330 (*cph*B-s-c-pII) was amplified from *cph*B-s and flanked by *Bgl*II and *Sal*I restriction sides. This fragment was integrated into pEXA2-*cph*B-s-c-pI, resulting in the vector pEXA2-*cph*B-s-c. S-*cph*B-s was constructed via integration of the coding region *cph*B-s into pICH26201. For vector pS-c*cph*B-sA2, the Ala-Ser-Ser AA sequence at position +4 to +12 was adapted from GCT TCC TCC to GCC ATT GGA using primer *cph*B-sA2-BsaI-fw. For propagation, all constructed vectors were transformed into *E. coli* TG1 and validated by sequencing (Eurofins MWG Operon, Ebersberg, Germany). For plant infiltration, the vectors were transformed into *A. tumefaciens* strain ICF320 (Bendandi *et al.*, 2010).

Agroinfiltration of *Nicotiana benthamiana* plants

Transient expression in *N. benthamiana* plants was carried out as described by Nausch *et al.* (2012b) (cultivation and growth of bacteria and plants) and Leuzinger *et al.* (2013) (infiltration process) with modifications (for details see Data S1). Plants were infiltrated using vacuum infiltration with a freeze dryer (Alpha 1-4 Freeze Dryer, Christ, Germany) at 100 mBar for 2 min. Noninfiltrated leaves were removed, and plants were incubated in the dark overnight before they were returned to their regular growth conditions with a 16h day and 8h night cycle at 20–22 °C.

Sample preparation and analyses of protein content and RNA

Samples were taken 7, 8 and 9 days post infiltration (dpi), frozen immediately with liquid N_2 and stored at −80 °C. All leaves were harvested and pooled. Two to three plants per day and constructs were analysed as independent replications. TSP was measured according to Bradford (1976) using Pierce reagent and bovine serum albumin (BSA) (Thermo Scientific) as the standard. Isolation and preparation of total RNA were conducted as described previously (Nausch *et al.*, 2012b), and Northern blot analysis was performed as described in Data S1 using the primers *Bsa*I-*cph*B-b-fw, *cph*B-b-*Bsa*I-rv, *cph*B-s-*Bsa*I-rv and *cph*B-s-N-fw (Table S2).

Western blot analysis

Sample preparation and Western blot analysis were carried out as described by Nausch *et al.* (2012b) with modifications (for details see Data S1). CPHB-B isolated from *E. coli* was used as a positive control. The primary anti-CPHB antibody was produced in two Zika rabbits. Serum was obtained via centrifugation of the

collected blood, and 0.04% sodium azide was added for storage. A commercial secondary antibody was used (goat anti-rabbit POD, Dianova, Hamburg Germany), and signals were detected using the ECL chemiluminescence system.

Analysis of enzyme activity in crude plant extracts

The pooled leaf sample was mixed with chilled phosphate-buffered saline (PBS) and homogenized using a Polytron (Pt-MR 2100, Kinematica AG, Switzerland) at maximum speed. TSP (600 μg) was incubated with 100 and 200 μg CGP. The reaction was neutralized (Law *et al.*, 2009). Two samples per construct and CGP concentration were analysed. The reaction was stopped immediately or overnight using trichloroacetic acid precipitation. After centrifugation, the pellet was resolved in 300 μL 1× SDS sample buffer (Nausch *et al.*, 2012b), and 30 μL of the sample was analysed by 12% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue R250 (Carl Roth GmbH, Germany) for 20 min.

Isolation of CGP and CGPase and prediction of protein properties

CGP was isolated from *Solanum tuberosum* tubers (PsbY-cphA$_{TE}$-12) as described by Neubauer *et al.* (2012), and 10 mg was dissolved in 1 mL 0.1 M HCL pH < 2. CGPase was isolated from *E. coli* BL21 cells carrying pET22b-*cph*B-his and *N. benthamiana* leaves using Ni^{2+} NTA affinity chromatography as described in detail in the supplementary information (Data S1). The molecular weight of the proteins was predicted using the sequence manipulation suite home page (Stothard, 2000). Prediction of *in silico* protein folding was carried out using the Phyre2 server (Kelley and Sternberg, 2009; Kelley *et al.*, 2015).

Mouse study and plasma amino acid analysis

The procedures performed in this study were in accordance with the German animal protection regulations and were approved by the relevant authorities (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei, Mecklenburg-Vorpommern, Germany; permission No. 7221.3-1-017/14).

Male mice (age 49 days) of an unselected control strain (FZTDu; Dietl *et al.* (2004)) bred at the Leibniz Institute for Farm Animal Biology (FBN) in Dummerstorf were housed individually with sawdust bedding at 22 °C and a 12:12-h dark:light cycle. Mice were fed a standard rodent diet *ad libitum* (Altromin 1314, Altromin GmbH & Co. KG, Lage, Germany; 22.5% crude protein, 5% crude fat, 12.5 MJ ME/kg diet) and had free access to water. Balanced for litter and body weight (BW), the mice were randomly divided into four groups (n = 7/group) according to the type of test pellet fed: cyanophycin (CGP), cyanophycin + cyanophycinase (CGP+CGPase), free L-Arg and L-Asp (Asp+Arg) and control (CON).

Protein-free test pellets were based on 220 mg of a 1:1 mixture of corn starch (Backfee, OsnaNährmittel GmbH, Osnabrück, Germany) and powdered sucrose (Nordzucker AG, Braunschweig, Germany). The basal pellet mixture was supplemented with 30 mg CGP (from *S. tuberosum* tubers), 30 mg CGP + 10 mg CGPase (from *N. benthamiana* leaves), or 15 mg Arg (Degussa, Frankfurt/Main, Germany) plus 15 mg Asp (Reanal, Budapest, Hungary). This corresponded to 1 mg CGP, 0.33 mg CGPase, or 0.5 mg Arg or Asp, respectively, per g BW. The pellet for the control group (CON) contained none of these supplements. Dry matter was adjusted to 250 mg for each pellet with the starch/sucrose mixture to achieve comparable energy contents. A volume of 40–45 μL of pH 3 water was added to the mixtures, the mixture was filled in 1-mL plastic syringes (Omnifix® 40 Solo, Braun, Melsungen, Germany), and pellets were pressed manually with a plunger. Pressed pellets were stored overnight at 4 °C before consumption.

At the age of 79–92 days, the mice were transferred to cages without sawdust after overnight food withdrawal. Test pellets were offered to the mice for 15 min, the remaining food was collected, and the animals were transferred back to their original cages. The tips of their tails were snipped to collect blood in sodium-heparinized microhematocrit capillary tubes (Marienfeld, Lauda-Königshofen, Germany) at 0, 20, 40, 60, 120, 180 and 240 min after pellet administration. The capillaries were immediately put on ice and were centrifuged for 3 min at 10 000 × ***g*** and 4 °C. The isolated plasma was diluted with ultrapure water and stored at −20 °C. Immediately after the 240 min, blood samples were taken, mice were killed by cervical dislocation. The abdomen was opened, the small and large intestine were isolated, and the total contents were rinsed with 3 mL cold PBS, weighed, and stored frozen at −20 °C The residual CGP concentration of the small and large intestine in the CGP, CGP+CGPase and free Asp+Arg mice were quantified as described in Data S1. We used only mice with >40% intake of their respective pellets. Thus, valid observations were obtained for 6, 6, 7 and 7 mice in the CGP, CGP+CGPase, Asp+Arg and CON groups, respectively. Plasma AA were analysed by HPLC separation with fluorescence detection of o-phthaldialdehyde derivatives on a 250 × 4 mm HyperClone ODS (C18) 120 Å (Phenomenex, Aschaffenburg, Germany) as described (Kuhla *et al.*, 2010). Standard AA (A9906 Sigma, Munich, Germany) allowed assignment of retention times and quantification. The β-Asp-Arg dipeptide, eluting at a retention time of 3.8 min, was identified using isolated CGP degraded by CGPase (Fig. S2). Enzyme hydrolysis of CGP (200 μg) was performed as described (Law *et al.*, 2009) with minor modifications. CGP (10 mg/mL in 0.1 M HCL) was diluted in PBS (pH 7.2), and 50 μg of S-CPHB-S (1 mg/mL) isolated from *N. benthamiana* was added. Samples were incubated at RT overnight. CGPase was removed from the hydrolysis mixture by centrifugation with Roti®-Spin MINI-3 cartridges (3 kDa, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) for 20 min at 17 000 × ***g*** and 4 °C. The β-Asp-Arg dipeptide was quantified by the AUC using a four-point calibration. The detection limit was 0.5 μM. An aliquot of the β-Asp-Arg dipeptide filtrate (100 μL) was hydrolysed in 1 mL of 6 N HCl at 110 °C for 22 h with ascorbic acid as an antioxidant under a N_2 atmosphere to confirm the Asp and Arg constituents only. After HCl removal under a N_2 stream at 60 °C, the residue was diluted in 1 mL H_2O, centrifuged for 20 min at 17 000 × ***g*** and 4 °C and analysed for AA concentrations. Based on the concentrations of Arg and Asp, the amount of β-Asp-Arg was recalculated. Then, plasma concentrations of Asp, Arg, β-Asp-Arg dimer and Orn were quantified. For calculation of the pharmacokinetics of these metabolites, data were normalized for the pellet intake and BW after subtraction of the mean concentrations of the corresponding AA of the CON group. The AUC for the β-Asp-Arg dimer and Arg between 0 and 4 h after administration was calculated with TableCurve 2D V 5.01 software (SYSTAT Software Inc., Chicago, IL). The time (T_{max}) at which the maximal plasma concentration, C_{max}, was reached was computed using a best curve fit. Plasma clearance was calculated from the administered dose of CGP (converted to β-Asp-Arg equivalents) or Arg (μmol) divided by the AUC.

Statistical analysis of mouse study

Mouse data were evaluated with SAS 9.4 software (SAS Institute Inc. 2011, Cary, NC). A repeated-measures ANOVA implemented in a mixed model was used with the fixed effects of group (CGP+CGPase, CGP, Asp-Arg, CON) and time of blood sampling (20, 40, 60, 120, 180, and 240 min), as well as the interaction term. The effect of repeatedly sampled animals was considered random. The covariance structure was autoregressive (AR1). For singly measured variables, a one-way ANOVA was used with group as a fixed effect. Residual intestinal CGP contents were analysed by a two-factorial ANOVA with group and intestinal location (small and large intestine) as fixed factors and the interaction term. Pellet intake did not differ among groups and was not considered a factor. Effects were considered significant at $P \leq 0.05$, and group differences were tested using the Tukey–Kramer test. Significance levels of $P < 0.10$ were considered a statistical trend. Data are presented as least-square means (LSMEANS) and their standard errors (SE).

Acknowledgements

We are grateful for the support of Dr. Alain Steinmann and Bioserv GmbH Rostock in the production of anti-CPHB antibodies. Additionally, we thank Quentin L. Sciascia, PhD, for assistance during the mouse trial, particularly with collection of the blood samples. Care for the experimental animals by the team at the Tiertechnikum of the Leibniz Institute for Farm Animal Biology, Dummerstorf, is also greatly appreciated. The animal study was funded by the core budget to the Leibniz Institute for Farm Animal Biology, Dummerstorf. The plant work was funded by the scholarship of the 'Landesgraduiertenförderung' of the University of Rostock. The publication of this article was funded by the Open Access fund of the Leibniz Institute for Farm Animal Biology (FBN).

Conflict of interest

The authors declare no conflict of interest.

References

Allen, M.M., Morris, R. and Zimmerman, W. (1984) Cyanophycin granule polypeptide protease in a unicellular cyanobacterium. *Arch. Microbiol.* **138**, 119–123.

Barahimipour, R., Strenkert, D., Neupert, J., Schroda, M., Merchant, S.S. and Bock, R. (2015) Dissecting the contributions of GC content and codon usage to gene expression in the model alga *Chlamydomonas reinhardtii*. *Plant J.*, **84**, 704–717.

Bazer, F.W., Kim, J., Ka, H., Johnson, G.A., Wu, G. and Song, G. (2012) Select nutrients in the uterine lumen of sheep and pigs affect conceptus development. *J. Reprod. Develop.* **58**, 180–188.

Benchabane, M., Goulet, C., Rivard, D., Faye, L., Gomord, V. and Michaud, D. (2008) Preventing unintended proteolysis in plant protein biofactories. *Plant Biotech. J.* **6**, 633–648.

Bendandi, M., Marillonnet, S., Kandzia, R., Thieme, F., Nickstadt, A., Herz, S. and Frode, R., *et al.* (2010) Rapid, high-yield production in plants of individualized idiotype vaccines for non-Hodgkin's lymphoma. *Ann. Oncol.* **21**, 2420–2427.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.

Dietl, G., Langhammer, M. and Renne, U. (2004) Model simulations for genetic random drift in the outbred strain Fzt: DU. *Arch. Anim. Bree.* **47**, 595–604.

Egelkrout, E., Rajan, V. and Howard, J.A. (2012) Overproduction of recombinant proteins in plants. *Plant Sci.* **184**, 83–101.

Floss, D.M., Sack, M., Stadlmann, J., Rademacher, T., Scheller, J., Stoger, E. and Fischer, R. *et al.* (2008) Biochemical and functional characterization of anti-HIV antibody-ELP fusion proteins from transgenic plants. *Plant Biotech. J.* **6**, 379–391.

Gils, M., Kandzia, R., Marillonnet, S., Klimyuk, V. and Gleba, Y. (2005) High-yield production of authentic human growth hormone using a plant virus-based expression system. *Plant Biotech. J.* **3**, 613–620.

Gleba, Y., Klimyuk, V. and Marillonnet, S. (2005) Magnifection—a new platform for expressing recombinant vaccines in plants. *Vaccine* **23**, 2042–2048.

Gupta, M. and Carr, N.G. (1981) Enzyme-activities related to cyanophycin metabolism in heterocysts and vegetative cells of Anabaena Spp. *J. Gen. Microbiol.* **125**, 17–23.

Hejazi, M., Piotukh, K., Mattow, J., Deutzmann, R., Volkmer-Engert, R. and Lockau, W. (2002) Isoaspartyl dipeptidase activity of plant-type asparaginases. *Biochem. J.* **364**, 129–136.

Hondred, D., Walker, J.M., Mathews, D.E. and Vierstra, R.D. (1999) Use of ubiquitin fusions to augment protein expression in transgenic plants. *Plant Physiol.* **119**, 713–723.

Hühns, M., Neumann, K., Hausmann, T., Ziegler, K., Klemke, F., Kahmann, U. and Staiger, D. *et al.* (2008) Plastid targeting strategies for cyanophycin synthetase to achieve high-level polymer accumulation in *Nicotiana tabacum*. *Plant Biotech. J.* **6**, 321–336.

Hühns, M., Neumann, K., Hausmann, T., Klemke, F., Lockau, W., Kahmann, U. and Kopertekh, L. *et al.* (2009) Tuber-specific cphA expression to enhance cyanophycin production in potatoes. *Plant Biotech. J.* **7**, 883–898.

Jang, I.C., Niu, Q.W., Deng, S.L., Zhao, P.Z. and Chua, N.H. (2012) Enhancing protein stability with retained biological function in transgenic plants. *Plant J.* **72**, 345–354.

Kelley, L.A. and Sternberg, M.J.E. (2009) Protein structure prediction on the Web: a case study using the Phyre server. *Nat. Protoc.* **4**, 363–371.

Kelley, L.A., Mezulis, S., Yates, C.M., Wass, M.N. and Sternberg, M.J.E. (2015) The Phyre2 web portal for protein modeling, prediction and analysis. *Nat. Protoc.* **10**, 845–858.

Klang, J.E., Burnworth, L.A., Pan, Y.X., Webb, K.E. and Wong, E.A. (2005) Functional characterization of a cloned pig intestinal peptide transporter (pPepT1). *J. Anim. Sci.* **83**, 172–181.

Klimyuk, V., Benning, G., Gils, M., Giritch, A. and Gleba, Y. (2004) *Process of Producing a plastid-targeted protein in plant cells*. AG, I.G. WO 2004/101797 A1

Kuhla, B., Kucia, M., Goers, S., Albrecht, D., Langhammer, M., Kuhla, S. and Metges Cornelia, C. (2010) Effect of a high-protein diet on food intake and liver metabolism during pregnancy, lactation and after weaning in mice. *Proteomics* **10**, 2573–2588.

Law, A.M., Lai, S.W.S., Tavares, J. and Kimber, M.S. (2009) The structural basis of beta-Peptide-specific cleavage by the serine protease cyanophycinase. *J. Mol. Biol.* **392**, 393–404.

Leuzinger, K., Dent, M., Hurtado, J., Stahnke, J., Lai, H., Zhou, X. and Chen, Q. (2013) Efficient agroinfiltration of plants for high-level transient expression of recombinant proteins. *J. Vis. Exp.*, **77**, 1–9.

Marillonnet, S., Giritch, A., Gils, M., Kandzia, R., Klimyuk, V. and Gleba, Y. (2004) In planta engineering of viral RNA replicons: efficient assembly by recombination of DNA modules delivered by Agrobacterium. *Proc. Natl. Acad. Sci. USA* **101**, 6852–6857.

Marillonnet, S., Thoeringer, C., Kandzia, R., Klimyuk, V. and Gleba, Y. (2005) Systemic Agrobacterium tumefaciens-mediated transfection of viral replicons for efficient transient expression in plants. *Nat. Biotechnol.* **23**, 718–723.

Matthews, D.M. and Adibi, S.A. (1976) Peptide absorption. *Gastroenterology* **71**, 151–161.

Moreau, M.J.J., Morin, I. and Schaeffer, P.M. (2010) Quantitative determination of protein stability and ligand binding using a green fluorescent protein reporter system. *Mol. BioSyst.* **6**, 1285–1292.

Nausch, H., Mikschofsky, H., Koslowski, R., Meyer, U., Broer, I. and Huckauf, J. (2012a) High-Level transient expression of ER-targeted human interleukin 6 in Nicotiana benthamiana. *PLoS ONE*, **7**, 1–16.

Nausch, H., Mischofsky, H., Koslowski, R., Meyer, U., Broer, I. and Huckauf, J. (2012b) Expression and subcellular targeting of human complement factor C5a in Nicotiana species. *PLoS ONE*, **7**, 1–13.

Neubauer, K., Huhns, M., Hausmann, T., Klemke, F., Lockau, W., Kahmann, U. and Pistorius, E.K. *et al.* (2012) Isolation of cyanophycin from tobacco and potato plants with constitutive plastidic cphA(Te) gene expression. *J. Biotech.* **158**, 50–58.

Neumann, K., Neumann, K., Stephan, D.P., Ziegler, K., Huhns, 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 Biotech. J.* **3**, 249–258.

Patel, J., Zhu, H., Menassa, R., Gyenis, L., Richman, A. and Brandle, J. (2007) Elastin-like polypeptide fusions enhance the accumulation of recombinant proteins in tobacco leaves. *Transgenic Res.* **16**, 239–249.

Perlak, F.J., Fuchs, R.L., Dean, D.A., Mcpherson, S.L. and Fischhoff, D.A. (1991) Modification of the coding sequence enhances plant expression of insect control protein genes. *Proc. Natl. Acad Sci. USA* **88**, 3324–3328.

Pillay, P., Schluter, U., van Wyk, S., Kunert, K.J. and Vorster, B.J. (2014) Proteolysis of recombinant proteins in bioengineered plant cells. *Bioengineered* **5**, 15–20.

Piron, R., De Koker, S., De Paepe, A., Goossens, J., Grooten, J., Nauwynck, H. and Depicker, A. (2014) Boosting in planta production of antigens derived from the Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) and subsequent evaluation of their immunogenicity. *PLoS ONE*, **9**, 1–16.

Richter, R., Hejazi, M., Kraft, R., Ziegler, K. and Lockau, W. (1999) Cyanophycinase, a peptidase degrading the cyanobacterial reserve material multi-L-arginyl-poly-L-aspartic acid (cyanophycin) - Molecular cloning of the gene of Synechocystis sp PCC 6803, expression in *Escherichia coli*, and biochemical characterization of the purified enzyme. *Eur. J. Biochem.* **263**, 163–169.

Rubio-Aliaga, I. and Daniel, H. (2008) Peptide transporters and their roles in physiological processes and drug disposition. *Xenobiotica* **38**, 1022–1042.

Sallam, A. and Steinbuchel, A. (2009a) Cyanophycin-degrading bacteria in digestive tracts of mammals, birds and fish and consequences for possible applications of cyanophycin and its dipeptides in nutrition and therapy. *J. Appl. Microbiol.* **107**, 474–484.

Sallam, A. and Steinbuchel, A. (2009b) *Process for the preparation of dipeptides from cyanophycin employing the isolated Pseudomonas alcaligenes DIP1 CGPase CphEal*. Münster, W.W.-U.

Sallam, A. and Steinbuchel, A. (2010) Dipeptides in nutrition and therapy: cyanophycin-derived dipeptides as natural alternatives and their biotechnological production. *Appl. Microbiol. Biotechnol.* **87**, 815–828.

Sallam, A., Kast, A., Przybilla, S., Meiswinkel, T. and Steinbuchel, A. (2009) Biotechnological process for production of beta-dipeptides from cyanophycin on a technical scale and its optimization. *Appl. Environ. Microbiol.* **75**, 29–38.

Sawant, S.V., Kiran, K., Singh, P.K. and Tuli, R. (2001) Sequence architecture downstream of the initiator codon enhances gene expression and protein stability in plants. *Plant Physiol.* **126**, 1630–1636.

Sharp, P.M. and Li, W.H. (1987) The codon adaptation index – a measure of directional synonymous codon usage bias, and its potential applications. *Nucleic Acids Res.* **15**, 1281–1295.

Simon, R.D. (1987) Inclusion bodies in the cyanobacteria: cyanophycin, polyphosphate, polyhedra bodies. In *The Cyanobacteria* (Fay, P. and Van Baalen, C., eds), pp. 192–222. Amsterdam, New York, Oxford: Elsevier.

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

Stothard, P. (2000) The sequence manipulation suite: javaScript programs for analyzing and formatting protein and DNA sequences. *Biotechniques* **28**, 1102-+.

Ullrich, K.K., Hiss, M. and Rensing, S.A. (2015) Means to optimize protein expression in transgenic plants. *Curr. Opin. Biotechnol.* **32**, 61–67.

Utagawa, T. (2004) Production of arginine by fermentation. *J. Nutr.* **134**, 2854s–2857s.

Wang, Y.X. , Zhang, L., Zhou, G., Liao, Z., Ahmad, H., Liu, W. and Wang, T. (2012) Dietary L-arginine supplementation improves the intestinal development through increasing mucosal Akt and mammalian target of rapamycin signals in intra-uterine growth retarded piglets. *Br. J. Nutr.* **108**, 1371–1381.

Webster, D.E., Wang, L., Mulcair, M., Ma, C., Santi, L., Mason, H.S. and Wesselingh, S.L. *et al.* (2009) Production and characterization of an orally immunogenic Plasmodium antigen in plants using a virus-based expression system. *Plant Biotech. J.* **7**, 846–855.

Wenzel, U., Meissner, B., Doring, F. and Daniel, H. (2001) PEPT1-mediated uptake of dipeptides enhances the intestinal absorption of amino acids via transport system b(0,+). *J. Cell. Physio.* **186**, 251–259.

Wu, G.Y. (2014) Dietary requirements of synthesizable amino acids by animals: a paradigm shift in protein nutrition. *J. Anim. Sci. Biotechnol.*, **5, 34**, 1–12.

Wu, G., Knabe, D.A. and Kim, S.W. (2004) Arginine nutrition in neonatal pigs. *J. Nutr.*, **134**, 2783S–2790S; discussion 2796S–2797S.

Wu, G.Y., Bazer, F.W., Davis, T.A., Kim, S.W., Li, P., Rhoads, J.M., Satterfield, M.C., Smith, S.B., Spencer, T.E. and Yin, Y. (2009) Arginine metabolism and nutrition in growth, health and disease. *Amino Acids* **37**, 153–168.

Yagasaki, M. and Hashimoto, S. (2008) Synthesis and application of dipeptides; current status and perspectives. *Appl. Microbiol. Biotechnol.* **81**, 13–22.

Yamaguchi, R., Nakamura, M., Mochizuki, N., Kay, S.A. and Nagatani, A. (1999) Light-dependent translocation of a phytochrome B-GFP fusion protein to the nucleus in transgenic Arabidopsis. *J. Cell Biol.* **145**, 437–445.

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). *Eur. J. Biochem.* **254**, 154–159.

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. *J. Biosci.* **57**, 522–529.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. *In silico* secondary structure determination of S-CPHB-S and S-CPHB-A2 was carried out using the Phyre2 server (Kelley and Sternberg, 2009; Kelley *et al.*, 2015).

Figure S2. Chromatogram of the ß-Asp-Arg dipeptide after enzymatic degradation of cyanophycin with cyanophycinase and subsequent enzyme removal (blue); Asp and Arg signals after acid hydrolysis of the ß-Asp-Arg dipeptide (red); LU, luminescence units.

Table S1. Body weight, intakes of pellets, cyanophycin (CPG), cyanophycinase (CPGase), and free Asp and Arg, as well as concentrations of plasma Asp, ß-Asp-Arg dipeptide, Arg, and Orn in four groups of mice fed protein-free pellets supplemented with CPG, CPG+CPGnase, Asp+Arg, or none of these (control; CON).

Table S2. Sequence of primers used in this study[1]

Data S1. Detailed experimental procedure.

Supporting information to B2

Fig B2 S 1: *In silico* secondary structure determination of S-CPHB-S and S-CPHB-A2 was carried out using the Phyre2 server (Kelley *et al.* 2009, Kelley *et al.* 2015).

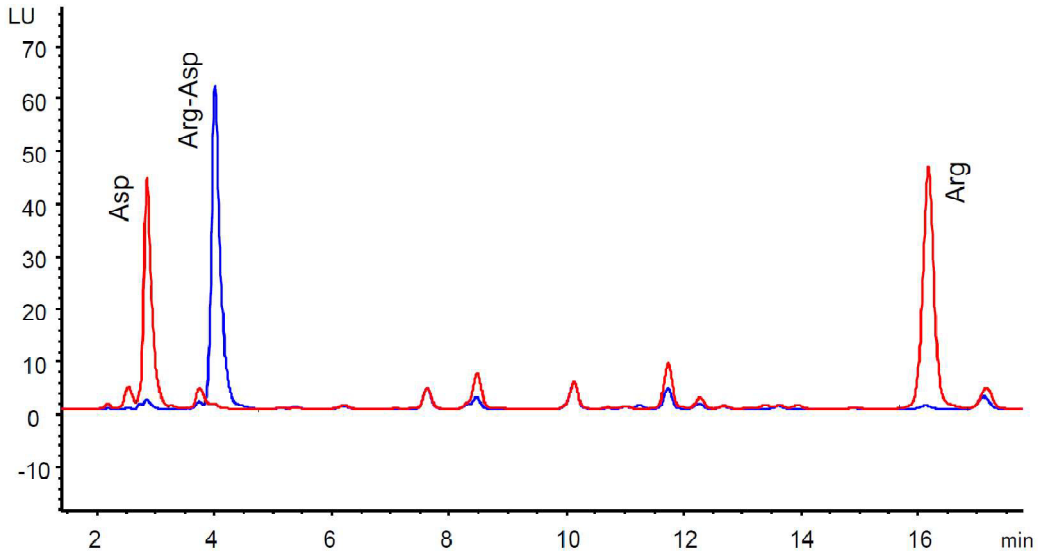


Figure B2 S 2: Chromatogram of the ß-Asp-Arg dipeptide after enzymatic degradation of cyanophycin with cyanophycinase and subsequent enzyme removal (blue); Asp and Arg signals after acid hydrolysis of the ß-Asp-Arg dipeptide (red); LU, luminescence units.

Table B2 S 1: Body weight, intakes of pellets, cyanophycin (CPG), cyanophycinase (CPGase), and free Asp and Arg, as well as concentrations of plasma Asp, ß-Asp-Arg dipeptide, Arg, and Orn in four groups of mice fed protein-free pellets supplemented with CPG, CPG+CPGnase, Asp+Arg, or none of these (control; CON).

	CPG	CPG+ CPGase	Asp+ Arg	CON	SE[1]	P≤		
						G*	T	G x T
BW*, g	39.4	39.5	39.0	41.8	1.74	0.615	-†	-†
Intakes								
Pellet, mg	223	207	210	186	20.4	0.603	-	-
CPG, mg · kg^{-1} BW	683.5^{a}	662.2^{a}	0^{b}	0^{b}	58.89	0.001	-	-
CPGase, mg · kg^{-1} BW	0^{b}	260^{a}	0^{b}	0^{b}	26.54	0.001	-	-
Asp, mg · kg^{-1} BW	290.6^{a}‡	281.1^{a}	324.1^{a}	0^{b}	29.75	0.001	-	-
Arg, mg · kg^{-1} BW	393.5^{a}	381.5^{a}	324.1^{a}	0^{b}	37.5	0.001	-	-
Plasma concentrations, µmol/L§								
Asp	7.3^{a}¶	10.1^{ab}	16.3^{b}	14.0^{ab}	1.92	0.012	0.019	0.091
ß-Asp-Arg	0^{a}	77^{b}	0^{a}	0^{a}	6.5	0.001	0.001	0.001
Arg	62.5^{a}	71.7^{ab}¶	106.9^{b}	63.7^{a}	9.79	0.008	0.001	0.001
Orn	95.8^{b}	97.3^{b}	230.9^{a}	123.2^{b}	14.2	0.001	0.275	0.001

Data are shown as LSMEANS ± SE, and *n* = 6 – 7 mice per group.

*G = group, T = time, G x T = group x time interaction, body weight (BW).

†Singly measured variables were analyzed with one-way ANOVA.

‡Values differing among groups have different letters (a, b) on the same line (Tukey, $P < 0.05$).

§Basal plasma Arg, Asp, and Orn concentrations in mice prior to pellet intake were 102 ± 5.1, 12.8 ± 1.5, and 99.5 ± 10.2 µmol · L^{-1}, respectively ($P > 0.5$).

¶Values sharing the sign on the same line tend to differ (Tukey, $P < 0.10$).

Table B2 S 2: Sequence of primers used in this study[1]

Name	Sequence	Vector
BsaI-cB-b-fw	5'-TTTT**GGTCTC**ACATGGCGATCGGGGGAGCGGAGG-3'	pcB-b
cB-b-BsaI-rv	5'-TTTT**GGTCTC**AAAGCTTAGTGGTGGTGGTGGTGGTGCTCG-3'	pcB-b
BsaI-cB-s-fw	5'-TTTT**GGTCTC**ACATGGCCATTGGAGGGGCAG-3'	pcB-s pScB-s
BsaI-cB-sAfw	5'TTTT**GGTCTCAC**ATGgcttcctccGCCATTGG-3'	pcB-sA
cB-s-BsaI-rv	5'-TTTT**GGTCTCAAAGC**TTAGTGATGGTGATGATGATGTTCC-3'	pcB-s(A) pScB-s(A2)
BamHI-cB-s-fw	5'-TTTT**GGATCC**ATGGCCATTGGAGGGGCAG-3'	pGFP-cB-s
BsaI-GFP-fw	5'-TTTTGGTCTCACATGGTGAGCAAGGGCGAGGAGCTGTTCACC-3'	pGFP-cB-s
GFP-BamHI-rv	5'-CCCCCGATCGCCAT**GGATCC**CTTGTACAGCTCGTCCATGC-3'	pGFP-cB-s
BsaI-cBs-c-pI-fw	5'-TTTTGGATCC**GGTCTC**ACATGTTGTACAGAATTC-3'	pcB-s-c
cBs-c-pI-SalI-rv	5'-TTTT**GTCGAC**GAATATTGTAGGAG-3'	pcB-s-c
BglII-cBs-c-pII-fw	5'-TTTTGC**AGATCT**TGACTACCTTTTGCCAAAGAGC-3'	pcB-s-c
cB-s-c-pII-SalI-BsaI-rv	5'-TTTT**GTCGAC**GGATCC**GGTCTC**AAAGCTTAGTGATGGTGATGATGATG-3'	pcB-s-c
BsaI-cB-sA2-fw	5'-TTTT**GGTCTC**ACATGgcctctagcGCCATTGG-3'	pScB-sA2
cB-s-N-fw	5'TTTTCAGAAGATAAGGTTAGAGGAAGGCAG-3	Northern

[1]Restriction sites used in this study are shown in bold. Start and stop codons of *cph*B are underlined. The added amino acids A-S-S are shown in small letters. cB= abbreviation for *cph*B

Text S1 Experimental Procedure

Agroinfiltration of N. benthamiana plants

Modifications: the volume of infiltration buffer was increased from 50 mL to 4 L. Therefore, 400 mL of an *A. tumefaciens* secondary overnight culture was grown and treated as described for the primary culture (Nausch *et al*. 2012). The resulting pellet was dissolved in 4 L infiltration buffer (Marillonnet *et al*. 2004)), and 1 mL of the detergent Silwet Gold (Spieß-Urania; Hamburg, Germany) was added.

RNA analysis

A 3 µg aliquot of RNA was separated by gel electrophoresis on a 1.5% agarose gel in MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, 5% formaldehyde, pH 7). Blotting, hybridization and signal detection were performed as described for Southern blot analysis by Nausch *et al.* (2012) with modifications. The membrane was prehybridized for 3 h at 51°C, DIG-labeled probes were amplified using the primers BsaI-cB-b-fw, cB-b-BsaI-rv, cB-s-BsaI-rv and cB-s-N-fw (Table S2) and the membrane was hybridized to the probe at 51°C.

Western blot

Modifications during sample preparation: PMSF in the protein extraction buffer was replaced with protease inhibitor cocktail tablets (Complete Tablets, Mini, EASYpack, Roche, Mannheim, Germany) according to the manufacturer's recommendations. Total soluble protein (TSP) was precipitated using trichloroacetic acid at 4°C for 2.5 h and dissolved in 30 µL 1x sample buffer (10% glycerin, 150 mM Tris (pH 6.8), 3% SDS, 1% mercaptoethanol, and 2.5% bromophenol blue).
After separation, the samples were blotted on a 0.45 µm nitrocellulose Amersham Hybond ECL membrane (GE Healthcare Life Science, Darmstadt, Germany), which was blocked overnight at room temperature (RT) in TBS+T buffer (10 mM Tris, 15 mM NaCl, pH 7.6, 0.05% Tween) containing 5% skim milk powder.

Antibody concentration and incubation: the primary serum was used at a 1:6000 dilution and incubated for 2 h at RT. After 3 washing steps, the secondary antibody (goat anti-rabbit POD, Dianova Hamburg, Germany) was diluted 1:20,000 and incubated for 1 h at RT. After another 3 washing steps, the signals were detected using the ECL chemiluminescence system, and the membrane was exposed to Amersham Hyperfilm^{TM} ECL films (GE Healthcare Limited, Darmstadt Germany) for 2 and 5 min.

Analysis of enzyme activity in crude plant extracts

PBS: 140 mM NaCl, 10 mM KCl, 6.4 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2.

Ni^{2+} NTA affinity purification of cyanophycinase (CPGase)

A single colony of *E. coli* BL21 carrying pET22b-*cph*B-His (Lockau unpublished) was grown in 50 mL Luria-Bertani (LB) medium (Carl Roth GmbH, Karlsruhe, Germany) containing 1 % glucose, 125 $\mu g \cdot ml^{-1}$ ampicillin at 37°C for 3 h . After centrifugation (10 min, 4,600·g, RT), the pellet was dissolved in 100 mL of the same medium and incubated for 2 h at 37°C. Following centrifugation (10 min, 4,600 g, RT), the pellet was dissolved in 200 mL LB medium, containing 250 $\mu g \cdot ml^{-1}$ ampicillin and 1 mM IPTG and grown overnight at RT. This culture was centrifuged (15 min, 4,600 g, RT), and the pellet was dissolved in 30 mL NPI buffer (50 mM $NaH_2PO_4 \cdot H_2O$, 300 mM NaCl, pH 8) containing 10 mM imidazole. Cells were lysed using an ultrasonic unit on ice (2x30 s, output 120) and subsequently centrifuged for 15 min at 4°C, 4,600 g. The supernatant was transferred to a new tube and centrifuged again for 1 h, 4°C, 12,000 g. A 2.7 mL ProBond®Resin (Novex® Life Technologies™, Van Allen Way Carlsbad, CA, USA,) Ni^{2+}-NTA matrix was equilibrated with 25 mL NPI buffer. Then, 20 ml of supernatant was loaded on the column, which then was washed twice with 25 mL NPI buffer containing 20 and 30 mM imidazole. Protein was eluted from the matrix surface using 6 fractions of NPI buffer containing 300 mM imidazole. Fractions 2, 3 and 4 were desalted using a PD-10 Sephadex® G-25M column (Pharmacia Biotechnology, Uppsala, Sweden), which was equilibrated with 50 mL PBS (pH 7.5) and eluted with 3.5 mL of the same buffer. Aliquots of 200 µL were stored at -20°C or 4°C for next day use. CPGase was isolated from *N. benthamiana* leaves by homogenization of *leaf material* from one whole plant with a Polytron (2x30 s, 19,000 rpm) in cold 50 mM $Na_2HPO_4 \cdot H_2O$ buffer (pH 8) containing 0.3 M sucrose and 0.24 M NaCl. Homogenized material was filtered through 3 layers of Miracloth (Merck Millipore, Germany) and centrifuged (4°C, 45 min, 12,000 g). CPHB was isolated as described above, but 15 mL column matrix was used. Therefore, the amounts of buffers were adjusted, and all fractions were rebuffered with PBS (pH 7.5) using Vivaspin 20 concentration tubes according to the manufacturer's protocol (Sigma Aldrich, Steinheim, Germany).

CGP quantification in the large and small intestine

Intestinal digesta samples were stored at -20°C. Cyanophycin (CGP) analysis was conducted as described by Hühns *et al*. (2008) with modifications. Freeze-dried intestinal digesta samples (20-35 mg) were analyzed. Soluble proteins were extracted with 1 mL 50 mM Tris (pH 8) for 30 min in a shaker (1,000 rpm). After a centrifugation step (13,200 rpm, RT, 15 min), the supernatant was discarded and the pellet resuspended in 1 mL of 0.1 M HCl. After another centrifugation step, 800 µL of the supernatant was collected and used for CGP analysis. Tubes with 1 to 10 µL of sample were filled with 0.1 M HCl to a final volume of 800 µL. After addition of 200 µL of 5 x RotiQuant Bradford reagent (Cat. No. K015.1, Carl

Roth GmbH + Co. KG, Karlsruhe, Germany) and a 5 min incubation, samples were measured at 595 nm in microcuvettes (Cat. No.: 67.742; Sarstedt AG & Co., Nümbrecht, Germany). A calibration curve was prepared with purified CGP from tobacco leaves or potato tubers, extracted as described by Neubauer (2012), with a concentration range of 1 to 5 µg · mL^{-1} CGP. OD values of the control mice were subtracted from OD values of samples from the CGP and CGP+CGPase groups.

B3: Coexpression and comparison of two cyanophycinases in transgenic, cyanophycin producing tobacco plants.

Daniel Ponndorf, Inge Broer, Henrik Nausch (2017): Coexpression and comparison of two cyanophycinases in transgenic, cyanophycin producing tobacco plants. Transgenic Research, 1-9

Author's contribution: Together with I.B. and H.N. I designed the experiments. Together with H.N. I performed the plant experiments and the molecular analysis of the plants. Western blots and comparison of enzyme activity was performed by H.N. I performed the statistical analysis and interpretation of the data and wrote the article.

Transgenic Res
DOI 10.1007/s11248-017-0019-0

ORIGINAL PAPER

Expression of CphB- and CphE-type cyanophycinases in cyanophycin-producing tobacco and comparison of their ability to degrade cyanophycin in plant and plant extracts

Daniel Ponndorf · Inge Broer · Henrik Nausch

Received: 8 November 2016 / Accepted: 17 April 2017

Abstract Increasing the arginine (Arg) content in plants used as feed or food is of interest, since the supplementation of food with conditionally essential Arg has been shown to have nutritional benefits. An increase was achieved by the expression of the Arg-rich bacterial storage component, cyanophycin (CGP), in the chloroplast of transgenic plants. CGP is stable in plants and its degradation into β-aspartic acid (Asp)-Arg dipeptides, is solely catalyzed by bacterial cyanophycinases (CGPase). Dipeptides can be absorbed by animals even more efficiently than free amino acids (Matthews and Adibi 1976; Wenzel et al. 2001). The simultaneous production of CGP and CGPase in plants could be a source of β-Asp-Arg dipeptides if CGP degradation can be prevented *in planta* or if dipeptides are stable in the plants. We have shown for the first time that it is possible to co-express CGP and CGPase in the same plant without substrate degradation *in planta* by transient expression of the cyanobacterial CGPase CPHB (either in the plastid or cytosol), and the non-cyanobacterial CGPase CPHE (cytosol) in CGP-producing *Nicotiana tabacum* plants. We compared their ability to degrade CGP *in planta* and in crude plant extracts. No CGP degradation appeared prior to cell homogenization independent of the CGPase produced. In crude plant extracts, only cytosolic CPHE led to a fast degradation of CGP. CPHE also showed higher stability and in vitro activity compared to both CPHB variants. This work is the next step to increase Arg in forage plants using a stable, Arg-rich storage protein.

Keywords Cyanophycinase CPHB · CPHE · Cyanophycin · Transient coexpression · *Nicotiana tabacum*

Introduction

Arginine (Arg) is important for animal nutrition and has shown beneficial effects on growth, health, reproduction and meat quality (Ma et al. 2015; Wang et al. 2015; Wu et al. 2014) while no adverse effects of long-term Arg supplementation were found in pigs, sheep and rats (Hu et al. 2015; Wu et al. 2007). Since Arg also plays an important role in human bio-vital processes, it is used in medicine and as an additive in the food industry (Sallam and Steinbuchel 2010). Arg is commonly produced by fermentation (Utagawa 2004) and is supplemented as a free amino acid. Enhancing the Arg content in forage crops could lead to a cheaper, easier and sufficient Arg supply for livestock. To our best knowledge, no successful

D. Ponndorf · I. Broer (✉) · H. Nausch
Department of Agrobiotechnology and Risk Assessment for Bio- and Gene Technology, Faculty of Agricultural and Environmental Sciences, University of Rostock, Justus-von-Liebig Weg 8, 18059 Rostock, Germany
e-mail: inge.broer@uni-rostock.de

Published online: 21 April 2017

breeding attempt to increase the content of free Arg in plants has been described so far. One reason for this failure might be the feedback inhibition of Arg synthesis (Sancho-Vaello et al. 2009; Winter et al. 2015). In order to prevent this, newly synthesized Arg needs to be bound, keeping the free Arg content at the endogenous level.

Storage can be achieved by incorporating Arg in the cyanobacterial storage polypeptide cyanophycin (multi-L-arginyl-poly-L-aspartic acid, CGP). CGP is a nitrogen, carbon and energy storage protein, which is synthesized by most cyanobacteria and also several non-photosynthetic bacteria (Allen et al. 1984; Simon 1987; Simon and Weathers 1976; Ziegler et al. 2002). It is created via non-ribosomal biosynthesis by the enzyme cyanophycin synthetase (*cph*A) (Ziegler et al. 1998) and consists of an L-aspartic acid (Asp) backbone with linked L-Arg residues (Simon and Weathers 1976). CGP proved to be stable and resistant to common eukaryotic and prokaryotic proteases (Simon and Weathers 1976), and its degradation is restricted to cyanophycinases (CGPases), which are produced by prokaryotes and occur in two classes: (1) Intracellular, called CPHB, mainly produced by cyanobacteria (Allen et al. 1984; Gupta and Carr 1981; Richter et al. 1999), and (2) extracellular CPHE, produced by non-cyanobacterial prokaryotes (Obst et al. 2002). CPHB and CPHE catalyze the degradation of CGP into β-Asp-Arg dipeptides (Gupta and Carr 1981; Obst et al. 2002; Richter et al. 1999). In human trials, these dipeptides had an improved nutritional effect compared to free AA, since they are taken up more efficiently (Matthews and Adibi 1976; Wenzel et al. 2001).

The chloroplast-targeted expression of the *cph*A coding region from *Thermosynechococcus elongatus* BP-1 in tobacco and potato led to the stable production of CGP in plants and an increase in the amount of total Arg (Hühns et al. 2008, 2009; Nausch et al. 2016; Neumann et al. 2005). Although CPHE-producing bacteria were found in the colon of mammals (Sallam and Steinbuchel 2009a), feeding of CGP should not result in an increase in Arg in the blood, since Arg uptake is mainly restricted to the small intestine (Bröer 2008). This has been confirmed in a previous study where we showed that CGP can only be degraded by mice when degrading enzymes have been added to the feed (Ponndorf et al. 2016). Thus the co-expression of CGPase and CGP might enable the release of β-Asp-Arg dipeptides in the gut, making the addition of the enzyme unnecessary.

As already shown for bacteria (Richter et al. 1999; Sallam et al. 2009; Sallam and Steinbuchel 2009b, 2010), CPHB and CPHE were successfully produced in *Nicotiana benthamiana*. In case of CPHB, it was necessary to improve translation and stability for successful enzyme accumulation in plants, while CPHE was produced in high amounts in a stable manner without any adaptations. In addition, CPHE demonstrated a higher CGP degrading activity. Nevertheless, when added to crude *N. benthamiana* extracts, both CPHB and CPHE were able to degrade purified CGP at room temperature (RT) (Nausch and Broer 2016; Ponndorf et al. 2016).

In this study we analyzed the co-expression of different CGPases in CGP-producing *Nicotiana tabacum* var. Badischer Geudertheimer plants (BG) (Nausch et al. 2016) to allow the release of dipeptides during digestion of the feed.

Materials and methods

Transient expression in *Nicotiana tabacum* var. badischer geudertheimer (BG) and sample preparation

We used the constructs S-*cph*B-s and *gfp*-*cph*B-s for the production of S-CPHB and GFP::CPHB (Ponndorf et al. 2016) and *cphE241syn* (p*cph*E-s) for the production of CPHE (Nausch and Broer 2016), respectively. Clones of one parental BG plant were used for transient expression [Event 176 (Nausch et al. 2016)]. Four-week-old clones were transferred from tissue culture, containing Murashige–Skoog medium, to peat soil and grown under greenhouse conditions for another 2 weeks before vacuum infiltration as described by Ponndorf et al. (2016). The vectors were transferred into the *Agrobacterium tumefaciens* strain ICF320, cultivated in LB with 50 μg × ml^{-1} rifampicin and kanamycin, each. Cell cultures were centrifuged and diluted in infiltration buffer (100 mM MES (pH 5.5), 10 mM $MgCl_2$, 0.02% Silwet Gold). BG plants were submerged into the infiltration buffer and vacuum was applied (50 mbar, 5 min), using a freeze drier. Infiltrated plants were kept in the dark for one night. After 10 days post infiltration (dpi) 3–5 leaves per plant were harvested and cut vertically. One half was frozen immediately without

homogenization to analyze the *in planta* state, while the other half was homogenized using a PT2100-Homogenizer (Kinematica AG, Littau-Lucerne, Switzerland) (30.000 rpm; 30–45 s) and incubated overnight at 22–24 °C.

Quantification of CGP

CGP was quantified as described by Nausch et al. (2016). Freeze dried leaf material (30–35 mg) was homogenized with ceramic pills using a Precellys 24 homogenisator (VWR International GmbH, Erlangen, Germany) and incubated in 1 mL 50 mM Tris (pH 8) for 30 min. After centrifugation, the pellet was resuspended in 1 mL of 0.1 M HCl and incubated for 1 h at room temperature. After another centrifugation step, 800 mL of the supernatant was used for CGP analysis. For that 1–10 µL of sample were diluted with 0.1 M HCl to a final volume of 800 and 200 mL of 5 × RotiQuant Bradford reagent (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) was added. Samples were measured at 595 nm. A calibration curve was prepared with purified CGP from potato tubers, as described in 2.3. OD values of leaf samples from non-transgenic plants that were infiltrated with the corresponding *Agrobacterium* strain were subtracted from OD values of samples of infiltrated transgenic plants. Complete Protease Inhibitor Cocktail Tablets EASYpacks (Roche) were added to the 50 mM Tris buffer according to the manufacturer's advice. Additionally, we added 2 mM Pefabloc® (Sigma-Aldrich), 1 µg ml^{-1}Aprotinin and 1 mM PMSF (Sigma-Aldrich) to prevent unwanted CGP degradation during protein extraction.

Isolation of CGP and CGPase

Isolation of CGP from *Solanum tuberosum* tubers was conducted as described by Neubauer et al. (2012). Ground potatoes were stirred in 1% (w/v) $NaHSO_3$ for 1 h and the homogenate decanted through a sieve (≤0.5 mm pore diameter). The aqueous CGP-containing flow-through was passed again through a sieve (70 µm pore diameter) and the residue treated with 0.1 M HCl to solubilize CGP. CGP was separated from insoluble starch via centrifugation (4466×*g*, 15 min) and CGP precipitated in the supernatant by adjusting to pH 5 with NaOH.

CGPase from *E. coli* and *N. benthamiana* were isolated as described by Ponndorf et al. (2016) for CPHB and by Nausch and Broer (2016) for CphE. Since all CGPases contain a His-Tag, they were purified via Ni–NTA purification. The ProBond™ Purification System (Thermo Fisher Scientific) and a Glass Econo-Column® (BioRad, Hercules, USA), that were packed with the nickel resin was used. Crude *E. coli* or leaf extracts were centrifuged at (16,260×*g*, 4 °C) and after an initial equilibration of the resin with NPI buffer [50 mM NaH_2PO_4 (pH 8), 300 mM NaCl] containing 10 mM Imidazol, the supernatant was loaded onto the column. After two washing steps with buffer, containing 20 and 40 mM imidiazol, the His-tagged target protein was eluted with NPI buffer and 300 mM imidazol.

The eluted proteins were desalted via Sephadex G25 M Desalting Columns (Column PD-10; GE Healthcare Europe GmbH; Freiburg, Germany) and desalting buffer [20 mM Tris (pH 8) and 1 mM DTT].

In planta, semi in vivo and in vitro activity assays

The CGPase activity assay *in planta* was carried out as described above. The activity in vitro and in crude extracts was determined as described by Nausch and Broer (2016). Purified CGP (200 µg) was diluted either in PBS or in 1000 µg of crude TSP extracts of BG and 500 ng or 5 µg of purified CGPase were added respectively, diluted with PBS to a final volume of 5 ml and incubated at 22–24 °C. At 15 min time points, one tenth of the original reactions was collected and the reaction stopped by TCA precipitation. Pellets were resuspended in 25 µl SDS-PAGE loading buffer, separated in a 12% SDS-PAGE and subsequently Coomassie stained.

SDS PAGE and western blot

Samples were resuspended in loading buffer, (10% glycerin, 150 mM Tris (pH 6.8), 3% SDS, 1% β-mercaptoethanol, and 2.5% bromophenol blue) and denaturated at 95 °C for 5 min prior to separation in a 12% SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane, using a BioRad Trans-Blot semi-dry transfer cell. Two mA/cm2 were applied for 1 h using 50 mM Tris, 40 mM glycine, 0.01% SDS, and 20% methanol as the transfer buffer (pH 8.5). CPHB variants were detected via a self-made rabbit anti CPHB

antibody (Ponndorf et al. 2016) and a goat anti-rabbit, POD-conjugated antibody (Dianova, Hamburg, Germany). For the detection of GFP, a rabbit anti-GFP antibody (SySy GmbH, Göttingen, Germany) and the same secondary antibody were used as previously described. CPHE was detected as described by Nausch and Broer (2016) via its His-Tag using a mouse anti-His and a donkey anti-mouse, POD conjugated antibody (both Dianova). Proteins were detected via chemiluminescence using a Kodak Biomax light X-ray film (VWR; Darmstadt, Germany).

Calculation and prediction of protein properties

Molecular weight of proteins was predicted using the sequence manipulation suite homepage (Stothard 2000).

Statistical evaluation

Exploratory data analysis, the comparison of means and creation of box plots was carried out using IBM SPSS Statistics 22. Tests were chosen depending on the data properties. Normal distribution was tested using the Shapiro–Wilk test with $p > 0.05$ defined as normally distributed. Homogeneity of variation was tested using the Levene statistic with $p > 0.05$ defined as homogenous. Depending on these requirements and the respective dataset the corresponding statistical tests were chosen.

Results

The MagnICON® transient expression system (Marillonnet et al. 2005) was used to co-express CGPase in transgenic *N. tabacum* var. BG plants (event BG 176) producing CGP in the plastid (Nausch et al. 2016). We analyzed three CGPase variants, which had been successfully produced in plants before and were confirmed to be active in plant material. The intercellular CGPase CPHB from *Thermosynechococcus elongatus* BP-1 was targeted to the chloroplast by the fusion to the transit peptide of the small subunit of RuBisCO (S-CPHB) or stabilized in the cytosol by the fusion to the green fluorescence protein (GFP) (GFP::CPHB) (Ponndorf et al. 2016). The intercellular CGPase CPHE from *Pseudomonas alcaligenes* (Nausch and Broer 2016; Sallam et al. 2011) was expressed in the cytosol.

Infiltrated clones of BG 176 were harvested at 10 dpi, and the CGP content was determined in freeze-dried material. Leaves were harvested at 10 dpi to allow even small amounts of CGPase to degrade CGP in the chloroplast (S-CPHB), or in the case that the separation might not be complete (GFP::CPHB, CPHE). The CGP content varied between independent experimental replicates (data not shown), but these differences did not occur between plants of the same replicate.

CPHE shows higher activity compared to S-CPHB under in vitro conditions

As shown previously in *N. benthamiana,* CPHB variants and CPHE were active in vitro, and S-CPHB and GFP::CPHB did not differ in their activity in *N. benthamiana* (Nausch and Broer 2016; Ponndorf et al. 2016). Therefore, purified S-CPHB (5 µg) and CPHE (0.5 µg) isolated from *N. benthamiana* were incubated with purified CGP (20 µg) either under in vitro conditions or in crude BG extracts, containing 100 µg of total soluble protein (TSP, Fig. 1). The reaction was stopped every 15 min and analyzed with SDS-PAGE by Coomassie staining. Experiments were conducted at least twice. Within the time investigated, the activity of CPHE in BG crude extracts was much higher than S-CPHB. Although the amount of S-CPHB added to the samples was 10 times higher than CPHE, its degradation of the same amount of CGP was approximately 2 times slower (Fig. 1).

Additionally, when the amount of S-CPHB was reduced to 2.5 µg per sample, no visible decrease in CGP content occurred over the time investigated (data not shown). In contrast, only 100 ng of CPHE per sample are sufficient for a detectable CGP degradation (Nausch and Broer 2016).

Expression of chloroplast-targeted CGPase does not result in CGP degradation *in planta*

In order to analyze whether the storage of Arg in β-Asp-Arg dipeptides is superior to CGP storage in the chloroplasts, CGPases should be transported to the chloroplast where CGP is present. Since CPHE could not be targeted to the plastids of *N. benthamiana* (Nausch and Broer 2016), we only investigated the effect of chloroplast targeting for S-CPHB in BG. At 10 dpi comparable amounts of CGP were analyzed in uninfiltrated plants [median value, 6.7 µg mg^{-1} dry

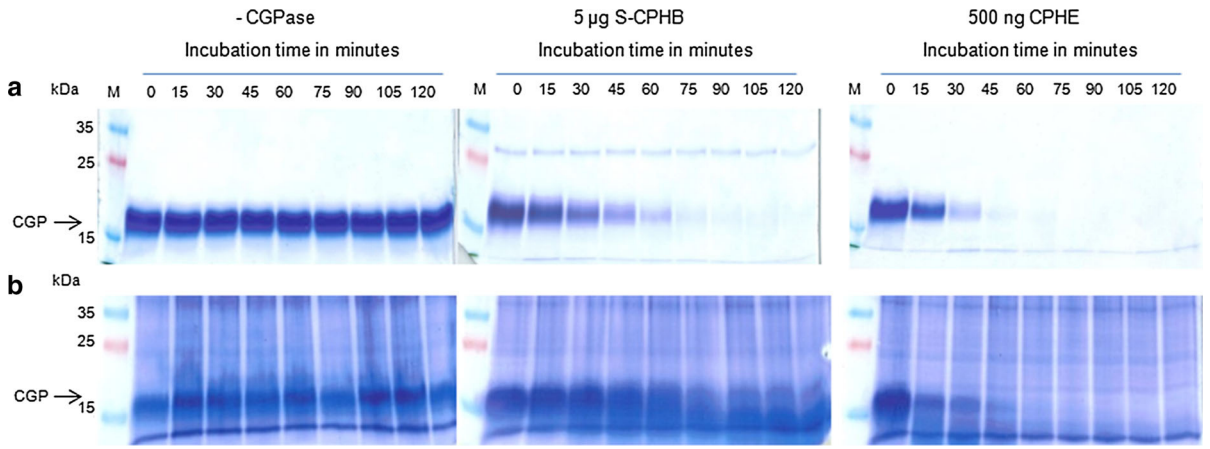


Fig. 1 Comparison of enzyme activity of S-CPHB and CPHE **a** in vitro (CPHE already shown by Nausch and Broer 2016). **b** Degradation of 20 µg isolated CGP in 100 µg total soluble protein (TSP) isolated from *N. tabacum* Badischer Geudertheimer M = PageRuler^{TM} Plus Prestained Protein ladder; -CGPase = control without the addition of S-CPHB and CPHE respectively

weight (dw)], the empty vector control (10.0 µg mg^{-1} dw) and in plants transfected with the S-CPHB expressing vector (8.0 µg mg^{-1}dw); (Fig. 2). The observed differences were not significant (Dunnet-T3 test). The presence of S-CPHB was verified by western blot (Fig. 3). The two bands represent the expected size of the monomer [29 kilo Dalton (kDa)] and dimer (ca 70 kDa) of the mature protein. The expected size of the unprocessed monomer including the signal peptide is about 35 kDa. Stronger signal intensity was observed for S-CPHB compared to GFP::CPHB in the anti-CPHB western blot, but was not detectable at all when using anti-His antibodies, probably due to the lower sensitivity of this antibody. Because only the mature CPHB was detected in the western blot, it is likely that the enzyme enters the chloroplast. However, this expression did not decrease the amount of CGP in the plastids. After homogenization and incubation of the plant material for 24 h, no S-CPHB signals could be detected (Fig. 3) indicating instability of the enzyme after cell homogenization.

Transient expression of CPHE in CGP producing *N. tabacum* BG plants is sufficient to completely degrade CGP in homogenized leaf tissue

The spatial separation of CGP and CGPase should prevent degradation of CGP in the chloroplast *in planta* and might enable the controlled degradation of CGP after cell homogenization. Therefore, BG 176 clones were infiltrated with vectors *gfp-cph*B-s and *cphE241syn* encoding GFP::CPHB and CPHE to allow cytosolic enrichment of CGPase. Leaf material was harvested at 10dpi, homogenized and incubated for 24 h at RT. The CGP content was measured directly after harvest and after cell homogenization and incubation. The expression of cytosolic GFP::CPHB did not lead to a measurable degradation of CGP in comparison to the GFP control, neither before nor after cell homogenization (Fig. 2). The expression of CPHE in the cytosol did not result in CGP degradation in intact leaves, but a significant decrease ($p = 0.00$, Dunnet T3 Test, $\alpha = 0.01$) was measured in homogenized tissue. While the mean CGP content averaged 21 µg CGP mg dw^{-1} directly after homogenization, only 1 µg CGP mg dw^{-1} was present after 24 h (Fig. 2).

In order to investigate whether the absence of CGP degradation for most constructs was caused by the absence of the CGPase or its inactivity, western blots were conducted (Fig. 3). Anti-CPHB and anti-GFP western blots seem to be more sensitive compared anti-His western blots, since 5 times more purified protein was necessary to give the same signal intensity in anti-His blots compared to anti-CPHB and anti-GFP blots.

As shown in Fig. 3, GFP::CPHB was detected in samples directly after harvest (T0), while a decrease in protein was observed after cell homogenization and incubation (T24). Using anti-GFP antibodies, a protein corresponding to the size of the GFP protein (29 kDa) was detected. Although GFP and CPHB monomers

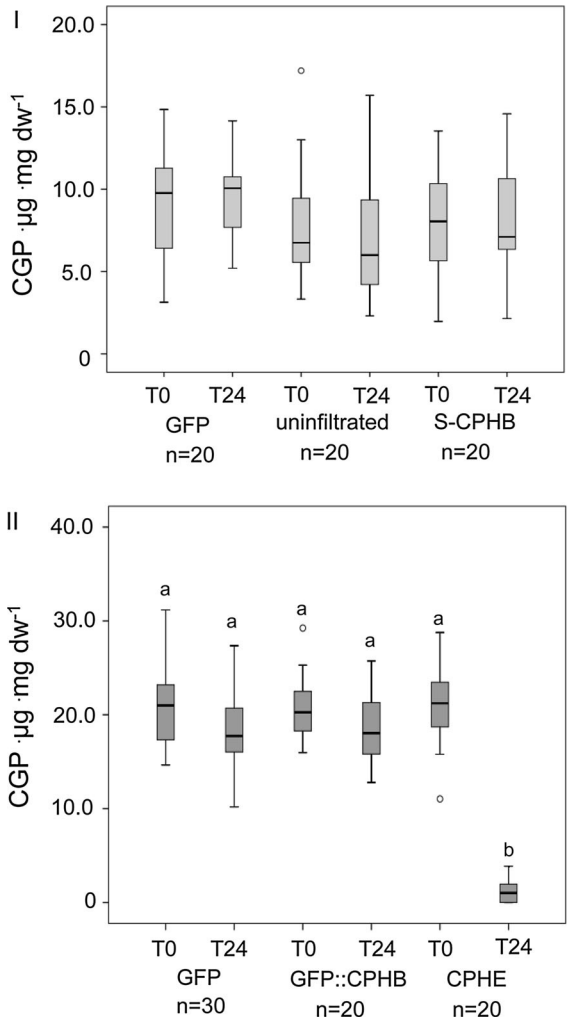


Fig. 2 *In planta* and crude plant extract activity assay of different cyanophycinase (CGPase) variants at two different experimental time points (**I** and **II**). Infiltrated *N. tabacum* Badischer Geudertheimer leaves were harvested at 10 days post-infiltration (dpi), and vertically cut into halves. One half was frozen immediately without homogenization (T0). The other half was homogenized and incubated for 24 h at room temperature (T24). The material was freeze dried and CGP content was measured. Circles in the *box plot* show outliers; CGP, cyanophycin; dw, dry weight; NIC, *N. tabacum* Badischer Geudertheimer-176 near isogenic control; GFP, empty vector control: pICH18711 expressing the green fluorescent protein (Marillonnet et al. 2005). Values with different letters (*a*, *b*) significantly differ between groups (Dunnet T3, $p < 0.01$)

have an equal size we assume this band is specific for GFP, because no CPHB signals were detected in the anti-CPHB western blots for the same sample

In contrast to S-CPHB and GFP::CPHB, CPHE was clearly detectable in T0 as well as T24 samples.

Discussion

Here we could show for the first time that sufficient storage of arginine and timely delivery of β-Asp Arg dipeptides is possible when CGP and CPHE are coexpressed in a commercial tobacco cultivar but produced in separate compartments. The commercial usage of CGP producing plants to supplement Arg in feed depends on the storage of high amounts of CGP or β-Asp-Arg dipeptides in the plant and its controlled and complete degradation to β-Asp-Arg dipeptides in the extract. The fact that the separation of CGP (chloroplast) and CGPase (cytosol) is sufficient to prevent premature degradation and that the co-expression of CPHE in the CGP-producing commercial *N. tabacum* variety BG leads to complete CGP degradation 24 h after homogenization supports the assumption that the valorization of feed is possible without additional effort. Considering that the degradation of CGP in the gut after co-delivery of isolated CGP and CGPase is possible, and that β-Asp-Arg dipeptide uptake has been proven (Ponndorf et al. 2016), this result represents another crucial step for the enrichment of β-Asp-Arg dipeptides in feed.

Degradation of CGP could not be observed after targeting CPHB to the chloroplast. In contrast to CPHE, where only unprocessed protein was found when targeted to plastids (Nausch and Broer 2016), in this study the presence of processed CPHB after infiltration of BG indicated an import of at least parts of the enzyme (Abad et al. 1989; Lamppa and Abad 1987; Richter and Lamppa 1998; Robinson and Ellis 1984). In a previous study, we demonstrated that different CPHB variants are unstable outside of the chloroplast (Ponndorf et al. 2016). Here we show in addition that CGP degradation depends on the amount of CGPase, hence the most obvious cause for non-degradation of CGP in the chloroplast is low CPHB activity or low protein content, which is in line with the results obtained for the cytosol.

All cytosolic CGPase variants could be detected but, as expected, no CGP degradation was shown without cell homogenization. As desired, the spatial separation of CGP and CGPase into different cell compartments prevents the degradation of CGP. The

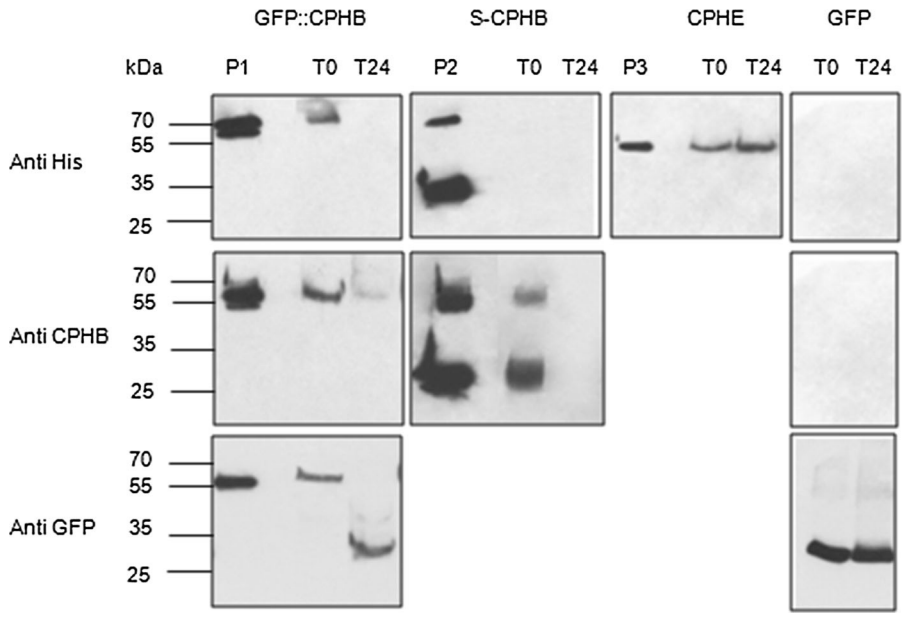


Fig. 3 Western blot analysis of 100 µg total soluble protein (TSP) isolated from *N. tabacum* Badischer Geudertheimer leaf material. Leaves were cut vertically. One half was frozen immediately (T0) the other half was homogenized and incubated for 24 h (T24). GFP, expressed by vector pICH18711 (Marillonnet et al. 2005) was used as control. kDa, kilodalton; P1, positive control GFP::CPHB isolated from *E.coli*; P2, positive control CPHB isolated from *E.coli*; P3, positive control: CPHE isolated from *E.coli*; anti-his western: 5 ng of P1, P2 and P3; anti-CPHB western: 1 ng of P1 and P2, anti-GFP western: 1 ng of P1

protection of recombinant proteins from cytosolic proteases via localization in different plant compartments has often been described as reviewed by Pillay et al. (2014). Hence storage and accumulation of CGP in chloroplasts is possible in parallel to the accumulation of the degrading enzyme in the cytosol.

After cell homogenization, nearly the complete pool of CGP was degraded in plants expressing CPHE after 24 h. This is accompanied by stable and high expression of CPHE similar to that described by Nausch and Broer (2016). In contrast to this, GFP::CPHB did not cause a measurable degradation of CGP and proved to be instable in crude plant extract. This instability seems to be due to a degradation of CPHB, since only the GFP domain of the fusion protein was still detectable, likely due to its high stability in plant cells (Sheen et al. 1995). This instability of GFP::CPHB was not observed in previous studies in *N. benthamiana* (Ponndorf et al. 2016), probably due to the substantially higher expression levels of the MagnICON vectors in *N. benthamiana* (Nausch et al. 2012a).

In addition to the protein stability, CPHE is more active compared to both S-CPHB and GPF::CPHB and therefore more suitable for the expression in CGP-producing plants. The data presented here show for the first time that it is possible to express sufficient amounts of active CPHE in plants to degrade the complete pool of CGP present in the same plant.

However, the high amounts of CPHE observed after transient expression will probably not be achieved in stably transformed plants (Gleba et al. 2005; Nausch et al. 2012b). Therefore, it remains to be seen whether stably transformed plants can produce sufficient amounts of CGPase to degrade CGP into β-Asp-Arg dipeptides. Stable expression might also demand another expression system such as seeds. Huckauf et al. (personal communication) could demonstrate that the expression of the viral antigen VP60 in pea seeds was significantly higher compared to transient expression in *N. benthamiana*. Due to their high nutritional value, peas would be a perfect feed additive. Stable accumulation of CGP has already been shown by Baars et al. (in preparation) in this plant species. Hence the coproduction of CGP and CPHE in pea seeds might be a promising strategy to enhance the Arg content in food and feed. Nevertheless, at least in mice, Arg

derived from β-Asp-Arg dipeptides were not bioavailable (Ponndorf et al. 2016).

However positive effects of β-Asp-Arg dipeptide supplementation on fish body mass have been described in aquaculture (Dr. Martin Krehenbrink, personal communication). Hence β-Asp-Arg dipeptides might also be bioavailable for other species, and it remains to be ascertained whether this holds true for other mammals. The coexpression of a suitable isoaspartyl dipeptidase might also increase bioavailability.

Acknowledgements We would like to express our very great appreciations to Alex Rajewski for proof-reading and the helpful suggestions and Dr. Martin Krehenbrink, Cysal GmbH, for sharing their aquaculture results.

Funding This study was funded by the University of Rostock.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Abad MS, Clark SE, Lamppa GK (1989) Properties of a chloroplast enzyme that cleaves the chlorophyll a/B binding-protein precursor—optimization of an organelle-free reaction. Plant Physiol 90:117–124. doi:10.1104/Pp.90.1.117

Allen MM, Morris R, Zimmerman W (1984) Cyanophycin granule polypeptide protease in a unicellular cyanobacterium. Arch Microbiol 138:119–123

Bröer S (2008) Amino acid transport across mammalian intestinal and renal epithelia. Physiol Rev 88:249–286

Gleba Y, Klimyuk V, Marillonnet S (2005) Magnifection—a new platform for expressing recombinant vaccines in plants. Vaccine 23:2042–2048. doi:10.1016/j.vaccine.2005.01.006

Gupta M, Carr NG (1981) Enzyme-activities related to cyanophycin metabolism in heterocysts and vegetative cells of Anabaena spp. J Gen Microbiol 125:17–23

Hu SD, Li XL, Rezaei R, Meininger CJ, McNeal CJ, Wu GY (2015) Safety of long-term dietary supplementation with largening in pigs. Amino Acids 47:925–936. doi:10.1007/s00726-015-1921-5

Hühns M et al (2008) Plastid targeting strategies for cyanophycin synthetase to achieve high-level polymer accumulation in *Nicotiana tabacum*. Plant Biotechnol J 6:321–336. doi:10.1111/j.1467-7652.2007.00320.x

Hühns M et al (2009) Tuber-specific cphA expression to enhance cyanophycin production in potatoes. Plant Biotechnol J 7:883–898. doi:10.1111/j.1467-7652.2009.00451.x

Lamppa GK, Abad MS (1987) Processing of a wheat light-harvesting chlorophyll a/B protein-precursor by a soluble enzyme from higher-plant chloroplasts. J Cell Biol 105:2641–2648. doi:10.1083/jcb.105.6.2641

Ma X, Zheng C, Hu Y, Wang L, Yang X, Jiang Z (2015) Dietary L-arginine supplementation affects the skeletal longissimus muscle proteome in finishing pigs. PLoS ONE 10:1–16

Marillonnet S, Thoeringer C, Kandzia R, Klimyuk V, Gleba Y (2005) Systemic agrobacterium tumefaciens-mediated transfection of viral replicons for efficient transient expression in plants. Nat Biotechnol 23:718–723. doi:10.1038/Nbt1094

Matthews DM, Adibi SA (1976) Peptide absorption. Gastroenterology 71:151–161

Nausch H, Broer I (2016) Cyanophycinase CphE from *P. alcaligenes* produced in different compartments of *N. benthamiana* degrades high amounts of cyanophycin in plant extracts. Appl Microbiol Biotechnol. doi:10.1007/s00253-016-8020-8

Nausch H, Mikschofsky H, Koslowski R, Meyer U, Broer I, Huckauf J (2012a) High-level transient expression of ER-targeted human interleukin 6 in *Nicotiana benthamiana*. PLoS ONE. doi:10.1371/journal.pone.0048938

Nausch H, Mischofsky H, Koslowski R, Meyer U, Broer I, Huckauf J (2012b) Expression and subcellular targeting of human complement factor C5a in Nicotiana species. PLoS ONE. doi:10.1371/journal.pone.0053023

Nausch H et al (2016) Tobacco as platform for a commercial production of cyanophycin. New Biotechnol. doi:10.1016/j.nbt.2016.08.001

Neubauer K et al (2012) Isolation of cyanophycin from tobacco and potato plants with constitutive plastidic cphA(Te) gene expression. J Biotechnol 158:50–58. doi:10.1016/j.jbiotec.2011.12.008

Neumann K, Stephan DP, Ziegler K, Huhns M, Broer I, Lockau W, Pistorius EK (2005) Production of cyanophycin, a suitable source for the biodegradable polymer polyaspartate, in transgenic plants. Plant Biotechnol J 3:249–258. doi:10.1111/j.1467-7652.2005.00122.x

Obst M, Oppermann-Sanio FB, Luftmann H, Steinbuchel A (2002) Isolation of cyanophycin-degrading bacteria, cloning and characterization of an extracellular cyanophycinase gene (cphE) from *Pseudomonas anguilliseptica* strain BI—the cphE gene from *P. anguilliseptica* BI encodes a cyanophycin-hydrolyzing enzyme. J Biol Chem 277:25096–25105. doi:10.1074/jbc.M112267200

Pillay P, Schluter U, van Wyk S, Kunert KJ, Vorster BJ (2014) Proteolysis of recombinant proteins in bioengineered plant cells. Bioengineered 5:15–20. doi:10.4161/Bioe.25158

Ponndorf D et al (2016) Stable production of cyanophycinase in *Nicotiana benthamiana* and its functionality to hydrolyse cyanophycin in the murine intestine. Plant Biotechnol J. doi:10.1111/pbi.12658

Richter S, Lamppa GK (1998) A chloroplast processing enzyme functions as the general stromal processing peptidase. Proc Natl Acad Sci USA 95:7463–7468

Richter R, Hejazi M, Kraft R, Ziegler K, Lockau W (1999) Cyanophycinase, a peptidase degrading the cyanobacterial reserve material multi-L-arginyl-poly-L-aspartic acid (cyanophycin)—molecular cloning of the gene of *Synechocystis* sp. PCC 6803, expression in *Escherichia coli*, and biochemical characterization of the purified enzyme. Eur J Biochem 263:163–169. doi:10.1046/j.1432-1327.1999.00479.x

Robinson C, Ellis RJ (1984) Transport of proteins into chloroplasts. Partial purification of a chloroplast protease involved in the processing of important precursor polypeptides. Eur J Biochem 142:337–342

Sallam A, Steinbuchel A (2009a) Cyanophycin-degrading bacteria in digestive tracts of mammals, birds and fish and consequences for possible applications of cyanophycin and its dipeptides in nutrition and therapy. J Appl Microbiol 107:474–484. doi:10.1111/j.1365-2672.2009.04221.x

Sallam A, Steinbuchel A (2009b) Process for the preparation of dipeptides from cyanophycin emplyoing the isolated *Pseudomonas alcaligenes* DIP1 CGPase CphEaI. European Patent Application, Publication No. 2 133 419 A1 (16.12.2009)

Sallam A, Steinbuchel A (2010) Dipeptides in nutrition and therapy: cyanophycin-derived dipeptides as natural alternatives and their biotechnological production. Appl Microbiol Biotechnol 87:815–828. doi:10.1007/s00253-010-2641-0

Sallam A, Kast A, Przybilla S, Meiswinkel T, Steinbuchel A (2009) Biotechnological process for production of beta-dipeptides from cyanophycin on a technical scale and its optimization. Appl Environ Microbiol 75:29–38. doi:10.1128/AEM.01344-08

Sallam A, Kalkandzhiev D, Steinbuchel A (2011) Production optimization of cyanophycinase ChpEal from *Pseudomonas alcaligenes* DIP1. AMB Express 1:38. doi:10.1186/2191-0855-1-38

Sancho-Vaello E, Fernandez-Murga ML, Rubio V (2009) Mechanism of arginine regulation of acetylglutamate synthase, the first enzyme of arginine synthesis. FEBS Lett 583:202–206. doi:10.1016/j.febslet.2008.12.001

Sheen J, Hwang SB, Niwa Y, Kobayashi H, Galbraith DW (1995) Green-fluorescent protein as a new vital marker in plant-cells. Plant J 8:777–784. doi:10.1046/j.1365-313X.1995.08050777.x

Simon RD (1987) Inclusion bodies in the cyanobacteria: cyanophycin, polyphosphate, polyhedra bodies. In: Fay P, Van Baalen C (eds) The cyanobacteria. Elsevier, Amsterdam, pp 192–222

Simon RD, Weathers P (1976) Determination of structure of novel polypeptide containing aspartic-acid and arginine which is found in cyanobacteria. Biochem Biophys Acta 420:165–176

Stothard P (2000) The sequence manipulation suite: JavaScript programs for analyzing and formatting protein and DNA sequences. Biotechniques 28:1102

Utagawa T (2004) Production of arginine by fermentation. J Nutr 134:2854s–2857s

Wang B et al (2015) Effects of dietary arginine supplementation on growth performance, flesh quality, muscle antioxidant capacity and antioxidant-related signalling molecule expression in young grass carp (*Ctenopharyngodon idella*). Food Chem 167:91–99. doi:10.1016/j.foodchem.2014.06.091

Wenzel U, Meissner B, Doring F, Daniel H (2001) PEPT1-mediated uptake of dipeptides enhances the intestinal absorption of amino acids via transport system b(0, +). J Cell Physiol 186:251–259. doi:10.1002/1097-4652(200102)186:2<251:AID-JCP1027>3.0.CO;2-F

Winter G, Todd CD, Trovato M, Forlani G, Funck D (2015) Physiological implications of arginine metabolism in plants. Front Plant Sci. doi:10.3389/Fpls.2015.00534

Wu G et al (2007) Pharmacokinetics and safety of arginine supplementation in animals. J Nutr 137:1673S–1680S

Wu GY, Bazer FW, Dai ZL, Li DF, Wang JJ, Wu ZL (2014) Acid nutrition in animals: protein synthesis and beyond amino. Annu Rev Anim Biosci 2:387–417. doi:10.1146/annurev-animal-022513-114113

Ziegler K, Diener A, Herpin C, Richter R, Deutzmann R, 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). Eur J Biochem 254:154–159

Ziegler K, Deutzmann R, 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 section C-a. J Biosci 57:522–529

C Discussion

In this work we describe a new and promising approach for the supply of the conditional AA Arg to the chow of livestock: The production of CGP, a bacterial storage protein which sequence contains high amounts of Arg (Simon and Weathers, 1976) and the expression of the corresponding enzyme CGPase in plants. CGPase catalyzes the degradation of CGP to β-Asp-Arg dipeptides (Gupta and Carr, 1981; Richter et al., 1999) which is of benefit, because the uptake of dipeptides is not only more efficient, but also promotes the uptake of single AA (Matthews and Adibi, 1976; Wenzel et al., 2001). This approach shows two possibilities to supplement Arg to the diet.

I: The production of both proteins in plants and supplementation of the isolated proteins to the feed, and

II: The coexpression of both proteins in one plant either produced in different cell compartments or in the chloroplast. The supply of the β-Asp-Arg dipeptides is then possible during feed uptake, when the cells are homogenized or are accumulated in the chloroplast during plant growth, which makes it unnecessary to isolate the proteins.

The first approach is already described in bacterial cell cultures (Sallam et al., 2009; Sallam and Steinbuchel, 2010; Santos et al., 2012), but the use of plants could be more promising, because plant production can be easily scaled up and needs only soil or substrate, light, water and fertilizers. In addition it requires less investment compared to fermentation. Plants, depending on the cultivar, also can produce a higher biomass per time, space and energy supply and hence, a higher yield of protein could be possible (Fischer et al., 1999; Ma et al., 2003; Merlin et al., 2014; Sabalza et al., 2014). It has to be kept in mind that lower investment applies for stably transformed plants but that this is not necessarily true for transiently produced proteins, because the MagnICON® transient expression system requires bacteria, the respective growth media and fermentation equipment, as well as the equipment for the vacuum infiltration. In this case, the suitability of plants compared to bacteria depends on the produced amount of CGPase in combination with the produced biomass of *N. benthamiana.* Because we did not evaluate the overall production of CPHB or CPHE in plants, it is not yet known whether the CGPase production in plants could be more efficient than in bacteria.

The coexpression of CGP and CGPase in plants seems to be more interesting than the first approach, because this could make an isolation of the proteins unnecessary which will safe material costs, chemical waste and labour. Up to date the coexpression of a storage protein

and a corresponding degrading enzyme has not been described for the biofortification of plants with AA and presents a new scientific strategy.

For both approaches it is necessary to meet a couple of requirements. For the first approach it is important to produce both proteins in industrial scale and provide an easy and cheap possibility for isolation. Furthermore, the degradation in the animal has to be possible without any allergenic potential or negative influences on the health of animals caused either by CGP or CGPase. Meeting the requirements of the second approach is more complicated. Beside the aforementioned demands and the main goal, the increase of bound Arg in plants, it is important to consider any possible influences on the endogenous metabolism of the host plant, which could change the composition of the diet. Effects of the increase of AA by different strategies like altered AA composition (Zhu and Galili, 2003), aberrant phenotypes which negatively influenced the agronomic performance of plants (Angelovici et al., 2011; Liu et al., 2016) and a change in the composition of endogenous proteins (Cohen et al., 2016; Liu et al., 2016) were described before and their possible occurrence has to be considered for CGP as well as CGPase. In the following, I want to discuss how our approaches meet the named requirements and provide an overview about remaining experiments and work. Finally, I want to discuss, if one or both approaches are suitable to replace the conventional Arg supplementation in the diets of livestock.

C1 Can cyanophycin be produced in high amounts in plants and which cultivars are most promising for a commercial production?

The first important requirement for both approaches described, is a high CGP and CGPase production in plants to supply sufficient amounts of Arg. Hereby, the total production of the protein does not only depend on the total accumulation in the plant, but also on the produced biomass, the time for cultivation and m² needed which varies between cultivars.

For the first approach, the production of CGP and CGPase in plants and supplementation of the isolated proteins to the feed, *N. tabacum* is a promising cultivar, because it is industrial scalable and already used for the production of a broad range of recombinant proteins (Tremblay et al., 2010; Tuse et al., 2014; Xu et al., 2012). So far CGP was produced in PH, STA and STD, where the highest amounts of CGP were 6.8 % CGP of the DW in PH (Hühns et al., 2008) and up to 7.5 % DW in STA (Hühns et al., 2009). However, PH is not suitable for the commercialized production of CGP, because this cultivar does not produce a high biomass. Hence other *N. tabacum* cultivars which were bred for a commercial application were used to produce CGP. As described in Chapter B1 we showed that the cultivars BG and VG, which produced 170 % more biomass, are more promising than PH. In BG a higher CGP amount was measured with up to 8.8 % DW^{-1}. Crossbreeding of the PH elite event and

BG even led to 9.4 % CGP·DW^{-1}, but the biomass was reduced to 76-84 % compared to the BG controls. After backcrossing with BG the biomass increased, but the CGP content decreased. Hence, the production of CGP in directly transformed plants was more suitable. Furthermore, BG showed a higher homogeneity of the CGP content compared to VG, which is another advantage of this cultivar. A low variation between parents and descendants as well as between siblings promotes the development of a master seed bank, which enables a stable CGP yield between generations. It was also shown that the transient expression of CGPase in BG and *N. benthamiana* is possible and leads to high amounts of recombinant protein which is discussed in detail later on.

BG also produces more CGP than the potato cultivars STD and STA. STA furthermore showed a negative effect of high CGP amounts on the tuber phenotype, which was expressed in brown staining of the tubers (Hühns et al., 2008), while no influence of CGP production on the phenotype of BG was visible. For the commercialized production of CGP it is also advantageous to decouple the harvest of the plants and the CGP isolation. Therefore it is important to enable long term storage of the plant material. In case of potato tubers this usually can easily be realized, but it was shown that the browning of the tubers increased during storage. However, the CGP content did not decrease (Hühns et al., 2008), but in high CGP lines the tubers went slate and it was impossible to isolate pure CGP (Ponndorf unpublished). During ensilage of BG plants the CGP content was also stable, showing that the long term storage of CGP in ensilaged tobacco material is possible.

Hence, BG seems to be the most promising cultivar for the commercialized production of CGP, but their might still be room for improvement by further selection and breeding, because it was already shown that conventional breeding can enhance the produced amount of recombinant proteins in transgenic plants (Hühns et al., 2008; Mikschofsky et al., 2009; Streatfield, 2007). In conclusion we can state that our first hypothesis: “The expression of cyanophycin synthetase (CPHA) in *N. tabacum* cv. Badischer Geudertheimer and cv. Virginia Golta leads to a higher production of cyanophycin compared to *Solanum tuberosum* and *N. tabacum* Petit Havanna SRI” was proven und can be accepted.

For the second approach, the direct supply of CGP and CGPase to the feed, *N. tabacum* BG and VG are not suitable, because both are non-food plants due to the high content of the alkaloid nicotine. Hence, the production in tobacco seeds, which can be administrated to animals (Rossi et al., 2014), or other cultivars is necessary. One cultivar that shows the potential to produce CGP is pea (*Pisum sativum*) (Baars unpublished). However, CGP production in pea leads to an aberrant growth and reduced germination of the seeds (Baars, personal communication) which limits the potential of this cultivar for our application, unless this problem can be solved. Additionally, CGP and CGPase could be produced in more

common food plants like corn. Since CGP production in maize is not published up to now, it is unknown whether this is possible. The use of low alkaloid tobacco (MSLA, Menassa et al. (2001) might be an alternative. This tobacco can be added to the chow of mice with up to 20% without negative influences (Menassa et al., 2007). Since it was shown that tobacco has the potential to produce CGP and CGPase it is likely that both proteins can be produced in MSLA which might then represent an alternative to BG and VG. However, in this work we focused on proof of concept research and used *N. tabacum* as model plant for time inexpensive analysis of our strategy and to determine the suitability of CGP as Arg source in plants by CGPase mediated degradation.

C2 Is cyanophycin a suitable Arginin store in plants?

The approach of using recombinant stable storage proteins to increase the AA content in plants is not new and was conducted successfully before. However, different requirements have to be considered when recombinant proteins are used as stable storage proteins. First, the protein has to be expressed in a sufficient amount and it must be stable in plants to increase the AA content. This was, for example, the limitation when synthetic proteins were used as AA source (Beauregard and Hefford, 2006; Galili and Amir, 2013). As described previously, CGP accumulates in high amounts and in a stable manner during plant growth and in consequence meets these demands.

Additionally, the used storage proteins have to lead to an accumulation of the desired AA which have to be available during digestion of the feed. The production of CGP in plants led to four times more Arg and twice the Asp compared to the controls in total AA content in VG and BG. This is one of the highest increases in the AA content described for stable storage proteins. So far the mean amounts ranged between 30-65 % (Altenbach et al., 1992; Lee et al., 2003; Liu et al., 2016; Wong et al., 2015; Yu et al., 2005; Yue et al., 2014), while the highest increase was described by Molvig et al. (1997), who expressed the sunflower 2S albumin in lupine and enhanced the Met content up to 97%. Other approaches which led to an up to 80fold enhancement of Lys in *A. thaliana* were associated with major drawbacks, which are discussed later (Zhu and Galili, 2003). To benefit from the increased Arg content in plants it is necessary that CGP can be digested in the GIT of animals. This requires the presence of CGPase, because we showed that CGP is not degradable in the intestine of mice (chapter B3) without the enzyme. Though the presence of bacteria that express CPHE in the colon of mammals, birds and fish was described (Sallam and Steinbuchel, 2009), the β-Asp Arg dipeptides will most likely not be available, because the AA uptake takes place in the small intestine prior to a possible degradation of CGP in the colon (Bröer, 2008). We also detected large amounts of CGP in the colon of mice after administration of CGPase free

pellets, what indicates that CGP is not degraded in the GIT. This is suggesting that either a degradation, mediated by CPHE expressing bacteria, is not possible *in vivo*, or that these bacteria do not exist in all mammals.

We showed that feeding of CGP and CGPase in mammals leads to the successful degradation of CGP and in consequence to the release β-Asp-Arg dipeptides and their absorption. Hence, CGP in combination with CGPase meets the demands according to the accumulation of AA and their ability during digestion of the food.

The uptake of the proteins with food and feed introduces another important requirement. The administration of CGP and CGPase should not have impacts on the health of the target organism such as toxicity and a possible allergenic potential. This was the major drawback of the Met rich storage protein 2S-serum albumin from Brazil nut which was used to increase the Met content in transgenic plants (Altenbach et al., 1992; Altenbach et al., 1989) and revealed to transfer the allergenic potential from Brazil nut to the host plants (Nordlee et al., 1996). We did not conduct any allergenicity test for CGPase so far, but tests for CGP provided no hints for an allergic potential (Steinmann, 2011).

Furthermore, the production of CGP and CGPase in plants has also to be possible without significant interference with the endogenous metabolism. For example, high amounts of Lys in seeds led to the reduction of seed germination in other approaches (Angelovici et al., 2011; Liu et al., 2016; Zhu and Galili, 2003). We did not observe a correlation between the CGP content and seed germination (chapter B1), but the toxicity of CGP in the cytosol of plants and a negative influence on the growth and germination was described previously (Neumann et al., 2005). Though it was possible to mitigate this negative influence by the targeting of the CGP production to the plastids (Hühns et al., 2009; Hühns et al., 2008) high CGP contents still had an influence on the phenotype of potato tubers and CGP also leads to reduced growth and germination in pea (Baars, personnel communication). This might be caused by a shift in the AA composition in the host plant which might result in an interference with the production of endogenous proteins (Nausch et al., 2016). Hühns et al. (2009) described a reduction in the free Asp and Glu pool, but no analysis of the proteome was conducted. Hence, it is not clear what exactly is the reason for the phenotypic differences. Since we did not analyze the composition of other AA but the total Arg and Asp content, we can't discuss whether other AA or proteins are influenced in BG and VG plants by CGP. Alterations of the AA composition and endogenous proteins have also been described in different other publications using storage proteins or other strategies to increase the AA composition. Biofortification of plants with AA such as Lys and Met did not only lead to an altered storage protein composition (reviewed by Galili and Amir (2013); Cohen et al. (2016)) but also to variations in the content of different AA such as Pro, Leu, Gln and Glu (Liu et al.,

2016; Sikdar et al., 2016; Wong et al., 2015). This suggests that biofortification of plants with AA influences the endogenous biosynthesis. Hence, total AA composition and the expression of endogenous proteins has to be considered in the future to characterize a possible influence of CGP in detail. This does also account for CGPase, which proteolytic activity might influence other proteins, though this is unlikely due to its high substrate specificity (Law et al., 2009).

We showed that CGP led to an increase of total Arg while Hühns et al. (2009) measured no decrease in free Arg. This is indicating that our approach can avoid the feed-back inhibition of the Arg synthesis. Furthermore, the bound Arg is available for mice by the co-administration of its degrading enzyme CGPase. Hence, CGP shows a high potential to serve as an Arg store in plants, though further investigations has to be done according possible interference with the endogenous metabolism.

While much effort was taken to improve the CGP production in plants, the expression of CGPase in plants is new and has to be analyzed from the beginning onwards.

C3 Are plants able to produce an active form of cyanophycinase?

To answer this question, we analyzed the production of CGPase in N. *benthamiana* and BG via transient expression. The MagnICON® expression method (Marillonnet et al., 2004) enables the fast production of recombinant proteins. Consequently, only a short period of time is necessary to analyze the expression and the properties of plant made recombinant proteins (Gleba et al., 2005; Marillonnet et al., 2005; Nausch et al., 2012a; Nausch et al., 2012b). These first approaches yielded high amounts of CGPase and showed promising results (Chapter B2 and B3 and Nausch and Broer (2016)).

A satisfying production of CPHB required different strategies to optimize the expression of the truncated *cph*B_{Te} gene. As described in chapter B3, the first step was to optimize the translation of the gene by the adaption to the codon usage to *N. tabacum* and the insertion of the sequence GCT TCC TCC, encoding for Alanin-Serine-Serine, behind the start codon as described by Sawant et al. (2001). Both steps increased the amount of CPHB substantially. The positive effect of the adaption of the codon usage was also shown for cytosolic *cph*E expression in *N. benthamiana* (Nausch and Broer, 2016). However, in contrast to CPHE, which turned out to be very stable, CPHB was degraded in crude plant extract during protein isolation. Hence, we used different strategies to stabilize the protein. Fusion of CPHB to GFP (GFP::CPHB) and the translocation of CPHB to the chloroplast (S-CPHB) increased the production and stability of the protein. The expression of the full CPHB, which carries a 55 AA elongation at its *N*-terminus was less successful. However, after expression of these enzymes in CGP producing BG plants, CPHB revealed to be instable in crude plant extract

despite all modifications. This might also have been the case in *N. benthamiana*, but was most likely not observed for GFP::CPHB and S-CPHB due to the high production rates, which can't be achieved in BG (Nausch et al., 2012a). In contrast CPHE remained stable in BG crude plant extracts for at least 24 hours (chapter B3). This stability is a major advantage of CPHE compared to CPHB in both of our strategies.

The analysis of the enzyme activity showed that both, plant made CPHE and CPHB are active *in vitro* and in crude plant extract of *N. benthamiana*, but that CPHE is more active compared to CPHB under the same conditions. Repetition of these experiments with crude extract from BG plants showed no activity for GFP::CPHB, but for CPHE. This was most likely caused by a combined effect of enzyme stability and different activity levels of the enzymes. *In planta* degradation in the chloroplasts was only analyzed with S-CPHB, because it was not possible to locate CPHE in the chloroplast up to now (Nausch and Broer, 2016). As shown in crude plant extracts of BG for GFP::CPHB, no CGP degradation was detected in the plastids of BG plants. This might be caused by low expression levels of S-CPHB in BG plants, because the enzyme was detected in Western blots and seems to be stable in the plastid, but its ability to degrade CGP depends on the enzyme concentration in the presence of CGP as shown in chapter B3. Purified S-CPHB proofed to be able to degrade plant made CGP in the GIT of mice. Because CPHE has been more stable and active compared to CPHB under the same conditions, I assume that CPHE might also lead to the degradation of CGP in animals. These results show that our strategies are promising and that CPHE is more suitable than CPHB, but more work is necessary to further improve the enzyme production.

To follow the approach of supplementation of the isolated proteins to food and feed requires plants which produce high amounts of both proteins for commercial production. As aforementioned this is already possible for CGP in stably transformed plants and the production of CPHE is promising, because transient expression in *N. benthamiana* is largely scalable for industrial purposes (Holtz et al., 2015; Leuzinger et al., 2013). The purification of CGPase via the 6* His-tag by nickel-nitrilotriacetic acid (Ni^{2+}-NTA) affinity chromatography, which is time and material expensive, is a drawback of this strategy. Alternative isolation methods like the oleosin fusion system which proofed to be less expensive and time consuming compared to chromatography purification methods (reviewed by Lojewska et al. (2016)) might solve this problem. For a commercialized production of CPHE it is also necessary to establish a reliable CPHE quantification method to conduct techno-economic analysis as described by Walwyn et al. (2015) and Nandi et al. (2016). This would also enable a comparison with other expression systems like bacteria cell cultures.

The coexpression of CGP and CGPase requires stably transformed plants which should produce high amounts of the enzyme, because we showed that the CGP degradation depends on the amount of CGPase (Chapter B3 and Nausch and Broer (2016)). While the transient expression system prooved to produce high amounts of CPHE it is not known, if this holds true for stably transformed plants, because transient expression usually leads to higher protein yields than stable transformation (Gleba et al., 2005; Nausch et al., 2012b). Hence, it has to be analyzed if sufficient amounts of the enzyme can be produced in stable transformants. If this is the case, the direct supply of β-Asp-Arg dipeptide during the food uptake might be possible. In contrast, the release of the dipeptides during plant growth seems not to be promising, because of the lack of *in planta* activity of CPHB and the fact that CPHE can't be translocated to the chloroplast. To further follow this strategy it might be possible to analyze the chloroplast targeting of CPHE with other transit peptides, because the efficiency of the transport depends on the coding sequence of the cargo protein and might work with other leader peptides (Hühns et al., 2008; Jang et al., 2002; Liu et al., 2004; Wong et al., 1992).

In conclusion we could show that plants are able to produce an active form of CGPase which degraded CGP *in vitro*, in crude plant extract and in the GIT of mice. Hence, we can state that our second hypotheses: "Plants are able to produce an active form of the intracellular cyanophycinase CPHB" can be accepted, but that CPHE is the more suitable enzyme for our approach, because of the higher stability and activity. In conclusion, this CGPase should be used in further attempts like generating stably transformed plants.

In contrast, our third hypothesis: "The coexpression of cynaophycin and cyanophycinase is possible and cyanophycin can be degraded *in planta* in the chloroplast as well as after cell homogenization in crude plant extracts" can only be partially accepted. We showed that CGP can't be degraded in the chloroplast by S-CPHB, but after cell homogenization in crude plant extract by CPHE.

C4 Does the release of β-Asp-Arg dipeptiedes lead to an increased Arg level in the plasma?

To analyze if the degradation of plant made CGP in the murine GIT is possible by the co-administration of plant made CGPase and to analyze whether the released β-Asp-Arg dipeptides lead to an increased Arg level in the plasma, feeding studies were performed. Four Groups of mice were analyzed according the respective pellet which contained either CGP, CGP+CGPase, Arg+Asp or no protein as the control (CON). As described previously the degradation of CGP by CGPase in the murine GIT was possible and it was also shown, that the degradation required the presence of the enzyme, because notably higher CGP

amounts were found in the intestine samples of mice after administration of CGPase free pellets. The high amount of CGP in the colon samples of the CON group is also an indication for the absence of bacteria which express CPHE as shown for mammal species as well as for birds and fish (Sallam and Steinbuchel, 2009).

Mice fed with pellets containing CGP+CGPase showed an increase in β-Asp-Arg dipeptides in the plasma which was not detected in the other groups. Unexpectedly no increase in Arg, Asp or Orn was measured as shown for the mice fed with the pellets containing free AA. This indicates that the β-Asp-Arg dipeptides are absorbed by peptide transporters (PepT1 and PepT2) (Klang et al., 2005; Rubio-Aliaga and Daniel, 2008), but that they were not hydrolyzed later on as suggested. Usually the majority of di- and tripeptides is cleaved in the cytosol after the transport into the enterocyte by peptidases and free Arg is transported into the vascular circulation and to the extra-intestinal tissue by 4F2/y$^+$LAT1 and 4F2/y+LAT2 transporters as pictured in Fig. C 1 (reviewed by Bröer (2008)).

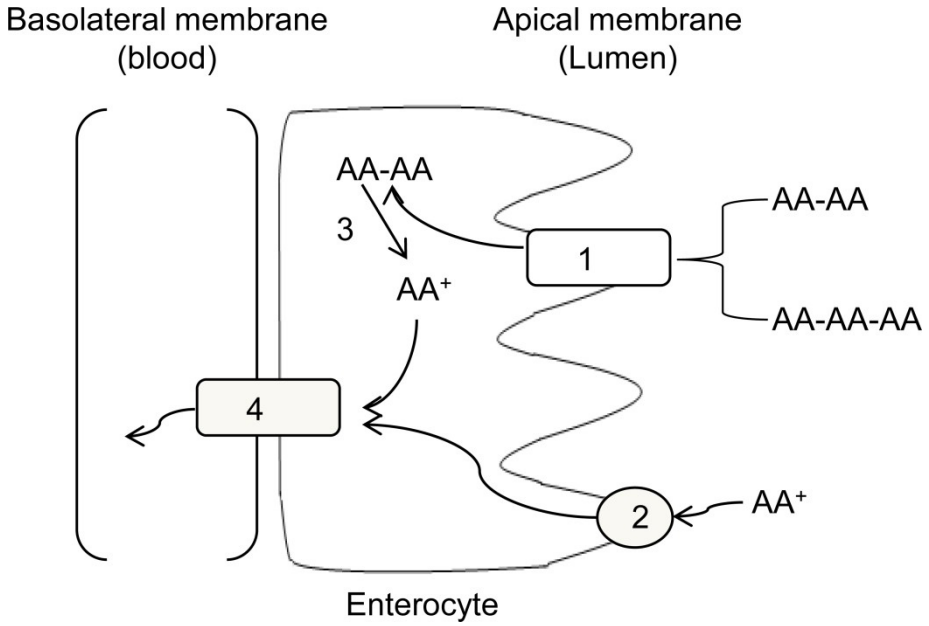


Fig C 1: Absorption of cationic amino acids (AA^+) and di- and tripeptides in the small intestine according to Bröer (2008). 1: Di- and tripeptides are absorped via PepT1 and PepT2 transporters into the enterocyte ((Rubio-Aliaga and Daniel, 2008) and hydrolyzed by peptidases to single AA (3). 2: Arg monomers are transported via cationic AA transporter of the $b^{0,+}$ system. Singl e cationic AA are either metabolized or transported into the blood stream via 4F2/y+ LAT1/LAT2 transporters (4)

This observation is also in contrast to the work of Dorer et al. (1968) who showed that aspartyl di-and tri peptides in rats are rather metabolized than excreted. He isolated the responsible β-aspartyl peptidase from rat livers and kidneys (Dorer et al., 1968) and further works showed that β-aspartyl peptides are hydrolyzed by isoaspartyl dipeptidase (Gary and Clarke, 1995), isoaspartyl aminopeptidase (Larsen et al., 2001) and plant type asparaginases from *E.coli*, *Anabena*, *Synechocystis* and *Arabidopsis thaliana* (Hejazi et al., 2002). The analysis of plant type asparaginases revealed that the substrate specificity differs

between species and that β-Asp-Arg dipeptides are hydrolyzed by the enzymes from *E.coli*, *Anabena* and *Synechocystis* but not by asparaginase isolated from *Arabidopsis thaliana* (Hejazi et al., 2002). This suggests that no appropriate dipeptidase is present in mice which is in line with the observation that some dipeptides are entering the bloodstream without being cleaved (Matthews and Adibi, 1976).

So far it seems like the dipeptides are not bioavailable in the blood stream. As shown in detail in chapter B2 the concentration of β-Asp-Arg dipeptides declined after 120 minutes and it seems like they are slowly released to the circulation and subsequently detoxified and excreted via the urine, though this theory has to be confirmed by the analysis of the amino acid composition of the urine and other tissues like the liver and kidney. In contrast to that, a positive effect of the administration of β-Asp-Arg dipeptides in fish was observed (Krehenbrink, personal communication) which shows the potential of our approach and further feeding studies in other species than mice seem to be justified. For example, it might be possible to determine a possible hydrolysis of the β-Asp-Arg dipeptides by adding isolated dipeptides to enterocyt cell cultures from various species and analyze a possible degradation. A similar experiment was successful with isolated bacteria from different species to analyze the presence of CGPase expressing bacteria in the digestive tract of mammals, birds and fish (Sallam and Steinbuchel, 2009). If mammals are not able to metabolize the β-Asp-Arg dipeptides it might also be possible to administrate CGP, CGPase and a suitable dipeptidase to the chow either in isolated form or coexpressed in plants. In this approach the beneficial effect of dipeptides compared to single AA might be lost, because CGP is degraded to β-Asp-Arg dipeptides which are then hydrolyzed to free Arg and Asp in the GIT. This would still be more sustainable than fishmeal, but it has to be analyzed if it is more beneficial than Arg from bacterial fermentation.

In conclusion, our fourth hypothesis: “Feeding of cyanophycin and cyanophycinase leads to the release of β-Asp-Arg dipeptides in the gastro intestinal tract of mammals which increases the arginine concentration in the plasma” can be accepted only with limitations. Indeed we showed that CGP is degraded in the GIT of mammals by CGPase, but we can’t explain what happens to the β-Asp-Arg dipeptides which unexpectedly appeared in the plasma instead of Arg.

C5 Final conclusion

So far I discussed my working hypotheses and how our strategy meets the existing demands. Now, I finally want to discuss the main hypothesis of this work. “The coexpression of cyanophycin and cyanophycinase in plants can replace the conventional Arg supplementation in the diets of mammals.”

Arg is conventionally supplemented in two forms: Isolated Arg from bacterial fermentation (Utagawa, 2004) and in the form of fishmeal (Li et al., 2011). Our strategies have two major advantages. The supply of β-Asp-Arg dipeptides by the CGPase mediated degradation of CGP might be beneficial compared to isolated Arg, because the uptake of dipetides is more efficient (Matthews and Adibi, 1976; Wenzel et al., 2001) and plants show advantages compared to bacterial fermentation (Merlin et al., 2014) and are more sustainable compared to the use of fishmeal (Hardy, 2010). However, plants have to produce sufficient amounts of CGP and CGPase to supply the necessary amounts of β-Asp-Arg dipeptides, which also have to be bioavailable, to represent a possible alternative.

We showed that the accumulation of CGP in plants can be optimized by the use of the cultivar BG and that an active form of CGPase can be produced in *N. benthamiana* and in CGP expressing BG plants. After homogenization of plant tissue, CPHE led to the degradation of CGP in crude BG extract, while it was shown that plant made CPHB degrades CGP in the GIT of mice, which shows the suitability of our approach. Unexpectedly no increase of Arg, but of β-Asp-Arg dipeptides was measured, indicating that the dipeptides are not bioavailable for mice.

Concluding, I can state that CGP is a suitable Arg store in plants and that the approach of the coexpression of CGPase, as a new scientific strategy, was successful and presents a possible alternative to conventional forms of Arg supplementation, if the released dipeptides are bioavialable. More work has to be done to optimize the CPHE isolation, to establish the coexpression of CGP and CPHE in stably transformed plants and to analyze the bio-availability of β-Asp-Arg dipeptides in other animals than mice. Hence, the main thesis of this work “The coexpression of cyanophycin and cyanophycinase in plants can replace the conventional Arg supplementation in the diets of mammals” can’t be accepted so far, but our work describes a high promising strategy to reach this aim.

D References

Abd-El-Karem, Y., Elbers, T., Reichelt, R. and Steinbuchel, A. (2011) Heterologous expression of Anabaena sp. PCC7120 cyanophycin metabolism genes cphA1 and cphB1 in Sinorhizobium (Ensifer) meliloti 1021. *Appl Microbiol Biotechnol* **89**, 1177-1192.

Aboulmagd, E., Voss, I., Oppermann-Sanio, F.B. and Steinbuchel, 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.

Allen, M.M., Morris, R. and Zimmerman, W. (1984) Cyanophycin Granule Polypeptide Protease in a Unicellular Cyanobacterium. *Archives of Microbiology* **138**, 119-123.

Altenbach, S.B., Kuo, C.C., Staraci, L.C., Pearson, K.W., Wainwright, C., Georgescu, A. and Townsend, J. (1992) Accumulation of a Brazil nut albumin in seeds of transgenic canola results in enhanced levels of seed protein methionine. *Plant Mol Biol* **18**, 235-245.

Altenbach, S.B., Pearson, K.W., Meeker, G., Staraci, L.C. and Sun, S.S.M. (1989) Enhancement of the Methionine Content of Seed Proteins by the Expression of a Chimeric Gene Encoding a Methionine-Rich Protein in Transgenic Plants. *Plant Molecular Biology* **13**, 513-522.

Angelovici, R., Fait, A., Fernie, A.R. and Galili, G. (2011) A seed high-lysine trait is negatively associated with the TCA cycle and slows down Arabidopsis seed germination. *New Phytologist* **189**, 148-159.

Avraham, T. and Amir, R. (2005) The expression level of threonine synthase and cystathionine-gamma-synthase is influenced by the level of both threonine and methionine in Arabidopsis plants. *Transgenic Res* **14**, 299-311.

Beauregard, M. and Hefford, M.A. (2006) Enhancement of essential amino acid contents in crops by genetic engineering and protein design. *Plant Biotechnol J* **4**, 561-574.

Bröer, S. (2008) Amino acid transport across mammalian intestinal and renal epithelia. . *Physiol. Rev.* **88**, 249-286.

Burillo, S., Luque, I., Fuentes, I. and Contreras, A. (2004) Interactions between the nitrogen signal transduction protein PII and N-acetyl glutamate kinase in organisms that perform oxygenic photosynthesis (vol 186, pg 3346, 2004). *Journal of Bacteriology* **186**, 5555-5555.

Cohen, H., Pajak, A., Pandurangan, S., Amir, R. and Marsolais, F. (2016) Higher endogenous methionine in transgenic Arabidopsis seeds affects the composition of storage proteins and lipids. *Amino Acids* **48**, 1413-1422.

Cortes-Giraldo, I., Megias, C., Alaiz, M., Giron-Calle, J. and Vioque, J. (2016) Purification of free arginine from chickpea (Cicer arietinum) seeds. *Food Chemistry* **192**, 114-118.

De Clercq, A., Vandewiele, M., Van Damme, J., Guerche, P., Van Montagu, M., Vandekerckhove, J. and Krebbers, E. (1990) Stable Accumulation of Modified 2S Albumin Seed Storage Proteins with Higher Methionine Contents in Transgenic Plants. *Plant Physiol* **94**, 970-979.

De Steur, H., Blancquaert, D., Strobbe, S., Lambert, W., Gellynck, X. and Van Der Straeten, D. (2015) Status and market potential of transgenic biofortified crops. *Nature Biotechnology* **33**, 25-29.

Dorer, F.E., Haley, E.E. and Buchanan, D.L. (1968) The hydrolysis of beta-aspartyl peptides by rat tissue. *Arch Biochem Biophys* **127**, 490-495.

Du, J., Li, L., Ding, X., Hu, H., Lu, Y. and Zhou, S. (2013) Isolation and characterization of a novel cyanophycin synthetase from a deep-sea sediment metagenomic library. *Appl Microbiol Biotechnol* **97**, 8619-8628.

Du, J., Li, L. and Zhou, S. (2016) Enhanced cyanophycin production by Escherichia coli overexpressing the heterologous cphA gene from a deep sea metagenomic library. *J Biosci Bioeng*.

Fischer, R., Drossard, J., Commandeur, U., Schillberg, S. and Emans, N. (1999) Towards molecular farming in the future: moving from diagnostic protein and antibody production in microbes to plants. *Biotechnol Appl Biochem* **30 (Pt 2)**, 101-108.

Forsyth, J.L., Beaudoin, F., Halford, N.G., Sessions, R.B., Clarke, A.R. and Shewry, P.R. (2005) Design, expression and characterisation of lysine-rich forms of the barley seed protein CI-2. *Biochim Biophys Acta* **1747**, 221-227.

Frizzi, A., Caldo, R.A., Morrell, J.A., Wang, M., Lutfiyya, L.L., Brown, W.E., Malvar, T.M. and Huang, S. (2010) Compositional and transcriptional analyses of reduced zein kernels derived from the opaque2 mutation and RNAi suppression. *Plant Mol Biol* **73**, 569-585.

Frizzi, A., Huang, S., Gilbertson, L.A., Armstrong, T.A., Luethy, M.H. and Malvar, T.M. (2008) Modifying lysine biosynthesis and catabolism in corn with a single bifunctional expression/silencing transgene cassette. *Plant Biotechnology Journal* **6**, 13-21.

Frommeyer, M. and Steinbuchel, A. (2013) Increased lysine content is the main characteristic of the soluble form of the polyamide cyanophycin synthesized by recombinant Escherichia coli. *Appl Environ Microbiol* **79**, 4474-4483.

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

Fuser, G. and Steinbuchel, A. (2007) Analysis of genome sequences for genes of cyanophycin metabolism: Identifying putative cyanophycin metabolizing prokaryotes. *Macromolecular Bioscience* **7**, 278-296.

Galili, G. and Amir, R. (2013) Fortifying plants with the essential amino acids lysine and methionine to improve nutritional quality. *Plant Biotechnology Journal* **11**, 211-222.

Galili, G., Amir, R. and Fernie, A.R. (2016) The Regulation of Essential Amino Acid Synthesis and Accumulation in Plants. *Annual Review of Plant Biology, Vol 67* **67**, 153-+.

Gary, J.D. and Clarke, S. (1995) Purification and characterization of an isoaspartyl dipeptidase from Escherichia coli. *J Biol Chem* **270**, 4076-4087.

Gleba, Y., Klimyuk, V. and Marillonnet, S. (2005) Magnifection - a new platform for expressing recombinant vaccines in plants. *Vaccine* **23**, 2042-2048.

Gupta, M. and Carr, N.G. (1981) Enzyme-Activities Related to Cyanophycin Metabolism in Heterocysts and Vegetative Cells of Anabaena Spp. *Journal of General Microbiology* **125**, 17-23.

Hai, T., Frey, K.M. and Steinbuchel, A. (2006) Engineered cyanophycin synthetase (CphA) from Nostoc ellipsosporum confers enhanced CphA activity and cyanophycin accumulation to Escherichia coli. *Appl Environ Microbiol* **72**, 7652-7660.

Hai, T., Oppermann-Sanio, F.B. and Steinbuchel, A. (2002) Molecular characterization of a thermostable cyanophycin synthetase from the thermophilic cyanobacterium

Synechococcus sp. strain MA19 and in vitro synthesis of cyanophycin and related polyamides. *Appl Environ Microbiol* **68**, 93-101.

Hardy, R.W. (2010) Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal. *Aquaculture Research* **41**, 770-776.

Hejazi, M., Piotukh, K., Mattow, J., Deutzmann, R., Volkmer-Engert, R. and Lockau, W. (2002) Isoaspartyl dipeptidase activity of plant-type asparaginases. *Biochemical Journal* **364**, 129-136.

Hoffman, L.M., Donaldson, D.D. and Herman, E.M. (1988) A modified storage protein is synthesized, processed, and degraded in the seeds of transgenic plants. *Plant Mol Biol* **11**, 717-729.

Holtz, B.R., Berquist, B.R., Bennett, L.D., Kommineni, V.J., Munigunti, R.K., White, E.L., Wilkerson, D.C., Wong, K.Y., Ly, L.H. and Marcel, S. (2015) Commercial-scale biotherapeutics manufacturing facility for plant-made pharmaceuticals. *Plant Biotechnol J* **13**, 1180-1190.

Hou, Y.Q., Yin, Y.L. and Wu, G.Y. (2015) Dietary essentiality of "nutritionally non-essential amino acids" for animals and humans. *Exp Biol Med (Maywood)* **240**, 997-1007.

Hournard, N.M., Mainville, J.L., Bonin, C.P., Huang, S., Luethy, M.H. and Malvar, T.M. (2007) High-lysine corn generated by endosperm-specific suppression of lysine catabolism using RNAi. *Plant Biotechnology Journal* **5**, 605-614.

Hu, S., Li, X., Rezaei, R., Meininger, C.J., McNeal, C.J. and Wu, G. (2015) Safety of long-term dietary supplementation with L-arginine in pigs. *Amino Acids* **47**, 925-936.

Huang, S., Adams, W.R., Zhou, Q., Malloy, K.P., Voyles, D.A., Anthony, J., Kriz, A.L. and Luethy, M.H. (2004) Improving nutritional quality of maize proteins by expressing sense and antisense zein genes. *J Agric Food Chem* **52**, 1958-1964.

Huang, S., Frizzi, A., Florida, C.A., Kruger, D.E. and Luethy, M.H. (2006) High lysine and high tryptophan transgenic maize resulting from the reduction of both 19- and 22-kD alpha-zeins. *Plant Mol Biol* **61**, 525-535.

Huang, S., Kruger, D.E., Frizzi, A., D'Ordine, R.L., Florida, C.A., Adams, W.R., Brown, W.E. and Luethy, M.H. (2005) High-lysine corn produced by the combination of enhanced lysine biosynthesis and reduced zein accumulation. *Plant Biotechnol J* **3**, 555-569.

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 cphA expression to enhance cyanophycin production in potatoes. *Plant Biotechnology Journal* **7**, 883-898.

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.

Hwang, P. and Willoughby, D.S. (2015) Intracellular Mechanistic Role of Nitric Oxide: A Comparative Analysis of the Effectiveness of L-Arginine and L-Citrulline Supplementation on Nitric Oxide Synthesis and Subsequent Exercise Performance in Humans. *International Journal of Food and Nutritional Science* **2**, 1-8.

Jang, I.C., Lee, K.H., Nahm, B.H. and Kim, J.K. (2002) Chloroplast targeting signal of a rice rbcS gene enhances transgene expression. *Molecular Breeding* **9**, 81-91.

Joentgen, W., Groth, T., Steinbüchel, A., Hai, T. and F.B., O. (2001) Polyasparaginic acid homopolymers and copolymers, biotechnical production and use thereof. (AG, B. ed). US.

Klang, J.E., Burnworth, L.A., Pan, Y.X., Webb, K.E. and Wong, E.A. (2005) Functional characterization of a cloned pig intestinal peptide transporter (pPepT1). *Journal of Animal Science* **83**, 172-181.

Krehenbrink, M., Oppermann-Sanio, F.B. and Steinbuchel, 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. *Arch Microbiol* **177**, 371-380.

Larsen, R.A., Knox, T.M. and Miller, C.G. (2001) Aspartic peptide hydrolases in Salmonella enterica serovar typhimurium. *J Bacteriol* **183**, 3089-3097.

Law, A.M., Lai, S.W.S., Tavares, J. and Kimber, M.S. (2009) The Structural Basis of beta-Peptide-Specific Cleavage by the Serine Protease Cyanophycinase. *Journal of Molecular Biology* **392**, 393-404.

Lee, T.T., Wang, M.M., Hou, R.C., Chen, L.J., Su, R.C., Wang, C.S. and Tzen, J.T. (2003) Enhanced methionine and cysteine levels in transgenic rice seeds by the accumulation of sesame 2S albumin. *Biosci Biotechnol Biochem* **67**, 1699-1705.

Leuzinger, K., Dent, M., Hurtado, J., Stahnke, J., Lai, H., Zhou, X. and Chen, Q. (2013) Efficient agroinfiltration of plants for high-level transient expression of recombinant proteins. *J Vis Exp*.

Li, X.L., Rezaei, R., Li, P. and Wu, G.Y. (2011) Composition of amino acids in feed ingredients for animal diets. *Amino Acids* **40**, 1159-1168.

Liu, X., Zhang, C., Wang, X., Liu, Q., Yuan, D., Pan, G., Sun, S.S. and Tu, J. (2016) Development of high-lysine rice via endosperm-specific expression of a foreign LYSINE RICH PROTEIN gene. *BMC Plant Biol* **16**, 147.

Liu, Y.J., Yuan, Y., Zheng, J., Tao, Y.Z., Dong, Z.G., Wang, J.H. and Wang, G.Y. (2004) Signal peptide of potato PinII enhances the expression of Cry1Ac in transgenic tobacco. *Acta Biochimica Et Biophysica Sinica* **36**, 553-558.

Lojewska, E., Kowalczyk, T., Olejniczak, S. and Sakowicz, T. (2016) Extraction and purification methods in downstream processing of plant-based recombinant proteins. *Protein Expr Purif* **120**, 110-117.

Ma, J.K., Drake, P.M. and Christou, P. (2003) The production of recombinant pharmaceutical proteins in plants. *Nat Rev Genet* **4**, 794-805.

Ma, X.Y., Lin, Y.C., Jiang, Z.Y., Zheng, C.T., Zhou, G.L., Yu, D.Q., Cao, T., Wang, J. and Chen, F. (2010) Dietary arginine supplementation enhances antioxidative capacity and improves meat quality of finishing pigs. *Amino Acids* **38**, 95-102.

Ma, X.Y., Zheng, C.T., Hu, Y.J., Wang, L., Yang, X.F. and Jiang, Z.Y. (2015) Dietary L-Arginine Supplementation Affects the Skeletal Longissimus Muscle Proteome in Finishing Pigs. *Plos One* **10**.

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

Mainieri, D., Rossi, M., Archinti, M., Bellucci, M., De Marchis, F., Vavassori, S., Pompa, A., Arcioni, S. and Vitale, A. (2004) Zeolin. A new recombinant storage protein constructed using maize gamma-zein and bean phaseolin. *Plant Physiol* **136**, 3447-3456.

Marillonnet, S., Giritch, A., Gils, M., Kandzia, R., Klimyuk, V. and Gleba, Y. (2004) In planta engineering of viral RNA replicons: Efficient assembly by recombination of DNA modules delivered by Agrobacterium. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 6852-6857.

Marillonnet, S., Thoeringer, C., Kandzia, R., Klimyuk, V. and Gleba, Y. (2005) Systemic Agrobacterium tumefaciens-mediated transfection of viral replicons for efficient transient expression in plants. *Nature Biotechnology* **23**, 718-723.

Mateo, R.D., Wu, G.Y., Bazer, F.W., Park, J.C., Shinzato, I. and Kim, S.W. (2007) Dietary L-arginine supplementation enhances the reproductive performance of gilts. *Journal of Nutrition* **137**, 652-656.

Matthews, D.M. and Adibi, S.A. (1976) Peptide Absorption. *Gastroenterology* **71**, 151-161.

Mckay, G. and Shargool, P.D. (1981) Purification and Characterization of N-Acetylglutamate 5-Phosphotransferase from Pea (Pisum-Sativum) Cotyledons. *Biochemical Journal* **195**, 71-81.

Menassa, R., Du, C., Yin, Z.Q., Ma, S., Poussier, P., Brandle, J. and Jevnikar, A.M. (2007) Therapeutic effectiveness of orally administered transgenic low-alkaloid tobacco expressing human interleukin-10 in a mouse model of colitis. *Plant Biotechnol J* **5**, 50-59.

Menassa, R., Nguyen, V., Jevnikar, A. and Brandle, J. (2001) A self-contained system for the field production of plant recombinant interleukin-10. *Molecular Breeding* **8**, 177-185.

Merlin, M., Gecchele, E., Capaldi, S., Pezzotti, M. and Avesani, L. (2014) Comparative evaluation of recombinant protein production in different biofactories: the green perspective. *Biomed Res Int* **2014**, 136419.

Mikschofsky, H., Schirrmeier, H., Keil, G.M., Lange, B., Polowick, P.L., Keller, W. and Broer, I. (2009) Pea-derived vaccines demonstrate high immunogenicity and protection in rabbits against rabbit haemorrhagic disease virus. *Plant Biotechnol J* **7**, 537-549.

Molvig, L., Tabe, L.M., Eggum, B.O., Moore, A.E., Craig, S., Spencer, D. and Higgins, T.J. (1997) Enhanced methionine levels and increased nutritive value of seeds of transgenic lupins (Lupinus angustifolius L.) expressing a sunflower seed albumin gene. *Proc Natl Acad Sci U S A* **94**, 8393-8398.

Mooibroek, H., Oosterhuis, N., Giuseppin, M., Toonen, M., Franssen, H., Scott, E., Sanders, J. and Steinbuchel, 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.

Nandi, S., Kwong, A.T., Holtz, B.R., Erwin, R.L., Marcel, S. and McDonald, K.A. (2016) Techno-economic analysis of a transient plant-based platform for monoclonal antibody production. *MAbs* **8**, 1456-1466.

Nausch, H. (2016) Regulated degradation of plant-derived Cyanophycin to release dipeptides as feed additives. . In: *The second conference of the International Society for Plant Molecular Farming* (Depicker, A. ed). Ghent: ISPMF.

Nausch, H. and Broer, I. (2016) Cyanophycinase CphE from P. alcaligenes produced in different compartments of N. benthamiana degrades high amounts of cyanophycin in plant extracts. *Appl Microbiol Biotechnol*.

Nausch, H., Huckauf, J. and Broer, I. (2016) Peculiarities and impacts of expression of bacterial cyanophycin synthetases in plants. *Appl Microbiol Biotechnol* **100**, 1559-1565.

Nausch, H., Mikschofsky, H., Koslowski, R., Meyer, U., Broer, I. and Huckauf, J. (2012a) High-Level Transient Expression of ER-Targeted Human Interleukin 6 in Nicotiana benthamiana. *Plos One* **7**.

Nausch, H., Mischofsky, H., Koslowski, R., Meyer, U., Broer, I. and Huckauf, J. (2012b) Expression and Subcellular Targeting of Human Complement Factor C5a in Nicotiana species. *Plos One* **7**.

Neubauer, K., Huhns, M., Hausmann, T., Klemke, F., Lockau, W., Kahmann, U., Pistorius, E.K., Kragl, U. and Broer, I. (2012) Isolation of cyanophycin from tobacco and potato plants with constitutive plastidic cphA(Te) gene expression. *Journal of Biotechnology* **158**, 50-58.

Neumann, K., Stephan, D.P., Ziegler, K., Huhns, 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.

Nordlee, J.A., Taylor, S.L., Townsend, J.A., Thomas, L.A. and Bush, R.K. (1996) Identification of a Brazil-nut allergen in transgenic soybeans. *N Engl J Med* **334**, 688-692.

Obst, M., Oppermann-Sanio, F.B., Luftmann, H. and Steinbuchel, A. (2002) Isolation of cyanophycin-degrading bacteria, cloning and characterization of an extracellular cyanophycinase gene (cphE) from Pseudomonas anguilliseptica strain BI - The cphE gene from P-anguilliseptica BI encodes a cyanophycin-hydrolyzing enzyme. *Journal of Biological Chemistry* **277**, 25096-25105.

Oliva, M., Ovadia, R., Perl, A., Bar, E., Lewinsohn, E., Galili, G. and Oren-Shamir, M. (2015) Enhanced formation of aromatic amino acids increases fragrance without affecting flower longevity or pigmentation in Petunia x hybrida. *Plant Biotechnology Journal* **13**, 125-136.

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

Reyes, A.R., Bonin, C.P., Houmard, N.M., Huang, S. and Malvar, T.M. (2009) Genetic manipulation of lysine catabolism in maize kernels. *Plant Molecular Biology* **69**, 81-89.

Richter, R., Hejazi, M., Kraft, R., Ziegler, K. and Lockau, W. (1999) Cyanophycinase, a peptidase degrading the cyanobacterial reserve material multi-L-arginyl-poly-L-aspartic acid (cyanophycin) - Molecular cloning of the gene of Synechocystis sp PCC 6803, expression in Escherichia coli, and biochemical characterization of the purified enzyme. *Eur J Biochem* **263**, 163-169.

Rose, W.C. and Rice, E.E. (1939) The significance of the amino acids in canine nutrition. *Science* **90**, 186-187.

Rossi, L., Dell'Orto, V., Vagni, S., Sala, V., Reggi, S. and Baldi, A. (2014) Protective effect of oral administration of transgenic tobacco seeds against verocytotoxic Escherichia coli strain in piglets. *Vet Res Commun* **38**, 39-49.

Rubio-Aliaga, I. and Daniel, H. (2008) Peptide transporters and their roles in physiological processes and drug disposition. *Xenobiotica* **38**, 1022-1042.

Sabalza, M., Christou, P. and Capell, T. (2014) Recombinant plant-derived pharmaceutical proteins: current technical and economic bottlenecks. *Biotechnol Lett* **36**, 2367-2379.

Sallam, A., Kast, A., Przybilla, S., Meiswinkel, T. and Steinbuchel, A. (2009) Biotechnological process for production of beta-dipeptides from cyanophycin on a technical scale and its optimization. *Appl Environ Microbiol* **75**, 29-38.

Sallam, A. and Steinbuchel, A. (2009) Cyanophycin-degrading bacteria in digestive tracts of mammals, birds and fish and consequences for possible applications of cyanophycin and its dipeptides in nutrition and therapy. *Journal of Applied Microbiology* **107**, 474-484.

Sallam, A. and Steinbuchel, A. (2010) Dipeptides in nutrition and therapy: cyanophycin-derived dipeptides as natural alternatives and their biotechnological production. *Applied Microbiology and Biotechnology* **87**, 815-828.

Sancho-Vaello, E., Fernandez-Murga, M.L. and Rubio, V. (2009) Mechanism of arginine regulation of acetylglutamate synthase, the first enzyme of arginine synthesis. *Febs Letters* **583**, 202-206.

Santos, S., Torcato, I. and Castanho, M.A. (2012) Biomedical applications of dipeptides and tripeptides. *Biopolymers* **98**, 288-293.

Sawant, S.V., Kiran, K., Singh, P.K. and Tuli, R. (2001) Sequence architecture downstream of the initiator codon enhances gene expression and protein stability in plants. *Plant Physiology* **126**, 1630-1636.

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. *Appl Microbiol Biotechnol* **75**, 751-762.

Shargool, P.D. and Jain, J.C. (1985) Use of N-2-Bromoacetyl-L-Ornithine to Study L-Ornithine and L-Arginine Biosynthesis in Soybean (Glycine-Max L) Cell-Cultures. *Plant Physiology* **78**, 795-798.

Sikdar, M.S., Bowra, S., Schmidt, D., Dionisio, G., Holm, P.B. and Vincze, E. (2016) Targeted modification of storage protein content resulting in improved amino acid composition of barley grain. *Transgenic Res* **25**, 19-31.

Simon, R.D. (1987) Inclusion bodies in the cyanobacteria: Cyanophycin, polyphosphate, polyhedra bodies. In: *The Cyanobacteria* (Fay, P. and Van Baalen, C. eds), pp. 192-222. Amsterdam, New York, Oxford: Elsevier.

Simon, R.D., Lawry, N.H. and Mclendon, G.L. (1980) Structural Characterization of the Cyanophycin Granule Polypeptide of Anabaena-Cylindrica by Circular-Dichroism and Raman-Spectroscopy. *Biochimica Et Biophysica Acta* **626**, 277-281.

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.

Steinle, A., Bergander, K. and Steinbuchel, A. (2009) Metabolic engineering of Saccharomyces cerevisiae for production of novel cyanophycins with an extended range of constituent amino acids. *Appl Environ Microbiol* **75**, 3437-3446.

Steinle, A., Witthoff, S., Krause, J.P. and Steinbuchel, A. (2010) Establishment of cyanophycin biosynthesis in Pichia pastoris and optimization by use of engineered cyanophycin synthetases. *Appl Environ Microbiol* **76**, 1062-1070.

Steinmann, A. (2011) Etablierung eines Modellsystems zum Nachweis eines allergenen Potenzials in transgenen Pflanzen [Establishment of a model system for the detection of an allergenic potential of transgenic plants]. In: *der Mathematisch-Naturwissenschaftlichen Fakultät [Faculty of mathematics and natural science]*. Rostock: University of Rostock

Streatfield, S.J. (2007) Approaches to achieve high-level heterologous protein production in plants. *Plant Biotechnol J* **5**, 2-15.

Sun, S.S.M. and Liu, Q.Q. (2008) Methods to produce desired proteins in plants. (The Chinese University of Hong Kong, H.K.C. ed). United States Patent.

Tan, B., Yin, Y.L., Liu, Z.Q., Li, X.G., Xu, H.J., Kong, X.F., Huang, R.L., Tang, W.J., Shinzato, I., Smith, S.B. and Wu, G.Y. (2009) Dietary l-arginine supplementation increases muscle gain and reduces body fat mass in growing-finishing pigs. *Amino Acids* **37**, 169-175.

Torrent, M., Alvarez, I., Geli, M.I., Dalcol, I. and Ludevid, D. (1997) Lysine-rich modified gamma-zeins accumulate in protein bodies of transiently transformed maize endosperms. *Plant Mol Biol* **34**, 139-149.

Tremblay, R., Wang, D., Jevnikar, A.M. and Ma, S. (2010) Tobacco, a highly efficient green bioreactor for production of therapeutic proteins. *Biotechnol Adv* **28**, 214-221.

Tuse, D., Tu, T. and McDonald, K.A. (2014) Manufacturing economics of plant-made biologics: case studies in therapeutic and industrial enzymes. *Biomed Res Int* **2014**, 256135.

Tzin, V., Malitsky, S., Ben Zvi, M.M., Bedair, M., Sumner, L., Aharoni, A. and Galili, G. (2012) Expression of a bacterial feedback-insensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase of the shikimate pathway in Arabidopsis elucidates potential metabolic bottlenecks between primary and secondary metabolism. *New Phytologist* **194**, 430-439.

Tzin, V., Rogachev, I., Meir, S., Ben Zvi, M.M., Masci, T., Vainstein, A., Aharoni, A. and Galili, G. (2013) Tomato fruits expressing a bacterial feedback-insensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase of the shikimate pathway possess enhanced levels of multiple specialized metabolites and upgraded aroma. *Journal of Experimental Botany* **64**, 4441-4452.

Utagawa, T. (2004) Production of arginine by fermentation. *Journal of Nutrition* **134**, 2854s-2857s.

Voss, I., Diniz, S.C., Aboulmagd, E. and Steinbuchel, A. (2004) Identification of the Anabaena sp. strain PCC7120 cyanophycin synthetase as suitable enzyme for production of cyanophycin in gram-negative bacteria like Pseudomonas putida and Ralstonia eutropha. *Biomacromolecules* **5**, 1588-1595.

Voss, I. and Steinbuchel, A. (2006) Application of a KDPG-aldolase gene-dependent addiction system for enhanced production of cyanophycin in Ralstonia eutropha strain H16. *Metab Eng* **8**, 66-78.

Walwyn, D.R., Huddy, S.M. and Rybicki, E.P. (2015) Techno-economic analysis of horseradish peroxidase production using a transient expression system in Nicotiana benthamiana. *Appl Biochem Biotechnol* **175**, 841-854.

Wenzel, U., Meissner, B., Doring, F. and Daniel, H. (2001) PEPT1-mediated uptake of dipeptides enhances the intestinal absorption of amino acids via transport system b(0,+). *Journal of Cellular Physiology* **186**, 251-259.

Wiefel, L., Broker, A. and Steinbuchel, A. (2011) Synthesis of a citrulline-rich cyanophycin by use of Pseudomonas putida ATCC 4359. *Appl Microbiol Biotechnol* **90**, 1755-1762.

Wiefel, L. and Steinbuchel, A. (2014) Solubility behavior of cyanophycin depending on lysine content. *Appl Environ Microbiol* **80**, 1091-1096.

Winter, G., Todd, C.D., Trovato, M., Forlani, G. and Funck, D. (2015) Physiological implications of arginine metabolism in plants. *Front Plant Sci* **6**.

Wong, E.Y., Hironaka, C.M. and Fischhoff, D.A. (1992) Arabidopsis-Thaliana Small Subunit Leader and Transit Peptide Enhance the Expression of Bacillus-Thuringiensis Proteins in Transgenic Plants. *Plant Molecular Biology* **20**, 81-93.

Wong, H.W., Liu, Q. and Sun, S.S. (2015) Biofortification of rice with lysine using endogenous histones. *Plant Mol Biol* **87**, 235-248.

Wu, G.Y. (2009) Amino acids: metabolism, functions, and nutrition. *Amino Acids* **37**, 1-17.

Wu, X., Ruan, Z., Gao, Y.L., Yin, Y.L., Zhou, X.H., Wang, L., Geng, M.M., Hou, Y.Q. and Wu, G.Y. (2010) Dietary supplementation with l-arginine or N-carbamylglutamate enhances intestinal growth and heat shock protein-70 expression in weanling pigs fed a corn- and soybean meal-based diet. *Amino Acids* **39**, 831-839.

Wu, Z., Hou, Y., Hu, S., Bazer, F.W., Meininger, C.J., McNeal, C.J. and Wu, G. (2016) Catabolism and safety of supplemental L-arginine in animals. *Amino Acids* **48**, 1541-1552.

Xu, J., Dolan, M.C., Medrano, G., Cramer, C.L. and Weathers, P.J. (2012) Green factory: plants as bioproduction platforms for recombinant proteins. *Biotechnol Adv* **30**, 1171-1184.

Yang, X.F., Jiang, Z.Y., Gong, Y.L., Zheng, C.T., Hu, Y.J., Wang, L., Huang, L. and Ma, X.Y. (2016) Supplementation of pre-weaning diet with L-arginine has carry-over effect to improve intestinal development in young piglets. *Canadian Journal of Animal Science* **96**, 52-59.

Yang, Y., Wu, Z.L., Jia, S.C., Dahanayaka, S., Feng, S., Meininger, C., McNeal, C. and Wu, G.Y. (2015) Safety of long-term dietary supplementation with L-arginine in rats. *Amino Acids* **47**, 1909-1920.

Yu, J., Peng, P., Zhang, X., Zhao, Q., Zhu, D., Sun, X., Liu, J. and Ao, G. (2005) Seed-specific expression of the lysine-rich protein gene sb401 significantly increases both lysine and total protein content in maize seeds. *Food Nutr Bull* **26**, 427-431.

Yue, J., Li, C., Zhao, Q., Zhu, D. and Yu, J. (2014) Seed-specific expression of a lysine-rich protein gene, GhLRP, from cotton significantly increases the lysine content in maize seeds. *Int J Mol Sci* **15**, 5350-5365.

Zeng, X.F., Wang, F.L., Fan, X., Yang, W.J., Zho, B., Li, P.F., Yin, Y.L., Wu, G.Y. and Wang, J.J. (2008) Dietary arginine supplementation during early pregnancy enhances embryonic survival in rats. *Journal of Nutrition* **138**, 1421-1425.

Zhu, X.H. and Galili, G. (2003) Increased lysine synthesis coupled with a knockout of its catabolism synergistically boosts lysine content and also transregulates the metabolism of other amino acids in Arabidopsis seeds. *Plant Cell* **15**, 845-853.

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. *Z Naturforsch C* **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). *Eur J Biochem* **254**, 154-159.

Personal communication:

Susanne Baars: Fachagentur Nachwachsende Rohstoffe e.V. Hofplatz 1, 18276 Gülzow-Prüzen, Germany (s.baars@fnr.de)

Dr Jana Huckauf: Universität Rostock, Agrar- und Umweltwissenschaftliche Fakultät, Justus-von-Liebig-Weg 8, 18059 Rostock (jana.huckauf@uni-rostock.de)

Dr. Martin Krehenbrink: Cysal GmbH Mendelstraße 11, 48149 Münster, Germany (m.krehenbrink@cysal.de)

E Appendix

E1 Index of abbreviations

%	Percent
AA	Amino Acid(s)
Arg	Arginine
Asp	Aspartate (Aspartic acid)
BG	*Nicotiana tabacum* cv. Badischer Geudertheimer
CDM	Cell dry matter
CGP	Cyanophycin
CGPase	Cyanophycinase
Cit	Citrulline
CON	Control
CPHA	Cyanophyin synthetase
CPHB	Intracellular Cyanophycinase
CPHE	Intercelleular Cyanophycinase
Dpi	Day post- infiltration
DW	Dry weight
GFP::CPHB	Fusion protein of the green fluorescent protein and CPHB
GIT	Gastrointestinal tract
Gln	Glutamine
Glu	Glutamic acid
His	Histidine
kDa	Kilo Dalton
Leu	Leucine
Lys	Lysine
Met	Methionine
NAGK	N-Acetylglutamate synthase

NAGS	N-Acetylglutamate kinase
NAOGAcT	N^2-Acetylornithine:glutamate acetyltransferase
Orn	Ornithine
PH	*Nicotiana tabacum* Petit Havanna SRI
Pro	Proline
S-CPHB	CPHB, targeted to the chloroplast by the Transit peptide from the small subunit of RuBisCO (S)
STA	*Solanum tuberosum* cv. Albatros
STD	*Solanum tuberosum* cv. Desirée
Thr	Threonine
Trp	Tryptophan
VG	*Nicotiana tabacum* cv. Virginia Golta

E2 Index of Figures

E3 Index of tables

E4 Danksagung

Die Entstehung dieser Arbeit wäre ohne die hier genannten Menschen nicht möglich gewesen und ich möchte ihnen allen von Herzen dafür danken.

Liebe Inge, ich danke dir für die Bereitstellung des Themas, das mir entgegengebrachte Vertrauen, die vielen Diskussionen, Anregungen und die Hilfe während meiner gesamten Zeit bei dir, insbesondere während meiner Promotion. Seit meiner Bachelorarbeit, bis zum Verlassen der Arbeitsgruppe, habe ich mehr von dir gelernt als nur fachliches Wissen. Auch dafür und für die schönen Jahre, vielen Dank!

Dank gebührt auch meinen Kollegen und den Studenten, die mir bei der Arbeit geholfen haben. Henrik, dir möchte ich von Herzen dafür danken, dass du mich zu jeder Zeit so gut unterstützt hast, wie es dir möglich war. Egal ob im Labor, oder beim Verfassen der Publikationen und der Dissertation. Ohne dich wäre mein Kopf nur halb so voll mit Wissen, wie er ist. Susanne, danke für die Hilfe, die offenen Ohren für alle meine Anliegen während der Mensa- und Kaffeepausen und die notwendige Ablenkung bei spontanen Besuchen im Zoo, wenn es mal nicht so lief wie geplant. Axel, Christoph, Jana, Kerstin, Sonja und Marko, euch möchte ich besonders für die vielen Anregungen, die Unterstützung im Labor und für die offenen Ohren danken. Ihr habt mir sehr geholfen. Sven und Patti, danke für euren Einsatz und eure Lernbereitschaft.

Dank gebührt auch den Kooperationspartnern des FBN Dummerstorf. Frau Metges, vielen Dank für die Korrekturen, Hinweise und das Ermöglichen der Fütterungsstudien. Solvig, dir möchte ich danken, dass du mit deinem Wissen und Einsatz die Analysen durchführtest die diese Arbeit sehr bereichern. Auch allen anderen Mitarbeitern des FBN, die im Rahmen dieser Arbeit an den Versuchen beteiligt waren, möchte ich danken.

Trotz aller Hilfestellungen im kollegialen Umfeld wäre mir das Geleistete nicht möglich gewesen, wenn ich nicht von besonderen Menschen umgeben wäre. Meiner ganzen Familie, danke ich von Herzen für den Rückhalt und die Ermutigungen, auch in schweren Zeiten weiter zu machen. Ihr wart immer da, wenn ich nicht wusste wohin.

Allen meinen Freunden, insbesondere Jan und Kathi. Danke für Alles. Auch wenn ich ohne euch vermutlich ein Jahr früher fertig gewesen wäre. Ihr wisst, wie ich das meine.

In Dankbarkeit

Daniel

E5 Declaration of independence / Eigenständigkeitserklärung

I herewith declare that I wrote and composed the presented PhD thesis independently. I did not use any other sources, figures or resources than the ones stated in the references.

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und ohne, außer die von mir angegebenen, Hilfsmittel angefertigt habe. Alle genutzten Quellen und Zitate sind als solche gekennzeichnet.

Daniel Ponndorf

Rostock, 22.06.2017

E6 Authors contribution

The results of this work are published in three peer reviewed journal articles. I contributed to these works as described below.

Henrik Nausch, Tina Hausmann, **Daniel Ponndorf**, Maja Hühns, Sandra Hoedtke, Petra Wolf, Annette Zeyner, Inge Broer (2016): Tobacco as platform for a commercial production of cyanophycin. New Biotechnology 33, 842-851.

Author's contribution: I performed Southern blot analysis, determination of biomass production and CGP quantification of *N tabacum* var Badischer Geudertheimer plants (Event 116, 128, 125, 137, 157, 176).

Daniel Ponndorf, Sven Ehmke, Benjamin Walliser, Kerstin Thoss, Christoph Unger, Solvig Görs, Gürbüz Daş, Cornelia C. Metges, Inge Broer, Henrik Nausch (2016 accepted): Stable production of cyanophycinase in Nicotiana benthamiana and its functionality to hydrolyse cyanophycin in the murine intestine. Plant Biotechnology Journal 15-5,605-613

Author's contribution: I designed all of the used constructs, except the empty infiltration vectors and *gfp*-*cph*B. I cloned the constructs *cph*B-b, *cph*B-s, *cph*B-sA and *cph*B-s-c and performed Western blot analysis of infiltrated plants together with S.E. except for *gfp*-*cph*B. Northern blot analysis was conducted by K.T. according to my instructions. I prepared the material for the production of anti-CPHB antibodies as well as for the feeding studies in mice and assisted during both experiments and interpreted the plant experiments and wrote the plant sections of the article.

Daniel Ponndorf, Inge Broer, Henrik Nausch (2017): Coexpression and comparison of two cyanophycinases in transgenic, cyanophycin producing tobacco plants. Transgenic Research, 1-9

Author's contribution: Together with I.B. and H.N. I designed the experiments. Together with H.N. I performed the plant experiments and the molecular analysis of the plants. Western blots and comparison of enzyme activity was performed by H.N. I performed the statistical analysis and interpretation of the data and wrote the article.

E7 Hypothesis

1. The expression of cyanophycin synthetase (CPHA) in *N. tabacum* cv. Badischer Geudertheimer and cv. Virginia Golta leads to a higher production of cyanophycin compared to *Solanum tuberosum* and *N. tabacum* Petit Havanna SRI.
2. Plants are able to produce an active form of the intracellular cyanophycinase CPHB.
3. The coexpression of cynaophycin and cyanophycinase is possible and cyanophycin can be degraded *in planta* in the chloroplast as well as after cell homogenization in crude plant extracts.
4. Feeding of cyanophycin and cyanophycinase leads to the release of β-Asp-Arg dipeptides in the gastro intestinal tract of mammals which increases the arginine concentration in the plasma.

Finally, the results will be summarized to discuss the main thesis of this work:

“The coexpression of cyanophycin and cyanophycinase in plants can replace the conventional Arg supplementation in the diets of mammals.”

E7 Thesen

Arbeitsthesen:

1. Die Expression des Enzymes Cyanophycin Synthetase (CPHA) in *N. tabacum* cv. Badischer Geudertheimer und cv. Virginia Golta führt zu einer gesteigerten Produktion von Cyanophycin im Vergleich zu *Solanum tuberosum* und *N. tabacum* Petit Havanna SRI.
2. Pflanzen sind in der Lage, eine aktive Form der intrazellularen Cyanophycinase CPHB zu produzieren.
3. Die Koexpression von Cyanophycin und Cyanophycinase in einer Pflanze ist möglich und Cyanophycin kann in den Chloroplasten und in homogenisiertem Pflanzenmaterial abgebaut werden.
4. Die Fütterung von Cyanophycin und Cyanophycinase führt zur Freisetzung von β-Asp-Arg Dipeptiden im Verdauungstrakt von Säugern und zu einer Erhöhung des Argininspiegels im Plasma.

Hauptthese:

“ Die Koexpression von Cyanophycin und Cyanophycinase in Pflanzen kann die Konventionelle Arginin Supplementierung in der Ernährung von Säugetieren ersetzen.“

E8 Curriculum vitae

Daniel Helmut Ponndorf, M.Sc.

Personal information

Date of birth: 017.01.1985 in Reutlingen/Germany

Professional experience

2017-today Research Scientist at the department for Biological Chemistry, John Innes Centre Norwich

2012-2017 scientific coworker at the professorship for Agrobiotechnology, agricultural- and environmental faculty, University of Rostock/Germany

- Project management and coordination of tasks with project partners
- Design, conduction, evaluation and publication of experiments
- Transient and stable expression of bacterial enzymes on plants
- Supervision of bachelor and master students

2010: Four month internship at the National Research Council in Saskatoon/Canada: „*Agrobacterium tumefaciens* mediated transformation of pea “

- Transformation of pea plants using *agrobacterium tumefaciens*
- *In vitro* culture of pea plants and selection of transformants

08/2008 Internship at Biovativ GmbH, Groß Lüsewitz Germany

- Cultivation of transgenic plants in the greenhouse (barley and sugar beets)
- field trails with rapeseed and potatoes

08/2007 Internship at Biovativ GmbH, Groß Lüsewitz Germany

- Cultivation of transgenic plants in the greenhouse (potatoes and barley)
- field trails with rapeseed

2005-2006 Civil service at the „Michaelshof Rostock“

Educational profile

2017-today Post-doctoral position at the Department for Biological Chemistry, John Innes Centre Norwich/UK

2012-2017	PhD studies at the professorship Agrobiotechnology, Agricultural- and Environmental Faculty University of Rostock/Germany with the topic: „Increasing the arginine content in plants by coexpression of cyanophycin and cyanophycinase“(expected graduation 12/2016)
2009-2012	Masterstudies and -examinationin Agricultural Ecology. Grade 1.3 at the university of Rostock/Germany
2006-2009	Bachelorstudies and-examination in Agricultural Ecology at the university of Rostock/Germany
96-2005	Qualification for the university entrance, „Abitur“ at the Maxim-Gorki-Gymnasium Heringsdorf/Germany

Methods

- Cloning, modification and analysis of DNA
- Transformation of bacteria
- Transient and stable transformation of plants (tobacco and pea)
- *In vitro* cultivation of plants and selection of transformants
- Analysis of transgenic plants (DNA, RNA, Proteom)
- Expression, isolation and analysis of recombinant proteins in plants
- Design, implementation, evaluation and publication of plant related research
- Assistance in mice feeding trails

Advanced education

09/07/2016	DEKRA certified quality management representative according to DIN EN ISO 9001:201
04/22/2016	Advanced education for project managers according to “§15 Abs.2 Satz 1 Nr.3 GenTSV”. Permits to work as person in authority for biological safety.

Languages

German	First language
English	Excellent
French	basics (high school class 7-11)

Computer skills

MS Office	Excellent
IBM-SPSS	Good
R	Basics

Hobbies

Member of the international society for plant molecular farming (ISPMF)

Regularly exchange with international scientists in the field of plant molecular farming

American Football, cardio training and cooking

Publications

Henrik Nausch, Tina Hausmann, **Daniel Ponndorf**, Maja Hühns, Sandra Hoedtke, Petra Wolf, Annette Zeyner, Inge Broer (2016): Tobacco as platform for a commercial production of cyanophycin. New Biotechnology 33, 842-851.

Daniel Ponndorf, Sven Ehmke, Benjamin Walliser, Kerstin Thoss, Christoph Unger, Solvig Görs, Gürbüz Daş, Cornelia C. Metges, Inge Broer, Henrik Nausch (2016): Stable production of cyanophycinase in Nicotiana benthamiana and its functionality to hydrolyse cyanophycin in the murine intestine. Plant Biotechnology Journal 15, 605-613

Daniel Ponndorf, Inge Broer, Henrik Nausch (2017): Coexpression and comparison of two cyanophycinases in transgenic, cyanophycin producing tobacco plants. Transgenic Research

Scientific talks and meeting publications

05/25-27/2016	Meeting of the ISPMF in Gent – Poster presentation: „Production and stabilization of cyanophycinase in *N. benthamiana*“
02/18/2014	Agrosnet – Meeting of PhD students in Halle/Saale – Talk with the topic: „Fortifying plants with amino acids. A new strategy to increase the arginine content in forage plants.“
09/12-13/2013	Meeting „Molecular Precision Breeding“ – New Horizons and Applications IPK Gatersleben – Poster presentation with the topic:„Fortifying plants with amino acids: a new strategy to increase the arginine content in transgenic plants.”

Rostock, 22.06.2017