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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

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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*

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

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

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

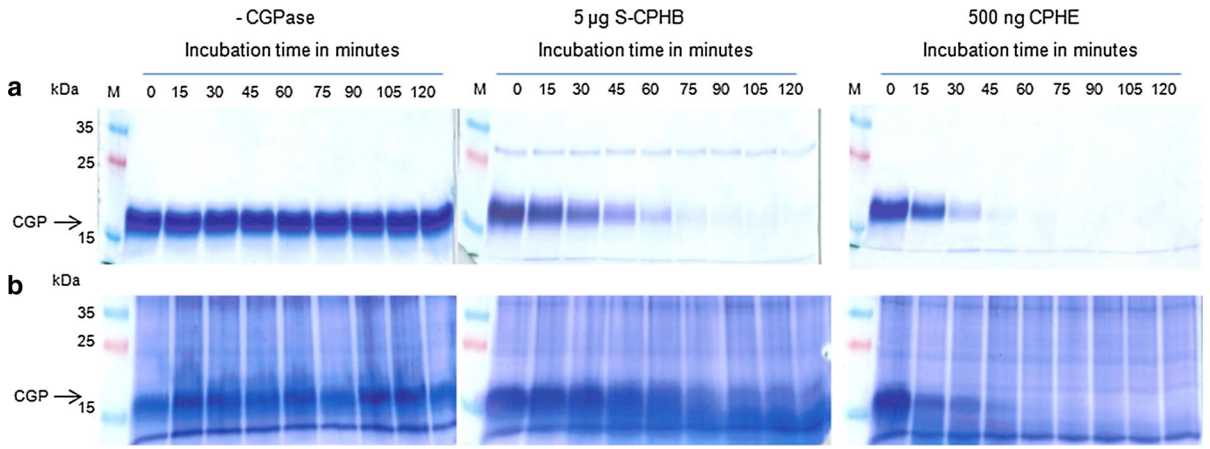

Fig. 1 Comparison of enzyme activity of S-CPHB and CPHE **a** in vitro (CPHE already shown by Nausch and Broer 2016). **b** Degradation of 20 µg isolated CGP in 100 µg total soluble protein (TSP) isolated from *N. tabacum* Badischer Geudertheimer M = PageRuler^{TM} Plus Prestained Protein ladder; -CGPase = 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

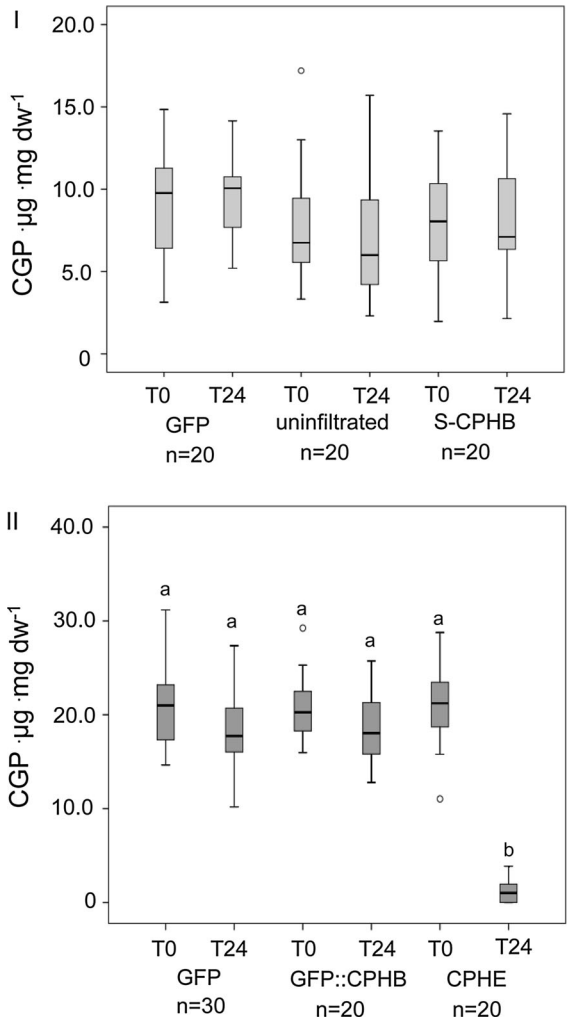

Fig. 2 *In planta* and crude plant extract activity assay of different cyanophycinase (CGPase) variants at two different experimental time points (**I** and **II**). Infiltrated *N. tabacum* Badischer Geudertheimer leaves were harvested at 10 days post-infiltration (dpi), and vertically cut into halves. 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

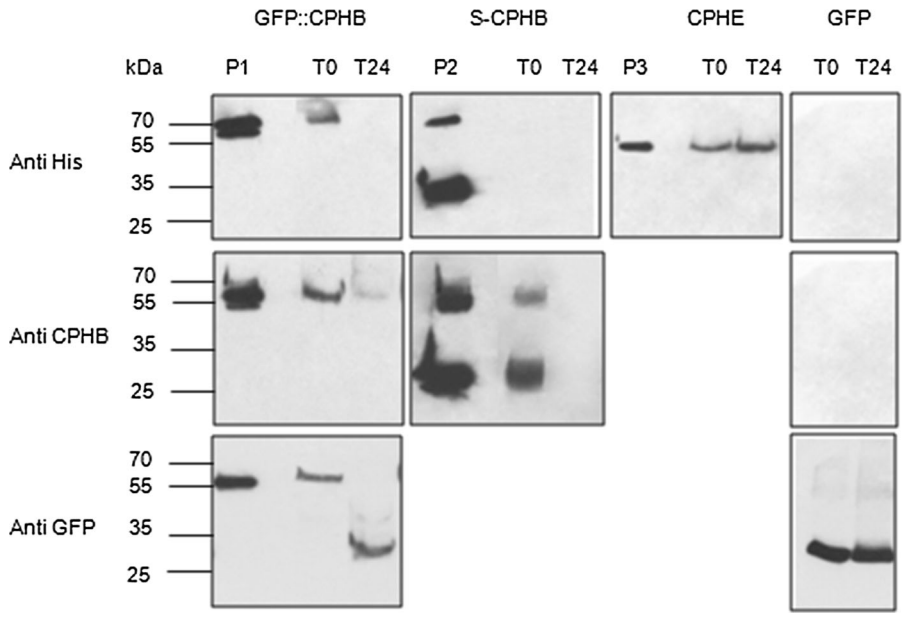

Fig. 3 Western blot analysis of 100 µg total soluble protein (TSP) isolated from *N. tabacum* Badischer Geudertheimer leaf material. Leaves were cut vertically. One half was frozen immediately (T0) the other half was homogenized and incubated for 24 h (T24). GFP, expressed by vector pICH18711 (Marillonnet 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.

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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.

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