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# From the lab to the field and closer to the market: Production of the biopolymer cyanophycin in plants

Jana Huckauf[a,*,1], Ursula Weisenfeld[b], Inge Broer[a]

[a] *Agrobiotechnology, University of Rostock, Rostock, Germany*
[b] *Institute of Management & Organisation (IMO), Leuphana University Lüneburg, Lüneburg, Germany*

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ABSTRACT

A range of studies has investigated the production of biopolymers in plants but a comprehensive assessment of feasibility and environmental safety and consumer acceptance is lacking. This review delivers such an assessment. It describes the establishment of the production in tobacco and potato, the analysis of lead events in the greenhouse and in the field, the establishment and upscaling of effective isolation processes and storage conditions, taking the cyanobacterial storage peptide cyanophycin (CGP) as an example. The paper lists several industrial and medical applications of CGP and its building blocks Arg-Asp-dipeptides. This production is especially interesting because the CGP content can exceed 10 % of the dry weight (dw) in the greenhouse and still deliver 4 g per plant in the field. Furthermore, risk assessment of CGP production in potatoes *in vitro*, *in vivo*, in the greenhouse, and in the field showed no relevant differences concerning environment or consumer safety compared with the near isogenic control. A consumer choice analysis in four European countries showed a preference for biodegradable CGP in food-wrapping materials over conventional plastic wrapping. Although data on economic feasibility is lacking, CGP as a renewable, biodegradable and $CO_2$-neutrally produced compound, is preferable over fossil fuels in many applications.

## 1. Introduction

Plants have been an important production platform for secondary metabolites but have been replaced in many cases by fossil fuels. With climate change and the rising cost of petroleum, plants have become more attractive again. Transgenic plants in particular are promising as a platform to increase the rate of potential products. Nevertheless, the sustainability of this production strategy has to be assessed. Plants provide an opportunity for mass production in the field, requiring only the addition of nutrients and water. In addition, their use of solar energy is extremely efficient. Plant cells are miniature factories that naturally produce biochemicals and biopolymers, such as starch, cellulose, hemicellulose, lignin, and rubber [1].

Nevertheless, during the 19th century, the low cost and high availability of fossil fuels triggered an industrial revolution, which led to increased production and efficiency, lower prices, more goods, improved wages, and migration from rural to urban areas. Accordingly, fuel-based chemistry has largely replaced plants as production platforms [2]. However, the widespread use of fossil fuels has caused a massive release of bound $CO_2$, which is a key driver of climate change. The price of fossil fuels fluctuates and the extraction and use of fossil fuels cause environmental and health damage [3]. Currently, the costs of fossil fuels are increasing, and climate change is causing severe problems. Consequently, plants have become the focus as production platforms for the sustainable synthesis of various materials and chemicals [4]. Oil, starch, and proteins are natural products used in biofuels, adhesives, cosmetics, detergents, paper, textiles, and pharmaceuticals [5].

As a special plant ingredient, biopolymers are of great interest because they are, in most cases, completely biodegradable and can serve as substitutes for fossil fuel-derived compounds [4,6]. Because their production in plants is $CO_2$ neutral, driven by solar energy and photosynthesis, they support the sustainable use of renewable resources.

However, attempts to produce biopolymers in agriculturally usable cultivars so far have resulted in no or low amounts of the target biopolymer. Economic viability still depends on governmental subsidies, and in order to produce significant amounts agricultural land use would have to be intensified, which might compete with food production [7]. The development of molecular biology and plant transformation

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* Corresponding author.
*E-mail address:* jana.huckauf@uni-rostock.de (J. Huckauf).

[1] Orcid-ID: 0000–0001-6054–6548

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techniques has opened possibilities of enhancing the yield and composition of traditional ingredients or of producing completely novel components of foreign origin in transgenic plants. The increase of the economic value of the plant and a more sustainable production of new and special ingredients can be expanded further by adding genes that encode enzymes, which convert an endogenous plant metabolite to a polymeric structure that is novel to the plant [8]. Several attempts to produce biopolymers have been successful (Table 1). One example is polyhydroxyalkanoate (PHA), a water-soluble biopolymer naturally produced by many bacteria as a carbon and energy reservoir that can be an alternative to petroleum-based plastics. However, bacteria can degrade PHA; hence, the concentration is limited [9]. Recent studies have shown that high amounts of PHA (more than 80 % of cell dry weight) could be produced from waste materials using halophilic microorganisms [10]. Plants cannot degrade PHA and have been proven to be effective PHA producers [11–15].

Combining natural plant-derived ingredients and products produced via transgenesis can lead to new applications. One example is the degradation of plant-derived lignin to the polyester precursor 2-pyrone-4,6-dicarboxylic acid (PDC) via enzymes encoded by bacterial genes. PDC is a promising building block for various biomaterials and polyesters [16].

Transgenic plants can also produce products in addition to natural ingredients (coproduction), such as starch, oil, or sugars, which can be isolated from different plant organs, such as leaves or seeds, or at different steps of the isolation process. Such multiple-use plants might increase the outcomes for farmers, reduce the amount of arable land used for non-food purposes, and reduce $CO_2$ emissions [17].

This study focuses on the production of the biopolymer CGP in transgenic plants. It delivers the first assessment of the feasibility, environmental safety and consumer acceptance of such a production, based on the reports in several studies (Fig. 1).

## 2. CGP - a natural storage compound from cyanobacteria with industrial potential

CGP (multi-L-arginyl-poly-L-aspartic acid) is the major nitrogen and carbon storage compound in most cyanobacterial species and in a few heterotrophic bacteria [42]. It is a branched polypeptide composed of L-aspartic acid and L-arginine (Fig. 2A) [43], sometimes with other amino acids, such as lysine, possibly replacing arginine [44,45]. Its high nitrogen-to-carbon ratio of 1:2 and stability make it an excellent nitrogen storage substance [46]. CGP accumulates in cytoplasmic membrane-less granules. The polymer is synthesized by only one enzyme, the cyanophycine synthetase (CphA), and homologous genes are found in a wide range of prokaryotes [47]. The enzyme catalyzes the ATP-dependent elongation of a CGP primer by the consecutive addition of the amino acids [45,48,49] using nonribosomal peptide synthesis [50]. First, the α-amino group of arginine is linked to the β-carboxylate group of aspartate via a β-iso-peptide bond to a β-arginine-aspartate dipeptide (β-Asp-Arg dipeptide). In the second step, the dipeptide is connected to the nascent CGP polypeptide via an α-peptide bond [51, 52].

Owing to its unusual composition, CGP exhibits interesting properties. It is insoluble under physiological conditions (neutral pH), soluble when diluted in an acid or base, and insensitive to commercially available proteases [46,53]. Degradation is possible by specific CGP hydrolases that are only present in bacteria. This activity leads to the release of arginine-aspartate dipeptides (Fig. 2C) [54]. These dipeptides have the potential for medical, nutritional, or cosmetic applications [55]. In

**Table 1**
Novel biopolymers produced in transgenic plants.

| Polymer | Native production host | Properties/ Application | Heterologous production host | References (first study) |
|---|---|---|---|---|
| Spider Silk | Spiders; produced for webs and wrapping of prey | Silk provide elasticity to tissues; combination with other polymers for new biomaterials, useful for industrial and medical purposes. | *Nicotiana tabacum*<br>*Solanum tuberosum* cv. Solara,<br>Desire *Arabidopsis thaliana* | [18]<br>[18]<br>[19] |
| Elastin | Mammals; extracellular matrix protein providing mechanical integrity to tissue | Prevent post-surgical adhesions and scars, tissue reconstruction, wound coverings, artificial pericardia, catheters, and programmed drug delivery | *Nicotiana tabacum* var. 'Petit Havana' | [20] |
| Collagen | Animals; found in connective tissue | Medical application including tissue engineering, surgical implants and drug delivery | Tobacco | [21] |
| PDC | Bacteria; intermediate that occurs during microbial degradation of lignin | Building block for diverse biomaterials and polyester | *Arabidopsis thaliana* | [16] |
| Phaseolin | In seeds of *Phaseolus vulgaris*; produced as storage protein | Applied in food formulation, fused to proteins or for protein biopolymers | Transplastomic tobacco | [22] |
| PHAs | Bacteria; produced as a carbon and energy storage polymer | Depending on polymer composition it is applicable in chemicals, plastics and feed supplements | *Arabidopsis thaliana*<br>*Gossypium* sp.<br>*Nicotiana tabacum* cv Samsun<br>*Brassica napus*<br>*Zea mays*<br>*Solanum tuberosum*<br>*Medicaco sativa*<br>*Beta vulgaris*<br>*Oryza* sp.<br>*Tamarix* sp.<br>*Linum usitatissimum*<br>*Saccharum officinarum*<br>*Panicum officinarum*<br>*Camelina sativa*<br>*Glycine max.*<br>*Populus sp.*<br>*Elaies guineensis* | [23]<br>[24]<br>[25]<br>[26,27]<br>[11]<br>[28]<br>[29]<br>[30]<br>[31]<br>[31]<br>[32]<br>[33]<br>[34]<br>[35]<br>[36]<br>[37]<br>[38] |
| Cyanophycin | Cyanobacteria and other photosynthetic and non-photosynthetic bacteria; produced as nitrogen storage compound | Medical applications of<br>copolymers of polyaspartate<br>can replace polyacrylatesL<br>-arginine stimulator of immune system | *Nicotiana tabacum* cv SRI<br>*Solanum tuberosum*<br>*Nicotiana tabacum* cv SL632<br>*Nicotiana tabacum* cv Virginia<br>Golta<br>*Nicotiana tabacum* cv<br>Badischer Geudertheimer | [39]<br>[40]<br>unpublished<br>[41]<br>[17,41] |

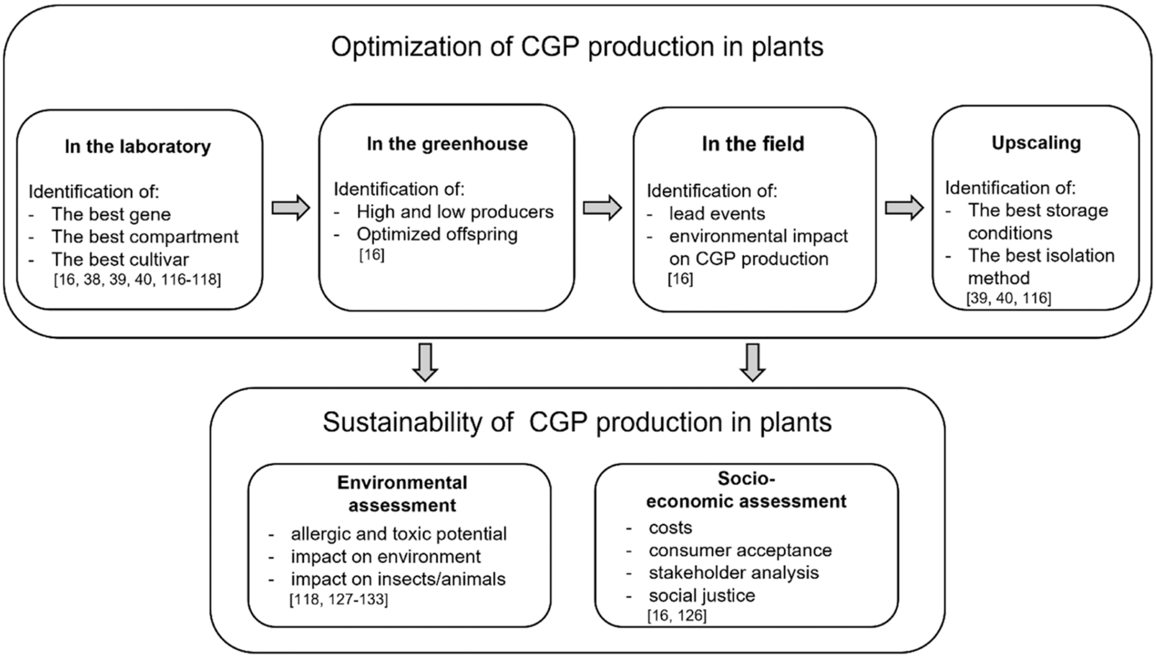

**Fig. 1.** Graphical overview.

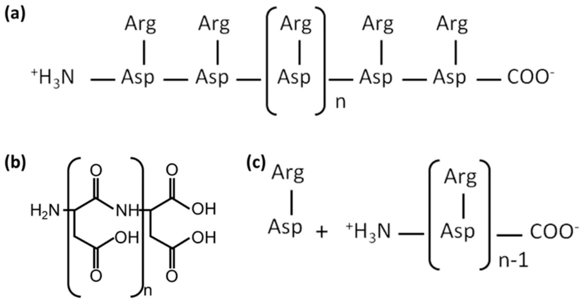

**Fig. 2.** A Structure of cyanophycin [46]. B Polyaspartate, which is the result of acid hydrolysis of cyanophycin after isolation. **C** β-Asp-Arg dipeptides resulted from enzymatic degradation of cyanophycin with cyanophycinase.

addition to the nutritional value, this might be due to the physiological functions of amino acids, such as immunomodulatory effects, which promote health and well-being [56,57]. In particular, although Arg is not an essential aminoacid, Arg dietary deficiency can result in metabolic, neurological, or reproductive dysfunction in animals such as pigs, poultry, and fish [56,58]. Dipeptides were also proposed to be tyrosinase inhibitors [59]. Although free amino acids inhibit each other's uptake [60], this is not the case for di-, tri-, and oligopeptides, and their intake results in a more balanced amino acid profile. In addition, a highly affine dipeptide absorption system in enterocytes of the small intestine supports a better uptake than that of free amino acids [55,61–68]. These beneficial effects are so pronounced that dipeptides can be applied in treating some human diseases [55,66]. L-Arg has been suggested to be a regulator of several physiological and immunological processes; for instance, it acts as a growth inducer [69–71] that stimulates the immune system and inhibits tumor cell growth [72–74]. Oligo-Arg has been studied for its ability to penetrate cell walls and may be a component of herbicides or antimalarial drugs [75].

CGP itself also has some medical applications; for example, if dissolved with HCl and cross-linked with glutaraldehyde, it shows potential for biomedical use in tissue engineering [76]. Acetylation can improve the permeability of the blood-brain barrier to various pharmaceuticals [77].

Although CGP is too brittle to be used alone [78] its modifications and combinations with other compounds extend its range of applications and are well-studied [78,79]. For instance, combined with hyaluronic acid or γ-polyglutamic acid, it can assemble polyelectrolyte multilayers with potential applications in wound dressings [80]. These layers support cell growth and migration. When conjugated with polyethylene glycol, CGP can form self-assembled nanovesicles that can be used to encapsulate anticancer drugs [81]. They can also serve as antibacterial agents, and adding diverse side chains with various properties can further expand the field of biomedical applications [51].

The fact that CGP is built from an Asp backbone with Arg residues suggests other potential applications. Mild hydrolysis of CGP is thought to result in the formation of homopolymers of polyaspartate (Fig. 2B) [40,82,83]. Polyaspartate is soluble, non-toxic, and biodegradable and can replace petrochemical polyacrylates [84] with wide applications in industry, agriculture, and medicine. It is currently chemically synthesized and is a biodegradable and biocompatible polymer with multiple biomedical [85] and industrial [86] applications, such as green antiscalant or water softener [87]. L-aspartic acid can also be converted into β-alanine via α-decarboxylation, which subsequently leads to the formation of acrylamide. Polyacrylamide is mainly used in wastewater treatment and is currently produced via the hydrolysis of acrylonitrile by the energy-intensive ammoxidation of propene [88]. The remaining free L-arginine can be hydrolyzed to L-ornithine and urea. While urea can be directly used as a fertilizer, the decarboxylation of L-ornithine leads to the formation of 1,4-diaminobutane, a monomer in nylon-4,6. Currently, 1,4-diaminobutane is mainly synthesized via hydrogenation of succinonitrile, which is produced by adding hydrogen cyanide to acrylonitrile [83].

As the CGP productivity of native producers at the industrial scale is limited [89], different biotechnological strategies have been employed to improve the process, such as optimization of the production media with additives (arginine) or renewable carbon sources, downstream processes, and heterologous expression in various microbial strains of diverse cyanobacterial CphA's. One such example is the optimization of CGP synthesis in cyanobacteria. A mutated PII signaling protein increases CGP production in *Synechocystis* sp. PCC 6803 up to 40 % dw [90]. Further experiments confirmed that CGP production in cyanobacteria can be raised under phosphorus limitation. In *Nostoc* sp. PCC

7120, the overall biomass was maximized by adaptation of nitrogen availability and parallel phosphorous limitation, which boosts CCP production up to 15 $mg_{CGP}$ $L^{-1}$ $d^{-1}$ [91,92]. Nevertheless, several authors still concluded that cyanobacteria are not suitable for the large-scale production of CGP because the growth of photosynthetic bacteria is slow, and only low cell densities are obtained [88,89,93].

Hence, for large-scale production, cyanobacterial cphA genes have been heterologously expressed in *Escherichia coli*, *Pseudomonas putida*, *Corynebacterium glutamicum*, *Cupriavidus necator* (formally *Ralstonia eutropha*), *Lactococcus lactis*, *Rhizopus oryzae*, a filamentous fungus, and eukaryotes such as *Saccharomyces cerevisiae*, *Pichia pastoris*, and plants (Table 2).

### 2.1. Plants as a platform for the production of CGP

Plant CGP production was first reported in 2005 [39]. CGP synthetase isolated from *Thermosynechococcus elongatus* ($CphA_{Te}$) is constitutively expressed in the cytosol of tobacco and potato plants. The choice of this gene was excellent because - CGP synthetases typically require existing segments of cyanophycinwhile $CphA_{Te}$ does not depend on such a primer for CGP synthesis [98]. Tobacco was chosen as the production platform because it is a non-food crop already used to produce therapeutic proteins [109,110]. In addition to its suitability for producing high-value compounds, tobacco also meets the needs of large-scale industrial production of low-value materials [111]. Tobacco can be grown without special demands on soil, fertilizers, or the environment, and it rapidly forms large amounts of leaf biomass [112–114]. Solaris, a special tobacco variety, is grown for oil production because of its high seed production [115]. In addition to oil, similar varieties can be used to produce CGP as a secondary product without additional demand for arable land, fertilizer, or other inputs [115].

The same is true for potatoes. For example, approximately 20 % of the potatoes harvested in Germany are used for starch production [116]. CGP could be the second product in the tuber isolated after starch isolation from the waste material [117]. During acid isolation from the tubers, CGP is isolated together with solanine, the main alkaloid of the potato. Even though the amount is low, production in engineered potato lacking key enzymes in the glycoalkaloid biosynthesis pathway should

**Table 2**

Examples of heterologous hosts for CGP production. $cphA_{6803}$ from *Synechocystis sp*. PCC 6803; $cphA_{6308}$ from *Geminocystis herdmanii sp*. PCC 6308; $cphA_{587}$ from *Acinetobacter sp*. Strain DSM 587; $cphA_{ADP1}$ from *Acinetobacter calcoaceticus* ADP1; $cphA_{7120}$ from *Anabaena sp*. PCC 7120; $cphA_{10664}$ from *Desulfitobacterium hafniense* DSM 10664; $cphA_{TE}$ from *Thermosynechococcus elongatus* BP-1; $cphA_{49}$ from deep-sea sediment metagenomis library (fss49 fosmid); $cphA_{MA19}$ from *Synechococcus sp*. MA19; $cphA_{6308}\Delta1$ from *Geminocystis herdmanii sp*. PCC 6308.

| Expression host | Strain/Variety | Cyanophycin synthetase gene | CGP production | References |
|---|---|---|---|---|
| *Escherichia coli* | DH1 | $cphA_{6803}$ | 24.0 % (w/w) | [94] |
| | | $cphA_{6803}$ | 28.0 % (w/w) | [95] |
| | | $cphA_{587}$ | 7.5 % (w/w) | [47] |
| | BL21 | $cphA_{6803}$ | 12.0 % (w/w) | [96] |
| | | $cphA_{6308}$ | 30.0 % (w/w) | [97] |
| | | $cphA_{ADP1}$ | 14.0 % (w/w) | [97] |
| | | $cphA_{7120}$ | 12.5 % (w/w) | [97] |
| | | $cphA_{10664}$ | not specified | [48] |
| | | $cphA_{TE}$ | not specified | [98] |
| | | $cphA_{49}$ | 16.0 % (w/w) | [99] |
| | TOP10 | $cphA_{6308}$ | 26.6 % (w/w) | [44,100] |
| | | $cphA_{7120}$ | 21.0 % (w/w) | [100] |
| | HMS174 (DE3) | $cphA_{6308}$ C595S mutant | 36.2 % (w/w) | [101] |
| *Pseudomonas putida* | *GPp104* | $cphA_{6803}$ | 9.7 % (w/w) | [100] |
| | | $cphA_{7120}$ | 24.0 % (w/w) | [100] |
| | | $cphA_{MA19}$ | 17.5 % (w/w) | [47] |
| | | $cphA_{6308}$ | 10.0 % (w/w) | [100] |
| | *KT2440* | $cphA_{6308}$ | 9.0 % (w/w) | [100] |
| | | $cphA_{7120}$ | 23.0 % (w/w) | [100] |
| | | $cphA_{MA19}$ | 15.8 % (w/w) | [100] |
| | | $cphA_{6803}$ | 6.8 % (w/w) | [100] |
| | *ATCC 4359* | $cphA_{6308}$ | 43.4 % (w/w) | [102] |
| *Corynebacterium glutamicum* | | $cphA_{6803}$ | 3.6 % (w/w) | [100,103] |
| | | $cphA_{10664}$ | 17.1 % (w/w) | [104] |
| | | $cphA_{BP-1}$ | 13.6 % (w/w) | [104] |
| *Cupriavidus necator (Ralstonia eutropha)* | $PHB^{-4}$ | $cphA_{6803}$ | 8.9 % (w/w) | [100] |
| | | $cphA_{6308}$ | 13.8 % (w/w) | [100] |
| | | $cphA_{MA19}$ | 16.0 % (w/w) | [100] |
| | | $cphA_{7120}$ | 22.0 % (w/w) | [100] |
| | H16 | $cphA_{6803}$ | 7.3 % (w/w) | [100] |
| | | $cphA_{6308}$ | 12.6 % (w/w) | [100] |
| | | $cphA_{MA19}$ | 14.1 % (w/w) | [100] |
| | | $cphA_{7120}$ | 20.0 % (w/w) | [100] |
| | HF39 | $cphA_{6803}$ | 7.0 % (w/w) | [103] |
| *Lactococcus lactis* subsp. *Cremoris* | NZ3900 | $cphA_{6803}$ with a glycine residue at the C-terminus | 20.4 % (w/w) | [96] |
| *Rhizopus oryzae* | *M16* | $cphA_{6803}$ | 0.5 % (w/w) | [105] |
| | | $cphA_{7120}$ | no CGP activity | [105] |
| *Saccharomyces cerevisiae* | *Arg4* | $cphA_{6308}$ | 6.2 % (w/w) | [106] |
| | | $cphA_{6308}$ | 12.0 % (w/w) | [107] |
| *Pichia pastoris* | GS115 | $cphA_{6308}$ | 13.0 % (w/w) | [108] |
| | GS115 | $cphA_{6308}\Delta1$ | 23.3 % (w/w) | [108] |
| | *KM71H* | $cphA_{6308}\Delta1$ | 11,6 % (w/w) | [108] |
| *Nicotiana tabacum* | SRI | $cphA_{TE}$ | 1.1 % dw | [39] |
| | SL632 | $cphA_{TE}$ | 10.4 % dw | unpublished |
| | Virginia Golta | $cphA_{TE}$ | 6.6 % dw | [41] |
| | Badischer Geudertheimer | $cphA_{TE}$ | 11.4 % dw | [17,41] |
| *Solanum tuberosum* | *Albatros* | $cphA_{TE}$ | 7.5 % dw | [40] |

be pursued, if CGP is to be used for medical applications.

In most tobacco and potato transformants, the production of CGP was relatively low, and the transgenic plants showed several adverse symptoms, such as growth reduction or variegated leaves. Nevertheless, the highest production was observed in tobacco plants. The best performer contained 1.14 % CGP in the dw, which was high but showed strong growth reductions [39]. New production systems must be developed to obtain healthy plants with high CGP content. Since cyanobacteria can survive with high CGP content, CGP synthase was guided to the chloroplasts. Here, three different leader peptides were tested. Only the psby leader encouraged the transport of CGP synthase to the plastid [118]. While plants containing the other constructs showed symptoms similar to those expressing the protein in the cytosol, tobacco and potato plants expressing the psbyCphA$_{Te}$ gene showed no phenotypically obvious stress symptoms. Nevertheless, potato production remained quite low. With constitutive expression, the CGP content in the tubers reached approximately 1 % CGP dw in the greenhouse and the field (Fig. 3A) [117,119]. As CGP is a nitrogen storage compound that the plant cannot use, the amount of CGP in the tuber might be limited by N-availability. Nevertheless, an increase in nitrogen fertilization in the greenhouse and field trials was found to positively affect biomass production in CGP-producing plants and near-isogenic lines, but cyanophycin accumulation was unchanged [119]. Similar results have been reported in cyanobacteria [91]. Therefore, CGP production was not limited by N availability. This is also supported by the fact that the amount of free amino acids is not reduced in the CGP-producing lines [39]. In conclusion, CGP production does not need additional N fertilization. Using a tuber-specific promoter increased the amount of CGP in the tuber to 7.5 %/dw [40,118]. However, the production of more than 3 % dw was accompanied by massive damage, which reduced the storage capacity [40,118]. Hence, potatoes are unsuitable for CGP production.

This was completely different for the tobacco plants. Among the primary transformants (T0), the best performer expressing the psbyCphA$_{Te}$ gene produced 1.73 % of CGP/dw. Through self-fertilization and selective breeding, the CGP content could be increased to 6.8 % dw in the T2 generation.

The first transformations used *N. tabacum* SRI [39], a small and commercially uninteressting laboratory variety, as the target. To increase biomass, the gene was used to transform two commercial tobacco varieties, Virginia Golta (VG) and the German variety Badischer Geudertheimer (BG). The primary transformants contained up to 6.6 % CGP/dw (VG) and 11.4 % CGP/dw (BG) [17,41].

A trial was conducted in Argentina to test the CGP production capacity in the field [17]. The T1 generation of BG events (with low, medium, and high expression levels) was chosen to analyze the environmental influence on different genotypes (Fig. 3B).

While the T0 events contained < 5 % CGP/dw (low), 6–7 % CGP/dw (medium), and 10 % CGP/dw (high), the T1 generation showed a maximum content of approximately 4 % CGP/dw, both in the greenhouse and first field trial. Because tobacco plants produce significantly more biomass in the field, the CGP yield/plant with a maximum of 4 g CGP/plant was considerably higher than in the greenhouse with 1 g/plant.

One of the main factors influencing the extent of sustainability in production is the CGP yield/hectare. Independent of the CGP content, most transgenic plants exhibited a slight but not significant yield penalty in leaf biomass, especially under stress conditions in the greenhouse and field trials. The decrease in seed yield observed in the greenhouse indicates that the production of CGP is an additional burden that, although insignificant for the leaf yield under optimal conditions, may lead to decreased stress resilience in transgenic plants. Since the storage of CGP-producing tobacco is possible via ensilage and CGP is stable under these conditions [41] the leaves were either sundried or ensiled. The resulting material was used to evaluate the extraction of CGP and compared with the laboratory protocol. Improved strategies using dried or ensilaged plant materials were developed [17]. The labor-intensive harvest of tobacco leaves can also be replaced by mechanized harvesting, which is already practiced by some tobacco farmers in Europe, yielding up to 3.5 t leaf dw per year and ha [112,113]. Farm-level analysis indicated that extracting CGP from tobacco plants can provide alternative income opportunities for tobacco farmers and that the CGP yield/ha might render CGP production economically feasible in plants.

Nevertheless, data on large scale production costs (e.g., cultivation and extraction costs) is lacking, hence, economic feasibility cannot be evaluated.

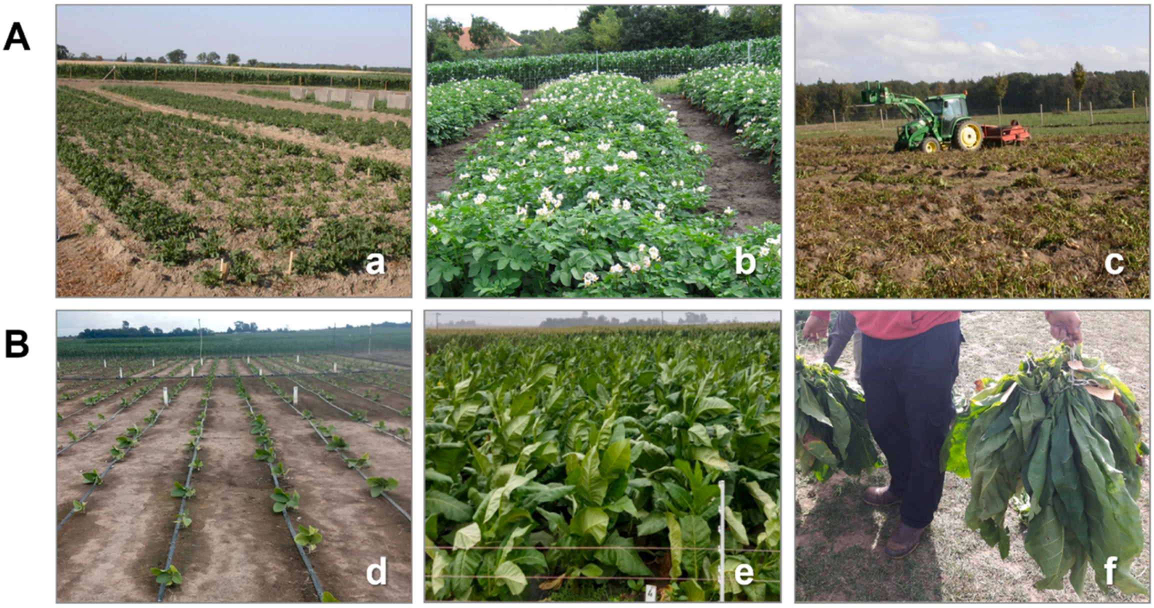

**Fig. 3.** Experimental release of: (A) two independent potato clones of PsbY-*cph*A 2006–2008 at different time points in the growth period, (B) six independent tobacco events of PsbY-*cph*A 2019/20 at different time points in the growth period. a, July sprouting of potatoes; b, August main growth period; c, October harvest; d, growth in the field with irrigation; e, plants with flowers; f, leaf harvest (photo a-c: chair Agrobiotechnology, University of Rostock; d-f: photos INDEAR, Rosario Argentina field trial 2019–2020).

When ultra-low nicotine tobacco varieties are used, the CGP produced in the tobacco can also be used as an Arg-Asp-dipeptide source. Bacteria able to digest CGP into dipeptides were identified in the gut, but amino acid uptake occurs already in the small intestine, so degradation occurs too late [54]. Hence, cyanophycinase must be added to the feed to make dipeptides available. One possibility is the expression of cyanophycinases in plants. Nevertheless, premature degradation of CGP in plants must be avoided if dipeptides are unstable. The production of CGP in chloroplasts offers the possibility of storing cyanophycinase in the cytosol, so both the enzyme and substrate are spatially separated, and degradation should only occur when the plant cell is destroyed. The cytosolic expression of CphE, a cyanophycinase produced by non-CGP-producing bacteria, led to the complete degradation in extracts of plants with 2.7 % CGP in the dw [120] within 1 h, whereas a CphB, a cyanophycinase from cyanobacteria, could not degrade in the extract. This was due to the higher stability of CphE [121].

Studies in mice have shown that plant-expressed CGP fed in combination with plant-made CGPase was hydrolyzed in the intestine, and high levels of beta-Asp-Arg dipeptides were found in the plasma, demonstrating dipeptide absorption [122]. Intestinal degradation of CGP by CGPase led to a low intestinal CGP content 4 h after consumption [122].

## 2.2. *Sustainability of CGP-producing plants*

While most of the work described above refers to the natural science and economic aspects of CGP production in plants, it is increasingly important to look at sustainability, which has become a key goal since safe earth system boundaries are increasingly exceeded [123], and climate and biodiversity crises point to the urgency of technological and institutional change. Historically applied to the long-term use of forests as a natural resource [124], environmental sustainability is related to the ability to maintain natural resources. Environmental sustainability has been identified as an important topic in science and technological development, but it needs to be complemented by institutional, political, and behavioral changes [125].

The social dimension of sustainability addresses societal benefits and risks (e.g., to health) and can be assessed through a participatory stakeholder approach [126].

Perceived societal benefits and risks influence the public acceptance of CGP applications. This has been addressed [127]. The authors explored the acceptance of hypothetical CGP-based food wrapping as a replacement for conventional plastics in various applications. They presented a choice-based conjoint analysis in four European countries, showing that CGP-based wrapping materials were preferred over conventional plastic wrapping.

Considerable research has been undertaken to identify potential risks for consumer health [128]. The consumer risk primarily consists of toxicity and allergenicity. Hence, several studies have analyzed the effects of CGP addition to feed on different rodents. CGP could not be detected after acidic extraction in the feces of rats fed with 20 % CGP-containing potatoes added to the feed [129]. This is in accordance with Salam and Steinbüchel [54], who reported CGPase activity in the digestive system but not until the colon was reached. Hence, intact CGP is thought to be present in the intestine, where it might pass through the membrane and enter the bloodstream. *In vitro* experiments using live pig gut mucosal membranes demonstrated that CGP could not enter the bloodstream [128]. Potential allergenic effects were excluded by analyzing blood sera isolated from systemically sensitized mice and orally fed rats. CGP-specific IgE, typical of an allergic reaction, was not detected. Nevertheless, a specific IgG was detected in the serum [129]. This indicates an immune reaction to CGP but no allergenic response.

Environmental risks were analyzed in different experiments using CGP-producing potatoes in a field trial [119]. Regarding substantial equivalence to the NIC, starch content was not significantly reduced [40, 119]. Neubauer [130] analyzed the potential changes in approximately 60 compounds from various substance classes (sugars, sugar alcohols, amino acids, organic acids, fatty acids, and sterols) compared with the baseline of six different potato varieties using gas chromatography–mass spectrometry. They identified uncommon variations in substance composition; however, their biological relevance remained to be determined [130]. In addition, a study showed that the amino acid content in CGP-producing potatoes and the NIC varied between years, and the significant effects found in 2006 were undetectable in 2007 and 2008 [119]. The same holds for the effects measured in 2008 or 2007. The only effect found in all three years was a decrease in bound glutamate, which was supposed to be due to Arg-Asp enrichment and an increase in Arg, except in the event of low CGP content in 2007. Hence, no significant unexpected changes in substantial equivalence were detected during the three-year field trial [119]. Nevertheless, one significant difference between the CGP-containing potatoes and NIC was the number and size of tubers. While the weight of CGP-producing tubers was significantly lower than that of NIC, the number of tubers per plant was significantly higher [119]. Another significant result of CGP production in potatoes was the occurrence of brown spots in the tubers. Hühns [40] reported that greenhouse tubers increased in brown spots after 32 weeks of storage. However, the tubers germinated. As they are not meant for consumption, this is not disadvantageous. Nevertheless, this could be a sign of an increased defense reaction that might influence the survival of tubers during winter and their subsequent reappearance in the next growing season. Therefore, CGP-producing tubers and the two NIC varieties Albatros and Desiree were stored in the soil at 10 and 30 cm depth during the three winter periods [131]. An increase in peroxidase and phenol content was identified in CGP-producing lines. Instead of increasing the frost resistance, the resistance decreased with an increase in peroxidase and phenols. Hence, the survival of CGP potatoes in soil did not increase compared with that of the NIC varieties [131].

The potential effects of genetically modified potato tubers producing CGP, along with altered enzyme activity in the tubers, were investigated with regard to the biomass, enzyme activity, and structural diversity of microorganisms. The caulosphere and bulk soil were sampled three to five times in field experiments during each of the three consecutive winter seasons [132]. The microbial biomass and enzyme activities involved in the C, N, and P cycles were analyzed, and the microbial community structure was determined based on phospholipid fatty acid (PLFA) analyses [131]. When potato tubers decomposed during winter, the microbial biomass in the caulosphere of all potato events increased independent of genetic modifications. Enzyme activities and PLFA markers in the caulosphere differed significantly among the three winter periods; however, the influences related to genetic modification or CGP production were not significant [131]. In summary, transgenic CGP-producing potato tubers exhibited no internal enzyme activity or effects on soil microorganisms that differed from those recorded in non-transgenic cultivars [131,132]. The effect of decomposing CGP potatoes on the earthworm *Lumbricus terrestris* (L.) was analyzed in a microcosm experiment. The potatoes were easily degradable and neither inhibited nor stimulated earthworm growth, reproduction, or activity [133].

CGP was also expressed in the transgenic roots of composite *Vicia hirsuta* plants grown in Petri dishes for semi-sterile growth or in small containers filled with non-sterile soil. The structural diversity of the bulk soil microbial community between transgenic and non-transgenic cultivars was determined using PLFA. Significant differences in the PLFA ratios of bacteria:fungi and of gram-positive:gram-negative bacteria were associated with dissimilarities in the quantity and molecular composition of rhizodeposits, as revealed using Py-FIMS analyses [134], especially between planted and unplanted soils. The effects of transgenic plants on a single PLFA were not significantly different from those of the NIC.

In summary, cphA expression had no significant influence on root induction, growth, biomass, or interaction with symbionts such as rhizobia (number, size, and functionality of nodules, selection of nodulating strains or arbuscular mycorrhizal fungi [134].

Although several aspects of environmental and consumer safety have been addressed, the analysis is by far not complete. For example, a $CO_2$ food print has not been calculated. In addition, the economic aspect as another dimension of sustainability needs to be addressed more thoroughly, also taking into account the yield penalty observed under stress conditions in tobacco.

## 3. Concluding remarks

To the best of the authors' knowledge, this is the most comprehensive analysis of the sustainability of transgenic plants developed at a university and not by a company. The production of CGP in transgenic tobacco is a promising strategy for producing several biodegradable compounds that can substitute fossil fuels in many applications. This could be a sustainable path in several respects: From an environmental perspective, it provides advantages because the production in the plant is $CO_2$-neutral, it is renewable, no additional fertilizer is necessary, and, as co-production, no additional land for cultivation or other agricultural input is needed. For potatoes, CGP production did not show any risks to consumers or the environment that differ from the NIC, and there is no reason to assume this is different for tobacco. From a social perspective, it offers an additional market for tobacco farmers to substitute the decreasing demand for tobacco as a smoking device. A consumer study conducted in four European countries supports the hope that a transgenic commodity will be accepted in the European market, at least if it is a non-food feed product with proven environmental advantages. From the economic perspective, the assessment to date is mainly based on production efficiency, but data on large scale production and extraction costs as well as on pricing is still missing. In addition, substituting compounds already used in industry is complicated and expensive, and the profit must be high and safe to merit such efforts. However, since CGP is renewable, biodegradable and $CO_2$-neutrally produced, it is a promising avenue for sustainable production.

## CRediT authorship contribution statement

**Broer Inge:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Weisenfeld Ursula:** Writing – review & editing, Writing – original draft. **Huckauf Jana:** Writing – review & editing, Writing – original draft, Data curation, Conceptualization.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Data availability

No data was used for the research described in the article.

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