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Tobacco as platform for a commercial production of cyanophycin

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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.

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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.

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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-*cph*A_{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′-AGC-CAGGAGCGGCATTGACC-3′) for the coding region of *cph*A_{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.

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