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Research Article

Electrophoretic and chromatographic evaluation of transgenic barley expressing a bacterial dihydrodipicolinate synthase

Nutritional quality of human and animal foodstuffs is determined by the content of essential amino acids. Barley is the fourth most important cereal of the world and the second most important cereal grown in the Czech Republic. Cereal grains such as barley contain insufficient levels of some essential amino acids, especially lysine. Dihydrodipicolinate synthase is the key enzyme involved in the regulatory step for lysine biosynthesis. Two constructs pBract214::sTPdapA and pBract214::mdapA containing the dapA gene from *Escherichia coli* coding for the bacterial dihydrodipicolinate synthase were used for transformation of barley. An Agrobacterium-mediated technique was used for transformation of immature embryos of spring barley cv. Golden Promise. Transgenic barley plants of the T0 and T1 generations were evaluated by PCR, real-time PCR, gel electrophoresis, and Western blot. Amino acid content was analyzed by HPLC after HCl hydrolysis. The lysine content in leaves of the T1 generation plant no. 5/5 was 50% higher than in wild-type plants; the lysine content in seeds of T2 generation plant no. 5/16 was 30% higher than in wild-type seeds of spring barley cv. Golden Promise.

Keywords:
Foodomics / Gel electrophoresis / Polymerase chain reaction / Transgenic barley / Western blot
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1 Introduction

Plants are of great importance to mankind since they are sources of human nutrition and provide animal feed as well as other important biomaterials. Nutritional quality of plant-derived human and animal foodstuffs is determined by the content of some compounds including essential amino acids. Lysine is one of the essential amino acids and due to its chemical properties, its presence in the peptide chains of many proteins is a very important factor affecting their function in mammalian metabolism [1–3]. Therefore, man is dependent on the intake of this amino acid from crops such as barley [4], wheat [5] or maize [6]. Lysine as well as threonine and methionine are synthesized by the aspartate biosynthetic pathway (Fig. 1A), which is predominantly regulated by feedback inhibition of aspartate kinase (AK) and dihydrodipicolinate synthase (DHDPS) [7]. DHDPS is the key enzyme in the regulatory step for lysine biosynthesis (Fig. 1B). This pathway can be found in plants and bacteria but the bacterial enzyme is much less sensitive (50-fold) to feedback inhibition by lysine than its equivalent plant enzyme.

Cereal grains such as wheat, barley, and maize contain insufficient levels of lysine. Modern genetics and biotechnology provide a number of tools that can be utilized for the development of higher food quality. The introduction of gene transfer technologies opened up new ways to alter the amino acid composition of the seed proteins in cereals including Spring barley (*Hordeum vulgare* L.), which is one of the most agronomically important crops in Europe [8]. Based on these facts, it is possible to express bacterial DHDPS in agronomically important crops and therefore increase free-lysine content [6, 9]. Transgenic plants with increased-free lysine levels in seeds have been obtained in *Arabidopsis*, tobacco, potato, barley, wheat, rice, corn, oil seed rape, pigeonpea, and soybean [10, 11], however, transformation strategies and analytical and biochemical tools for evaluation of the yield of the inserted compounds require optimizing [12]. Due to the

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Abbreviation: DHDPS, dihydrodipicolinate synthase

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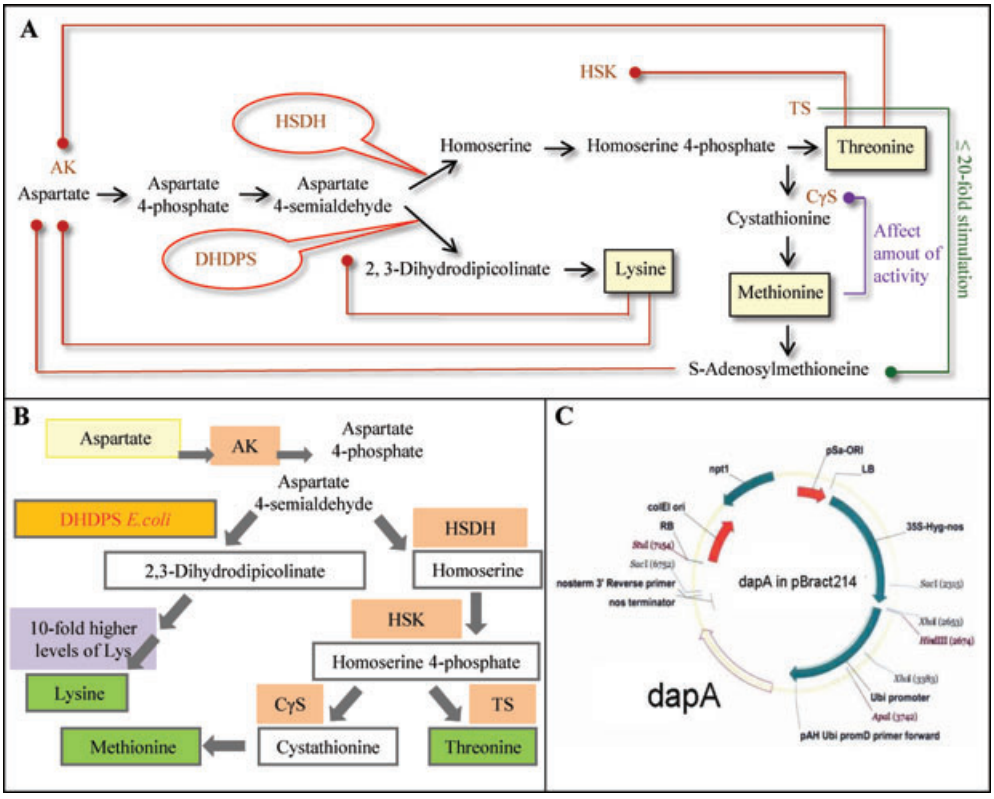

Figure 1. (A) Biochemical pathway of synthesis of lysine in higher plants adopted and modified according to Vauterin et al. [5]. (B) Diagram of dihydrodipicolinate synthase (DHDPS) regulation of the accumulation of lysine in plants by using the *E. coli* gene. (C) Diagram of vector pBract 214 containing the dapA gene for DHDPS synthesis.

timeliness of this topic, a new scientific branch called foodomics has been formed [13].

Foodomics has been defined as a new discipline that covers the scientific fields touching food and nutrition together with sophisticated modern analytical methods. Application of foodomics include other "omics" such as genomics, transcriptomics, proteomics, and other methods of compound identification related to food quality, food safety, or development of new transgenic food [14, 15]. Foodomics is also applying quantitative analysis for the determination of nutrition using different analytical methods [16]. Electrophoretic techniques where development is associated with technology based on the principle of lab-on-chip and microfluidic devices are suitable for such studies [17, 18]. The versatility of electrophoretic techniques and the ability to connect to a mass detector increases the application range of these methods in bioanalysis [19–22]. Thanks to the many available approaches, one may monitor many of the analytes from small organic and inorganic ions through simple organic molecules to peptides and proteins. Other methodologies are applicable for studying biomolecules such as DNA in foods [23]. Moreover, approaches for detection of the presence of transgenic food material are also of great interest [24–26].

In this study, Spring barley (variety Golden Promise) was genetically modified using a dapA construct to increase protein synthesis of DHDPS with subsequent enhancement of lysine production (Fig. 1C). For monitoring the success of genetic transformation electrophoretic methods were used. At the level of mRNA, the presence of the dapA transgene was monitored using PCR and agarose gel electrophoresis. Subsequently, determination of protein expression level by SDS-PAGE and Western blotting was carried out. To support the electrophoretic results, ion exchange chromatography with postcolumn derivatization by ninhydrin was used to quantify total lysine content in transgenic barley. These approaches allowed us to confirm the functionality of the chosen experimental scheme to engineer improved-free lysine content in barley.

2 Materials and methods

2.1 Chemicals

Working solutions of buffers or standard solutions of amino acids were prepared daily by diluting stock solutions. Standards of amino acids and others were of ACS purity and purchased from Sigma-Aldrich (USA) unless noted otherwise. The chemicals for Aminoacid analyzer (Ingos, Czech Republic) were prepared according the manufacturer's instructions and were purchased from Ingos (Czech Republic). All solutions were prepared in deionized water obtained using reverse osmosis equipment (Aqual 25, Czech Republic). The deionized water was further purified by using apparatus MiliQ Direct QUV equipped with the UV lamp. The resistance was 18 MΩ. The pH was measured using pH meter WTW inoLab (Weilheim, Germany).

2.2 Preparation of the plant expression vector

Two constructs pBract214::sTPdapA and pBract214::mdapA containing the *E. coli dap*A gene (gene of interest) coding for the bacterial DHDPS were used for transformation of barley. The cassette sTPdapA also includes the transit peptide Rubisco (*Hordeum vulgare* ribulose-1,5-bisphosphate carboxylase small subunit, Genbank U43493), which was prepared synthetically (Mr. Green, Germany). The *dapa* gene was under the control of the Ubi promoter from maize and nos terminator (Fig. 1C).

Prior to cloning into the destination vector intended for transformation of plant cells, DHDPS protein expression was verified in bacterial cells. The prepared expression vector pET160-DES, which contained a specific sequence Lumino (Lumino tag, Invitrogen), which is expressed together with the protein of interest, was transformed into chemo-competent *E. coli* BL21 (DE3) STAR cells. After a 5-h long cultivation, lysates were purified using NiNTA columns (Qiagen), which attach HIS-labeled proteins. After adding the luminescent materials (Lumino Green Detection Reagent, Invitrogen) the presence of the desired protein was evaluated by SDS-PAGE.

Each cassette containing the *dapA* gene was digested with KpnI and XhoI (NEB) and subcloned into the vector pENTER1A (Invitrogen), between the attL1 and attL2 specific recombination sites. Using LR specific recombination (Gateway® LR Clonase™ II, Enzyme Mix, Invitrogen cat. no. 11791-020) we further subcloned the *dapA* gene into the destination vector pBRACT214, specifically prepared for expression studies in barley (http://www.bract.org). Gene of interest was under the control of maize Ubi promoter, the construct also contains a gene conferring the resistance to the antibiotic hygromycin (selective marker) under the control of the 35S promoter (35S-Hyg-nos), (Fig. 1C). Both prepared expression vectors pBract214::sTPdapA and pBract214::mdapA were verified by restriction analysis. Each vector was transferred with helper plasmid pSoup to *Agrobacterium*, stain AGL1. pSoup contains the repA gene which enables pBRACT214 to replicate in *Agrobacterium*.

2.3 Plant material

Barley transformation is very genotype dependent. For our transformation experiments we used the responsive spring cultivar Golden Promise. Donor plants were grown in a controlled environment room at 15°C day and 12°C night temperatures, 80% relative humidity, and with light levels of 500 $\mu E.m^{-2}s^{-1}$ at the mature plant canopy level provided by high pressure sodium bulbs (Plantastar 400W, OSRAM, Czech Republic). Barley spikes were collected when immature embryos were 1.5–2 mm in diameter. The immature seeds were removed from the spike and firstly sterilized in 70% (v/v) ethanol for 30 s and then with 6% (v/v) sodium hypochloride (Sigma-Aldrich, USA) for 4 min. They were rinsed four times with sterilized water. The embryo was isolated from seed with the aid of two pairs of fine forceps and the embryonic axis was removed. The isolated embryos without embryogenic axis were plated scutellum side up on callus induction medium [27, 28].

2.3.1 *Agrobacterium*-mediated transformation

Agrobacterium strain AGL1 containing the appropriate pBract214 vector, with the *dap*A gene under the control of the maize ubiquitin promoter (ubi1), and the *hpt* gene conferring hygromycin resistance under a 35S promoter was used. An *Agrobacterium* culture was prepared overnight by adding a standard inoculum to 10 mL of liquid MG/L medium without any antibiotics, and incubating on a shaker at 180 rpm at 28°C (approximately 20 h). A small amount of *Agrobacterium* culture (approximately 200 μL for 25 embryos) was dropped onto each embryo so that the surface was just covered. Once all 25 embryos on a plate had been treated, the plate was tilted to allow any excess *Agrobacterium* culture to run off the embryos. After 3 min, the embryos were transferred to fresh callus induction medium scutellum side down and incubated at 23–24°C for 3 days. After 3 days cocultivation, the embryos were transferred to fresh callus induction medium, containing hygromycin (50 mg/L) as the selective agent and timentin (160 mg/L) to suppress *Agrobacterium* growth. Embryos were cultured scutellum side down at 23–24°C (selection 1) in the dark. Embryos were transferred twice to fresh selection plates with callus induction medium at 2 weeks intervals (selection 2 and 3). Callus derived from one embryo was not split up. Following 6 weeks callus induction, embryo-derived callus was transferred to transition medium containing hygromycin and timentin. The plates were incubated for 2 weeks, at 24°C under low light conditions, achieved by covering the plates with a thin layer of paper. Embryo-derived material was then transferred to regeneration medium contained the same levels of hygromycin and timentin but no growth regulators. The small regenerated plantlets were carefully removed from the plates and transferred to flasks containing 25 mL of regeneration medium. The rooted plants were transferred to the soil [27].

2.4 PCR analysis

Genomic DNA was used as a template for standard PCR analyses (Eppendorf, Germany). The analysis was performed with genomic DNA isolated from leaf tissue of putative transgenic plants using the procedure of Edwards et al. [29]. For PCR reaction premix REDTaq® ReadyMix™ PCR Reaction Mix (R2523-100RXN, Sigma-Aldrich, USA) was used. The presence of the *dapA*-cassette mdapA was determined by amplifying an 892 bp fragment (primers F: 5′-CTG CAG GAT CCA TGT TCA CGG-3′ and R: 5′-GAG CTC CCT AAA CTT TAC TGC-3′). Cassette sTPdapA was determined by amplifying a 980 bp fragment (primers F: 5′-AGC GCC ACT TCT GTT GCT CCA-3′ and R: 5′-AGC CAG CGT GCT TCA GAG

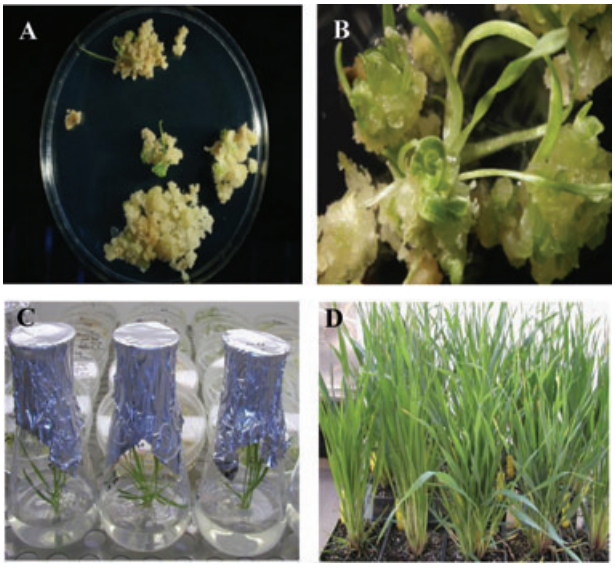

Figure 2. (A) Barley embryogenic callus 6 weeks after transformation. (B) Regeneration of plantlet on selection medium, 8 weeks after transformation. (C) Putative transgenic plants regenerated from regular calluses, containing dapA genes. (D) Regenerated transformed plants growing in GM containment glasshouse.

CAG-3′). The presence of selection gene *hpt* was determined by amplifying a 960 bp fragment (primers F: 5′-ACT CAC CGC GAC GTC TGT-3′ and R: 5′-GCG CGT CTG CTG CTC CAT-3′).

The concentration of the isolated genomic DNA obtained from transgenic plants was from 300 to 600 ng/mL. For both DNA amplicons we used 20 μL reaction volumes. The thermocycler PTC-200 (MJ Research, USA) was used for each amplicon. Amplified products were run in a 1% agarose gel, which was stained with ethidium bromide. Separation of fragments was performed on Elecrophoresis system (Bio-Rad 170-4486, USA) and photographed using an image analyzer (Syngene, UK).

2.5 Real-time PCR

RNA for Real-time PCR was isolated from leaf tissue of PCR positive plants by Ambion RNAqueous™ Kit, and treated with TURBO DNA-*free*. cDNA synthesis was done using reverse transcriptase by RevertAid H Minus M-MuLV, Oligo (dT) primers (Fermentas, USA). The expression of the following three genes were monitored: dapA gene (mdapA: primers F: 5′-GGT GAT GAT GAC GCT GGA TCT-3′ and R: 5′-GGT AAT TGC CGG GAC CG-3′; sTPdapA F: 5′-GGC CAT GGC GTG ATT TCT-3′ and R: 5′-AGA GAC ATG GCT CAA ATG TGC A-3′), for hygromycin resistance gene hpt (F: 5′-CGA GGT CGC CAA CAT CTT CT-3′ and R: 5′-GCG TCT GCT GCT CCA TAC AA-3′) and house-keeping gene for elongation factor (F: 5′-CCG CAC TGT CAT GAG CAA GT-3′ and R: 5′-GGG CGA GCT TCC ATG TAA AG-3′). To a 96-well plate, 5 μL of SYBR® Green Power PCR mix (Applied Biosystems, USA), 2.5 μL of primer mix (1.2 μM forward and reverse primers) and 2.5 μL of diluted cDNA were pipetted. Reactions were run in default set up on StepOnePlus™ Real-Time PCR System (Applied Biosystems). Relative expression in all samples was estimated by the delta delta Ct method with respect to the chosen housekeeping gene and the sample with the lowest detected expression used as a calibrator.

2.6 Western blot analysis

The plants where the *dap*A gene was verified by PCR and RT-PCR were also analyzed for DHDPS expression by Western blot. Proteins extracted from barley leaves (100 mg) from plants shown to have high expression following RT-PCR were analyzed. Homogenized samples in liquid nitrogen were extracted with extraction buffer (Tris-HCl, PMSF, Triton X-100 $C_{14}H_{22}O(C_2H_4O)_n$) for 1 h on ice. The extracts were centrifuged at 14 000 rpm for 15 min at 4°C. The protein concentration was measured with a BCA Protein assay Kit (PIERCE 23225, Bio-Rad). Samples (10 μg per lane) were loaded and fractionated by SDS-PAGE on an acrylamide gel (Running gel 10%, Stacking gel 5%) on a Bio-Rad Mini-PROTEAN Tetra Cell, and blotted onto a PVDF membrane PVDF. Gels were stained with Coomassie Brilliant Blue.

Specific rabbit antibody was prepared to detect the protein of interest (DHDPS) by the Department of Cell Biology and Genetics, Palacky University in Olomouc, Czech Republic. Primary antibody was used at a dilution of 1:750, and we used anti-rabbit IgG-HRP (Santa Cruz Biotechnology, USA) as a secondary antibody at the dilution of 1:5000. The ECL Plus Western Blotting Detection Reagents (Amersham, cat. no. RPN2132, USA) were used for detection of the final product of this assay.

2.7 Ion exchange liquid chromatography and acid hydrolysis

For determination of amino acids Lys, Met, and Thr an ion-exchange liquid chromatography (Model AAA-400, Ingos) with postcolumn derivatization with ninhydrin and VIS detector was used. A glass column with inner diameter of 3.7 mm and 350 mm in length was filled manually with a strong cation exchanger in sodium cycle LG ANB (Ingos) with approximately 12-μm particles and 8% porosity. The column was tempered within the range from 35 to 95°C. The elution of the amino acids of interest was carried out with the column temperature set to 74°C. A double channel VIS detector with inner cell of volume 5 μL was set to two wavelengths, 440 and 570 nm. A solution of ninhydrin (Ingos) was prepared in 75% v/v methylcelosolve (Ingos) and in 2% v/v 4 M acetic buffer (pH 5.5). Tin chloride ($SnCl_2$) was used as a reducing agent. The prepared solution of ninhydrin was stored under an inert atmosphere (N_2) in the dark at 4°C. The flow rate was 0.25 mL/min and the reactor temperature was 120°C.

2.7.1 Acid hydrolysis

A sample (approximately 0.1 g) was dissolved in the presence of 500 μL 6 M HCl. Acid hydrolysis was carried out in a microwave reactor (Anton Paar, Germany). The experimental parameters were as follows: power 80 W, Ramp 15 min, Hold 120 min, max 120°C, max pressure 25 bar, Rotor-XF-100-6 (Anton Paar, Germany). The other parameters were optimized.

2.8 Descriptive statistics and estimation of detection limits

Data were processed using MICROSOFT EXCEL® (USA) and STATISTICA.CZ Version 8.0 (Czech Republic). Results are expressed as mean ± standard deviation (SD) unless noted otherwise (EXCEL®). Statistical significances of the differences were determined using STATISTICA.CZ. Differences with $P < 0.05$ were considered significant and were determined by using a one way ANOVA test (particularly Scheffe test), which was applied for comparison of the means. The detection limits ($S/N = 3$) were calculated according to Long and Winefordner [30], whereas N was expressed as standard deviation of noise determined in the signal domain unless stated otherwise.

3 Results and discussion

3.1 Production of transgenic barley plants

Barley scutella from immature embryos were transformed with the bacterial *dapA* gene under the regulation of the maize ubiquitin promoter (ubi1), and the *hpt* gene conferring hygromycin resistance under a 35S promoter. The cassette sTPdapA in the first vector, pBract214::sTPdapA, also included the transit peptide Rubisco *H. vulgare* (rbcS) before the bacterial dapA gene. The cassette of the second vector, pBract214::mdapA, did not contain the transit peptide Rubisco *H. vulgare* (rbcS). These plasmids were introduced into plant tissue by *Agrobacterium*-mediated transformation; 150 immature embryos were transformed with each plasmid. Explants were transferred after three days cocultivation on induction medium. Three different basic plant tissue culture media were used during the transformation and regeneration process: callus induction, transition and regeneration media. During all cultivation stages, hygromycin (Roche 10843555001) as the selection agent, and Timentin (Duchefa T0190) to remove *Agrobacterium* from the cultures were added to all media following the cocultivation step. After 6 weeks selection on callus induction medium, the embryo-derived callus was transferred to transition medium (Fig. 2A). During this 2-week transition culture period, transformed calluses started to produce small shoots (Fig. 2B). After the 2 weeks on transition medium the embryo-derived material was transferred to regeneration medium without any growth regulators (Fig. 2C). From 300 transformed immature embryos with both plasmids, 350 putative transgenic plants were regenerated. These were subsequently transferred to soil (Fig. 2D). Most plants, 192 (1.28 plants/one embryo) were regenerated from scutella, which were transformed by construct pBract214::mdapA; 158 (1.05 plants/one embryo) plants regenerated after transformation with vector pBract214::sTPdapA.

3.2 Evaluation of expression of the transgene at the DNA, RNA, and protein level using electrophoresis

During the growing season of plants of the T0 and T1 generations, their characterization using PCR, RT-PCR, and SDS-PAGE was carried out. At the DNA level, 325 plants of the T0 generation, which regenerated under in vitro conditions, were analyzed (Fig. 3). Two hundred twenty-four plants were PCR positive (72% mdapA, 62% sTPdapA). For RT-PCR analysis 122 plants were selected, from which 109 plants showed expression at the RNA level. Based on these results, the plants were divided into four groups: (i) high expression of dapA and selective hpt gene, (ii) medium expression, (iii) low expression, and (iv) different gene expressions of dapA and hpt. From each group representative plants (samples) were selected, from which proteins were isolated and DHDPS expression was examined by Western blot analysis. The size of the desired protein, DHDPS, is 38 kDa. In total 109 plants were analyzed of which 39 plants showed the presence of the desired protein.

Analyses using SDS-PAGE of eight samples of the T0 generation, which were positive in the analysis of expression at the level of RNA, are shown in Fig. 4A. Western blot analysis of DHDPS from *E. coli* and from transgenic barley is shown in Fig. 4B. In total, 109 transgenic plants of the T0 generation were analyzed. Of 57 plants that were transformed with vector pBract214: mdapA, only seven plants (12%) showed an increased of the presence of DHDPS. Of 52 plants that were transformed with vector pBract214: sTPdapA, 32 plants (62%) showed the presence of DHDPS (Fig. 4C). Transformation efficiency, in the T0 generation, for both dapA cassettes was evaluated using leaf samples and the final differences between the two transformation approaches are shown in Fig. 5. In spite of the fact that pBract214: sTPdapA showed worse results in the case of transformed embryos and regenerated plants and both PCR analyses, the presence of DHDPS in these transgenic plants was almost five times higher compared to pBract214: mdapA transformed plants.

Based on the results of all analyses, seeds of selected progeny were sown in the greenhouse. In total 436 plants were analyzed at the level of DNA and RNA. Different patterns of segregation for the dapA transgene were found in different T1 generation progeny plants. The ratio of transgenic and nontransgenic plants significantly differed depending on the vector ($P < 0.05$). In the offspring of plants, which were transformed with pBract214: sTPdapA vector, only 3.6% of the

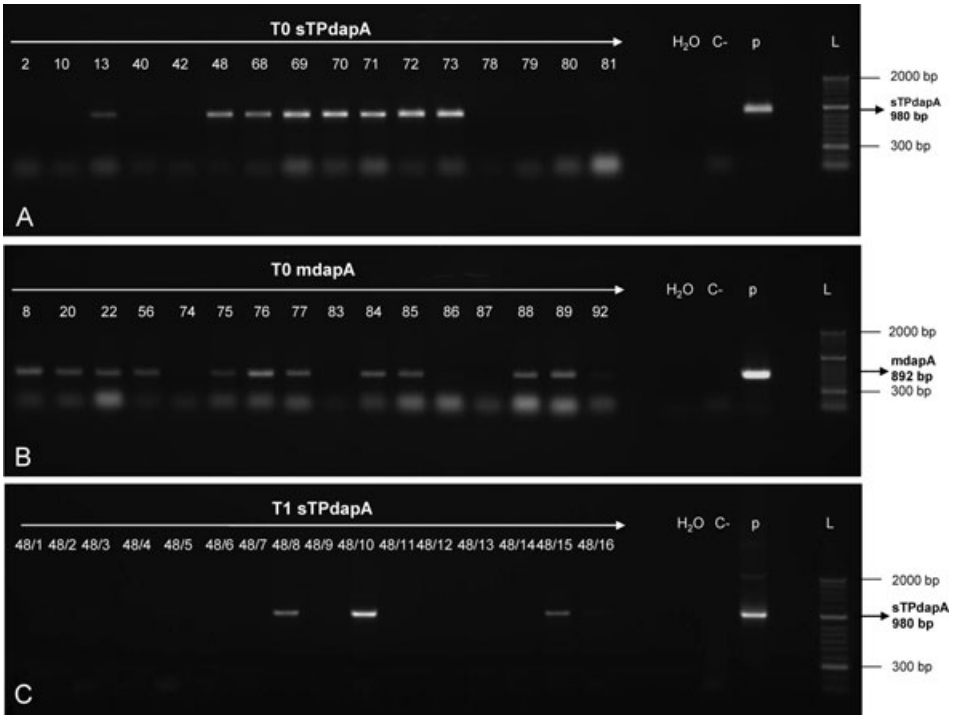

Figure 3. PCR analysis of T0 generation of transgenic plants after transformation with (A) vector pBract214::sTPdapA: plants no. 2, 10, 13, 40, 42, 48, 68, 69, 70, 71, 72, 73, 78, 79, 80, 81; H_2O control without DNA; P vector with transgene; (B) vector pBract214::mdapA: plants no. 8, 20, 22, 56, 74, 75, 76, 77, 83, 84, 85, 86, 87, 88, 89, 92. (C) Evaluation of the presence of the dapA transgene in the T1 progeny of plant 48, nos 1–16 after transformation with vector pBract214::sTPdapA. L: molecular weight markers HyperLadder II (Bioline, BIO-33040). Other experimental details see in Section 2.3.

plants showed the presence of the dapA transgene in the T1 generation and the ratio of nontransgenic to transgenic plants was 30:1. In the offspring of plants, which were transformed with the pBract214: mdapA vector, 29.8% of plants showed the presence of the dapA transgene and the ratio of nontransgenic to transgenic plants was 3:1 (Fig. 3). Characterization using RT-PCR was performed in selected PCR positive plants of the T1 generation. Seventy-two percent of the plants analyzed showed gene expression of dapA at the RNA level. Using PCR, analysis of the T2 generation was carried out. The presence of the transgene at the DNA level was tested in 240 T2 plants. Within each progeny, segregation of the dapA transgene still occurred. The percentage of positive plants in the T2 generation ranged from 5 to 24% and varied greatly between individual progenies from different transformation events.

3.3 Optimization of ion exchange chromatography for determination of lysine

For ensuring that DHDPS was not acting only as a protein but as an enzyme and was able to catalyze lysine synthesis even after transformation, we determined the total content of lysine, methionine and threonine, which are the main

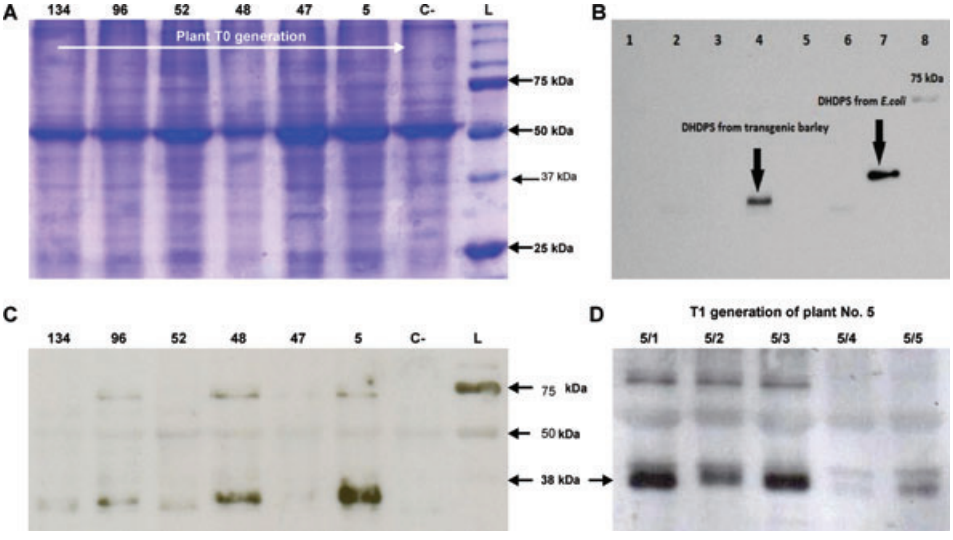

Figure 4. (A) SDS-PAGE electrophoretogram of the transgenic barley. Plants no. 134, 48, 47, 5 transformed with pBract214::sTPdapA, plants no. 96, 52 transformed with pBract214::mdapA. (B) Western blot analysis of DHDPS from *E. coli* and from transgenic barley. (C) Western blot analysis of T0 generation of different transgenic plants. (D) Western blot analysis of T1 progeny from transgenic plant No. 5 transformed with pBract214::sTPdapA. L: protein Standards (Bio-Rad, 161-0374).

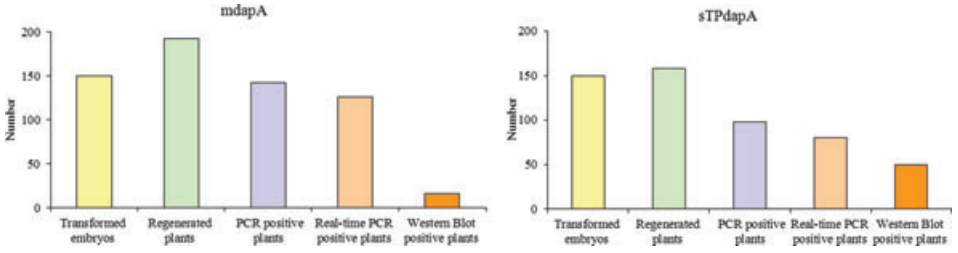

Figure 5. Summary of the transformation efficiency of both cassettes, mdapA and sTPdapA, in plasmid pBract214, at the DNA level by PCR, the RNA level by RT-PCR and by detection of DHDPS protein by Western blot, 150 immature embryos where transformed of each vector.

products of the DHDPS gene (Fig. 1A and B). Amino acids content in leaves of T1 generation plants, showing DHDPS expression, was determined. As a negative control, the non-transgenic variety Hiproly, which is used in hybridization programs aimed at increasing lysine in the grain due to its high content of lysine, was used. To determine content of these amino acids, sample preparation, and the method for analysis were optimized. Amino acid detection following elution from the chromatographic column was carried out using postcolumn derivatization by ninhydrin and detection by photometric detector at 570 nm. Using ion exchange chromatography, the analysis of lysine within the concentration range from 1 to 1000 μM was performed reaching the RSD of 3.6% ($n = 3$). The overlay of the chromatographic signals of calibration for lysine is shown in Fig. 6A. The calibration curve determined as a dependence of the peak area on the concentration, exhibited an excellent linearity ($R^2 = 0.999$, Fig. 6B). The limit of detection for lysine of 3 μM was calculated as S/N = 3. Parameters for the other two detected amino acids, methionine and threonine, were as follows. LODs (S/N = 3) were 7 μM for methionine ($R^2 = 0.998$, $n = 3$, RSD = 3.4%) and 5 μM for threonine ($R^2 = 0.997$, $n = 3$, RSD = 4.2%). For optimal yield of lysine during preparation of the plant sample by microwave-assisted digestion, we studied the influence of time of microwave-assisted hydrolysis on the total response of lysine. The intensity of microwave 80 W and total amount of sample 0.5 mL were used. We tested various times for the hydrolysis reaction 40, 60, 80, 100, 120, and 140 min. We found that the optimal yield of lysine was reached after 120 min hydrolysis. Prolonging the time of hydrolysis further did not show an enhancing trend in the yield as it is shown in Fig. 6C.

3.4 Determination of amino acids in transgenic barley plants

Leaf samples were prepared by hydrolysis in 6M HCl by microwave-assisted digestion, as optimized in Section 3.3. Using the above optimized ion exchange chromatography, we analyzed samples, which had shown positive expression of DHDPS during protein analysis. Three groups of samples with various rate of expression of the DHDPS gene were analyzed: low, medium, and high expression (Fig. 7A, B, and C, respectively). All groups were compared to the negative control represented by Hiproly commercial barley variety to examine the effect of genetic modification. The differences in amino acid content between the studied groups were not as high as the differences between individual samples were. The highest lysine contents of 500 μM were observed in three

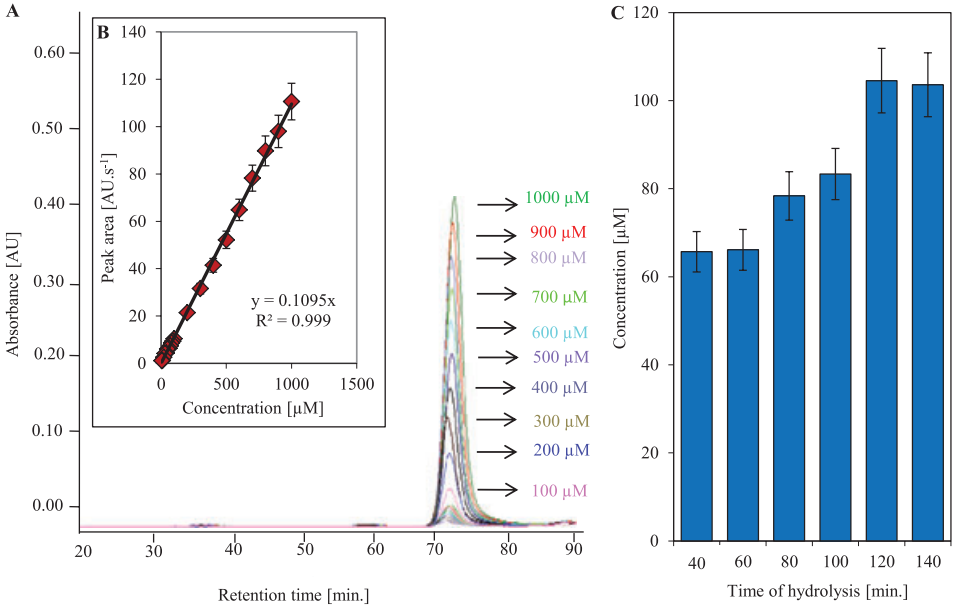

Figure 6. (A) Overlay chromatograms showing the calibration of lysine within the range 1 to 1000 μM. (B) Calibration curve of lysine for the range 1–1000 μM showing the linearity of the calibration curve. (C) Influence of time of hydrolysis on the yield of lysine in μM, the plateau of the yield of lysine is from time of hydrolysis 120 min. Other experimental details see in Section 2.8.

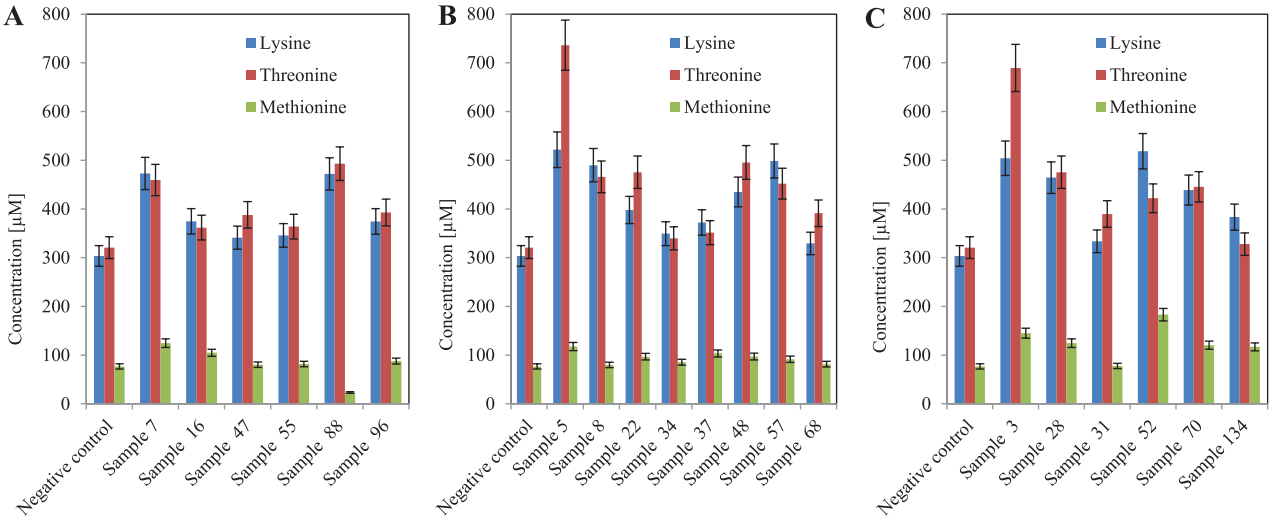

Figure 7. Total amount of lysine, threonine, and methionine in transgenic barley leaves with (A) low expression, (B) medium expression and (C) high expression. All compared to a negative control represented by Hiproly commercial Barley variety. Other experimental details see in Section 2.8 and caption for Fig. 5

samples number 3, 5, and 52 (Fig. 7). The lowest, and very similar concentrations of detected amino acids compared to the negative control Hiproly were detected in samples 34, 37, and 55. A lower concentration of methionine than the negative control was detected in sample number 88. All other samples showed higher amounts of the studied amino acids than the negative control. The highest concentrations of threonine were detected in samples number 3 and 5 and there were only a few cases where lysine concentration was above the level of methionine. The highest concentrations of lysine per sample were determined in samples 7, 16, 37, 52, and 134. The results obtained were in good agreement with the electrophoretic results above and give evidence of the functionality of the genetic modification approach. On the other hand, there was relatively high variability in total amino acid content between the individual plants.

4 Concluding remarks

The results obtained suggest that the efficiency of transformation in our experiments (percentage of plants in which the desired protein was detected) was very high, averaging 24%, in spite of the fact that the efficiency often ranges from 1 to 8%. After insertion of the transgene mdapA, without the transit peptide, 12% of the plants produced the DHDPS protein, and, after insertion of the transgene containing the small subunit of Rubisco transient peptide, referred to as sTPdapA, 36% of plants produced the desired protein. One of the important factors that significantly affected the efficiency of transformation was using an appropriately chosen donor vector pBract214. In this vector the dapA gene was under a strong constitutive plant promoter (ubiquitin). The selective *hpt* gene that forms part of the plasmid, was under the CaMV 35S promoter and this allowed a sufficient level of expression of the *hpt* gene for selection. Recombinant att1 and att2 sites in the vector allowed the proper orientation of the inserted gene. It was also verified that the *Agrobacterium tumefaciens* AGL1 strain is very suitable for the transformation of barley and reliably ensures the incorporation of the desired gene into the genome of barley. From the point of view of analytical chemistry, both electrophoretic and chromatographic methods enabled us to analyze transformed samples and monitor the success of the transformation. Based on the results, it can be concluded that a combination of modern biological approaches with robust bioanalytical tools represent a powerful tool in foodomics.

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