Electrophoretic and Chromatographic Evaluation of Transgenic Barley Expressing a Bacterial Dihydrodipicolinate Synthase

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Abstract

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 polymerase chain reaction, Real-Time polymerase chain reaction, gel electrophoresis and Western blot. Amino acid content was analysed by high performance liquid chromatography 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 contain 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

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 feed-back 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 DHPS in agronomically important crops and therefore increase free-lysine content [6, 9]. Transgenic lines withincreased 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 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 sodium dodecyl sulphate polyacrylamide

gel electrophoresis and Western blotting was carried out. To support the electrophoretic results, ion exchange chromatography with post column 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. Experimental Section

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 analyser (Ingos, Czech Republic) were prepared according the manufacturer's instructions and were purchased from Ingos (Czech Republic). All solutions were prepared in deionised water obtained using reverse osmosis equipment (Aqual 25, Czech Republic). The deionised 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-labelled 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 cloned into the vector pENTER1A containing specific recombinant sites (attL1 and attL2) for insertion of this gene into the pBract214 vector by restriction endonucleases (KpnI and XhoI, NEB). This vector based on pGreen, is Gateway™ compatible and has been designed for crop transformation, specifically for expression studies in barley (http://www.bract.org) . The *DapA* gene was inserted between the segments attR1, attR2 under the control of the Ubi promoter (maize ubiquitin 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). Both prepared donor vectors pBract214::sTPdapA and pBract214::mdapA were verified by restriction analysis. Each vector was transferred with helper plasmid pSoup, containing the virulence genes, into competent cells of Agrobacterium strain AGL1 by electroporation (Fig. 1C).

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].

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 co-cultivation, the embryos were transferred to fresh

callus induction medium, containing hygromycin (50mg/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 two 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 two 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 a 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 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 analyser (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 house-keeping 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 analysed for DHDPS expression by Western blot. Proteins extracted from barley leaves (100 mg) from plants shown to have high expression following RT-PCR were analysed. Homogenized samples in liquid nitrogen were extracted with extraction buffer (Tris HCl, phenylmethylsulfonyl fluoride, 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, BioRad). 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 polyvinylidene 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 Inc., 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 aminoacids Lys, Met and Thr an ion-exchange liquid chromatography (Model AAA-400, Ingos) with post column 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 aminoacids 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.

Acid hydrolysis

A sample (app. 0.1g) 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 (S.D.) 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 (3 signal/noise, S/N) 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 resistence 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 co-cultivation 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 co-cultivation step. After six 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 analysed (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 analysed of which 39 plants showed the presence of the desired protein.

307 Analyses using SDS-PAGE of eight samples of the T0 generation, which were positive
308 in the analysis of expression at the level of RNA, are shown in Fig. 4A. Western-blot
309 analysis of DHDPS from *E. coli* and from transgenic barley is shown in Fig. 4B. In
310 total, 109 transgenic plants of the T0 generation were analysed. Of 57 plants that were
311 transformed with vector pBract214: mdapA, only 7 plants (12 %) showed an increased
312 of the presence of DHDPS. Of 52 plants that were transformed with vector pBract214:
313 sTPdapA, 32 plants (62 %) showed the presence of DHDPS (Fig. 4C). Transformation
314 efficiency, in the T0 generation, for both dapA cassettes was evaluated using leaf
315 samples and the final differences between the two transformation approaches are shown
316 in Fig. 5. In spite of the fact that pBract214: sTPdapA showed worse results in the case
317 of transformed embryos and regenerated plants and both PCR analyses, the presence of
318 DHDPS in these transgenic plants was almost five times higher compared to pBract214:
319 mdapA transformed plants.
320 Based on the results of all analyses, seeds of selected progeny were sown in the
321 greenhouse. In total 436 plants were analysed at the level of DNA and RNA. Different
322 patterns of segregation for the dapA transgene were found in different T1 generation
323 progeny plants. The ratio of transgenic and non-transgenic plants significantly differed
324 depending on the vector ($p < 0.05$). In the offspring of plants, which were transformed
325 with pBract214: sTPdapA vector, only 3.6% of the plants showed the presence of the
326 dapA transgene in the T1 generation and the ratio of non-transgenic to transgenic plants
327 was 30:1. In the offspring of plants, which were transformed with the pBract214:
328 mdapA vector, 29.8% of plants showed the presence of the dapA transgene and the ratio
329 of non-transgenic to transgenic plants was 3:1 (Fig. 3). Characterization using RT-PCR
330 was performed in selected PCR positive plants of the T1 generation. 72% of the plants

analysed 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 catalyse lysine synthesis even after transformation, we determined the total content of lysine, methionine and threonine, which are the main products of the DHDPS gene (Figs. 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 post-column 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 relative standard deviation (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 3 S/N. Parameters for the other two detected amino acids, methionine and threonine, were as follows. Limits of detection (3

S/N) 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 80W 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 minutes. We found that the optimal yield of lysine was reached after 120 minutes 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 analysed 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 analysed: low, medium and high expression (Figs. 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 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. Conclusion

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 (JIC). In this vector the dapA gene was under a strong constitutive plant promoter (ubiquitin). The selective *hpt* gene which 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

403 chemistry, both electrophoretic and chromatographic methods enabled us to analyse
404 transformed samples and monitor the success of the transformation. Based on the
405 results, it can be concluded that a combination of modern biological approaches with
406 robust bio-analytical tools represent a powerful tool in foodomics.
407
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Caption for Figures

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.

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.

Figure 3

Polymerase chain reaction (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.

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.

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 120min. Other experimental details see in chapter 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 chapter 2.8 and caption for Figure 5.

References

[1] Rodgers, J. T., Lerin, C., Haas, W., Gygi, S. P., Spiegelman, B. M., Puigserver, P., *Nature* 2005, *434*, 113-118.

[2] Kim, S. C., Sprung, R., Chen, Y., Xu, Y. D., Ball, H., Pei, J. M., Cheng, T. L., Kho, Y., Xiao, H., Xiao, L., Grishin, N. V., White, M., Yang, X. J., Zhao, Y. M., *Mol. Cell* 2006, *23*, 607-618.

[3] Biolo, G., Fleming, R. Y. D., Wolfe, R. R., *J. Clin. Invest.* 1995, *95*, 811-819.

[4] BrinchPedersen, H., Galili, G., Knudsen, S., Holm, P. B., *Plant Mol.Biol.* 1996, *32*, 611-620.

[5] Vauterin, M., Frankard, V., Jacobs, M., *Plant Mol.Biol.* 1999, *39*, 695-708.

[6] Huang, S., Frizzi, A., Florida, C. A., Kruger, D. E., Luethy, M. H., *Plant Mol.Biol.* 2006, *61*, 525-535.

[7] Azevedo, R. A., Arruda, P., Turner, W. L., Lea, P. J., *Phytochemistry* 1997, *46*, 395-419.

[8] Kraakman, A. T. W., Niks, R. E., Van den Berg, P., Stam, P., Van Eeuwijk, F. A., *Genetics* 2004, *168*, 435-446.

[9] Huang, S. S., Kruger, D. E., Frizzi, A., D'Ordine, R. L., Florida, C. A., Adams, W. R., Brown, W. E., Luethy, M. H., *Plant Biotechnol. J.* 2005, *3*, 555-569.

[10] Falco, S. C., Guida, T., Locke, M., Mauvais, J., Sanders, C., Ward, R. T., Webber, P., *Bio-Technology* 1995, *13*, 577-582.

[11] Azevedo, R. A., Lea, P. J., *Amino Acids* 2001, *20*, 261-279.

[12] Puiggros, F., Sola, R., Blade, C., Salvado, M. J., Arola, L., *J. Chromatogr. A* 2011, *1218*, 7399-7414.

[13] Cifuentes, A., *J. Chromatogr. A* 2009, *1216*, 7109-7109.

[14] Herrero, M., Simo, C., Garcia-Canas, V., Ibanez, E., Cifuentes, A., *Mass Spectrom. Rev.* 2012, *31*, 49-69.

[15] Herrero, M., Simo, C., Garcia-Canas, V., Ibanez, E., Cifuentes, A., *Mass Spectrom. Rev.* 2012, *31*, 49-69.

[16] Rezzi, S., Ramadan, Z., Fay, L. B., Kochhar, S., *J. Proteome Res.* 2007, *6*, 513-525.

[17] Cifuentes, A., *Electrophoresis* 2010, *31*, 2091-2091.

[18] Castro-Puyana, M., Garcia-Canas, V., Simo, C., Cifuentes, A., *Electrophoresis* 2012, *33*, 147-167.

[19] Simo, C., Dominguez-Vega, E., Marina, M. L., Garcia, M. C., Dinelli, G., Cifuentes, A., *Electrophoresis* 2010, *31*, 1175-1183.

[20] Simo, C., Ibanez, C., Gomez-Martinez, A., Ferragut, J. A., Cifuentes, A., *Electrophoresis*, *32*, 1765-1777.

[21] Levandi, T., Leon, C., Kaljurand, M., Garcia-Canas, V., Cifuentes, A., *Anal. Chem.* 2008, *80*, 6329-6335.

[22] Hernandez-Borges, J., Neususs, C., Cifuentes, A., Pelzing, M., *Electrophoresis* 2004, *25*, 2257-2281.

[23] Herrero, M., Garcia-Canas, V., Simo, C., Cifuentes, A., *Electrophoresis* 2010, *31*, 205-228.

[24] Garcia-Canas, V., Mondello, M., Cifuentes, A., *Electrophoresis* 2010, *31*, 2249-2259.

[25] Leon, C., Rodriguez-Meizoso, I., Lucio, M., Garcia-Canas, V., Ibanez, E., Schmitt-Kopplin, P., Cifuentes, A., *J. Chromatogr. A* 2009, *1216*, 7314-7323.

[26] Rodriguez-Nogales, J. M., Cifuentes, A., Garcia, M. C., Marina, M. L., *Food Chem.* 2010, *120*, 1229-1237.
[27] Dahleen, L. S., *Plant Cell Tissue Organ Cult.* 1995, *43*, 267-269.
[28] Bartlett, J. G., Alves, S. C., Smedley, M., Snape, J. W., Harwood, W. A., *Plant Methods* 2008, *4*, 1-12.
[29] Edwards, K., Johnstone, C., Thompson, C., *Nucleic Acids Res.* 1991, *19*, 1349-1349.
[30] Long, G. L., Winefordner, J. D., *Anal. Chem.* 1983, *55*, A712-A724.

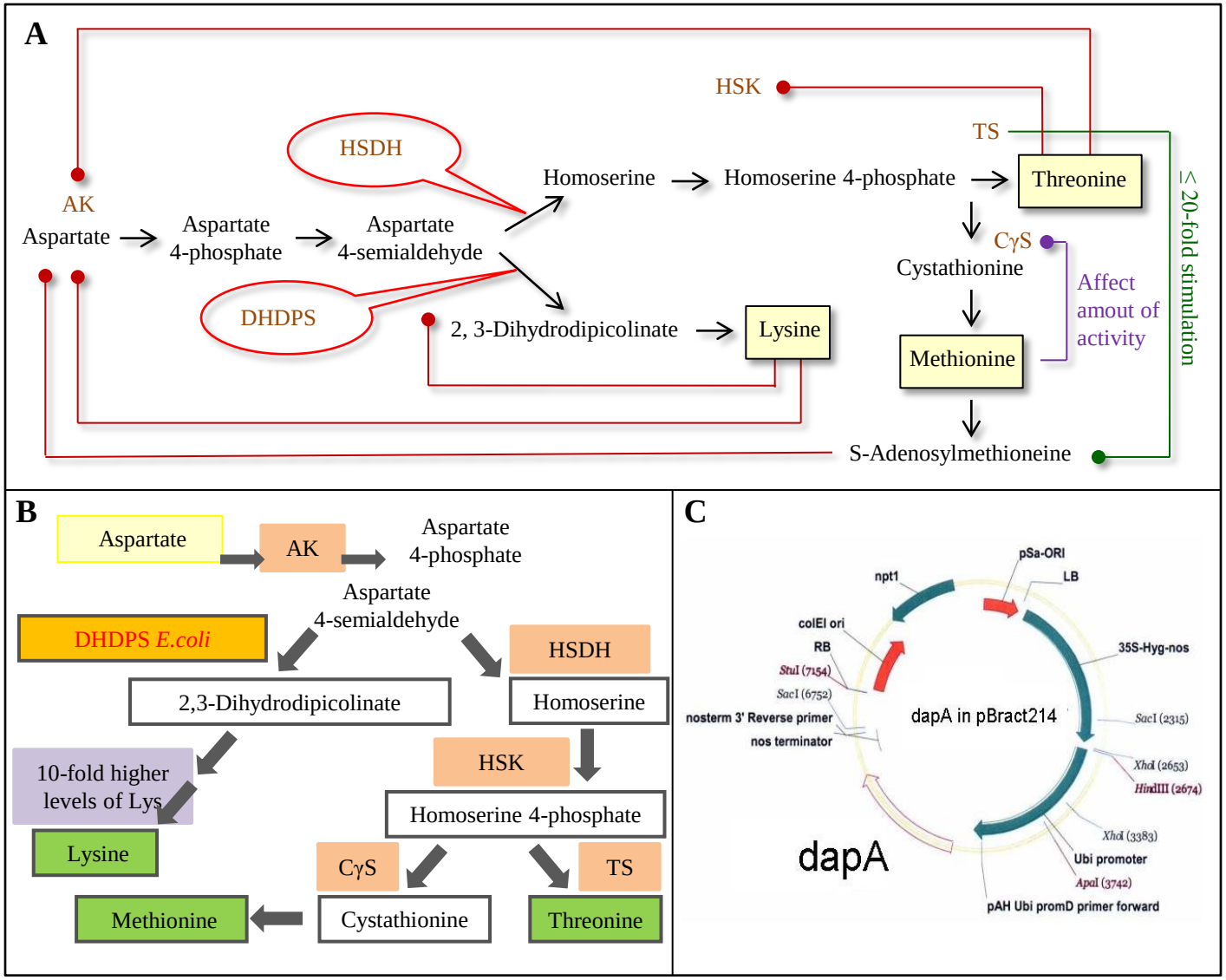

A
HSK
TS
HSDH
Homoserine
Homoserine 4-phosphate
Threonine
AK
Aspartate
Aspartate 4-phosphate
Aspartate 4-semialdehyde
≤ 20-fold stimulation
CγS
Cystathionine
Affect amout of activity
DHDPS
2, 3-Dihydrodipicolinate
Lysine
Methionine
S-Adenosylmethioneine
B
Aspartate
AK
Aspartate 4-phosphate
Aspartate 4-semialdehyde
DHDPS E.coli
HSDH
2,3-Dihydrodipicolinate
Homoserine
10-fold higher levels of Lys
HSK
Homoserine 4-phosphate
Lysine
CγS
TS
Methionine
Cystathionine
Threonine
C
pSa-ORI
npt1
LB
colEI ori
RB
35S-Hyg-nos
StuI (7154)
SacI (6752)
nosterm 3' Reverse primer
nos terminator
dapA in pBract214
SacI (2315)
XhoI (2653)
HindIII (2674)
XhoI (3383)
Ubi promoter
ApaI (3742)
dapA
pAH Ubi promD primer forward

Figure 1

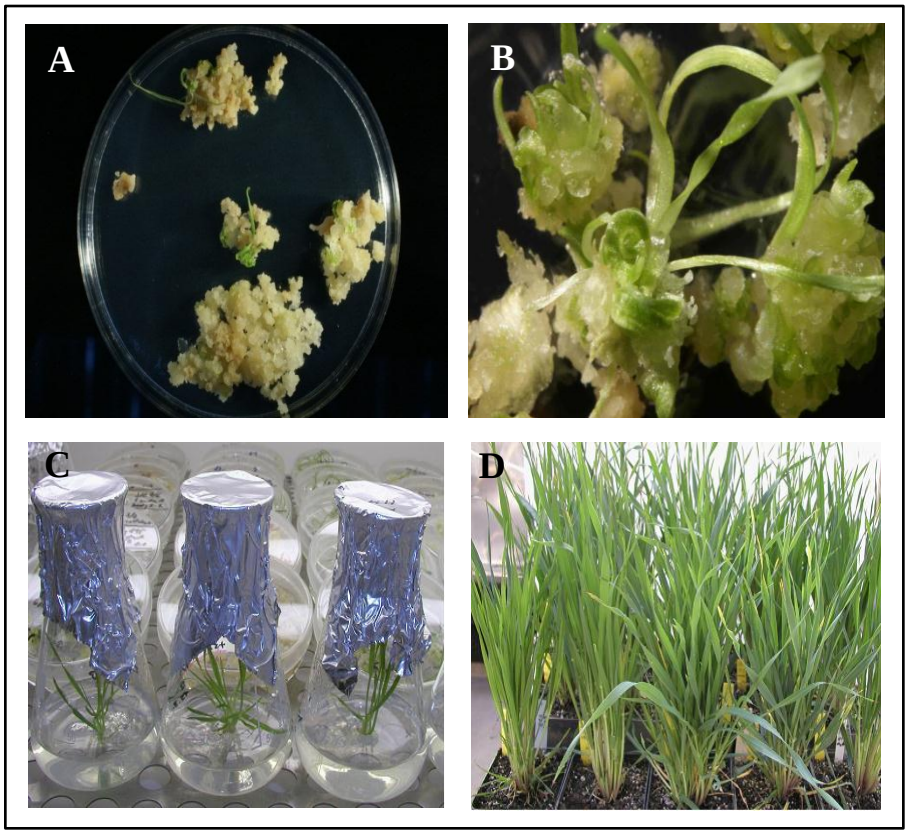


Figure 2

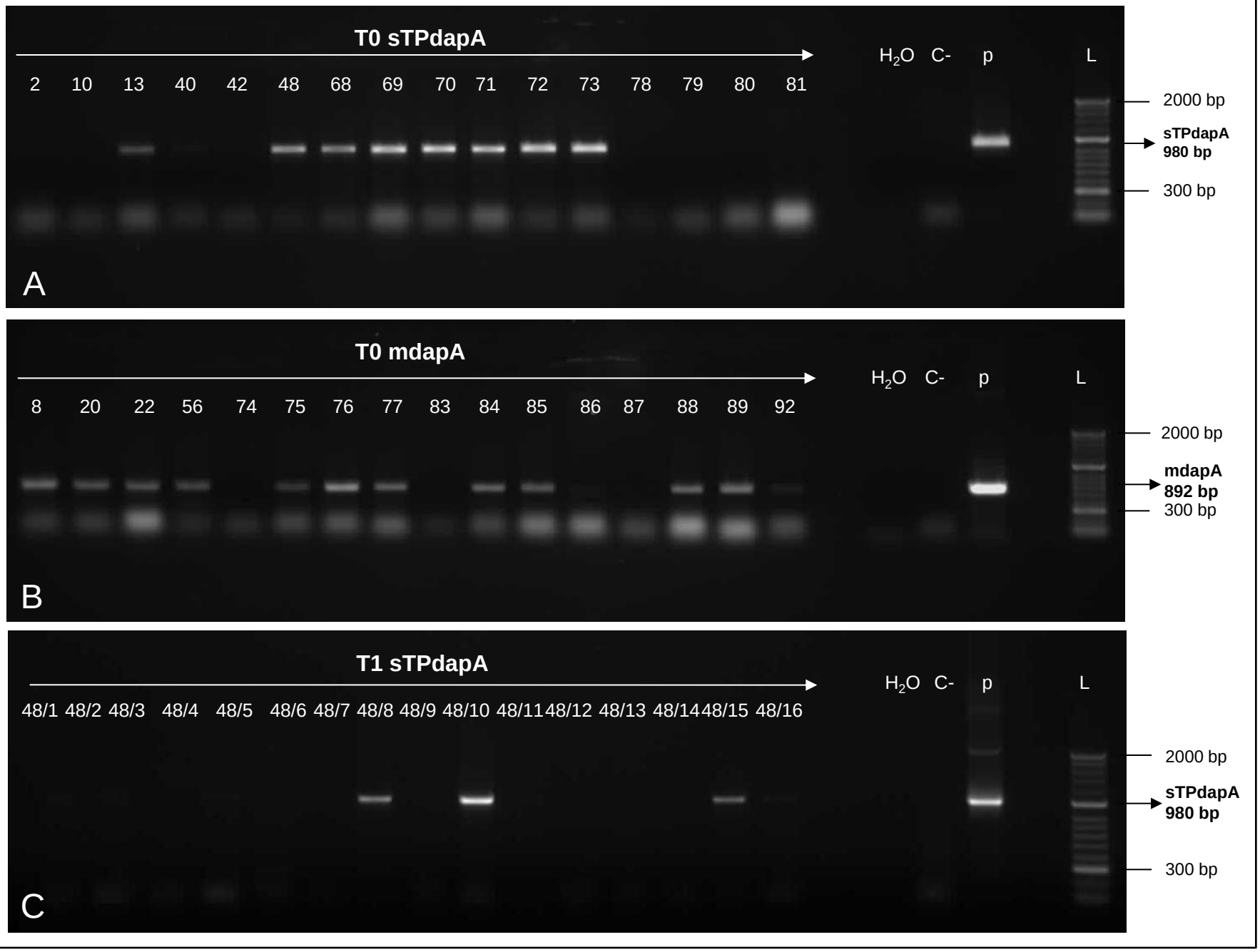

T0 sTPdapA
2 10 13 40 42 48 68 69 70 71 72 73 78 79 80 81
H2O C- p L
2000 bp
sTPdapA
980 bp
300 bp
A
T0 mdapA
8 20 22 56 74 75 76 77 83 84 85 86 87 88 89 92
H2O C- p L
2000 bp
mdapA
892 bp
300 bp
B
T1 sTPdapA
48/1 48/2 48/3 48/4 48/5 48/6 48/7 48/8 48/9 48/10 48/11 48/12 48/13 48/14 48/15 48/16
H2O C- p L
2000 bp
sTPdapA
980 bp
300 bp
C

Figure 3

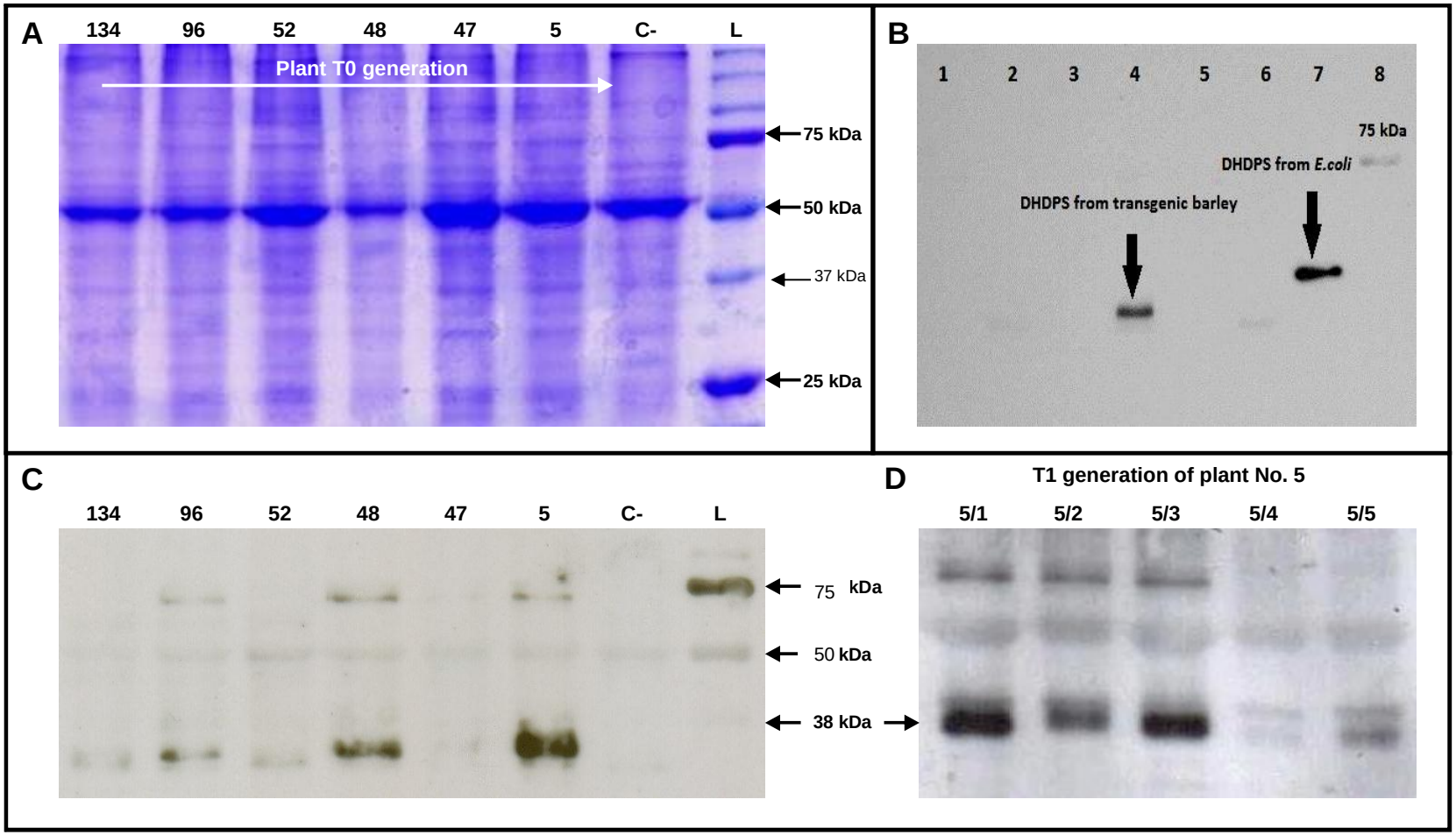
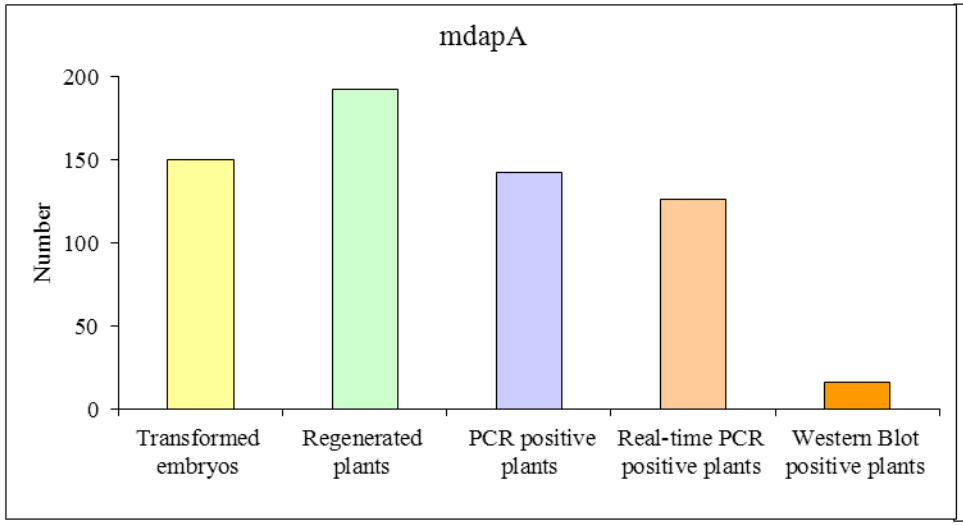

A
134
96
52
48
47
5
C-
L
Plant T0 generation
75 kDa
50 kDa
37 kDa
25 kDa
B
1
2
3
4
5
6
7
8
75 kDa
DHDPS from E.coli
DHDPS from transgenic barley
C
134
96
52
48
47
5
C-
L
75 kDa
50 kDa
38 kDa
D
T1 generation of plant No. 5
5/1
5/2
5/3
5/4
5/5

Figure 4


mdapA
Number
200
150
100
50
0
Transformed embryos
Regenerated plants
PCR positive plants
Real-time PCR positive plants
Western Blot positive plants

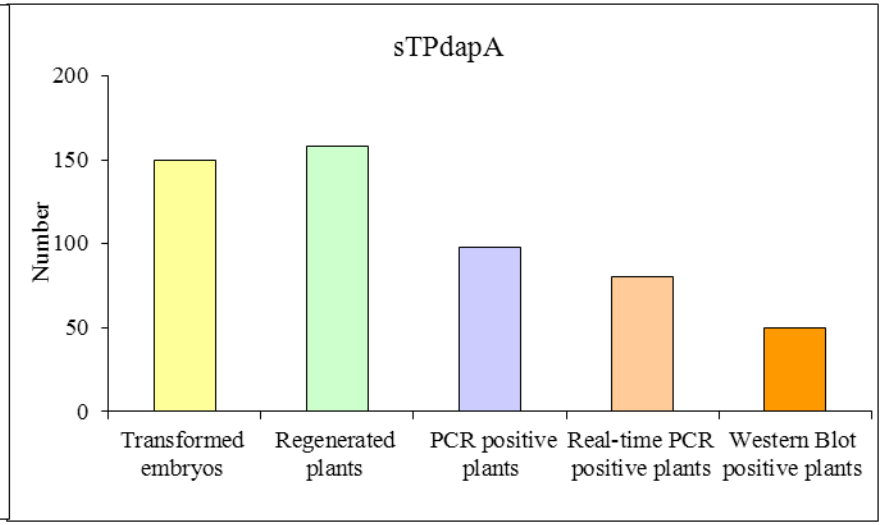

sTPdapA
Number
200
150
100
50
0
Transformed embryos
Regenerated plants
PCR positive plants
Real-time PCR positive plants
Western Blot positive plants

Figure 5

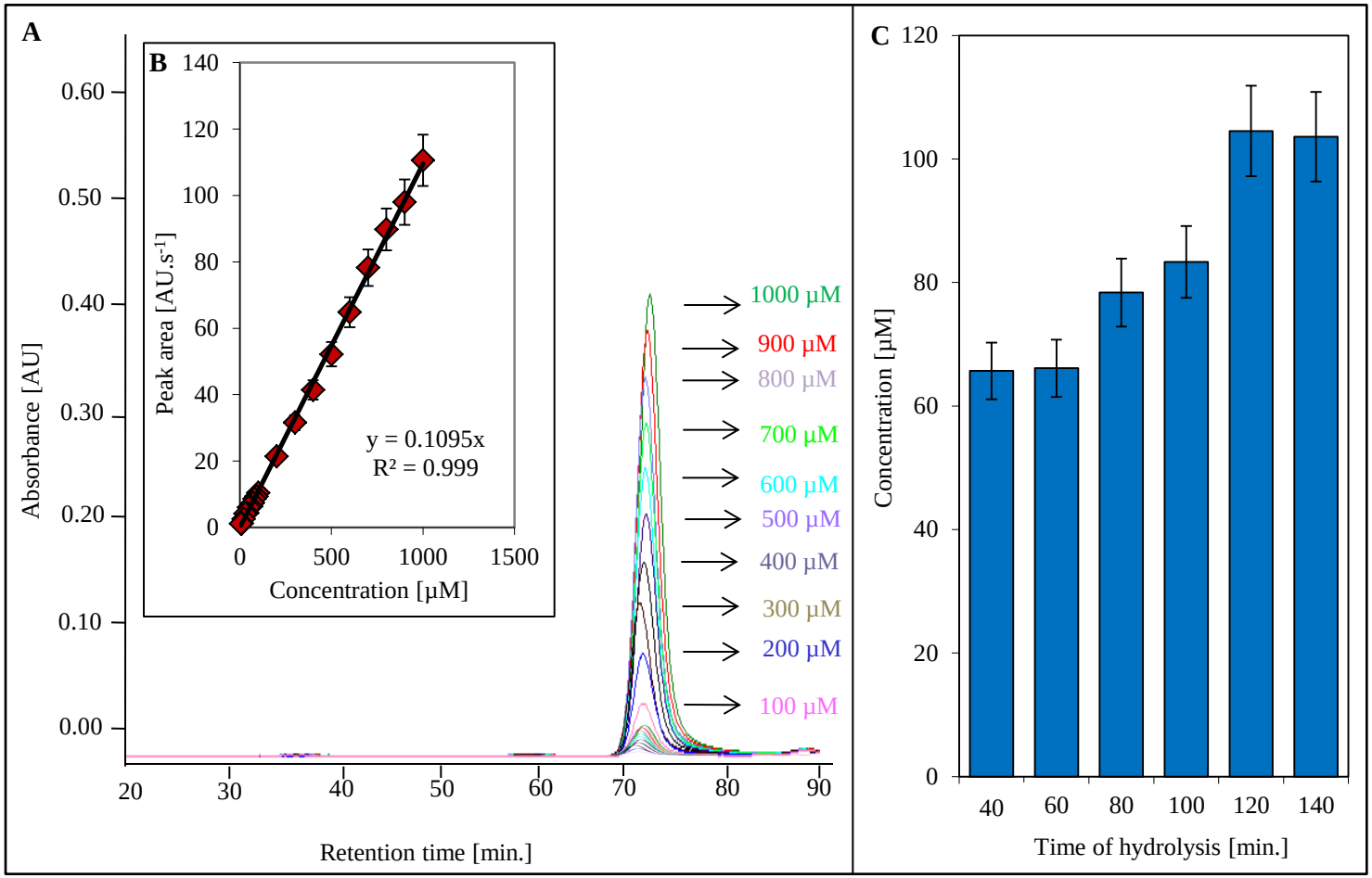

A
B
C
Absorbance [AU]
Retention time [min.]
Peak area [AU.s-1]
Concentration [µM]
y = 0.1095x
R² = 0.999
1000 µM
900 µM
800 µM
700 µM
600 µM
500 µM
400 µM
300 µM
200 µM
100 µM
Concentration [µM]
Time of hydrolysis [min.]

Figure 6

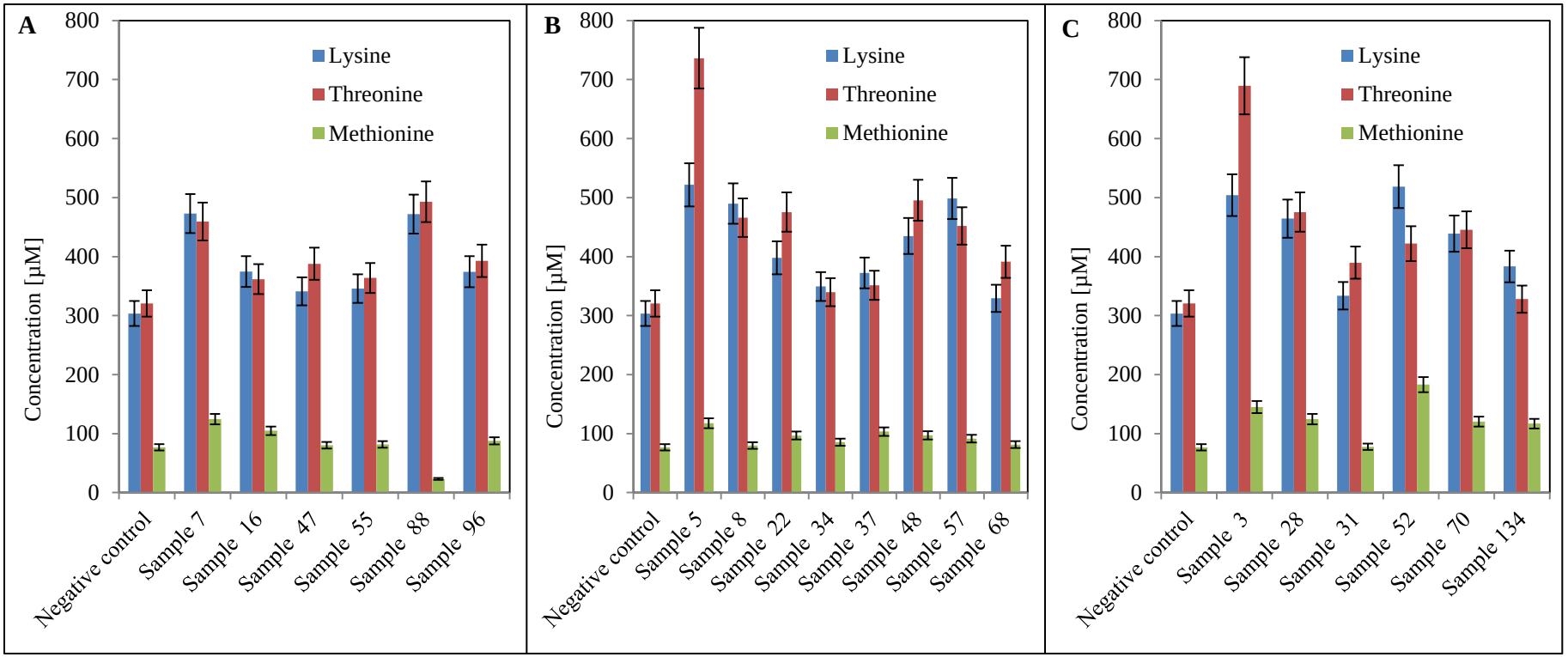

A
B
C
Lysine
Threonine
Methionine
Concentration [µM]
0
100
200
300
400
500
600
700
800
Negative control
Sample 7
Sample 16
Sample 47
Sample 55
Sample 88
Sample 96
Sample 5
Sample 8
Sample 22
Sample 34
Sample 37
Sample 48
Sample 57
Sample 68
Sample 3
Sample 28
Sample 31
Sample 52
Sample 70
Sample 134

Figure 7