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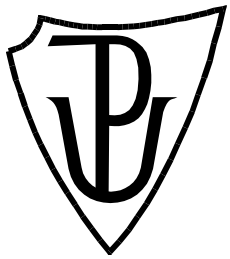

ANALYSIS OF TRANSGENIC BARLEY WITH ENRICHED LYSINE CONTENT

MASTER THESIS

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Study program:	N1406 Biochemistry
Study branch:	Biochemistry
Study mode:	Full-time
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Submitted:	26.4.2010

I hereby declare that this thesis has been written solely by myself and that all the sources used in this thesis are cited and included in the references part.

In Olomouc .....

Katarína Mrízová

Acknowledgments

I would like to say thank you to my advisor Petr Galuszka and my co-advisor Ludmila Ohnoutkova for their willingness and time they spent when helping me and their valuable advices. My thanks belong to Dr.Wendy Harwood for her cooperation in barley transformation, to Petr Tarkowski for MALDI-TOF MS analysis, and to all staff of Department of Biochemistry and Division of Molecular Biology for their help.

Bibliografická identifikácia

Meno a priezvisko autora	Katarína Mrízová
Názov práce	Analýza transgénnych rastlín jačmeňa so zvýšenou tvorbou lyzínu
Typ práce	Diplomová
Pracovisko	Katedra Biochemie
Vedúci práce	doc. Mgr. Petr Galuszka, Ph.D
Rok obhajoby práce	2010

Abstrakt

Lyzín je esenciálna aminokyselina, ktorá patrí medzi najviac limitované aminokyseliny v rastlinách. Biosyntéza lyzínu je regulovaná hlavne enzýmom dihydrodipikolinát-syntázou (DHDPS). Tento enzým je v rastlinách silno inhibovaný koncovým produktom lyzínom, zatial čo u baktérií je táto inhibícia asi 50-krát slabšia. S cieľom zvýšiť produkciu lyzínu v jačmeni bol bakteriálny *dapA* gén, ktorý kóduje DHDPS enzým, transformovaný do jačmeňa pomocou *Agrobacterium tumefaciens*. Pre transformáciu jačmeňa boli použité dva konštrukty: *pBract214::sTPdapA* a *pBract214::mdapA.* Konštrukt pBract214::*mdapA* obsahuje *dapA* gén z *E.coli*, ktorý bol 3-krát mutovaný, pretože obsahoval 3 bodové mutácie oproti pôvodnej sekvencii. Kazeta pre konštrukt *pBract214::sTPdapA* bola umelo pripravená (Mr.Gene) a pred začiatok *dapA* génu bola nasyntetizovaná signálna sekvencia malej podjednotky enzýmu Rubisco z jačmeňa, ktorá mala nasmerovať proteín do chloroplastov. Zo 150 transformovaných embryí zregenerovalo 330 rastlín, ktoré boli podrobené analýze prostredníctvom real-time PCR a Western blottingu. Zo 14 analyzovaných rastlín, ktoré boli transformované konštruktom *pBract214:mdapA*, bola expresia *dapA* génu potvrdená v 11 z nich a dve rastliny boli pozitívne na DHDPS proteín. Z 34 analyzovaných rastlín, ktoré boli transformované konštruktom *pBract214*::*sTPdapA*, bola expresia dapA génu potvrdená v 31 rastlinách a 18 z nich bolo pozitívných na DHDPS

proteín. Lyzín bol v transgenných rastlinách transformovaných konštruktom *pBract214::sTPdapA* zvýšený o 20 % oproti netransformovaným rastlinám.

Kľúčové slová	Dihydrodipikolinátsyntáza, *Agrobacterium tumefaciens*, lyzín,
Počet strán	71
Počet príloh	
Jazyk	Anglický

Bibliographical identification:

Author´s first name and surname	Katarína Mrízová
Title	Analysis of transgenic barley with enriched lysine content
Type of thesis	Master
Department	Department of Biochemistry
Supervisor	Dr. Petr Galuszka
The year of presentation	2010
Abstract	Lysine is an essential amino acid and is one of the most limited amino acids in plants food-stocks. Main regulation point of lysine biosynthesis is enzyme dihydrodipicolinate synthase (DHDPS). DHDPS is strongly inhibited by lysine excess in plants whereas bacterial DHDPS are less sensitive (50-fold) to the lysine inhibition. In order to increase of lysine biosynthesis in barley, *dapA* gene encoding DHDPS was transformed into barley genome by *Agrobacterium*-mediated transformation. Two construct were used for barley transformation*: pBract214::sTPdapA* a *pBract214::mdapA*. The construct *pBract214::mdapA* contains *E.coli dapA* gene that was engineered due to three detected point mutation. The cassette for construct *pBract214::sTPdapA* was synthesized by Mr.Gene and upstream of *dapA* gene signal sequence of small Rubisco subunit was added. This signal sequence should direct the protein into chloroplast. From 150 transformed embryos, 330 regenerated into plants and they were subsequently analyzed by real-time PCR and Western blotting. Out of 14 analyzed plants transformed with construct *pBract214::mdapA*, 11 plants were confirmed to contain bacterial *dapA* transcript and two of them were positive on DHDPS protein. From 34 analyzed plants transformed with construct *pBract214::sTPdapA* the expression of *dapA* gene was confirmed in 31 plants and 18 plants were positive on DHDPS protein. The biosynthesis of lysine was approximately increased up to

	20 % in transgenic plants transformed with construct *pBract214::sTPdapA*, compared to wild type plants.
Keywords	Dihydrodipicolinate synthase, *Agrobacterium tumefaciens*, lysine
Number of pages	71
Number of appendices	
Language	English

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5. Analysis of transgenic barley by Western blotting and real-time PCR

2 INTRODUCTION

Considering nutrition value of cereals, such as barley, wheat and maize, they are one of the most important food sources for human life. The Food and Agriculture Organization (FAO) estimated a global production of some 700 megatonne of maize, about 600 megatonne of wheat and 137 megatonne of barley for 2007. This data indicated the significance of these crops. While the first research works, focusing on the cereals improvement, used mostly conventional breeding, in these years new technologies have been developed. Because of growing world population, increasingly exhausted fossil energy sources, availability or global warming, the developing of new technologies that will lead to increasing nutrition values and higher tolerance to stress of cereals is a challenge. The genetic engineering of cereals have started to improve from 1990s with arrival of faster and more directed modifications of agronomical useful traits (Repellin et al., 2001; Jones, 2005; Shrawat & Lörz, 2006; Goedeke et al., 2007; Kumlehn et al., 2009). First successful genetic transformation events in cereal species was based on direct gene transfer, however it was associated with a number of disadvantages. In 1994, Hiei et al. was first to publish a protocol of *Agrobacterium*–mediated transformation of rice, which represents a beginning of a new epoch in cereal transformation. In this method, T-DNA is integrated in the nuclear genome and the transgenes are expressed. In the following years, similar protocols for all major cereal crops including barley and wheat (Tingay et al., 1997; Cheng et al., 1997) were published. Many particular conditions influence the successful interaction of *Agrobacterium tumefaciens* with the target cells. Because of cereals are untypical *Agrobacterium* hosts, small deviations from optimal conditions can be crucial for gene transfer events. Nutrient concentration, temperature, pH, presence and concentration of phenolic compounds and antioxidants should be strictly controlled during the process (Hensel et al., 2009).

Dihydrodipicolinate synthase (DHDPS) is a key enzyme in lysine biosynthesis. It occurs at the branch point of the threonine and methionine biosynthetic pathway and is feedback regulated by lysine. According to its function, DHDPS is of interest to biotechnologists aiming to engineer crops rich in lysine, often the limiting nutrient in staple crops (Miflin et al., 1999). Bacterial DHDPS are less sensitive to lysine inhibition. In order to increase lysine biosynthesis in barley, bacterial *dapA* gene, which encodes DHDPS protein, was transformed into barley using *Agrobacterium tumefaciens* (figure 1). Constructs containing *dapA* gene (*pBract 214*::*mdapA* and *pBract 214*::s*TPdapA*) for transformations were prepared. For detection of transgenic plants, the antibodies

against DHDPS protein were raised in rabbits. For this purpose, recombinant DHDPS protein was produced in *E.coli* according to protocol optimalised in previous work (Mrízová 2008, Palacky University Olomouc).

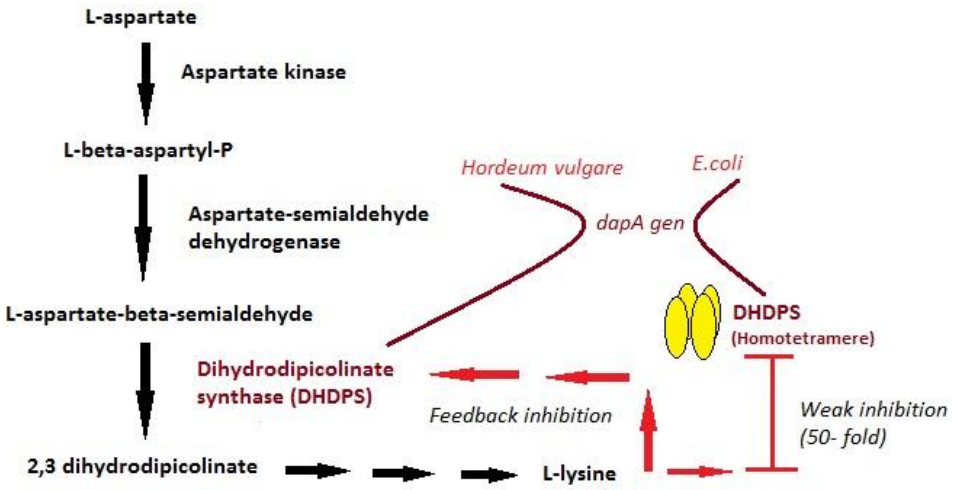


Figure 1 Scheme of lysine biosynthesis. Introduction of *dapA* gene from *E.coli* into barley should result in increasing lysine content.

3 THEORETICAL PART

3.1 Dihydrodipicolinate synthase, structure and function

Dihydrodipicolinate synthase (DHDPS, EC 4.2.1.52) catalyses the branch point reaction of lysine biosynthesis in plants and microbes, the condensation of (S)-aspartate-β-semialdehyde ((S)-ASA) and pyruvate to form an unstable heterocyclic product, (4S)-4-hydroxy-2,3,4,5-tetrahydro-(2S)-dipicolinic acid (HTPA) (figure 2).

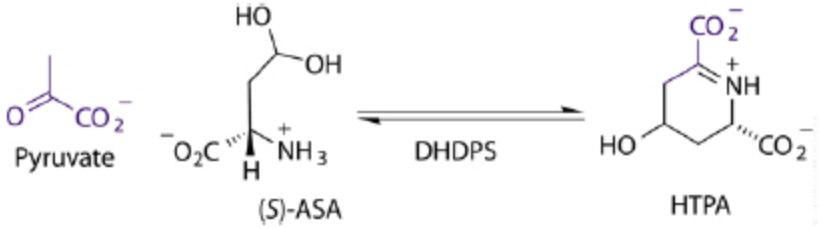


Figure 2 The reaction catalysed by DHDPS (Dobson et al., 2004).

The currently accepted mechanism of DHDPS is outlined in figure 3, in which the structure of (S)-ASA is presumed to be the hydrate (Blickling et al., 1997). In the first step of the mechanism, lysine 161 in the active site of *E.coli* DHDPS forms a Schiff base with pyruvate, as has been clearly demonstrated in several studies (Shedlarski & Gilvarg, 1970; Borthwick et al., 1995; Laber et al., 1992). Subsequent binding of the second substrate, (S)-ASA, is followed by dehydration and then cyclization to form the product, which is released into free solution. Based on the X-ray crystal structure of the *E.coli* enzyme (Blickling et al., 1997; Mirwaldt et al., 1995) and sequence homologies with DHDPS from other sources (Lawrence et al., 1997), it was proposed that a catalytic triad of three residues Tyr 133, Thr 44 and Tyr 107, act as a proton shuttle to transfer protons to and from the active site. In 2004, Dobson et al. strongly supported this hypothesis using site-directed mutagenesis to remove each of these residues in turn.

DHDPS from *E.coli* is a homotetramer (Shedlarski & Gilvarg, 1970). The monomer has a structure of $(\beta/\alpha)_8$ -barrel and the active site is situated within the centre of the β-barrel each of monomer (figure 4). The monomers are organized into two dimers with strong connections between monomers A and D, and B and C, in contrast to the weak connections between dimers. Lys 161 is situated within the β-barrel and Tyr 133 sits above this residue. Thr 44 is hydrogen bounded to both Tyr 133 and Tyr 107 of the monomer in the tight conformation, which reaches into adjacent

active site. Each of these residues is highly conserved in all DHDPS enzymes known to date (Dobson et al., 2004).

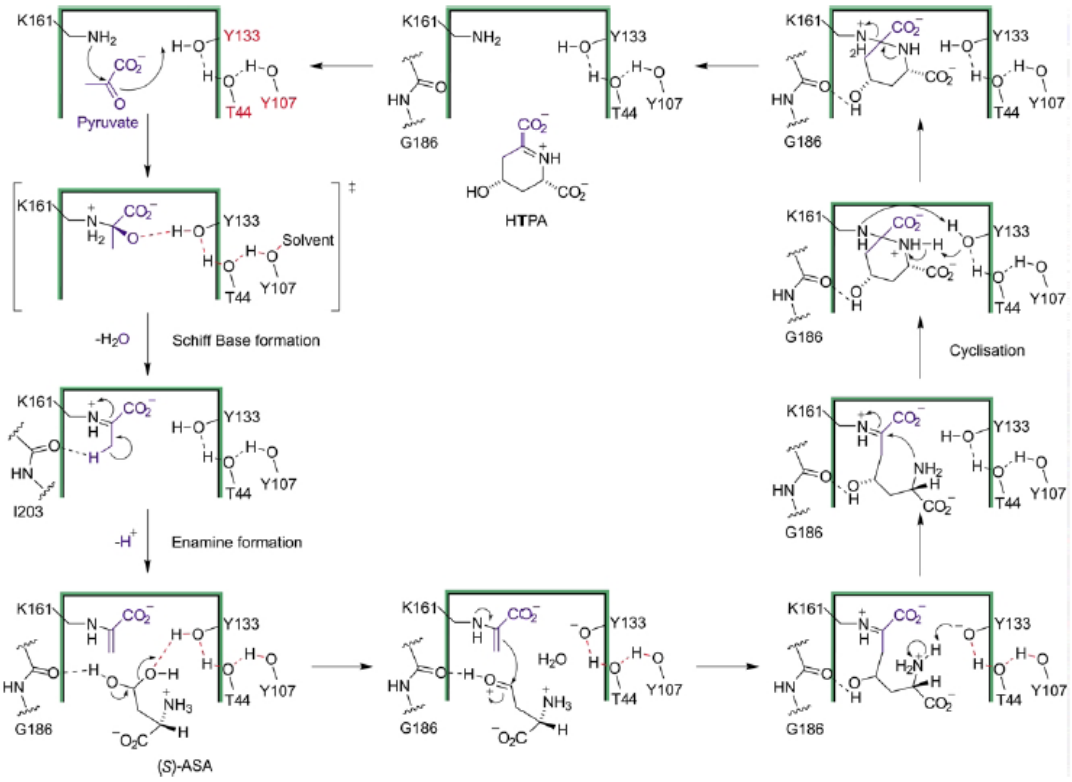


Figure 3 Mechanism of DHDPS (Dobson et al., 2004).

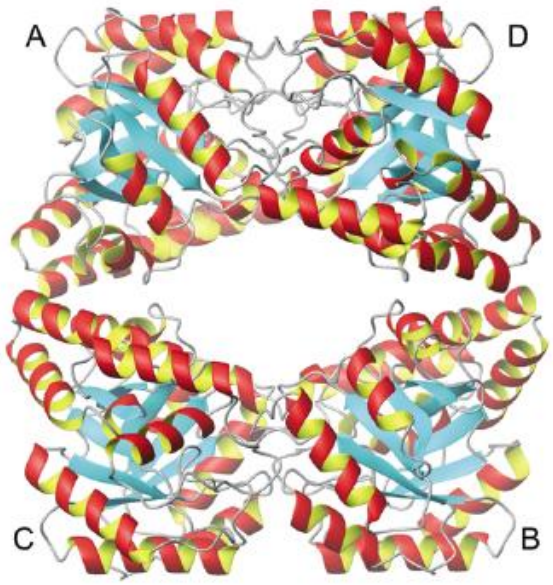


Figure 4 Stereo-view showing the quaternary structure DHDPS. B-strands are coloured light blue, α-helices are yellow and red, and turns are grey (Dobson et al., 2004).

3.2 Different techniques of barley transformation

Barley is one of the world's major cereal crops, ranking fourth behind wheat, rice and maize in terms of agronomic importance. Main effort in genetic engineering of barley is centred on improving the malting quality and disease resistance. The development of techniques for the genetic modification of barley has, in general, been slower than for other cereals. Transformation is restricted to a limited number of genotypes and occurs at low frequencies (Harwood et al., 2000). Successful transformation of barley (*Hordeum vulgare*) has been obtained through DNA uptake, electroporation, microinjection, particle bombardment and the *Agrobacterium tumefaciens* method. For successful stable genetic transformation of plants, ability to formation of shoots originating from totipotent cells is prerequisite. It was proven that for transformation of cereals it is better to use immature embryos, embryogenic pollen cultures and isolated ovules as explants target than leaf tissues, in contrast to dicotyledonous plants (Hensel et al., 2009)

3.2.1 Transformation of barley by direct DNA transfer into protoplasts

First successful genetic transformation events in cereal species was based on direct gene transfer (Toriyama et al., 1988). All major cereals, including japonica rice (Toriyama et a., 1988; Shimamoto et al., 1989), indica rice (Datta et al., 1990), maize (Rhodes et al., 1988; Omirulleh et al., 1993) and barley (Lazzeri et al., 1992; Funatsuki et al., 1995, Salmenkallio-Marttila et al., 1995) were successful transformed via DNA transfer into protoplasts. This method is not efficient for cereals with low regeneration capability and it is connected with technical problems. Protoplast transformation is not efficient in most cereals because it is inconvenient to establish cell suspension from which protoplasts capable of division are usually obtained and so it is more reliable to produce the embryonic suspensions yielding to protoplasts competent to regenerate plants. Other reason is increased accumulation of genetic aberration (somaclonal variation) in fast-growing cultures, such as cell suspension (Wang et al., 1992; Karp & Lazzeri, 1992). This means that suspensions typically lose their shoot regeneration capacity over a matter of months (Lührs & Lürz, 1988) and that suspension protoplast-derived transformants are frequently variant. Protoplasts method does not require the highly specialised equipment and from this reason it was successful used in first transformation attempts.

The generation of transgenic barley plants was enabled via polyethyleneglycol-mediated DNA uptake into protoplasts. Protoplasts were first isolated from embryogenic cell suspension of barley (Funatsuki et al., 1995) and in later approach directly from scutella (Nobre et al., 2000). Three plasmid constructs were used in mentioned reports; *pAct1Dneo*, containing the neomycin phosphotransferase (NPT II), gene under the control of the rice actin promoter and the nos teminator (figure 5) (Funatsuki et al., 1995), *pAct1Dgus* containing the *uidA* gene, coding for glucurodinase, driven by the rice actin promoter, and the *pCaIneo* containing *NPT II*, conferring kanamycin resistance, gene under the control of the CaMV35S promoter and the Adh 1 intron from maize. Transformations were performed according to a procedure described previously (Lazzeri et al., 1992) for protoplasts isolated from barley cell suspensions (in the study of Lazzeri et al., 1992 plants were no recovered). Protoplasts-derived calli were selected on medium containing the antibiotic G418 (analogue to kanamycin). Fertile plants were regenerated from a callus line in both described studies, Funatsuki et al. 1995 and Nobre et al. 2000. NPT II protein was detected using an ELISA kit and Southern hybridization.

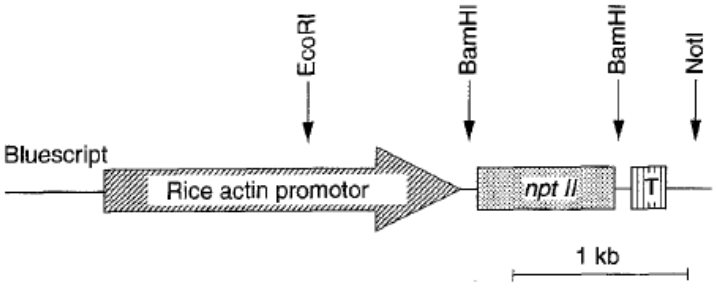


Figure 5 Construction of pAct1Dneo. The arrows show the restriction sites. T means the terminator from the nopaline synthase gene (Funatsuki et al., 1995).

Positive results, which were obtained (Funatsuki et al., 1995; Nobre et al., 2000) have proven the applicability of the protoplast transformation method in barley transformation, but low efficiency suggested that was necessary to develop more suitable transformation method applicable in cereals.

The method of direct DNA transfer into protoplasts was used for example for production of transgenic barley that produces engineered β-amylase with increased thermostability (Kihara et al., 2000). The protocol developed by Funatsuki et al. in 1995 was used for transformation of barley protoplasts. The original barley β-amylase gene was engineered using site-directed mutagenesis and random mutagenesis and these mutations led to increased thermostability by 11.6 °C compared to the unaltered enzyme. The mutant gene was introduced into barley under the control of barley its

native promoter and fertile plants were generated from 9 independent transgenic lines. The expression of mutant gene was confirmed and the transgene was transmitted to progeny. Increasing in thermostability could be of benefit for wider industrial utilization (Kihara et al., 2000).

3.2.2 Transformation of barley by microinjection

Microinjection of DNA into the pronuclei of fertilized eggs is the method that has been successful used for many of years for generation of transgenic animals. From this reason in 1990s substantial effort were put into development of this technique for plants. High transformation frequencies were reported for protoplasts (Crossway et al., 1986; Reich et al., 1986; Neuhaus et al., 1987). In 1986 Crossway et al., in their research compared intracellular versus cytoplasmic microinjection in tobacco protoplasts. They found the higher frequency of integration in tobacco protoplasts for intracellular microinjection (14 %) in contrast to cytoplasmic microinjection (6 %). In next attempts, microinjections into meristematic cells with intact cell walls or unicellular microspores were tried, but this technique was proved to be difficult (Neuhaus et al., 1987; Jones-Villeneuve et al., 1995). Only in single study (Schnorf et al., 1991), where tobacco protoplasts were microinjected, transgenic plants regenerated. They used concentrations 5, 50 and 500 µg/mL of intact plasmid DNA and the trait was transmitted to the next generation in a Mendelian fashion. This technology was subsequently optimised for tobacco protoplasts (Kost et al., 1995) and it was found that higher DNA concentration (1 mg/mL) resulted in very high transformation frequencies.

In following years efficient techniques for isolation of unfertilized egg cells and zygotes have been developed for a number of cereals (maize: Kranz et al., 1991; Faure et al., 1993; barley and wheat: Holm et al., 1994; Kovacs et al., 1994). Zygote protoplasts have been then used in next studies of barley transformation by microinjection. In 2000 Holm et al. published study about transformation of barley by microinjection into isolated zygote protoplasts. In this study authors used barley plants of the winter cultivar Igri and the spring malting barley cultivar Alexis. Zygote protoplasts were isolated by mechanical dissection from ovaries harvested 1 – 28 hours after pollination (according to study Holm et al., 1994) and they were embedded in agarose droplets. For transformation they used construct *pAct1-D* (Zhang et al., 1991) that contains the *E.coli gusA* gene for β-glucuronidase (GUS) under the control actin1 promoter from rice and *Agrobacterium* nopaline synthetase (nos) terminator (figure 6). The plasmid was linearized by digestion with *Sca*I. The microinjection set-up consisted of a Zeiss Axiovert microscope with a motor driven objective revolver,

mounted on a separate table within a laminar flow cabinet. From regenerated calli and leaves DNA was isolated and analysed by PCR and Southern blotting. The activity of GUS was measured according to method of Jefferson et al. (1987) using the X-gluc and methyl umbelliferone glucuronide substrates.

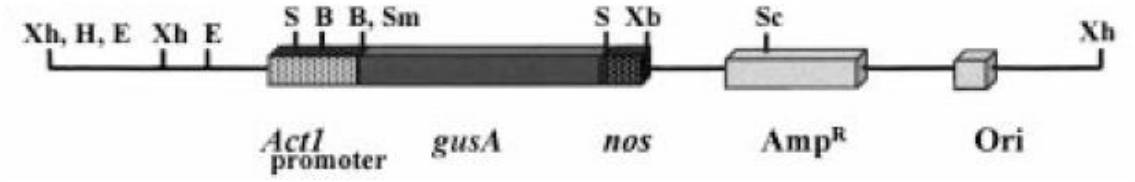


Figure 6 The construct designed by Zhang et al. 1991 and used for transformation in study of Halm et al. 2000. The restriction sites are shown above the construct (B: *Bam*HI, E: *Eco*RI, H: *Hind*III, S: *Sst*I or *Sac*I, Sc: *Sca*I, Sm: *Sma*I, Xb: *Xba*I, Xh: *Xho*I). Ori means origin of replication of gene and Amp^R means gene for resistance to ampicilin.

On average 62 % of the protoplasts survived the microinjection and of these 55 % continued development into embryo-like structures and eventually to plants. GUS analysis did not prove transient or stable GUS expression, but two zygote-derived structures assayed 9 days after injection showed a distinct blue coloration after histochemical analysis. PCR screening confirmed the presence of the *gusA* gene fragment in 21 % of the derived structures. Two lines of T1 plants were subjected to Southern blot analysis and for one line there was a strong hybridization to BamHI/SacI probe, comprising the code region of the *gusA* gene.

This study has shown that transformation of barley by microinjection is feasible but only rarely gene expression was achieved. The possible reason could be degradation of introduced DNA (Holm et al., 2000).

3.2.3 Transformation of barley by particle bombardment

Particle bombardment is a physical gene transfer method basically without target material limitations. One of the first successful transformations of barley with using this method was described in 1994 by Wan & Lemaux. They bombarded immature zygotic embryos, young callus and microspore-derived embryos with a plasmid containing *bar* gene and *gusA* gene alone or in combination with another plasmid barley yellow dwarf virus coat protein (*BYDVcp*) gene. *Bar* gene encodes phosphinothricin acetyltransferase (PAT) isolated from *Streptomyces hygroscopicus* which acetylates and inactivates component of herbicides Basta (phosphinothricin) and Herbiace (bialaphos). The expression of PAT was confirmed in 91 independent bialaphos-resistant callus lines. The integration of *bar* gene was confirmed by DNA hybridization in 67 analyzed lines. From 36 transformed callus lines regenerated more than 500 green, fertile, transgenic plants. From 41 lines albino plants were regenerated. Transformation frequency reported in this study was 7.9 independent lines per 100 immature embryo halves and 3 independent lines per 100 microspore-derived embryos. All plants, regenerated from transformation with using embryogenic callus as target explants were albino. According to results from this study, immature embryos seemed to have been the best target tissue for transformation of barley by particle bombardment (Wan & Lemaux, 1994).

In 1998, Cho and al. developed the protocol that enabled successful transformation of previously recalcitrant commercial cultivars. In this study they tested the effect of different composition of callus induction medium on callus induction frequency, growth rate and quality of three spring cultivars, Golden Promise (GP), Galena (GL), and Harrington (HT). They found that addition of up to 0.1 mg/mL of cytokinin 6-benzylaminopurine (BAP) and 5.0 µM cupric sulphate to callus induction medium resulted in shinier, more compact and slightly brown-coloured callus, which was more regenerable compared to callus induction medium only with auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) or dicamba (table 1). The next important findings in this study was that when the highly regenerable structures were exposed to dim light and maintained on 0.1 mg/mL BAP, they could be cultured for more than a year without a marked loss of regenerability or evidence of albinism.

Table 1 Callus induction frequency, initial growth rate and callus quality of two barley genotypes, Golden Promise (GP) and Galena (GL) on different callus induction media (CIM) (Cho et al., 1998).

Genotype	Medium	Composition of medium			Callus induction frequency (%)	Initial callus growth rate (mg/embryo per day)	Callus quality
		2,4-D (mg/L)	BAP (mg/L)	$CuSO_4$ (µM)			
GP	D	2.5	0	0.1	100 ± 0	12.8 ± 2.0	++
	DC	2.5	0	5.0	100 ± 0	10.6 ± 0.9	++++
	DB	2.5	0.1	0.1	96.7 ± 5.8	7.0 ± 0.5	+++(+)
	DBC1	2.5	5.0	5.0	100 ± 0	11.5 ± 1.2	+++++
	DBC2	2.5	5.0	5.0	100 ± 0	9.1 ± 1.0	++++(+)
	DBC3	1.0	5.0	5.0	86.7 ± 15.3	6.5 ± 1.2	++
GL	D	2.5	0	0.1	96.7 ± 5.8	15.0 ± 1.2	+
	DC	2.5	0	5.0	90.0 ± 10.0	13.6 ± 1.0	+(+)
	DB	2.5	0.1	0.1	66.7 ± 5.8	5.7 ± 1.6	++(+)
	DBC1	2.5	5.0	5.0	80.0 ± 17.3	8.3 ± 1.6	++
	DBC2	2.5	5.0	5.0	46.7 ± 11.5	5.8 ± 2.2	+++
	DBC3	1.0	5.0	5.0	46.7 ± 15.3	3.1 ± 1.5	++

Ten immature embryos from each genotype were transferred to each CIM. Each CIM contains different combination of hormones and copper. Values for callus-induction frequency and initial callus growth rate were measured 24 days after the initial callus induction and represent mean ± S.D. of three replicates for each treatment. Callus quality was assessed microscopically and scored with +++++ being the highest quality and being + the lowest.

They also tested the capability of BAP and copper to induce multiple shoot primordia in cultures initiated only on auxin in both, transgenic and nontransgenic GP callus cultures. In this experiment nontransgenic GP callus tissues were initiated on either D medium (table 1) or DM medium (2.5 mg/L dicamba) and transgenic GP calli were initiated on DM medium with bialaphos (selective agent). The both types of callus tissues were then transferred to regeneration medium either directly or with an intermediate culturing step on DBC2 medium or DBC3 medium (compositions of media are in table 1). The amount of green shoots from tissues cultured with an intermediate step increased 2.3 – 6.4 times in nontransgenic callus tissues and 2.8 – 11.4 in transgenic callus tissues. This increase in regenerability along with other changes in transformation protocol resulted in successful transformation of previously recalcitrant GL and HT cultivars (Cho et al., 1998).

In 2000, Harwood et al. examined six different methods for the preparation of the DNA coated gold particles (table 2) and three different particle delivery devices for their use in barley transformation systems. The embryos (Golden Promise) were bombarded with Accell™ electric discharge device (Christou et al., 1991), PDS 1000 He device (BioRad), and prototype of the Helios™ gene gun system (BioRad). Only one of these three delivery systems, the PDS 1000 He device, yielded to generation of transgenic plants. From six different gold / DNA preparation methods transformed plants were obtained using preparations 3, 4, and 5 (Harwood et al., 2000).

Table 2 Different gold / DNA preparations (1. Christou et al., 1991; 2. Sanford et al., 1993; 3. Wan & Lemaux, 1994; 4. Modified Wan & Lemaux, 1994; 5. Becker et al., 1994; 6. Modified Becker et al., 1994)

Number	Gold size	Gold/shot	DNA/shot	Volume/shot
1	0.95 µm	0.16 mg	0.32 µg	163.0 µL
2	0.95 µm	0.38 mg	0.63 µg	6.0 µL
3	0.95 µm	0.70 mg	8.3 µg	10.0 µL
4	0.95 µm	0.70 mg	2.1 µg	10.0 µL
5	0.95 µm	0.16 mg	0.41 µg	3.5 µL
6	gold mix	0.16 mg	0.41 µg	3.5 µL

Particle bombardment was, for example in 1996, used for production of transgenic barley that express a (1,3 -1,4)-β-glucanase that survives the high temperatures used for kiln drying of green malt. This heat stable enzyme synthesized during germination might eliminate the requirement of complete endosperm wall depolymerisation in the malting schedule and thereby provides new opportunities for the application of malting and mashing in production of conventional and novel biotechnological commodities (Jensen et al., 1996).

Nowadays is particle bombardment widely used as method not only for improvement of quality yield and resistance to disease of the cereals but transgenic cereals obtained by this method can be used for medical purpose. In this year Kim et al. published the study about transgenic rice that produce *E.coli* heat-labile toxin B subunit. Enterotoxigenic *E.coli* is one of the leading causes of diarrhoea in developing countries, and this disease can be fatal in the absence of treatment. Enterotoxigenic *E.coli* heat-labile toxin B subunit (LTB) can be used as an adjuvans, as a carrier of fused antigens, or as an antigen itself. The synthetic gen for LTB was transformed into rice by particle bombardment. Mice were orally immunized with lyophilized transgenic rice calli, which contain LTB, and they generated significant IgG antibody titres against bacterial LTB. The sera of immunized mice inhibited the binding of bacterial LTB to receptor. These results suggested the utilization of transgenic rice callus as a plant-based vaccine to provide effective protection against enterotoxigenic *E.coli* heat-labile toxin (Kim et al., 2010).

In 2008 the study about transgenic rice cell suspension with generation and accumulation human growth hormone was published. Human growth hormone (hGH) is an important hormone with lot of biological functions as a protein synthesis, cell proliferation and metabolic functions. Gene for hGH was transformed into rice calli by particle bombardment. This gene was introduced into rice callus under the control of rice amylase 3D promoter that is induced by sugar starvation. The integration of the synthetic hGH into the chromosome of the transgenic rice callus was confirmed by genomic DNA PCR and expression was confirmed by Northern blotting. Authors also detected this protein in transgenic rice callus by Western blotting after induction of amylase 3D promoter by incubation on sugar-free medium. The recombinant protein was accumulated in transgenic rice callus in amount 57 mg/L and it evidenced a biological activity (Kim et al., 2008).

Mentioned studies together with lot of others have proven that particle bombardment is method of transformation with a high potential in the future.

3.2.4 Transformation of cereals by *Agrobacterium*-mediated method

Agrobacterium tumefaciens causes crown gall disease of a wide variety of plants, for example apple, pear, cherry, almond and others. In 1907 Smith & Townsend discovered the bacterial origin of this disease and lot of studies started to focus on understanding of mechanism of oncogenesis. In 1988 Binns and Thomashaw revealed that *Agrobacterium tumefaciens* is capable of transferring the DNA segment (T-DNA) that is part of tumour-inducing (Ti) plasmid, to nucleus and integrate it into host genome. This finding suggested that *Agrobacterium tumefaciens* can be used for transformation of plants. In initial studies, T-DNA transfer process demonstrated three important facts for practical use of this process in plants. Firstly, during tumour formation, T-DNA is transferred to nucleus and integrated genes are subsequently expressed. Secondly, T-DNA genes are transcribed only in plants cell and do not participate in the transfer process. Thirdly, it is possible to introduce any kind of DNA between the T-DNA borders and it can be transferred to plant cells. This method was firstly used for transformation of dicotyledonous plant, because naturally *Agrobacterium* infects only this kind of plants. Other economically important plants, including cereals were transformed using alternative methods which are subscribed above (Opabode, 2006).

First successful *Agrobacterium*-mediated transformation of cereals was published in 1994 (Hiei et al., 1994). Authors transformed rice with gene for β-glucuronidase and the normal fertile plants were obtained. Some of the first transformation events in monocotyledonous plants are summarized in table 3. The most important factors which influence the transfer of T-DNA and its integration are plant genotype, explants, type of plasmid, bacteria strain, addition of *vir*-gene inducing synthetic phenolic compounds, culture media composition, tissue damage suppression and elimination of *Agrobacterium* infection after co-cultivation (Alt-Morbe et al., 1989; Bidney et al., 1992; Hoekama et al., 1993; Hiei et al., 1994, Komari et al., 1996; Nauerby et al., 1997; Klee, 2000). There are also other factors which influence this process of transformation. In 1997 Uze et al. observed that plasmolysis with 292 mM sucrose improved T-DNA delivery into precultured immature embryos rice but osmotic treatment did not have the same effect on precultured immature embryos of wheat (Uze et al., 2000). In 2003 Cheng et al. reported that desiccation of precultured immature embryos, suspension culture cells, embryonic calli of wheat and maize greatly improved T-DNA delivery and

plant tissue recovery after co-culture with *Agrobacterium* and it led to increased stable transformation frequency (Opabode, 2006).

Table 3 *Agrobacterium*-mediated transformation of some monocotyledonous plants (Opabode, 2006)

Cultivar	Strain (plasmid)	Marker	Explant	TF (%)	Reference
Banana (*Musa* spp.)					
Grand Nain (AAA)	LBA4404 (pBI141)	*npt*II	MCS	2.0	May et al. 1995
Barley (*Hordeum vulgare* L.)					
Winter (igri)	LBA4404 (pSBI:VG35PAT)	*hpt*	PC	2.2	Kumlehn et al. 2006
Rice (*Oryza sativa* L.)					
Indica (basmati 370)	EHA101 (pIGI21Hm)	*hpt*	EC	22	Rashid et al. 1996
Japonica (Taipei 309)	LBA4404 (pTOK233)	*hpt*	PCIE	3.0	Uze et al. 1997
Rye (*Secale cereale* L.)					
Spring (L22)	AGLO (pJFnptII)	*npt*II	PCIE	3.5	Popelka & Altpeter 2003
Sugarcane (*Saccharicum officinarium* L.)					
Ja60-5	LBA4404 (pBI141)	*hpt*	SC	0.94-1.15	Arencibia et al. 1998
Sorghum (*Sorghum bicolor* L.)					
C401	EHA101 (pPZP201)	*pmi*	IE	3.3	Gao et al. 2005
Pioneer 8505	EHA101 (pPZP201)	*pmi*	IE	2.8	Gao et al. 2005
Maize (*Zea mays* L.)					
A188	EHA101 (pTF102)	*Bar*	FIIE	5.5	Frame et al. 2002
A188	LBA4404 (pTOK233)	*hpt*	FIIE	11.8-30.6	Ishida et al. 1996
Wheat (*Triticum aestivum* L.)					
Spring (Bobwhite)	ABI (pMIN18365)	*npt*II	EC	10.5	Cheng et al. 2003
Winter (Candenza)	AGLI (pAL151)	*Bar*	IE	1.7	Wu et al. 2003

TF – transformation frequency, *npt*II – neomycin phosphotransferase, *hpt* – hygromycin phosphotransferase, pmi – phosphomannose isomerase, B*ar* – bialaphos resistant gene, PCIE –precultured immature embryo, EC – embryogenic calluses, FIIE – freshly isolated immature embryo, SC – suspension culture, IE – immature embryo, MCS – meristem corm slices, PC – pollen culture

Recently research article was published with improved protocols for generation of stable transgenic barley, wheat, triticale and maize (Hensel et al., 2009). Authors used hygromycin resistance gene as selectable marker in barley, wheat and triticale and selection of maize relied on bialaphos. The transformation efficiencies obtained in this study ranged between 20 to 86 % in barley, 2 to 10 % in wheat, 2 to 4 % in triticale and 0.5 to 24 % in maize. Period time needed for entire process from growing donor plants until harvest of mature grains from primary transgenic lines are shown in figure 7. The published protocols can be applied on research concerning improving of quality, disease resistance of cereals as well as for comprehensive functional analyses of recombinant nucleotide sequences on a large scale (Hensel et al., 2009).

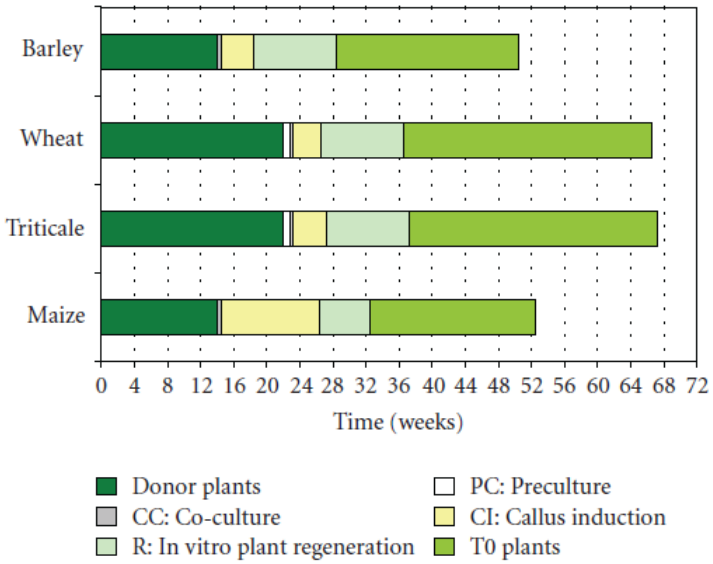


Figure 7 Time lines covering the entire process from growing the donor plants until mature grains can be harvested from primary transgenic plants (Hensel et al., 2009).

This *Agrobacterium*-mediated method is together with particle bombardment the most exploited method of transformation of cereals.

The method was used for production of engineered rice containing larger amounts of nicotinanamine (NA). NA is a metal chelator in higher plants and it is also used as an antihypertensive substance in humans. Hypertension can lead to severe health problems, such as cerebral stroke and cardiovascular disease. NA inhibits angiotensin-I-converting enzyme, key enzyme in hypertension, and it leads to reduced blood pressure. In the study of Usada et al. NA synthase gene from barley was transformed into rice by *Agrobacterium*-mediated method. The gene was introduced under the

control of *pGluB-1* promoter that induces strong expression in the endosperm of rice seeds. The content of NA was increased 4-fold in transgenic rice seeds compared to nontransgenic seeds. Authors removed the antibiotic resistance gene (selectable marker) using Cre/*loxP* DNA excision system and crossed NA producing plants with a cleistogamous mutant in order to prevent gene transfer via pollen dispersal. The trait was stably inherited by the progeny. The transgenic rice with increased NA content can serve as a novel functional food for prevention and treatment of hypertension (Usada et al., 2009).

Sorghum, one of the most important crops in the world besides wheat, rice, maize, soybean and barley, was successfully transformed with insecticidal *Bacillus thuringiensis cry1Ab* gene (*Bt*) using *Agrobacterium*-mediated method. Together 52 transgenic plants regenerated and transformation efficiency was 1.9 %. The expression of *Bt* gene was confirmed by Western blotting and ELISA assay. The transgenic plants with high expression of *Bt* gene displayed resistance to pink rice borer (*Sesamina inferens*) (Zhang et al., 2009).

Agrobacterium-mediated technique offers a lot of advantages over biolistic-mediated techniques in terms of efficiency and the quality of the transformed plants produced. This method led to the production of transgenic lines with lower numbers of transgene copies and they showed more stable transgene expression over generations and fewer cases of transgene silencing. It is ideal for studies of gene function in cereal crop system (Travella et al., 2005).

4 PRACTICAL PART

4.1 Equipment

Chemicals and samples were weight by analytical balance PR352 (Denver Instrument, USA) and solutions were autoclaved by autoclave HST 5.6.8 (Zirbus technology, Germany). Samples were centrifuged by centrifuge Mikro 200R (Hettich, Germany). The concentration of protein and nucleic acids were measured by spectrophotometer 8453 (Agilent, USA). Temperature dependent incubations were performed using shaker UNIMAX 1010 (Heidolph, Germany). The samples were heated by Thermomixer Comfort (Eppendorf, Germany) and thermocycler T– Personal (Biometra, Germany). The gels were visualised by UV transluminator (Vilbert Lourmat, Germany). The transcript level was quantified by real-time PCR (Applied Biosystems, USA). The plant samples were homogenized by homogenizer IKA yellow line DI 18. For chemiluminiscent detection of Western blotting, Chemiluminiscent detection film (Lumi-film 100 NIF, 8 x 10 inches, 20.3 x 25.4 cm) was used (Roche, USA). The chromatography was performed using low pressure liquid chromatographic system BioLogic (Bio-Rad, USA).

4.2 Chemicals, enzymes and kits

Chemicals were from Sigma (USA), Merck (Germany) and Duchefa (Netherlands). Cloning vectors were from Invitrogen (USA). RevertAid H Minus M-MULV reverse transcriptase and buffer for reverse transcriptase were from Fermentas Life Science (Lithuania). NiNTA Kit was from Qiagen (USA). Small Scale Phenol-Free Total RNA Isolation (RNAqueous®) Kit, TRI Reagent® Kit, and TURBO DNA-free™ Kit were from Applied Biosystems. Chloroplast Isolation Kit was from Sigma – Aldrich (Germany). WB luminol reagent SC - 2048 (substrate for luminescence detection) was from Santa Cruz Biotechnology (USA). Protein Ladder Mark 120 kDa was from Fermentas Life Science, Precision Plus Protein™ Standard and Bradford reagent for protein assay were from Bio-Rad, Lowry and Phenol Reagent were from Pierce (USA).

4.3 Media and buffers

Luria-Bertani (LB) medium: 1 % (w/v) peptone, 0.5 % (w/v) yeast extract, 1 % (w/v) NaCl, pH 8.5

Buffers for purification of DHDPS protein:

Lysis buffer: 8 M Urea, 0.1 M NaH_2PO_4, 0.01 M Tris, pH 8.0

Washing buffer: 8 M Urea, 0.1 M NaH_2PO_4, 0.01 M Tris, pH 6.3

Elution buffer: 8 M Urea, 0.1 M NaH_2PO_4, 0.01 M Tris, pH 4.5

Buffers and gels for SDS-PAGE electrophoresis:

Loading buffer: 50 % (v/v) glycerol, 0.05 M EDTA, 0.125 % (w/v) bromphenol blue, 0.125 % (w/v) xylene violet, pH 8.0

Running buffer: 0.025 M Tris, 0.192 M glycine, pH 8.3

Acrylamide – bisacrylamide: 30 % (w/v) acrylamide, 0.8 % (w/v) bisacrylamide

Buffer for running gel: 2.25 M Tris/HCl, pH 9.2

Buffer for stacking gel: 0.75 M Tris/HCl, pH 6.8

SDS – stock solution: 10 % (w/v) SDS

Washing buffer: 0.375 M Tris/HCl, 0.01 % (w/v) SDS, pH 9.2

Butanol for overlay of running gel: 50 % (v/v) butanol, 50 % (v/v) washing buffer

Ammonium persulphate $(NH_4)_2S_2O_8$ (APS): 10 % (w/v) ammonium persulphate

Running gel: 2.5 mL acrylamide – bisacrylamide, 1.25 mL buffer for running gel, 3.63 mL H_2O, 0.075 mL SDS – stock solution, 0.0075 mL TEMED, 0.0375 mL APS

Stacking gel: 1 mL acrylamide – bisacrylamide, 1.25 mL buffer for stacking gel, 5.13 mL H_2O, 0.075 mL SDS – stock solution, 0.0075 mL TEMED, 0.0375 mL APS

Buffers for Coomassie Brilliant Blue R-250 staining:

Fixing buffer: 40 % (v/v) methanol, 10 % (v/v) acetic acid

Coomassie Brilliant Blue R-250 solution: 0.1 % (w/v) Coomassie Brilliant Blue R-250, 15 % (v/v) acetic acid, 45 % (v/v) methanol

Destaining buffer: 40 % (v/v) methanol, 10 % (v/v) acetic acid

Buffers for silver staining:

Fixing buffer 1: 50 % (v/v) ethanol, 10 % (v/v) acetic acid

Fixing buffer 2: 5 % (v/v) ethanol, 1 % (v/v) acetic acid

Staining solution 1: 0.8 mM $Na_2S_2O_3.5H_2O$

Staining solution 2: 0.012 M $AgNO_3$, 0.75 % (v/v) 37 % formaldehyde

Developing solution: 0.566 M Na_2CO_3, 0.5 % (v/v) 37 % formaldehyde, 0.8 mM $Na_2S_2O_3.5H_2O$

Buffers for purification of antibodies:

Coupling buffer: 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3

Blocking buffer: 0.2 M glycine, pH 8.0

Acetate buffer: 0.1 M acetic acid, 0.5 M NaCl, pH 4.0

PBS buffer: 0.0685 M NaCl, 0.005 M Na_2HPO_4, 0.005 M NaH_2PO_4, pH 7.4

Washing buffer 1: 0.05 M Tris/HCl, 0.5 M NaCl, 0.1 % (v/v) Triton X-100, pH 8

Washing buffer 2: 0.05 M Tris/HCl, 0.5 M NaCl, 0.1 % (v/v) Triton X-100, pH 9

Washing buffer 3: 0.05 M Na_2HPO_4, 0.5 M NaCl, 0.1 % (v/v) Triton X-100, pH 6.3

Elution buffer: 0.05 M glycine, 0.15 M NaCl, 0.1 % (v/v) Triton X-100, pH 2.5

Media for barley transformation (Harwood et al., 2009):

Bacterial culture medium (MG/L): 5.0 g/L tryptone, 5.0 g/L mannitol, 2.5 g/L yeast extract, 1.0 g/L L-glutamic acid, 250 mg/L KH_2PO_4, 100 mL/L NaCl, 100 mg/L $MgSO_4.7H_2O$, and 10 µL biotin (0.1 mg/L stock), pH 7.2 was adjusted with NaOH, 15 g /L agar was added for plates

Callus induction medium: 4.3 g/L Murashige and Skoog modified plant salt base, 30 g/L maltose, 1.0 g/L casein hydrolysate, 350 mg/L myo-inositol, 690 mg/L proline, 1.0 mg/L thiamine HCl, 2.5 mg/L dicamba, 3.5 g/L phytagel, hygromycin 50 mg/L, timentin 160 mg/L, pH 5.8 was adjusted with NaOH

Transition medium: 2.7 g/L Murashige and Skoog modified plant salt base (without NH_4NO_3), 20 g/L maltose, 165 mg/L NH_4NO_3, 750 mg/L glutamine, 100 mg/l myo-inositol, 0.4 mg /L thiamine HCl, 2.5 mg/L 2,4-dichlorophenoxy acetic acid, 0.1 mg/L 6-benzylaminopurine, 3.5 g/L phytagel, hygromycin 50 mg/L, timentin 160 mg/L, pH 5.8

Regeneration medium: 2.7 g/L Murashige and Skoog modified plant salt base (without NH_4NO_3), 20 g/L maltose, 165 mg/L NH_4NO_3, 750 mg/L glutamine, 100 mg/l myo-inositol, 0.4 mg/L thiamine HCl, 3.5 g/L phytagel, hygromycin 50 mg/L, timentin 160 mg/L, pH 5.8

Extraction buffer for proteins: 0.2 M Tris/HCl, 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.3 % (v/v) Triton X – 100, pH 8

RNA - storage buffer: 0.8 M Na/citrate, 1.2 M NaCl, pH 6.4

Buffers for Western blot:

Transfer buffer: 0.2 M Tris, 1.2 M glycine, pH 8.5

Blotting buffer: 100 mL transfer buffer, 540 mL water, 160 mL methanol

Blocking buffer: 0.02 M Tris, 0.5 M NaCl, 5 % (w/v) milk, pH 7.5

Washing buffer: 0.02 M Tris, 0.5 M NaCl, 0.1 % (v/v) Tween - 20, pH 7.5

Primary antibody buffer: 0.02 M Tris, 0.5 M NaCl, 1 % (w/v) powdered milk, pH 7.5, primary antibody (dilution from 1 / 500 to 1 / 5000)

Secondary antibody buffer: 0.02 M Tris, 0.5 M NaCl, 1 % (w/v) powdered milk, pH 7.5, secondary antibody (dilution 1 / 4000)

Phosphate substrate buffer: 0.1 M Tris, 0.005 M $MgCl_2$, 0.1 M NaCl, pH 9.5

Staining buffer: nitro-blue-tetrazolium chloride (NBT) in 70 % (v /v) dimethylformamide (7.5 mg per 100 µL of 70 % dimethylformamide), 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 100 % dimethylformamide (2.5 mg per 100 µL), 45 µL NBT and 70 µL BCIP for 20 mL phosphate substrate buffer

MALDI digestion buffer: 50 mM NH_4HCO_3, 5 mM $CaCl_2$, 1.5 µM trypsin

4.4 Methods

4.4.1 Production of DHDPS protein

E.coli strain BL21 (DE3) was used for protein synthesis. The construct *pET160 DEST:: dapA* was prepared in the previous work (figure 8) (Mrízová 2008, Palacky University Olomouc). This plasmid contains C – terminal 6xHisTag, which allows easy purification using NiNTA columns. The *dapA* gene was placed under the control of isopropyl-β-D-thiogalactopyranoside (IPTG) inducible promoter.

The production of DHDPS protein was performed according to the protocol, optimized in the previous work (Mrízová 2008, Palacky University Olomouc). Erlenmeyer flask containing 40 mL of LB medium (100 μg/mL ampicilin) was inoculated with the glycerol stock of *E.coli* and cultivated overnight (ON), at 37°C, under gentle shaking (180 rpm). Then four 2 L Erlenmeyer flasks with 500 mL of LB medium (with addition 100 μg/mL ampicilin, 50 μg/mL carbenicilin, 1 % glucose) were inoculated with 10 mL of ON preculture. The optical density was measured at a wavelength of 600 nm (OD_{600}) every 30 min. The initial OD_{600} was approximately 0.1. When OD_{600} reached value of 0.5, the expression of protein was induced by IPTG (final concentration 0.1 mM). After 18 hours of additional cultivation at 37 °C, the culture was harvested by centrifugation at 4500 rpm, at 4°C, for 15 min. The cell pellet was stored at - 20 °C.

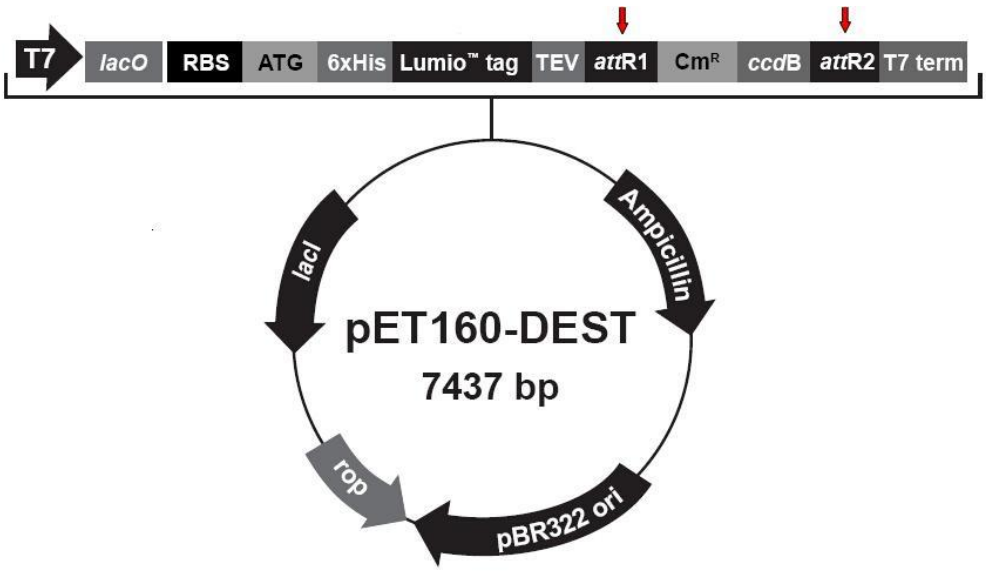


Figure 8 Map of the vector pET160 DEST. The coding sequence of *dapA* gene was inserted between sections attR1, attR2 under control of the T7 promoter.

4.4.2 Disruption of cells

The cell pellet was resuspended with 32 mL of lysis buffer and incubated for 1 hour, at room temperature, under gentle shaking (80 rpm). The suspension was then centrifuged at 4500 rpm, at 4°C, for 45 min, the supernatant was transferred to the fresh Eppendorf tubes and kept for following purification.

4.4.3 Purification of DHDPS protein

NiNTA columns were used for purification of DHDPS protein. The columns were equilibrated with 600 μL of lysis buffer (2000 rpm, 4°C). Then, 600 μL of lysate were loaded onto the columns and this step was repeated afterward with the first flow throw fraction. The columns were washed twice with 600 μL of wash buffer and protein was eluted four times with 200 μL aliquots of elution buffer. The elution buffer contained 40 mM imidazole in the first and second elution, and 250 mM imidazole in third and fourth elution. The purity of DHDPS protein was checked on SDS-PAGE gel and protein was then dialyzed against the elution buffer without urea (pH 7.0). The dialysis was performed stepwise, to avoid the aggregation of the protein. The concentration of urea in the dialysis buffer was stepped down in each overnight change in the range from 4 M to 0.1 M. Final dialysis buffer did not contain any urea. The produced protein was used for rabbit immunization and subsequent purification of specific antibodies from rabbit sera.

4.4.4 SDS-PAGE electrophoresis

SDS-PAGE was used to verify the correct molecular mass of the purified protein and estimate the purity of the protein. Two gels, stacking and running gel, were used for SDS-PAGE electrophoresis. Gels were polymerized in gel caster. First the running gel was poured and allowed to polymerize. After overlaying with a thin layer of 50 % butanol the stacking gel was poured and a comb was placed to create the wells. After the gel polymerization, the comb was removed and gel was ready for electrophoresis. The electrophoresis chamber was filled up with the running buffer and the gel was fixed into it. The protein samples were mixed with loading buffer (in ratio 4:1) and loaded into the wells with a syringe or pipette. Finally, the apparatus was plugged into a power supply set up to increasing current from 30 to 50 mA to separate proteins. After electrophoresis, gels were stained with suitable dye.

4.4.5 Coomassie Brilliant Blue R-250 (CBB) staining

The gel was first incubated in fixing buffer for 5 min, washed by water 2 x 2 min and incubated in CBB solution for 30 min. Next, the gel was washed 2 x 2 min by water and incubated in destaining buffer for 10 to 120 min.

4.4.6 Silver staining

The gel was first incubated in fixing buffer 1 for 30 min and then in fixing buffer 2 for 15 min. Next, it was washed 3 x 5 min by water and incubated in staining solution 1 for 1 min. The gel was then washed 3 x 30 s by water and incubated in staining solution 2 for 20 min. Then, it was washed 2 x 20 s by water and incubated in developing solution for 2 to 10 min.

4.4.7 Measurement of concentration of the protein

Bradford assay: 1 mL of Bradford Reagent (diluted 5 x) was mixed with 1 - 10 μL of sample (standard) until colour have changed to light blue (5 min). Next, an absorbance was measured at a wavelength of 595 nm.

Lowry assay: 150 μL of sample was mixed with 750 μL of Lowry Reagent and incubated for 10 min at room temperature. Next, 75 μL of Phenol Reagent (2-fold diluted) was added to the mixture and incubated for 30 min at room temperature. The absorbance was measured at a wavelength of 700 nm.

4.4.8 Preparation of antibodies against DHDPS protein

Two rabbits *Chinchilla* (weight up to 2 kg) were used for preparation of antibodies (marked as A and B). Purified DHDPS protein was divided into 5 doses with volume 1 mL and concentrations: first two 1 mg /mL; second two 0.2 mg /mL and last one 0.5 mg/mL. The rabbits were immunized 14 days, 21 days, 28 days and 42 days after the first dose. Approximately 3 months after immunization, the sera from rabbits were collected. Totally 40 mL of serum was obtained from two rabbits.

4.4.9 Preparation of column for antibody purification

Cyanogen bromide (CNBr) activated Sepharose was used for purification of antibodies by affinity chromatography. Processed agarose (Sepharose) has a primary structure consisting of alternating residues of D-galactose and 3-anhydrogalactose. These sugars provide an uncharged hydrophilic matrix. CNBr in base reacts with –OH groups on agarose to form cyanate esters or imidocarbonates. These groups react readily with primary amines under very mild conditions and the result is a covalent coupling of a ligand to the agarose matrix. The preferred resulting structure is an imidocarbonate, which has no charge (figure 9).

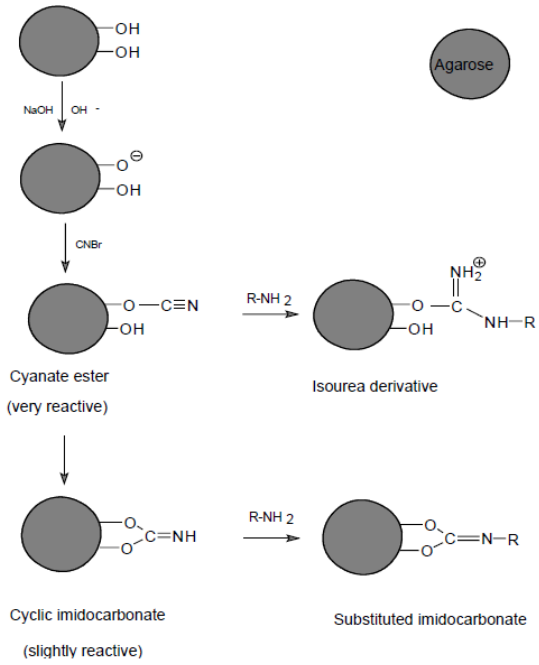


Figure 9 Activation of CNBr matrices.

The purified protein was dissolved in coupling buffer (930 µg of protein in total volume of 1 mL). One gram of CNBr-activated resin was washed in cold 1 mM HCl (200 mL per gram) for 30 min and supernatant was removed by gentle suction in a funnel. Next, the resin was washed with distilled water (5 – 10 mL) and then was washed with coupling buffer (5 mL). The resin was immediately transfer to a solution of the protein in coupling buffer and mixed ON, at 4 °C, using a paddle stirrer. Next, the unreacted ligand was washed away using coupling buffer. The unreacted groups were blocked with blocking buffer for 2 hours, at room temperature. The resin was then washed extensively to remove the blocking solution, first with basic coupling buffer at pH 8.5, then with acetate buffer. This washing cycle of high and low pH buffer solutions was repeated five times and the resin was ready for use.

4.4.10 Purification of antibodies

The column filled with above prepared resin was equilibrated with PBS buffer. 2 mL of serum were mixed with 4 mL of PBS buffer and loaded onto the column. The column was connected to BioLogic low-pressure chromatographic system and the flow was set up to 1 mL per min. The column was washed first with 30 mL of PBS buffer, then 30 mL of the washing buffer 1; 40 mL of the washing buffer 2 and finally with 50 mL of the washing buffer 3. The antibodies were eluted with elution buffer and immediately mixed with buffer containing 1 M Tris-Cl, pH 9 to shift pH to neutral range.

4.4.11 Tissue culture media preparation

Phytagel was prepared in advance at two times higher than the required concentration and sterilized by autoclaving. All tissue culture media were filter sterilized, therefore all media components, except those stored as sterile stocks, were added at two times higher than the required concentration, dissolved, the pH adjusted and the media filter sterilized (Steritop 0.22 µm filters, Milipore). Both the 2 x phytagel and the 2 x media were warmed to 60 °C in a water bath before use. The required stocks were added under sterile conditions to the warmed media, the media and the phytagel were mixed (in ratio 1:1 to get right concentration) and poured into 9 cm Petri dishes (Gama Group, Czech Republic). For regeneration, the media were poured into deeper dishes (tissue culture dish, 100 x 15 mm, Iwaki, USA) (Harwood et al., 2009).

4.4.12 Preparation of *Agrobacterium* standard inoculum

A single colony of *Agrobacterium* AGL1, containing the pBract 214 vector (figure 10) together with pSoup, was used to inoculate 10 mL of bacterial culture medium with 25 µg/mL rifampicin and 50 µg/mL kanamycin, incubated at 28 °C, for 40 h, at 180 rpm. 10 mL of sterile 30 % aqueous glycerol was added to the bacterial culture and mixed by inverting several times. Aliquots of 400 µL of the standard inoculum were placed into 0.5 mL Eppendorf tubes and were maintained at room temperature for 2 h mixing by inversion every 30 min. Standard inoculums were stored at – 80 °C ready for use (Harwood et al., 2009).

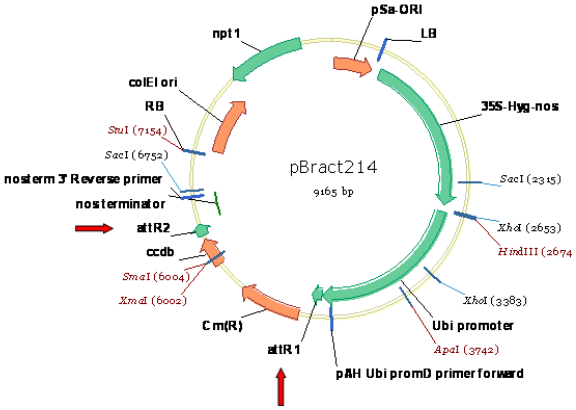


Figure 10 Vector *pBract 214* that was used for barley transformation. *DapA* gene was inserted between the segments attR1, attR2 under control of the Ubi promoter (maize ubiquitin promoter). A gene conferring the resistance to the antibiotic hygromycin (selective marker) is under control of the 35S promoter (35S-Hyg-nos).

4.4.13 Isolation of barley immature embryos

All 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 µmol / m^2 / s at the mature plant canopy level provides by metal halide lamps (HQl) supplemented with tungsten bulbs. Barley spikes were collected when immature embryos were 1.5 mm in diameter. The immature seeds were removed from the spike and the awns broken off without damaging the seed coat. The immature seeds were first sterilized in 70 % ethanol for 30 s. Next, they were washed 3 x by sterile distilled water and sterilized in a solution of sodium hypochloride (diluted 1 : 1 with water) for 4 min. They were then washed 4 x by sterile distilled water after which the immature seeds were drained but left wet in a screw top sterile jar. All procedures were performed in a laminar flow hood under sterile conditions. The embryo was isolated from seed with the aid of two pair of fine forceps and embryonic axis was removed. The embryo was then plated scutellum side up on callus induction medium. 25 embryos were placed on each 9 cm plate ready for *Agrobacterium* inoculation and stored at 23 – 24 °C in the dark (Harwood et al., 2009).

4.4.14 Transformation of barley

Two constructs were prepared for transformation of barley: *pBract 214::mdapA* (Mrízová 2008, Palacky University Olomouc) and *pBract 214::sTPdapA* (see below). First construct *pBract 214::mdapA* contained engineered DHDPS from *E.coli* and second one contained synthetic DHDPS with transit peptide (TP) of *Hordeum vulgare* ribulose-1,5-bisphosphate carboxylase small subunit. TP should direct gene to the chloroplast.

An *Agrobacterium* culture was prepared overnight by adding a standard inoculum to 10 mL of liquid MG/L medium without any antibiotics, incubated on shaker at 180 rpm, at 28 °C ON (approximately 20 h). A small amount of *Agrobacterium* culture (approximately 200 µL) 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 a fresh callus induction plate, scutellum side down. Plates were sealed with Microspore surgical tape and incubated at 23 – 24 °C for 3 days (Harwood et al., 2009).

4.4.15 Preparation of construct *pBract 214::sTPdapA*

The cassette *sTPdapA* (figure 11) used for construct *pBract 214::sTPdapA* was synthesized by Mr.Gene (Germany). The sequence of *E.coli dapA* gene was adjusted to plant codon in order to keep amino acid sequence. Upstreams of *dapA gene* TP of *Hordeum vulgare* ribulose-1,5-bisphosphate carboxylase small subunit was added (Genbank U43493) . The prepared construct was then subcloned into vector pENTR1A using restriction enzymes BamHI and NotI, followed by recombination into the vector pBract214 using recombination enzyme LR CLONASE™ II that can exchange specific fragments between two vectors.

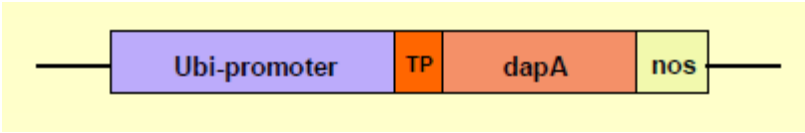


Figure 11 The cassette *stPdapA*. *DapA* gene is under the control of Ubi promoter from maize and nos terminator.

4.4.16 Selection and regeneration of transgenic plants

After 3 days co-cultivation, the embryos were transferred to fresh callus induction plates, containing hygromycin as the selective agent and timentin to suppress *Agrobacterium* growth. Embryos were cultured scutellum side down at 23 – 24 °C (selection 1) in the dark. Embryos were two times transferred to the fresh selection plates with callus induction medium in two weeks period (selection 2 and 3). Callus derived from one embryo was not split up. Next the transfer of embryo-derived callus to the transition medium containing hygromycin and timentin followed The plates were incubated for two weeks, at 24 °C under low light that was achieved by placing the plates in a tissue-culture room under light conditions but covering the plates with a thin layer of paper. Then, embryo-derived material was transferred to the regeneration medium in deep Petri dishes. The regeneration medium contained the same levels of hygromycin and timentin but no growth regulators. All regenerating calluses derived from a single embryo were kept together. Once shoots had been 2 – 3 cm in length and roots had formed, the small plantlets were carefully removed from the plates and transferred to Erlenmeyer flasks containing 12 mL of callus induction medium. This medium contained the same levels of hygromycin and timentin but no growth regulators. The rooted plants were transferred to the soil when had reached the top of the tubes. The leaf samples were collected for further analysis when plant had established well in soil (Harwood et al., 2009).

4.4.17 Extraction of proteins from barley

Leaves from barley were used for protein extraction. A plant material was weighted and grounded in a mortar with liquid nitrogen. Next, the extraction buffer (4 mL per gram of plant material) was added, the ground material was collected in an Eppendorf tube and incubated for 1 hour, on ice, with occasionally stirring by vortex. Then, the samples were centrifuged for 30 min, at 4 °C at 13000 x g. Supernatant was transferred to the fresh Eppendorf tubes and kept till use at – 20 °C.

4.4.18 Isolation of chloroplasts

The isolation of chloroplast was performed using the Chloroplast Isolation Kit. All steps were done at 2 °C to 4 °C, in dark. 30 grams of leaves from barley were washed with deionised water and the excess of water was removed. The midrib veins were removed with sharp scissors and the leaves were cut into small (1 - 3 cm) pieces. 120 mL of 1 x CIB buffer with 0.1 % (w/v) BSA were added (4 mL per gram of leaves). The leaves were processed by homogenizer DI 18 and filtered through the filter mesh 100, nylon into 50 mL tubes and the filter was squeezed in order to collect all of the liquid. The tubes were centrifuged for 3 min, at 200 x g to remove whole cells and cell wall debris as a white-green pellet. The supernatant was transferred to fresh 50 mL tubes and centrifuged for 7 min, at 1000 x g. The supernatant was discarded and the pellet was resuspended in 1 to 2 mL of 1 x CIB buffer with 0.1 % (w/v) BSA. For separation of the intact chloroplasts from the broken chloroplasts, 40 % percoll layer was used. 10 mL of 40 % percoll in a 50 mL tube were prepared (4 mL percoll, 6 mL of 1 x CIB buffer with 0.1 % (w/v) BSA). The chloroplast suspension was layered on the top of the 40 % percoll and it was centrifuged for 6 min at 1700 x g. The broken chloroplasts formed a band on top of the percoll layer and the intact chloroplasts were seated on the bottom as a small green pellet. The upper phases were carefully removed and the pellet was resuspended in 0.5 mL of 1 x CIB buffer without BSA.

4.4.19 Western blot (WB)

The gel was prepared according to SDS-PAGE protocol. The polyvinylidene membrane (PVDF) was cut (6 x 8.5 cm) and wetted in methanol for 30 sec. The porose pads and filter papers were wetted in blotting buffer. For transfer of proteins, the „sandwich“ was set up from bottom: black holder, porose pad, filter paper, gel, PVDF membrane, filter paper, porose pad, red holder. The „sandwich“ was put into the chamber filled with running buffer. The conditions were set up to 100 V, 350 mA and the transfer was run for 1 hour in fridge. Next, the membrane was blocked in blocking buffer ON, at room temperature. The membrane was then washed 2 x 10 min in washing buffer and incubated with primary antibody for 2 hours, at room temperature, under gentle shaking. Next, the membrane was washed 2 x 10 min in washing buffer and incubated with secondary antibody (type was chosen according to the type of the detection) for 1 hour, at room temperature, under gentle shaking. Finally, the membrane was washed 2 x 10 min in washing buffer.

Two types of detection were used. For chromogenic detection, the membrane was incubated 5 min in alkaline phosphate substrate buffer and then incubated in staining buffer for 5 – 30 min.

For luminescent detection, the membrane was covered with substrate for 2 min, foil-wrapped, put into the cassette and chemiluminescent detection film was developed. Time of developing depends on signal intensity (1 to 5 min).

4.4.20 Isolation of RNA

Two kits were used for isolation of RNA; Small Scale Phenol-Free Total RNA Isolation (RNAqueous®) Kit and TRI Reagent® Kit.

RNAqueous® Kit

A plant material (from leaves) was weighted and ground in a mortar with a liquid nitrogen. The sample was transferred to the Eppendorf tubes and 10 volumes of Lysis/Binding Solution were added (1 mL per 100 mg of the sample). The sample was centrifuged 2 min at 13000 x g in a microcentrifuge to remove debris. Next, an equal volume of 64 % ethanol was added and mix gently by inverting the tube several times. The lysate/ethanol mixture was applied to a filter cartridge assembled in a collection tube (maximum volume 700 µL) and centrifuged at 15000 x g for 1 minute. The flow-through was discarded and this step was repeated until all the sample had been drawn through the filter. The sample was then washed with 700 µl of wash solution 1 and the flow-through was discarded. Next, the sample was washed with 500 µL of wash solution 2 and the flow-through was removed (2 times). After discarding the wash solution, the sample was centrifuged to remove the last traces of wash solution. The filter cartridge was transferred to the fresh collection tube. The RNA was eluted two times, first with 60 µL and second with 40 µl of the elution solution (preheated to 80 °C) by centrifugation at 15000 x g.

TRI Reagent® Kit

The plant sample was prepared by the procedure described above. Then, 10 volumes of TRI Reagent was added to the sample and incubated for 5 min at room temperature. The homogenate was then centrifuged at 12000 x g for 10 min, at 4 °C, and supernatant was transferred to the fresh tube. Next, 200 µL of chloroform (per 1 mL of TRI Reagent) was added, mixed well and incubated at room temperature for 10 min. The mixture was then centrifuged at 12000 x g for 10 min, at 4 °C and the aqueous phase was transferred to a fresh tube. Then, 500 µL of isopropanol (per 1 mL of TRI Reagent) was added, mixed by vortex for 10 s and incubated at room temperature for 10 min. The mixture was centrifuged at 12000 x g for 8 min, at 4 °C

and supernatant was discarded (RNA forms white opaque pellet). The pellet was washed with 1 mL of 75 % ethanol (per 1 mL of TRI Reagent). The sample was centrifuged at 7500 x g for 5 min, ethanol was removed and RNA pellet was briefly air dried. RNA pellet was dissolved in RNA storage buffer.

The purified RNA was then treated with TURBO DNA-*free*™ Kit in order to remove DNA contaminants. To the RNA sample, 0.1 volume 10 x TURBO DNAse Buffer and 1 µL TURBO DNAse were added and mixed gently. The mixture was incubated at 37 °C for 1 hour. Subsequent 1 µL of TURBO Dnase was added, mixed gently and incubated at 37 °C for an hour. Next, 0.1 volume of Dnase Inactivation Reagent was added mixed by vortex for 2 min and centrifuged at 13000 x g for 7.5 min. The supernatant was transferred to the fresh Eppendorf tube and 0.5 volume of LiCl was added and mixed by vortex. The mixture was incubated at -20 °C for at least 30 min, and then centrifuged at 13000 x g for 20 min. The supernatant was discarded and sample was spun down for 1 minute at microcentrifuge to remove debris of LiCl. The pellet was washed with 500 µL of 70 % ethanol by centrifugation at 13000 x g for 5 min. Next, the pellet was air dried and dissolved in RNA storage buffer.

The concentration of isolated RNA was estimated by measurement of absorbance at a wavelength of 260 nm (when OD_{260} = 1 then sample RNA concentration is 40 µg per mL).

4.4.21 Reverse transcription

This method used RevertAid H Minus M-MULV reverse transcriptase for transcription RNA to cDNA. The samples were diluted by RNAse-free water to have equal concentration in each of samples. To 12 µL of sample, 1 µL of oligo (dT) primer was added, mixed and incubated at 70 °C for 5 min. Then, 4 µL of 5x reverse transcriptase buffer and 2 µL of 10 mM deoxynucleotides were added, mixed and incubated at 37 °C for 5 min. Next, 1 µL of reverse transcriptase was added, mixed and incubated at 42 °C for 90 min. Reaction was stopped by 10 min heat deactivation at 70 °C.

4.4.22 Real-time PCR

Real-time PCR is a technique used to monitor the progress of a PCR reaction in real time. Real-time PCR is based on the detection of the fluorescence produced by a reporter molecule, which increased, as the reaction proceeds. This occurs due to the accumulation of the PCR product with each cycle of amplification. In our case this fluorescent reporter molecule was dye that bind to the double-stranded DNA (SYBR®

Green). SYBR® Green binds to the minor groove of the DNA double helix. In the solution, the unbound dye exhibits very little fluorescence. The fluorescence is substantially enhanced when the dye is bound to double strand DNA.

To 20 µL of cDNA, 30 µL of EB buffer were added. To 96-well plate 5 µL of 2x SYBR® Green Power PCR mix (Applied Biosystems), 2.5 µL of primer mix (250 µL RNAse-free water, 3 µL of 100 µM forward primer, and 3 µL of 100 µM reverse primer) and 2.5 µL of sample were pipetted. The expression of 3 genes were monitored (40 cycles), *dapA* gene (figure 12), hygromycin resistance gene (figure 13), and house-keeping gene for elongation factor 1 (figure 14).

5' - GGT GAT GAT GAC GCT GGA TCT - 3' ***mdapA*_forward**
5' - GGT AAT TGC CGG GAC CG - 3' ***mdapA*_reverse**

5' - GGC CAT GGC GTG ATT TCT - 3' ***sTPdapA*_forward**
5' - AGA GAC ATG GCT CAA ATG TGC A - 3' ***sTPdapA*_reverse**

Figure 12 Sequence of primers for real-time PCR. First set of primers was designed for *mdapA* gene and second one was designed for *sTPdapA* gene.

5' - CGA GGT CGC CAA CAT CTT CT - 3' hygromycin_forward
5' - GCG TCT GCT GCT CCA TAC AA - 3' hygromycin_reverse

Figure 13 Sequence of primers designed for hygromycin resistance gene, which were used for real-time PCR.

5' - CCG CAC TGT CAT GAG CAA GT - 3' elong.factor1_forward
5' - GGG CGA GCT TCC ATG TAA AG - 3' elong.factor1_reverse

Figure 14 Sequence of primers designed for elongation factor 1, which were used for real-time PCR.

4.4.23 MALDI-TOF Mass Spectrometry (MS)

This type of MS uses MALDI (matrix assistant laser desorption ionisation) ionisation and TOF (time-of-flight) mass analyser. This method is used successfully in biochemical areas for the analysis of proteins, peptides, glycoproteins,

oligosaccharides, and oligonucleotides. MALDI ionisation is based on the bombardment of sample molecules with a laser light to bring about sample ionisation. The sample is pre-mixed with a highly matrix compound for the most consistent and reliable results, and a low concentration of sample to matrix works best. The matrix transforms the laser energy into excitation energy for the sample, which leads to sputtering of analyte and matrix ions from the surface of the mixture. TOF analyser separates ions according to their mass (m)-to-charge (z) (m/z) ratios by measuring the time it takes for ions to travel through a field free region known as the flight, or drift, tube. The heavier ions are slower than the lighter ones. The m/z scale of the mass spectrometer is calibrated with a known sample that can either be analysed independently (external calibration) or pre-mixed with the sample and matrix (internal calibration).

The protein sample was first separated on SDS PAGE gel and stained by CBB. The gel was rinsed with water. The band corresponding to DHDPS protein was excised from gel with scalpel and transferred to 1.5 mL eppendorf tube. The gel particles were washed with 100 – 150 µL of water for 5 min, spun down, and liquid was removed. Next, an acetonitril (approximately 3 – 4 times equal the volume of gel pieces) was added, and they were incubated until the gel pieces shrunk. The gel particles were then spun down, liquid was removed, and they were dried in a vacuum centrifuge. Next, the gel pieces were swollen in 10 mM dithiotreitol/0.1 M NH_4HCO_3 and they were incubated for 30 min, at 56°C to reduce the protein. The mixture was spun down and liquid was removed. The gel pieces were shrunk with acetonitril and acetonitril was then replaced with 55 mM iodoacetoamide/0.1 M NH_4HCO_3 and incubated for 20 min at room temperature in the dark. Then, iodoacetoamide solution was removed and gel particles were washed with 150 – 200 µL of 0.1 M NH_4HCO_3 for 15 min. The mixture was spun down and liquid was removed. Next, the gel pieces were shrunk with acetonitril, spun down, liquid was removed and they were dried in a vacuum centrifuge. Then, the gel pieces were rehydrated in 150 µL of 0.1 M NH_4HCO_3, incubated for 15 min, and 150 µL of acetonitril were added. The tube was mixed by vortex for 20 min, the gel particles were spun down, liquid was removed and they were dried in a vacuum centrifuge. The gel particles were then rehydrated in the digestion buffer and incubated at 4 °C for 45 min. Remaining supernatant was removed and 5 – 25 µL of the same buffer (without trypsin) were added to cover gel pieces. The sample was incubated at 37 °C ON, spun down and small aliquot of the supernatant was used for MALDI analysis.

4.4.24 Determination of amino acids in leaves

The composition of amino acids in the leaf extract was determined using a HPLC (High Pressure Liquid Chromatography) after hydrolysis of samples with HCl. This analysis was done in cooperation with Department of Chemistry and Biochemistry in Mendel's Agriculture and Forestry University in Brno, Czech Republic.

4.5 Results

4.5.1 Production of DHDPS protein

The protein was produced in *E.coli* strain BL21 DE3 growing in supplement LB medium under denatured conditions (37 °C). According to these conditions, the protein should be deposited in inclusion bodies. The cells and inclusion bodies were disrupted by 8 M urea and purified using NiNTA columns. Denatured protein was afterward renatured by stepwise dialysis. The right size and purity of the protein was checked on SDS-PAGE gel (figure 15). Identity of the protein was also checked using MALDI-TOF MS (figure 16). The protein was nearly pure after purification by NiNTA columns and dialysis, but there were still some impurities around 29 kDa (it was identified as Elongation Factor Tu from *E.coli*) and 45 kDa (it was identified as Ternary Complex-Bound *E.coli* 70s Ribosome) as it is shown on the gel used for MALDI-TOF MS. The band around 40 kDa was identified as DHDPS protein from *E.coli*. The expression in 2 L flasks was repeated several times in order to obtain adequate amount of protein for all experiments.

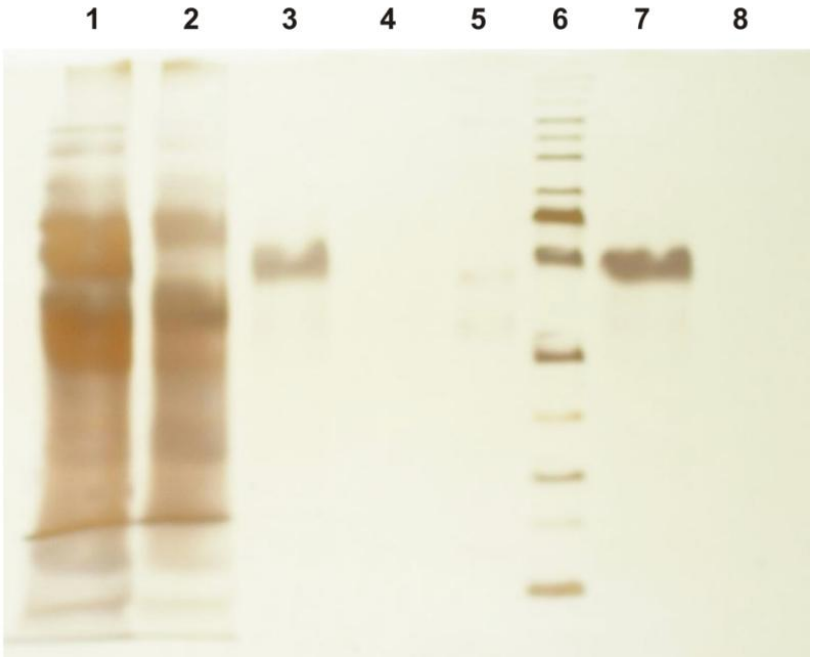


Figure 15 SDS-PAGE gel after expression and purification of DHDPS protein. **1.** DHDPS lysate; **2.** negative control lysate; **3.** first elution of DHDPS; **4.** first elution control; **5.** marker LUMIO; **6.** marker 120 kDa; **7.** second elution of DHDPS; **8.** second elution control.

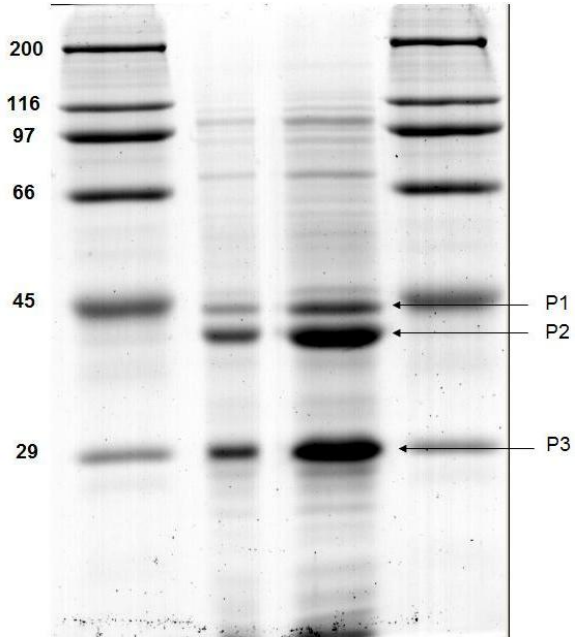


Figure 16 SDS-PAGE gel, proteins (P1, P2, P3) were analysed using MALDI-TOF MS. Protein P2 was identified as DHDPS protein from *E.coli.* Proteins P1 and P3 were identified as Elongation Factor Tu and Ternary Complex-Bound *E.coli* 70s Ribosome, both from *E.coli*.

4.5.2 Testing of purified antibodies

First of all, reactivity of antibodies with purified bacterial DHDPS protein was tested. Concentration range from 1 mg/mL to 0.002 mg/mL of DHDPS protein was tested. Two different dilutions of antibodies were used for reaction with bacterial DHDPS protein; 1/10000 and 1/5000 (figure 17 and 18). The best specificity antibody shows with the most diluted protein (0.002 mg/mL). The reaction was more specific for antibody dilution 1/10000 (figure 18).

Further, antibodies before and after purification were tested and compared. In this analysis, protein extract from plant was used. Two concentration of antibodies were used, 1/100 and 1/1000 (figure 19 and 20). The purified antibodies reacted with protein extract more specific than nonpurified antibodies.

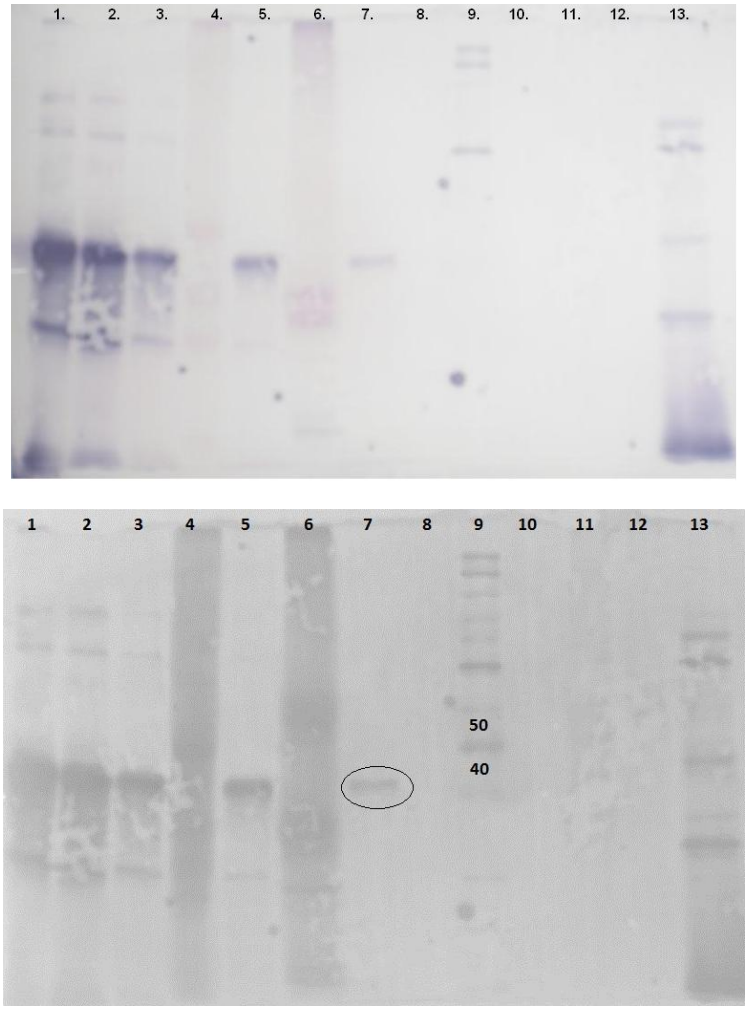


Figure 17 Membranes from WB. The dilution of primary antibodies was 1/5000. The upper membrane is before staining with amido black 10 B and membrane below is after staining. The samples were loaded on gel in order; **1.** DHDPS protein c = 1 mg/mL, **2.** DHDPS protein c = 0.2 mg/mL, **3.** DHDPS protein c = 0.05 mg/mL, **4.** negative control from untransformed barley leaves, **5.** DHDPS protein c = 0.01 mg/mL, **6.** negative control from untransformed barley roots, **7.** DHDPS protein c = 0.002 mg/mL, **8.** –, **9.** marker 120 kDa, **10.** –, **11.** negative control from untransformed barley leaves (10 times diluted), **12.** negative control from untransformed barley roots (10 times diluted), **13.** negative control proteins from bacteria (without *dapA* gene).

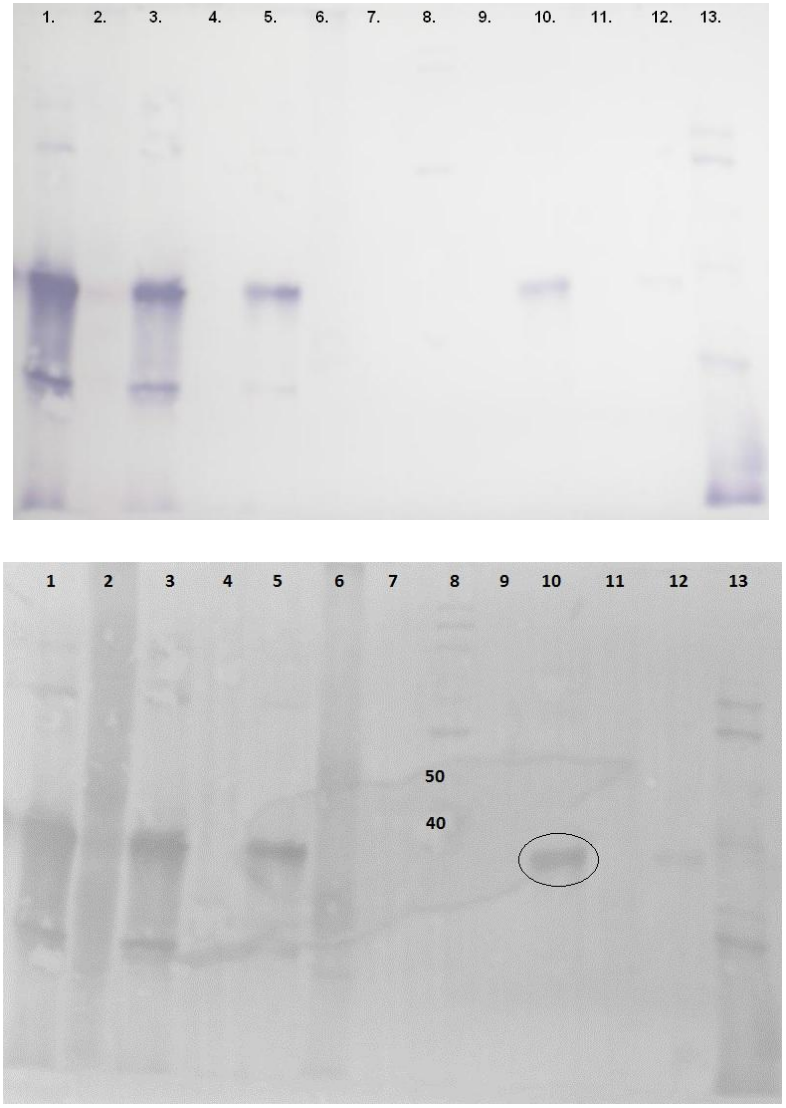


Figure 18 Membranes from WB. The dilution of primary antibodies was 1/10000. The upper membrane is before staining with amido black 10 B and membrane below is after staining. The samples were loaded on gel in order; **1.** DHDPS protein c = 1 mg/mL, **2.** negative control from untransformed barley leaves, **3.** DHDPS protein c = 0.2 mg/mL, **4.** negative control from untransformed barley roots, **5.** DHDPS protein c = 0.05 mg/mL, **6.** negative control from untransformed barley leaves (10 times diluted), **7.** -, **8.** marker 120 kDa, **9.** -, **10**. DHDPS protein c = 0.01 mg/mL **11.** negative control from untransformed barley roots (10 times diluted), **12.** DHDPS protein c = 0.002 mg/mL, **13.** negative control proteins from bacteria (without *dapA* gene).

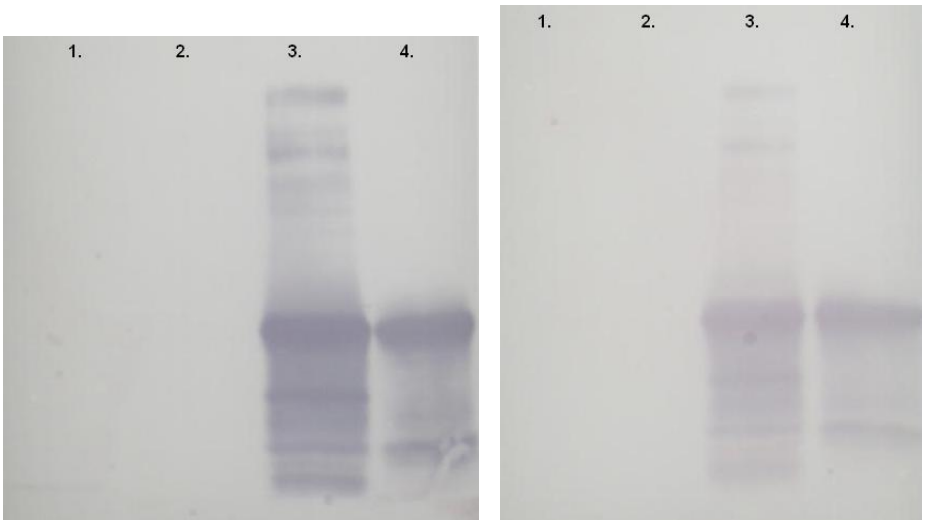


Figure 19 Membranes from WB. The purified primary antibodies in dilution 1/100 (left) and 1/1000 (right) were used. **1.** marker 120 kDa, **2.** protein extract from untransformed plants, **3.** DHDPS protein (before dialysis), **4.** DHDPS protein (after dialysis).

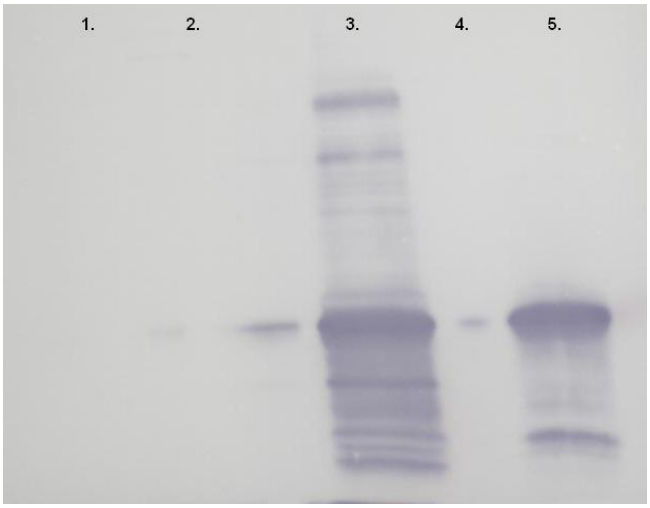


Figure 20 Membrane from WB. The non-purified primary antibodies in dilution 1/1000 was used. **1.** marker 120 kDa, **2.** protein extract from untransformed plants, **3.** DHDPS protein (before dialysis), **4.** -, **5.** DHDPS protein (after dialysis).

4.5.3 Transformation of barley

Together 150 embryos were transformed; 75 with construct *pBract 214::mdapA* and other 75 with construct *pBract214::sTPdapA* (figures 21, 22 and 23). The construct pBract214: mdapA contained *E.coli* DHDPS gene that was engineered by site directed mutagenesis because of 3 point mutations in original *dapA* gene obtained from Italy. This construct did not contain signal sequence thus expressed DHDPS protein should stay in cytoplasm. The construct *pBract214::sTPdapA* was synthesized (see Methods pg 36). 5' end of gene contained TP of *Hordeum vulgare* ribulose-1,5-bisphosphate carboxylase small subunit. The TP should direct DHDPS protein into chloroplast, where biosynthesis of lysine occurs. From 150 embryos, 330 plants regenerated and were analysed by WB and real-time PCR. Transformations of barley were done in cooperation with Dr. Ludmila Ohnoutková, Department of Cell Biology and Genetics, Faculty of Science, Palacky University and Dr. Wendy Harwood, Department of Crop Genetics, John Innes Centre, Norwich Research Park, United Kingdom.

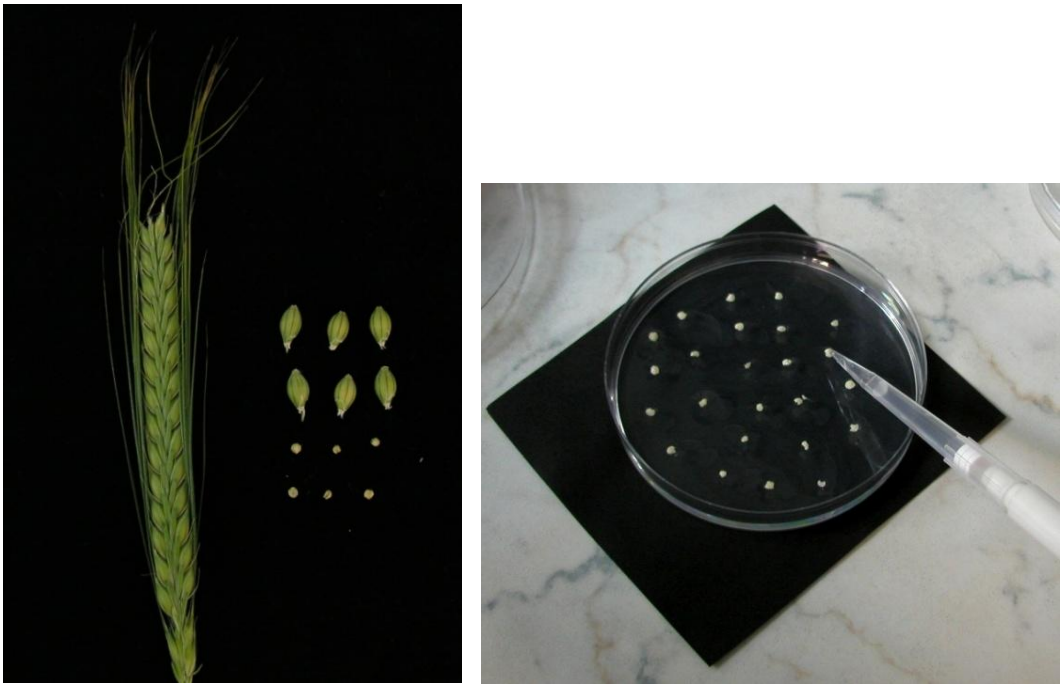


Figure 21 In the left figure are barley spike, immature seeds and immature isolated embryos. In the right figure, transformation of embryos with *Agrobacterium* is illustrated.

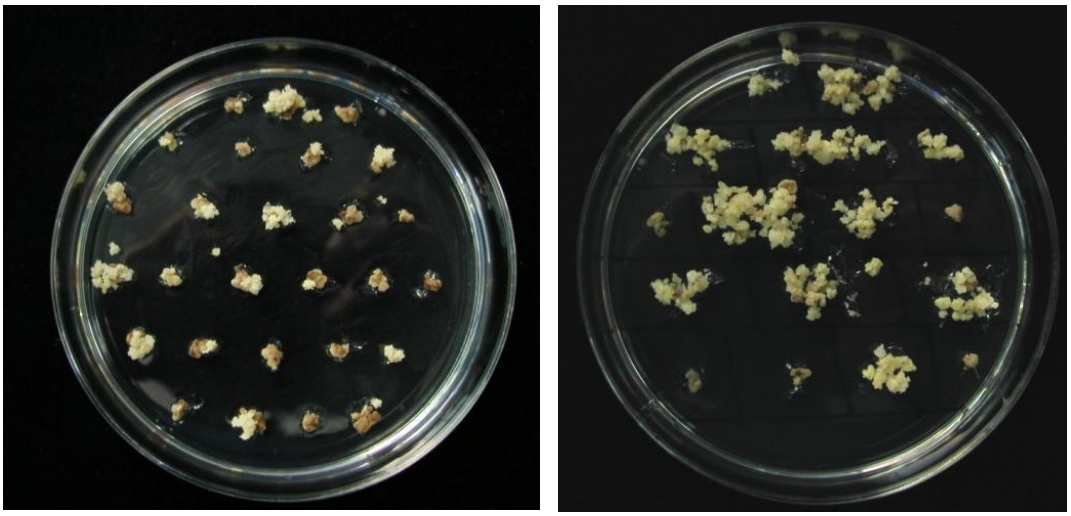


Figure 22 Selection of the positive embryos (left) and regeneration of the callus (left). Positive embryos generated the white callus.

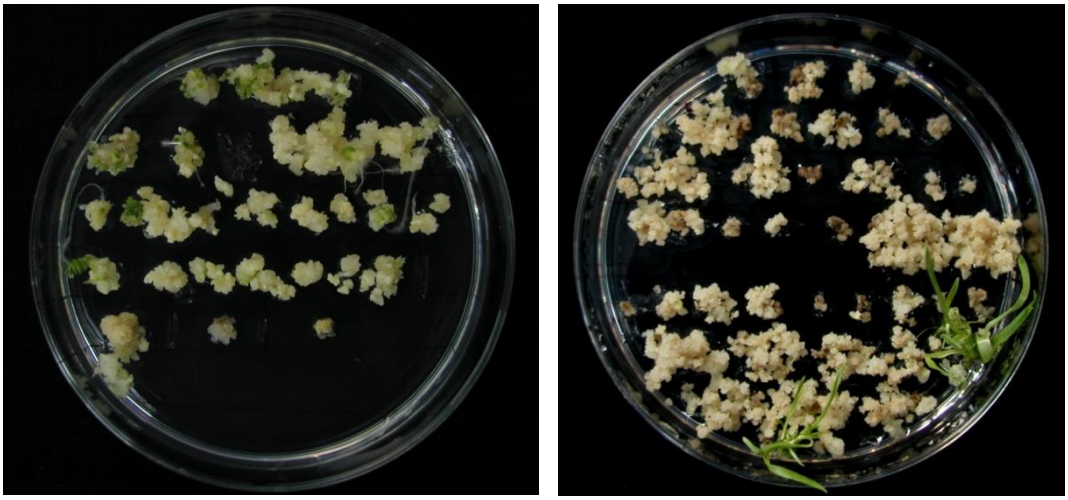


Figure 23 Regeneration of the transgenic plants. In the left figure generation of green shoots are shown and in the right figure, there are the first regenerated plantlets.

4.5.4 Analysis of transgenic barley

In order to detect the expression of DHDPS protein, the transgenic barley was analyzed by real-time PCR and WB. The results from real-time PCR and WB are summarized in table 4. In 14 analyzed plants transformed with construct *mdapA*, the expression of hygromycin gene was confirmed in all of them and expression of *dapA* gene was confirmed in 10 plants (table 5). In 34 analyzed plants transformed with construct *sTPdapA*, the expression of hygromycin gene was confirmed in 31 plants and expression of *dapA gene* was confirmed in 29 plants (table 6). The expression of *dapA* gene varied from 24 to 47009 fold in comparison to background expression of control

plant (unspecific amplification in non-transformed plants). As a reference gene, the expression of gene for elongation factor 1 was followed in all analyzed plants and relative changes of expression were calculated via delta delta Ct method by StepOnePlus Expression Software. The plants were then subjected to WB analysis. From 14 analyzed plants, transformed with construct *mdapA*, two were positive on DHDPS protein and 18 out of 34 plants transformed with construct *sTPdapA* were positive as well (figures 24 and 25). The antibodies from both rabbits were tested and reacted on DHDPS proteins with the same specificity rate.

Table 4 Summary of results from real-time PCR and WB analysis

Number of analysed plants with construct *mdapA*	Positive on *dapA* gene	%	Positive on hygromycin	%	Positive on WB	%
14	10	71	14	100	2	14
Number of analysed plants with construct *sTPdapA*						
34	29	85	31	91	18	53

Table 5 Summary of results from real-time PCR, construct *mdapA*

Number of sample construct *mdapA*	Expression *dapA*[1]	Expression hygromycin[1]	WB
57	6818 P	6887 P	N
8	10945 P	1636 P	N
16	536 P	442 P	N
21	1671 P	987 P	P
26	8837 P	4150 P	N
28	2295 P	2237 P	N
29	754 P	718 P	N
34	47009 P	11212 P	N
36	7036 P	1637 P	N
50	1164 P	1478 P	N
99	N	10P	N
98	N	88 P	P
24	N	16 P	N
20	N	32 P	N

[1] Levels of expression of *dapA* gene and hygromycin were normalized to the level of background unspecific expression in leaves of control plants, that was given the value 1
P - positive
N - negative

Table 6 Summary of results from real-time PCR, construct *sTPdapA*

Number of sample construct *sTPdapA*	Expression *dapA*	Expression hygromycin	WB
1	1864 P	11201 P	N
2	978 P	2631 P	P
3	1242 P	3789 P	P
5	1915 P	12195 P	P
7	1288 P	5486 P	P
57	N	N	N
9	109 P	1448 P	P
90	96 P	3767 P	P
59	N	4P	N
65	N	32 P	N
61	N	N	N
60	N	N	N
5	71 P	2009 P	N
39	52 P	2883 P	P
7	76 P	835 P	P
6	54 P	2317 P	P
47	50 P	36 P	P
45	37 P	44 P	P
55	29P	28 P	P
43	24 P	53 P	N
38	51 P	112 P	P
91	121 P	59 P	N
42	207 P	143 P	N
67	317 P	556 P	N
41	579 P	343 P	N
46	658 P	138 P	N
48	37377 P	2414 P	P
66	8742 P	3457203 P	P
68	3246 P	852 P	P
69	598 P	144 P	P
71	1181 P	281 P	N
79	7630 P	1303 P	N
81	523 P	1094 P	N
102	1460 P	634 P	N

[1] Levels of expression of *dapA* gene and hygromycin were normalized to the level of background unspecific expression in leaves of control plants, that was given the value 1

P - positive

N - negative

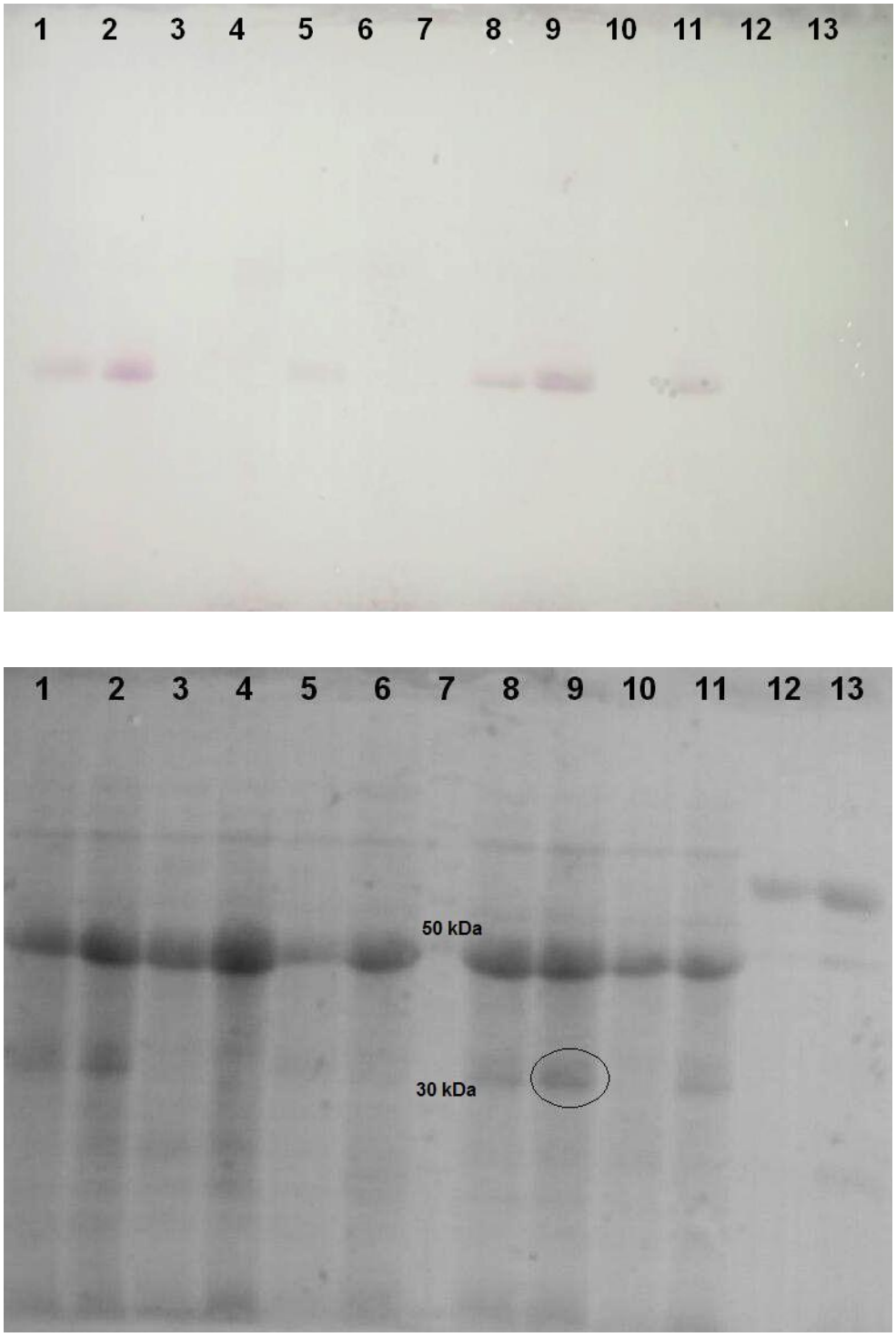


Figure 24 Results from WB. Primary antibody was from rabbit A, dilution 1/1000, this antibody was purified (described above). The upper figure is membrane before protein staining and figure below is after protein staining. Samples: **1.** sTPdapA 4, **2.** sTPdapA 3, **3.** negative control 1, **4.** mdapA 26, **5.** sTPdapA 6, **6.** negative control 2, **7.** marker 120 kDa, **8.** sTPdapA 2, **9.** sTPdapA 5, **10.** mdapA 36, **11.** mdapA 21, **12.** sTPdapA 2_extract from chloroplasts, **13.** negative control 1_extract from chloroplasts.

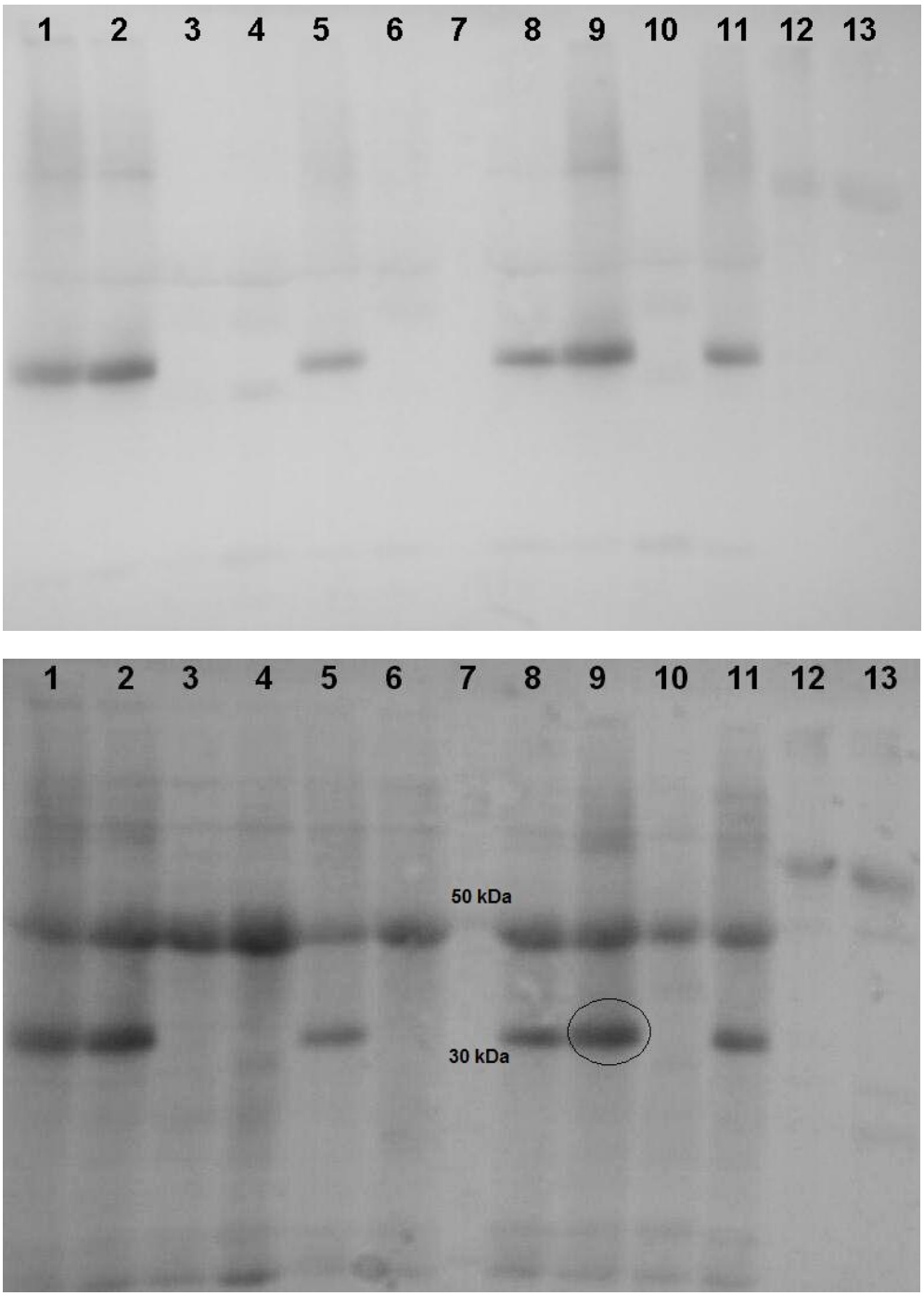


Figure 25 Results from WB. Primary antibody was from rabbit B, dilution 1/1000, this antibody was nonpurified. The upper figure is membrane before protein staining and figure below is after protein staining. Samples: **1.** sTPdapA 4, **2.** sTPdapA 3, **3.** negative control 1, **4.** mdapA 26, **5.** sTPdapA 6, **6.** negative control 2, **7.** marker 120 kDa, **8.** sTPdapA 2, **9.** sTPdapA 5, **10.** mdapA 36, **11.** mdapA 21, **12.** sTPdapA 2_extract from chloroplasts, **13.** negative control 1_extract from chloroplasts.

4.5.5 Analysis of chloroplasts from transgenic barley

Because of lysine biosynthesis is localized in chloroplasts, they were analyzed for presence of DHDPS protein by WB. Chloroplasts and their proteins were isolated from plants positive on expression of *dapA* gene. In this experiment, more sensitive luminescent detection was used (figures 26 and 27). Results did not prove presence of DHDPS protein in chloroplast of any of tested plants.

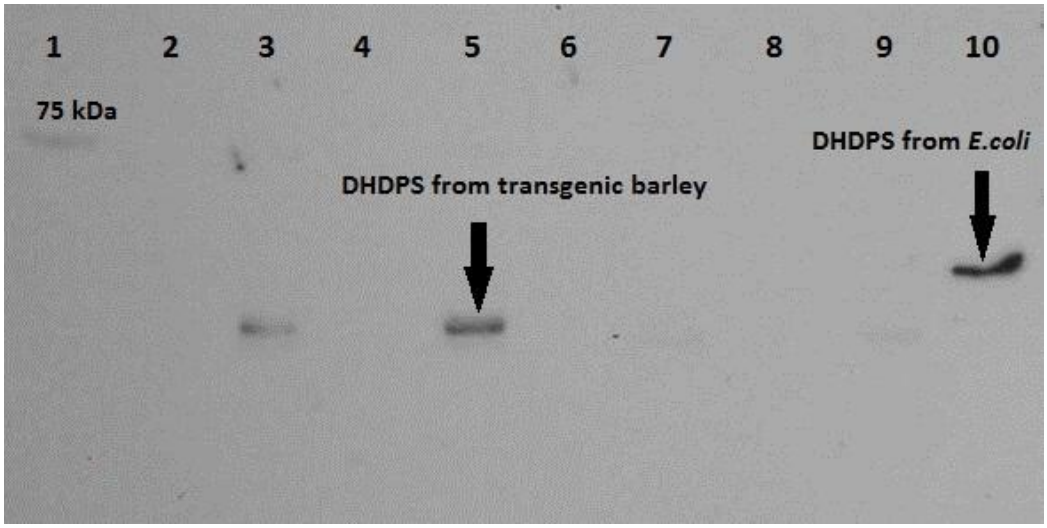


Figure 26 Membrane from WB. Samples were loaded: **1.** Precision Plus Protein standard, **2.** sTPdapA 68, chloroplasts (ch), **3.** sTPdapA 68, total proteins (P), **4.** sTPdapA 40 ch, **5.** sTPdapA 40 P, **6.** mdapA 57 ch, **7.** mdapA 57 P, **8.** negative control from barley ch, **9.** negative control from barley P, **10.** DHDPS from bacteria.

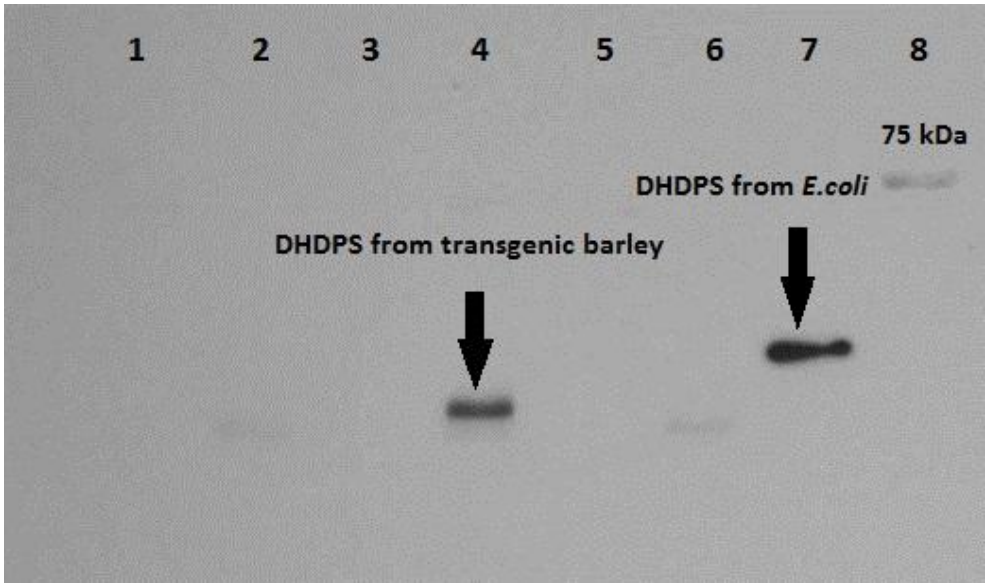


Figure 27 Membrane from WB. Samples were loaded: **1.** mdapA 21 ch, **2.** mdapA 21 P, **3.** sTPdapA 69 ch, **4.** sTPdapA 69 P, **5.** negative control from barley ch, **6.** negative control from barley P, **7.** DHDPS from bacteria, **8.** Precision Plus Protein standard.

4.5.6 Amino acids composition in leaves of transgenic barley

Together 26 plants were analyzed by HPLC in order to determine amino acids composition. According to results from real-time PCR were these plants divided into 4 group : with high *dapA* gene and hygromycin resistance gene expression, with medium *dapA* gene and hygromycin resistance gene expression, with low *dapA* gene and hygromycin resistance gene expression, and plants, which have different expression levels of hygromycin resistance gen and *dapA* gene. Results are summarized in table 7 and figure 28.

Table 7 Amino acids composition. Concentrations of amino acids are given in µmol/L.

	Asp	Thr	Ser	Glu	Pro	Gly
Negative control	742,25	349,79	631,06	565,92	974,71	578,14
High expression	1287,90	501,13	793,10	783,48	857,04	794,55
Medium expression	1092,15	439,55	538,75	634,59	1033,73	668,93
Low expression	1076,64	429,17	789,14	697,36	748,68	774,24
Different expression	1179,55	451,66	889,46	801,35	941,38	807,02
	Ala	**Cys**	**Val**	**Met**	**Ile**	**Leu**
Negative control	920,08	79,39	250,06	12,26	179,02	517,33
High expression	1393,32	70,87	331,59	81,73	269,28	754,05
Medium expression	1221,06	156,00	318,11	105,50	251,63	626,74
Low expression	1243,37	28,23	320,22	18,55	221,65	698,77
Different expression	1317,45	87,47	349,67	22,63	247,52	755,20
	Tyr	**Phe**	**His**		**Lys**	**Arg**
Negative control	178,76	356,87	293,11		334,98 ± 20	312,08
High expression	411,47	514,65	282,12		500,92 ± 40	484,85
Medium expression	311,45	472,64	327,60		461,64 ± 30	488,56
Low expression	302,49	478,58	271,59		427,25 ± 30	386,30
Different expression	299,68	527,04	355,77		489,97 ± 50	464,47

Each sample was measured three times and average value of lysine concentration ± standard deviation in all groups were calculated from average values of lysine concentrations of all samples in one group.

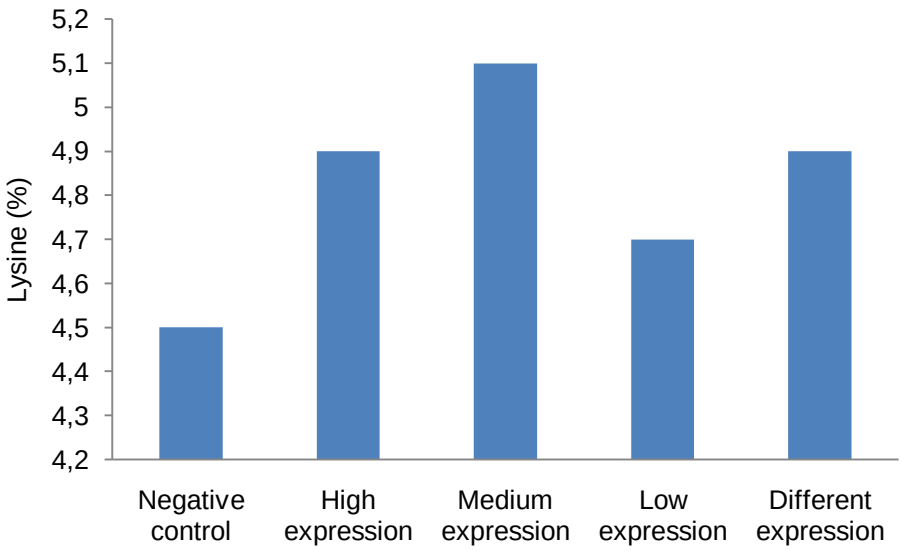


Figure 28 Comparison of lysine content between four groups and control. Amount of lysine is expressed as % of total amino acids that give the value 100 %.

5 DISCUSSION

The recombinant bacterial DHDPS protein was successfully produced in *E.coli* and purified by one step metal/chelate affinity chromatography. After high scale expression, we obtained enough protein to carry out other experiments (together 12.7 mg of pure DHDPS protein were obtained from 6.5 L of cultivation medium). The produced protein was used for immunization of rabbits and for purification of produced sera.

Two rabbits were used for production of antibodies against DHDPS protein. A sera from both rabbits were filtered and used for WB. In order to increase the specificity of antibodies, one serum was purified using recombinant protein linked to CNBr-activated matrix. During the process of immunization rabbit can produce antibody against plant proteins acquired from food, which could cross react with proteins from transgenic barley extracts. After purification, the antibodies were tested and compared with nonpurified antibodies. The reaction of purified antibodies was more specific to DHDPS protein than in nonpurified antibodies (figures 19 and 20, pg 48). The best results were obtained for dilution of antibodies 1/1000 and this dilution was used for next analyses of transgenic barley. The specificity of reaction toward DHDPS protein was comparable for sera from both immunized rabbits (figures 24 and 25, pgs 53 and 54). The molecular mass of DHDPS protein, produced in *E.coli* and in transgenic barley was different. The protein band produced in *E.coli* reacted with antibody had size around 37 kDa thus it is bigger compare to DHDPS protein, expressed in transgenic barley with molecular weight around 31 kDa. The size corresponds to theoretically calculated mass of bacterial DHDPS protein without plant transit peptide. The size differences can be explained that vector pET160 DEST, used for production of recombinant DHDPS protein contains except DHDPS coding sequence extra His-tag and Lumio sequence.

Transformation of barley was performed with *Agrobacterium*. From 150 transformed embryos, 330 plants regenerated and out of 48 analysed plants, 39 plants were positive for *dapA* gene expression. According to these results, applied transformation method was found to be optimal for production of bacterial protein in barley plants.

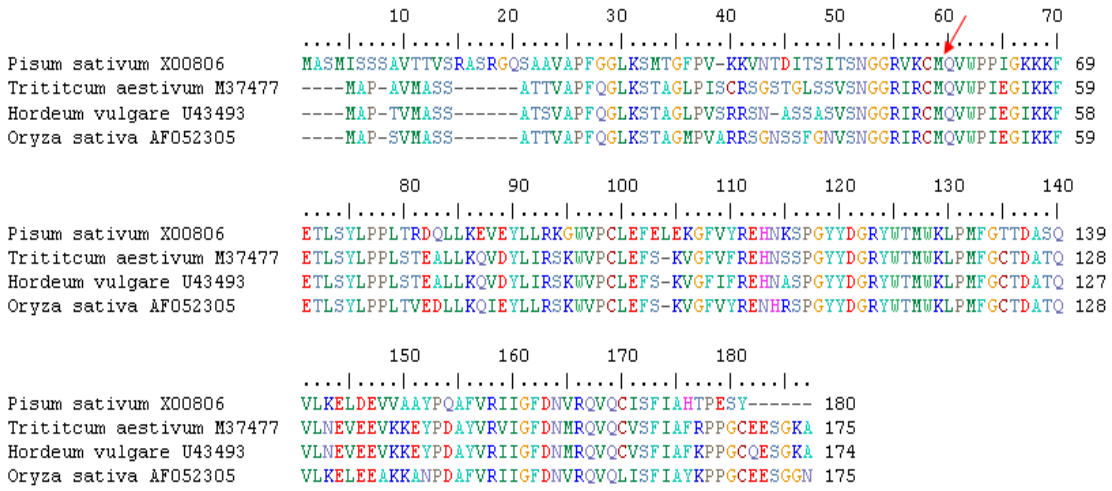
The transgenic plants were analyzed for the presence of transgene transcript by real-time PCR and bacterial DHDPS protein by WB. Only in 20 positive plants out of 39 with transgene expression, the bacterial DHDPS was detected by WB. This can be explained by the fact that not all produced mRNA is translated to functional protein.

There are various types of regulation in protein expression depend on transcript level. Level of active protein can be influenced also during translation of mRNA to amino acid sequence or during protein folding, which can lead to degradation of mRNA or protein. There were also observed differences in transcript level of hygromycin resistance gene and *dapA* gene. Not in all plants where hygromycin resistance gene was expressed, *dapA* gene detected. There can be differences in regulation of expression of these genes due to different promoters driving each of genes.

The analysis of chloroplasts should confirm the presence of DHDPS protein since transit peptide with plastid determination sequence has been used. In analyzed plants there were detected no band corresponding to DHDPS protein in chloroplast fractions. These results indicate that although the mature DHDPS protein is produced, it probably remains in cytoplasm. Transit peptide can be degraded early or there could be some disorders in expression. In previous studies (Karchi et al., 1994; Brinch-Pedersen, 1996; Zhu & Galili, 2003; Zhu & Galili, 2004) focused on increasing of lysine content of lysine in cereals, analogous TP from *Pissum sativum* was successfully used. The comparison of sequence homology for TP from barley and TP from pea is shown in figure 29. Because of pea belongs to family of dicotyledonous plants and barley is monocotyledonous plant we used TP from barley for this study to get the better results. The TP from barley have not been used yet and the sequence of TP could be cut from bad site that led to dysfunction of this TP.

In analyzed plants, lysine was increased up to 20 % in comparison with control plants. DHDPS protein was detected in cytoplasm, but not in chloroplast resulting probably in very low increasing of lysine content. Only few plants were analysed by Western blotting for presence of DHDPS protein in chloroplasts thus it is not clear if DHDPS protein is transported into the chloroplasts or its trafficking is limited by some unknown factor. There could be other regulation mechanism in lysine biosynthesis that causes only slight increase in lysine content. In the study of Pedersen et al. 1996, they increased lysine content in barley overexpressing mutated *E.coli* DHDPS and aspartate kinase (part of lysine biosynthetic pathway). As a result, leaves of primary transformants (T_0) exhibited 14-fold increase of free lysine and 8-fold increase in free methionine. These results suggested the role of aspartate kinase in regulation of lysine biosynthetic pathway. On the other hand, lysine catabolism can also be evolved in the regulation of lysine content when biosynthesis is enhanced. The role of lysine catabolism was investigated in study of Zhu & Galili 2003. They created the transgenic line of *Arabidopsis thaliana* that expressed *E.coli* DHDPS and crossed it with *Arabidopsis thaliana* knockout mutant in lysine α-ketoglutarate reductase (take part in

lysine catabolism). Transgenic plants expressing the bacterial DHDPS or with the knockout gene of lysine α-ketoglutarate reductase, contained 12-fold or 5-fold higher levels of seed free lysine than wild type plants. The combination of these two traits led to 80-fold increase in seed free lysine. These results showed that lysine catabolism plays the major role in lysine accumulation in *Arabidopsis* seeds (Zhu & Galili, 2003). In the future, approaches focused also on enzyme aspartate kinase and lysine α-ketoglutarate reductase can lead to significant improvement of lysine content in cereals.



Seq->	Pisum sativum	Trititcum aestivum	Hordeum vulgare	Oryza sativa
Pisum sativum	ID	0,355	0,355	0,338
Trititcum aestivum	0,355	ID	0,791	0,770
Hordeum vulgare	0,355	0,791	ID	0,791
Oryza sativa	0,338	0,770	0,791	ID

Figure 29 Comparison of sequences for TP of ribulose-1,5-bisphosphate carboxylase small subunit for pea (*Pisuv sativum*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*) and rice (*Oryza sativa*) (figure was created by BIOEDIT).

It could be also preferable if we did not have to introduce bacterial genes into plant genome. In the study od Seveneir et al. 2002, they isolated the potato gene encoding DHDPS and changed one amino acid residue to render the enzyme feedback-insensitive to lysine. Subsequent introduction of mutated potato gene back into potato led to dramatic increase of lysine content. The lysine content increased up to 15 % of the total amino acid level, whereas in untransformed plants this level is only 1 %. (Sévenier et al., 2002). This could be other way how to increase amount of lysine in cereals.

6 CONCLUSION

In the introduction of this work the importance of genetic improvement of cereals is discussed. In the theoretical part, fundamental informations about DHDPS are summarized. There are described different techniques used for cereals transformations including transformation by direct DNA transfer into protoplasts, transformation by microinjection, transformation by particle bombardment and *Agrobacterium*-mediated transformation. Some practical applications of these techniques are also described for diverse cereals especially barley.

In the practical part of this work, there is described applied method for barley transformation as well as other molecular-biology methods including Western blotting, reverse transcription, real-time PCR, affinity chromatography and MALDI TOF MS. There are summarized obtained results including production of recombinant DHDPS in bacterial host, preparation and purification of antibodies against DHDPS in rabbits, transformation of barley by bacterial *dapA* gene and analysis of transgenic barley plants by real-time PCR and Western blotting.

In this work I accomplished all required tasks and I achieved lot of theoretical knowledges about transformation methods as well as I learned new molecular-biology techniques as *Agrobacterium*-mediated transformation, real-time PCR and Western blotting.

7 ABBREVATIONS

A_{260}, A_{595}	absorbance at specified wavelength
APS	Ammonium persulphate $(NH_4)_2S_2O_8$
BAP	6–benzylaminopurine
Bar	bialaphos resistant gene
Bt	*Bacillus thuringiensis* cry1Ab gene
CBB	Coomassie Brilliant Blue R-250
CIM	callus induction medium
CNBr	Cyanogene bromide
dapA gene	gen encoding dihydrodipicolinate synthase
DHDPS	dihydrodipicolinate synthase
EC	embryonic calluses
E.coli	*Escherichia coli*
FAO	The Food and Agriculture Organization
FIIE	freshly isolated immature embryo
GL	Galena
GP	Golden Promise
GUS	β-glucuronidase
hGH	human growth hormone
HPLC	High Pressure Liquid Chromatography
hpt	hygromycin phosphotransferase
HQl	metal halide lamps
HT	Harrington
HTPA	(4S)-4-hydroxy-2,3,4,5-tetrahydro-(2S)-dipicolinic acid
IE	immature embryo
IPTG	isopropyl-β-D-thiogalactopyranoside
LB medium	Luria-Bertani medium
LTB	*E.coli* heat-labile toxin B subunit
MALDI	matrix assistant laser desorption ionisation
MS	mass spectrometry
MCS	meristem corn slices
NA	nicotinanamine
nos	nopaline synthetase
NPT II	neomycin phosphotransferase

PAT	phosphinothricin acetyltransferase
PC	pollen culture
PCIE	precultured immature embryo
PCR	polymeraze chain reaction
pmi	phosphomannose isomerase
PVDF	polyvinylidene membrane
(S)-ASA	(S)-aspartate-β-semialdehyde
SC	suspension culture
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Ti plasmid	tumour-inducing plasmid
TF	transformation frequency
TOF	time-of-flight mass analyser
TP	transit peptide
WB	Western blotting
2,4-D	2,4-dichlorophenoxyacetic acid

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