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Engineering of the aspartate family biosynthetic pathway in barley (*Hordeum vulgare* L.) by transformation with heterologous genes encoding feed-back-insensitive aspartate kinase and dihydrodipicolinate synthase

Henrik Brinch-Pedersen[1], Gad Galili[2], Søren Knudsen[1] and Preben B. Holm[1,*]
[1]*Carlsberg Research Laboratory, Gamle Carlsberg Vej 10, DK-2500 Copenhagen, Denmark (*author for correspondence);* [2]*Department of Plant Genetics, The Weizmann Institute of Science, Rehovot 76100, Israel*

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Abstract

In prokaryotes and plants the synthesis of the essential amino acids lysine and threonine is predominantly regulated by feed-back inhibition of aspartate kinase (AK) and dihydrodipicolinate synthase (DHPS). In order to modify the flux through the aspartate family pathway in barley and enhance the accumulation of the corresponding amino acids, we have generated transgenic barley plants that constitutively express mutant *Escherichia coli* genes encoding lysine feed-back insensitive forms of AK and DHPS. As a result, leaves of primary transformants (T_0) exhibited a 14-fold increase of free lysine and an 8-fold increase in free methionine. In mature seeds of the DHPS transgenics, there was a 2-fold increase in free lysine, arginine and asparagine and a 50% reduction in free proline, while no changes were observed in the seeds of the two AK transgenic lines analysed. When compared to that of control seeds, no differences were observed in the composition of total amino acids. The introduced genes were inherited in the T_1 generation where enzymic activities revealed a 2.3-fold increase of AK activity and a 4.0–9.5-fold increase for DHPS. T_1 seeds of DHPS transformants showed the same changes in free amino acids as observed in T_0 seeds. It is concluded that the aspartate family pathway may be genetically engineered by the introduction of genes coding for feed-back-insensitive enzymes, preferentially giving elevated levels of lysine and methionine.

Introduction

Aspartate is the direct precursor for the synthesis of three amino acids essential for non-ruminant animals, i.e. lysine, threonine, and methionine [9] as illustrated in Fig. 1. The aspartate family pathway has during the last few years received increasing interest in plant molecular biology and biotechnology since engineering of the pathway offers a means of improving the nutritional quality. The pathway is of particular importance during seed formation since asparagine, the direct precursor for aspartate, is the major source of nitrogen for the developing seed [21].

In higher plants and in many bacterial species, including *Escherichia coli*, aspartate kinase (AK) and dihydrodipicolinate synthase (DHPS) are the major regulatory enzymes in the synthesis of lysine and threonine [9, 12]. Both are regulated by end product feed-back inhibition, with AK being sensitive to inhibition with lysine and/or threonine, while DHPS activity is inhibited by lysine only. At least some plant AK isozymes appear to contain both aspartate kinase and homoserine dehydrogenase (HSD) activities [48, 2, 46, 20, 32]. This is also the case in *E. coli* where isozymes AK-I and AK-II but not AK-III are bifunctional [12].

Recent studies have documented that the content of free lysine and threonine can be increased drastically by generating transgenic plants expressing feed-back-insensitive bacterial AK and DHPS enzymes [19, 27, 15]. For example, constitutive expression of a mutated *E. coli lysC* gene and the *E. coli dapA* gene, encoding feed-back-insensitive AK-III and DHPS, respectively, resulted in 14- and 15-fold increases in free threonine and lysine in the leaves of tobacco transgenics.

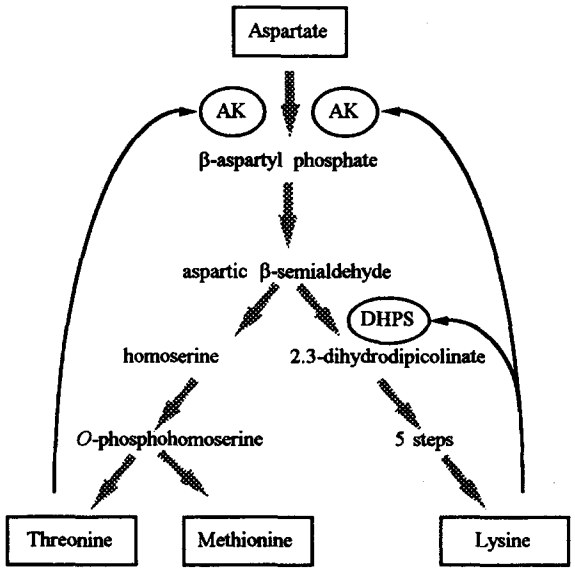

Figure 1. The aspartate-family biosynthetic pathway, where the curved arrows illustrate feed-back inhibition by end-product amino acids. AK, aspartate kinase; DHPS, dihydrodipicolinate synthase.

Expression of a feed-back-insensitive maize DHPS, caused by a single amino acid substitution, resulted in a four times elevation of free lysine in transgenic maize cell suspension cultures [4, 43]. Seed-specific expression of the mutant AK gene gave in tobacco a 7-fold increase in the free threonine content and a 3-fold increase in methionine [25, 19]. Dramatic changes have been reported for transgenic canola and soybean using a mutant *lysC* gene from *E. coli* and the *dapA* gene from *Corynebacterium* [15]. Canola seeds expressing the feed-back-insensitive *Corynebacterium* DHPS contained more than 100 times more free lysine than control seeds and the total lysine content was doubled. In soybean, co-expression of both genes resulted in a several hundred-fold increase in free lysine and an up to 5-fold increase in total lysine.

Seeds of barley are, as is the case for most cereals, especially deficient in the essential amino acids lysine and threonine, accounting for their poor nutritional quality for non-ruminant animals [8]. For this reason, the aspartate family pathway of barley has been intensively studied. Three AK isoenzymes have been identified of which AK-I is sensitive to feed-back inhibition by threonine, while AK-II and AK-III are sensitive to lysine only [7, 38]. AK mutants have been identified that exhibit increased levels of free threonine and methionine due to a reduced feed-back sensitivity, but these have not proven useful for commercial purposes [6, 7]. Selection for spontaneous and induced mutations that cause an increase in lysine content by changing the abundance of the different classes of protein in the barley grain has been more successful. This approach, however, has met difficulties such as yield depression and pleiotropic effects and extensive breeding has been required to obtain lines with a satisfactory performance [33].

The recent development of a transformation technique for barley [45] provides the opportunity to engineer the barley aspartate family pathway directly as described above for tobacco, canola and soybean. It has been the aim of the present study to generate transgenic barley with bacterial genes encoding feed-back-insensitive, recombinant AK and DHPS enzymes to evaluate their effect on the barley aspartate family pathway.

Materials and methods

Plasmids

Plasmids used for transformation of barley are schematically shown in Fig. 2. Construction of plasmids encoding feed-back-insensitive *E. coli* AK (Fig. 2A) and DHPS (Fig. 2B) has previously be described [40, 41]. Plasmid pDM803 (Fig. 2C) with the *uidA* gene encoding β-glucuronidase (GUS) and the *bar* gene for phosphinothricin acetyl transferase (PAT) was kindly provided by Dr David McElroy, Plant Gene Expression Center, Albany, NY.

Transformation of barley

Developing barley seeds of cv. Golden Promise were sterilized in 3.5% sodium hypochlorite for 10 min and rinsed in sterile water before embryos (1.0–1.5 mm) were dissected out under aseptic conditions. These were bisected longitudinally through the root and shoot meristems and placed with the sutellum side up on a modified Murashige and Skoog medium (MS) [34] supplemented with 2.5 mg/l Dicamba and 3.5 g/l Phytagel as previously described [45].

After 24 h in the dark at 25 °C, clusters of about 70 half-embryos were bombarded in a DuPont PDS 1000 Helium Biolistic Delivery system (BioRad) using 1100-psi rupture discs and 1 μm gold particles coated with plasmid DNA as detailed by the supplier.

One day after bombardment, the half-embryos were placed with the bombarded side of the scutellum in

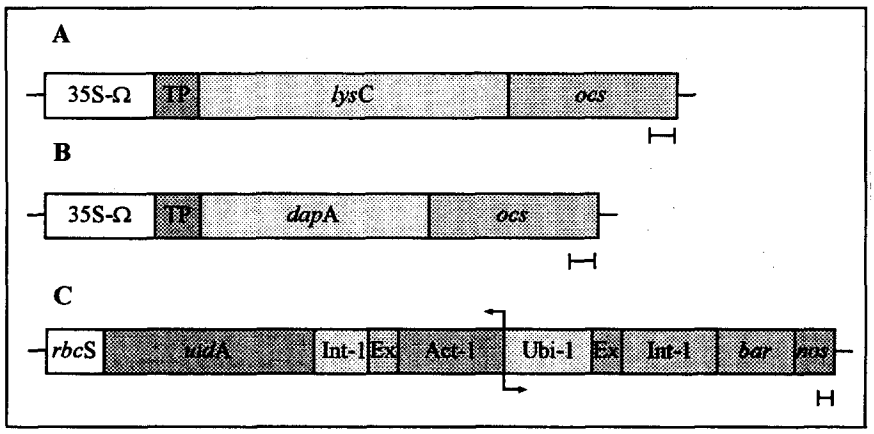

Figure 2. Expression casettes containing the gene for AK (A), DHPS (B). pDM803 directs expression of GUS and PAT (C). Bar = 100 bp. 35S Ω, the 35S promoter of the CaMV and leader sequence (Ω) from the tobacco mosaic virus mRNA [23, 18]; TP, the sequence coding for the chloroplast targeting sequence of the pea small subunit of ribulose 1,5-bisphosphate carboxylase (*rbcS*) [16]; *lysC*, the gene of *E. coli* encoding a desensitized AK-III [5]; *dapA*, the *E. coli* gene encoding DHPS [37]; *ocs*, the polyadenylation signal of the octopine synthase gene of *Agrobacterium tumefaciens* [22]; *Act*-1, the rice actin promoter, exon (Ex) and intron 1 (Int-1) [30]; *uidA*, the *E. coli* gene encoding GUS; *rbcS*, the transcription terminator and polyadenylation signal of *rbcS* [13]; *Ubi*-1, the maize ubiquitin promoter, exon (Ex) and intron 1 (Int-1) [11]; *bar*, the gene of *Streptomyces hygroscopicus* encoding PAT [44]; *nos*, terminator sequence of the *Agrobacterium tumefaciens* nopaline synthase gene [3].

contact with MS medium supplemented with dicamba and 5 mg/l bialaphos for selection of transformed cells. After 2 weeks, the callusing embryos were divided into 2–3 fragments and subjected to another 3–4 rounds of selection until rapidly proliferating cell lines were obtained. All structures developing from one half-embryo were regarded as the same cell line. Those cell lines resistant to bialaphos were subjected to histochemical analysis using the GUS assay [24].

Embryogenic, GUS-positive cell lines were then transferred to MS medium supplemented with 1 mg/l of bialaphos and 1 mg/l of benzyl amino purine for regeneration of plantlets. These were transferred to MS medium supplemented with 1 mg/l bialaphos, incubated for about 4 weeks, then planted in soil and cultured in a greenhouse (15 °C/10 °C, 16 h light period).

Embryogenic callus from bialaphos-resistant GUS-negative cell lines was handled in the same way except that regeneration was on 5 mg/l bialaphos.

Assays for enzyme activities

Assays for enzymic activities utilized leaf material from transgenic plants grown for 8 weeks in the greenhouse. Crude extracts of leaf proteins were prepared and assayed for AK and DHPS activities as described previously [40, 41].

Free and total amino acids in leaves and seeds of transgenic plants

The top leaf of plants grown for 6 weeks in the greenhouse, as well as mature seeds, were frozen in liquid nitrogen and free amino acids extracted as described by Shaul and Galili [41]. The total leaf amino acids were extracted by suspending 200 mg of homogenized leaf material in 1.5 ml of water. After 10 min of centrifugation (25 000 × *g*), the supernatant was removed, then lyophilized and dissolved in 200 ml 6 M HCl. The solution was hydrolysed under vacuum for 24 h (110°C), dried and dissolved in 50 mM sodium citrate buffer (pH 2.2). The composition of amino acids in the leaf and seed extracts was determined using an Alpha Plus (Pharmacia) amino acid analyser. Analyses were performed as duplicate samples and comprised 13 amino acids, i.e. alanine, arginine, asparagine, glycine, glutamine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tyrosine and valine. Plants originating from different lines showed some variation in the absolute amounts of the different amino acids. To facilitate comparisons and to eliminate potential sources of error such as different extraction yields, the amino acid compositions are expressed as percent of total amino acids. The total amino acid composition of the seeds was determined as described previously by Eggum *et al.* [14].

Analysis of plants using the polymerase chain reaction (PCR)

Genomic DNA, used as template for standard PCR analyses, was isolated from leaf tissue of putative transgenic plants using the procedure of Murray and Thompson [35]. The presence of *bar* was determined by amplifying a 429 bp fragment (the upper-strand primer, 5′-GCAGGAACCGCAGGAGTGGA-3′ and the lower-strand primer, 5′-ATCTCGGTGACGGGCAGGAC-3′, corresponded to bases 150–169 and 559–578 of the *bar* gene [47]). Analyses for the presence of the AK and DHPS expression cassettes utilized the same upper-strand primer, 5′-GGAGCATCGTGGAAAAAGA-3′, spanning bases 7283–7302 of the cauliflower mosaic virus (CaMV) 35S promoter [17]. The lower-strand primers, 5′-CGGCGGATTTTCAGTTTTAT-3′ and 5′GCTCAGCAGAACAAAATCATCT-3′ corresponded to bases 894–915 and 805–826 of the genes for AK and DHPS, respectively [10, 37]. The amplified fragments were 1320 and 974 bp long.

Results

Generation of transgenic plants

During the first of the biweekly selection periods, microprojectile-bombarded embryos responded primarily with undifferentiated growth. Thereafter some of the calli started to die or were arrested in further development while a few of the callus pieces continued their development. The following selection period provided the first well-growing, bialaphos-resistant embryogenic cell lines.

Bombardment of 690 embryos yielded 35 independent transgenic cell lines (Table 1), of which 63% produced active GUS enzyme. Eighteen percent of all cell lines were non-regenerable, while about 50% of the cell lines gave rise to albino plantlets. PCR analyses revealed that out of 13 cell lines regenerating green plants, 3 and 8 were co-transformed with the gene encoding AK and DHPS, respectively. Plants from one line transformed with the gene for AK and plants from two lines containing the gene for DHPS showed altered phenotypes with abnormal leaf morphology and sterility. Both abnormal DHPS-containing lines were excluded from further analyses.

Inheritance and expression of AK and DHPS in secondary transformants (T_1)

In order to overcome the dormancy of freshly harvested seeds, embryos were dissected from 77 mature kernels of primary transformants (T_0) and germinated on regeneration medium. The embryos were isolated from 2 and 6 cell lines transformed with the AK and DHPS gene, respectively. Although the transgenic plants eared later, they were phenotypically indistinguishable from wild-type plants. The inheritance and segregation of the introduced genes for AK and DHPS were tested by PCR for all 77 T_1 plants. Table 2 shows that lines TA1, TA3, TD1, TD2, and TD6 segregated close to a 3:1 ratio, indicating integration of the introduced trait at a single locus. For lines TD3, TD4 and TD5, two or more loci containing the transgene might be present.

Heterologous expression of AK and DHPS was analysed in leaf extracts derived from all of the T_1 plants. In line TA1 there was a 1.6–2.3-fold enhanced AK activity, while no activity increase was found in line TA3 (Table 2). As illustrated in Figure 3A, the increase in activity in individual plants from this line reflected the presence or absence of the transgene. For DHPS, there was an maximum increase in activity of 3.0–9.5-fold (Table 2). Also here a perfect correlation was observed between increased enzyme activity and the presence of the heterologous gene (Fig. 3B). It remains to be determined though if the differences in AK and DHPS activity in the segregating T_1 transgenics can be attributed to a dosage effect resulting from homozygozity for one or more loci containing the chimeric constructs or it results from an inherent variation between plants from the same line.

Amino acid composition in T_0 and T_1 plants

The leaves of 3 and 6 T_0 plants from independent cell lines transformed with the gene for AK and DHPS respectively, were analyzed for their free amino acids composition. Compared to wild-type seedlings at the same developmental stage, there were the same changes in free amino acid composition in plants expressing AK or DHPS (Table 3). The content of lysine and methionine was elevated 9–16-fold, and 6–8-fold respectively. Minor increases were observed for arginine, isoleucine, leucine and valine, while the glutamine content was half of that observed in wild-type leaves. Student's t-test showed, however, that only the lysine increase was significant at the 5% level.

Table 1. Summary of bombardment experiments.

Experiment No.	Number of embryos bombarded	Plasmids[a]	Number of GUS-positive cell lines[b]	Number of GUS-negative cell lines	Number of cell lines giving green plants
1	75	A + B	7	3	3
2	75	A + C	6	1	5
3	90	A + B	1	1	1
4	90	A + C	2	4	2
5	180	A + B	4	1	0
6	180	A + C	2	3	2
Total	690		22	13	13

[a] A represents pDM803; B and C correspond to AK and DHPS expression plasmids, respectively.
[b] GUS-positive as determined by staining.

Table 2. Segregation of the introduced heterologous genes as determined by PCR and enzyme activity increase for AK and DHPS in T_1 plants.

Cell line[a]	T_1[b] segregation, PCR positive: negative	Fold enzyme activity increase[c]
TA1	4:5:1	1.6–2.3
TA3	3.5:1	0
TD1	2.7:1	2.0–6.0
TD2	2.7:1	2.0–4.0
TD3	9.0:1	5.9–9.5
TD4	8.0:1	3.0–4.0
TD5	7:0:1	2.0–3.0
TD6	3.5:1	2.5–4.0

[a] Cell lines designated TA are transformed with the gene for AK, whereas cell lines designated TD are transformed with the DHPS encoding gene.
[b] Between 8 and 12 plants were anlysed per cell line by PCR.
[c] AK and DHPS activities (mean of at least two measurements) were normalized to the activity in leaves of control plants (mean of four measurements) that was given the value 1 (corresponding for AK to 2.19 ± 0.01 units per mg protein, for DHPS to 0.057 ± 0.012 units per mg protein).

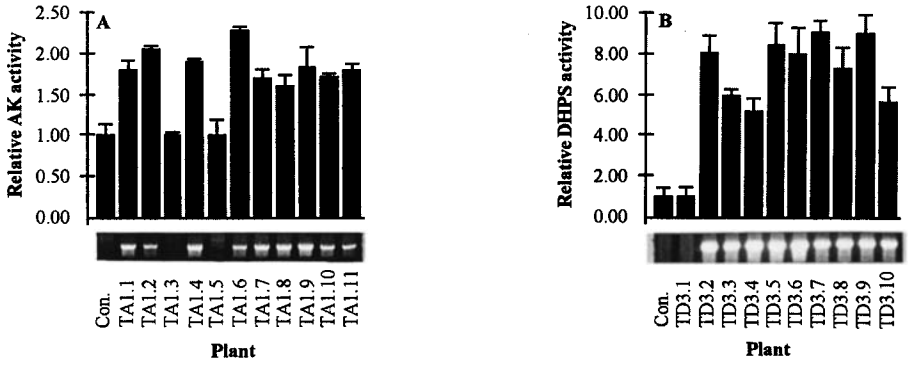

Figure 3. Relative levels of AK (A) and DHPS (B) enzyme activities in leaves from segregating T_1 plants. Plants with no PCR fragment detected, exhibit no alteration in enzyme activity. The level of enzyme activity (mean of at least two measurements) is plotted relative to the average level of enzyme activity in leaves of four control plants, which is given the value of 1. Con., control plants; TD3.1–TD3.10, T_1 plants representing offspring of plant TD3; TA1.1–TA1.11, T_1 plants representing offspring of plant TA1. Bars on top of each histogram represent the standard error.

Table 3. Relative increase in free amino acids in leaves of T_0 transgenic barley compared to wild type.

Plant[a,b]	Fold increase						
	Arg	Glu	Ile	Leu	Lys	Met	Val
TA1	3	−0.5	2	4	14*	8	2
TA2	2	−0.5	3	5	11*	6	3
TA3	3	−0.5	2	3	9	6	2
TD1	3	−0.5	3	3	16*	6	3
TD2	2	−0.5	2	4	12*	6	2
TD3	2	−0.5	3	5	13*	8	2
TD4	2	−0.5	2	3	9	6	2
TD5	2	−0.5	2	2	11*	7	3
TD6	3	−0.5	2	2	11	8	2

[a] TA1–TA3 were plants from independent cell lines transformed with the gene for AK; TD1–TD6 plants were from indepent cell lines transformed with the gene for DHPS.
[b] One plant was analyzed per cell line.
* Significant differences are given by $P < 0.05$, Student's t-test. The amino acid content was normalized to that found in the control plants which was given the value 1. Expressed as % (mol/mol) ± SE, the actual concentrations for the controls are: Arg, 0.51 ± 0.66; Glu, 41.59 ± 1.72; Ile, 0.73 ± 0.05; Lys, 0.23 ± 0.02; Met, 0.14 ± 0.03; Val, 1.71 ± 0.01.

Table 4. Relative changes in free amino acids in seeds of T_0 and T_1 plants transformed with the chimeric DHPS gene.

Plant[1]	Fold change			
	Arg	Asn	Lys	Pro
TD1	2	2	2	−0.5
TD2	3	2	2	−0.5
TD3	2	2	2	−0.5
TD3.2	2	2	2	−0.5
TD4	4	2	2	−0.5
TD4.8	2	2	2	−0.5

[1] TD1, TD2, TD3 and TD4 were plants from indepent cell lines transformed with the gene for DHPS. TD3.2 and TD4.8 were T_1 of TD3 and TD4. The amino acid content was normalized to that found in the control plants which was given the value 1. Expressed as % (mol/mol) ± SE, the actual concentrations for the controls are: Arg, 1.68 ± 0.02; Asn, 15.63 ± 0.71; Lys, 1.10 ± 0.02; Pro, 23.18 ± 1.67.

Although not statistically significant, the consistency with which the minor changes were obtained in different lines suggest that the deviations do reflect changes in the flux of molecules through the aspartate pathways.

The content of total amino acids, i.e. both free and protein-bound amino acids, was also determined in leaves of T_0 plants at the same developmental stage. Transgenic plants from 3 lines containing the heterologous *lysC* showed a 2-fold increase in methionine. Additionally, 2 of these lines (TA2 and TA3) revealed a 2-fold increase in arginine. T_0 plants representing 6 cell lines transformed with the gene encoding DHPS exhibited a 1.5–3-fold increase in methionine and a 5–8-fold increase in total arginine (data not shown).

Seeds of T_o plants from four lines containing the *dapA* gene were analysed for the composition of free amino acids (Table 4). The amounts of lysine, asparagine and arginine were doubled, while the proline content was only half of that observed in wild-type material. Seeds of plants from 2 cell lines containing the *lysC* gene were also analysed and no differences in amino acid composition were observed. Similarly, the total content of amino acids in seeds of lines TA1 and TA3 (transformed with the gene for AK) and TD5 and TD6 (containing the gene for DHPS) were similar to control plants (Table 5).

Seeds of two T_1 plants expressing bacterial DHPS were analyzed for the content of free amino acids (Table 4). The changes were similar to those described for the T_0 generation, i.e. they contained twice as much free lysine, asparagine and arginine, while the level of free proline was half of that found in wild-type seeds. Similar to T_0 plants, seeds of T_1 plants from the single

Table 5. Total T_0 seed amino acid composition (g/16 g N) in control, AK and DHPS transformants.

Amino acid	Control[a]	TA1[b]	TA3	TD5[c]	TD6
Alanine	3.14	3.09	3.17	3.45	3.17
Arginine	4.20	4.26	4.49	4.48	4.79
Aspartic acid	4.54	4.89	5.01	5.08	5.01
Cystine	1.76	1.63	1.70	1.70	1.70
Glutamic acid	25.53	25.30	24.95	23.31	24.44
Glycine	3.28	3.18	3.28	3.45	3.27
Histidine	2.02	1.99	2.08	2.03	2.08
Isoleucine	3.51	3.45	3.47	3.49	3.54
Leucine	6.33	6.11	6.15	6.14	6.15
Lysine	2.92	2.88	2.90	3.21	2.95
Methionine	1.39	1.34	1.40	1.50	1.41
Phenylalanine	5.63	5.63	5.53	5.39	5.48
Proline	13.06	12.79	12.69	11.72	12.42
Serine	3.86	3.83	3.83	3.83	3.73
Threonine	2.78	2.72	2.74	2.86	2.69
Tryptophan	1.12	1.11	1.09	1.11	1.10
Tyrosine	3.05	2.94	2.82	2.88	2.97
Valine	4.06	4.04	4.06	4.14	4.09
N, % of dry matter	3.51	3.97	3.73	3.64	4.36

[a] Control: seeds from plants transformed only with pDM803;
[b] TA 1 and TA3: seeds from two independent cell lines transformed with pDM803 and the gene for AK;
[c] TD5 and TD6: seeds from two cell lines transformed with pDM803 and the gene for DHPS.

line expressing the bacterial AK showed no changes in free amino acid composition.

Discussion

The present study confirms previous findings that particle bombardment of immature embryos followed by selection for expression of the *bar* gene is an efficient method for barley transformation [45]. About 5 transgenic cell lines were obtained per 100 embryos bombarded. Of these, 32% gave rise to green, transgenic plants. Co-transformation frequencies of unlinked genes were 84%, similar to the results of Wan and Lemaux [45].

The introduced genes encoding AK and DHPS were stably transmitted to the next generation. Although the number of progeny analyzed per line was too limited to establish precise segregation frequencies, the data suggest the presence of the transgene in one locus in some lines and possibly in two loci in other lines. Either of the heterologous genes expressed active enzyme in both the primary regenerants and in the T_1 plants, as apparent from enzyme assays (Table 2) and changes in amino acid composition (Table 4).

In contrast to tobacco plants expressing heterologous AK [41], no increase in the amount of threonine was measured in transgenic barley plants. Apparently, the increase in synthesis of threonine is rapidly compensated for by an accompanying increase in one or more of the reactions involved in the degradation or conversion of threonine. Threonine is cycled through the pathway for synthesis of isoleucine, valine and leucine, with the latter two amino acids also being synthesized from pyruvate in a parallel reaction. Since both pathways share several reactions that are catalyzed by enzymes possesing dual substrate specificities [29], it is likely that the observed 2–5-fold increase in isoleucine, valine and leucine (Table 3) may reflect an upregulation of the threonine catabolism. Accordingly, heterologous expression in barley of feed-back-insensitive *E. coli* AK affected the threonine as well as the lysine branch of the aspartate family pathway. This may also be the case in tobacco where expression of a feed-back insensitive AK besides increasing the threonine content also resulted in increased levels of isoleucine [41].

On the other hand Bright *et al.* [6, 7] have shown that barley seeds can accumulate threonine in lines expressing a mutated feed-back-insensitive form of AKII. This illustrates that other sources for genes coding for feed-back-insensitive AK may be used for increasing the threonine content of the barley seed.

In barley transformed with the gene for DHPS, changes in the composition of free amino acids in T_0 leaves were similar to those conferred by the bacterial AK enzyme. The 2–4-fold change in the asparagine content may reflect changes in the uptake of this amino acid imposed by an increased flux through the aspartate pathway. This may also influence the formation of the glutamate-derived amino acids, arginine and proline, since glutamate can be formed directly from aspartate by a transamination reaction with α-ketoglutarate.

The present results indicate that the levels of different amino acids related to the aspartate family pathway in barley may be modulated by introducing constitutively expressed genes coding for feed-back-insensitive bacterial AK and DHPS. The extent of such changes can be affected by tissue-specific expression of the corresponding genes. Thus, transgenic tobacco containing the bacterial gene for AK under the control of a seed specific promoter exhibited an increase in methionine in the seeds, but not in the leaves [25]. Co-expression in tobacco of AK and DHPS encoding genes resulted in the formation of excess lysine at the expense of threonine [42], indicating that DHPS enhances the flow of intermediates through the lysine branch of the aspartate family pathway. Overproduction of specific amino acids appears to enhance subsequent steps involved in the catabolism of these amino acids. For example, the absence of lysine overaccumulation in the mature seeds of transgenic tobacco plants expressing heterologous AK and DHPS, correlated with an increased activity of lysine ketoglutarate reductase, an enzyme that catabolizes lysine into saccharopine [26]. Transgenic canola and soybeans overproducing lysine also displayed elevated amounts of saccharopine and α-amino adipic acid, another intermediate in lysine catabolism [15]. Møller [36] showed by injection of ^{14}C-labelled lysine and other intermediates in lysine synthesis and catabolism into the endosperm of germinating barley that lysine was catabolized to saccharopine, 2-amino adipic acid and eventually to acetyl-CoA. Likewise, lysine-ketoglutarate reductase activity has been documented in developing maize endosperm. This activity probably accounts for the two-fold reduction in lysine content of the developing maize kernel when comparing the content of free lysine in the sap with that eventually incorporated into protein [1]. As suggested from the present results, deregulation of the flow through the aspartate family pathway may also influence the synthesis of amino acids, such as arginine and proline, by altering the flux through other interconnected pathways.

The changes in amino acid composition in the barley transgenics obtained in the present study was achieved by heterologous expression using the CaMV 35S promoter, which is a rather weak constitutive promoter in grasses [28, 31, 39, 49]. Accordingly, more drastic changes migth be expected if stronger constitutive or seed specific promoters are used for driving expression. Furthermore, it is apparent from the studies of tobacco and canola [25, 15] that homozygosity as well as the presence of additional copies of the bacterial genes confer higher expression levels. Changes in the amino acid composition may thus be modulated and optimized by classical breeding for plants possesing additional copies of the transgenes encoding AK and DHPS. As already illustrated for tobacco [25] and soybean [15], the lysine content of seeds can be increased dramatically in transgenics expressing both feed-back enzymes.

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