

## Additive effects of the feed-back insensitive bacterial aspartate kinase and the Brazil nut 2S albumin on the methionine content of transgenic narbon bean (*Vicia narbonensis* L.)

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### Abstract

So far two different strategies for engineering high methionine (Met) grain legumes were followed separately in several laboratories: a) The transfer of foreign genes encoding Met-rich proteins, and b) the engineering of Met biosynthesis pathways. In some cases a down regulation of the formation of endogenous sulfur-containing compounds was observed due to the expression of Met-rich foreign proteins. Since this might result from competition of the foreign protein with endogenous compounds for limited Met supply both strategies were combined in the present work. Double transformants of narbon bean (*Vicia narbonensis* L.) were generated which express seed-specifically the Met-rich Brazil nut 2S albumin (BNA) as well as a feed-back insensitive bacterial aspartate kinase (AK) known to stimulate Met biosynthesis in transgenic tobacco seeds. In order to produce double transformants a homozygous transgenic BNA line of narbon bean was either retransformed with the AK gene or crossed with an AK line. For the first time the influence of a deregulated AK on amino acids of the aspartate pathway was studied in seeds of a transgenic legume. Effects of expressing the foreign genes on inorganic sulphate, free and protein-bound Met and other amino acids of the aspartate pathway as well as on free sulphhydryl compounds of mature seeds were analysed. AK lines had 10 to 12 percent and the BNA line 80 percent increased Met in mature seeds. Double transformants showed additive but not synergistic effects of the expression of AK and BNA gene on seed Met. In their mature seeds protein-bound Met reached levels 2.0 to 2.4 times higher than in the wildtype. The Met level of best line corresponds approximately to the FAO standard for Met in a nutritionally balanced protein for human food or for feeding monogastric animals.

### Introduction

The nutritional value of seed protein for monogastric domestic animals is limited predominantly by its unbalanced essential amino acid composition. Methionine is the major limiting essential amino acid in grain legumes. Conventional breeding brought only limited progress in overcoming this nutritional limitation. Therefore, during the past ten years genetic engineering was employed to improve the amino acid composition of seeds. Mainly two strategies were indepen-

dently followed by several teams (Müntz et al. 1997, 1998): A) Engineering of the protein composition aiming at increased portions of Met-rich endogenous or foreign proteins, and B) engineering of amino acid biosynthesis pathways leading to increased amounts of total seed Met.

Following strategy A tobacco (Altenbach et al. 1989), canola (Altenbach et al. 1992), soybean (Townsend and Thomas 1994), lupin (Tabe et al. 1997) and narbon bean (Saalbach et al. 1994, 1995b; Pickardt et al. 1995) with strongly increased seed Met

contents have been engineered. In all cases genes encoding Met-rich 2S storage proteins were transferred to the target plant and there expressed under the control of seed-specific promoters from legume storage globulin genes. Almost doubling of seed Met was achieved in lupin and narbon bean whereas approximately 40 percent increase was reached in rape and soybean. In narbon bean containing the 2S Brazil nut albumin (BNA) (Sun et al. 1987; Altenbach et al. 1987), seed Met quantities reached approximately 80 percent of the FAO standard for nutritionally balanced food protein (Saalbach et al. 1995b, c) equal to wild type soybean which is the grain legume richest in seed Met. In lupin and narbon bean seed Met of different transgenic lines was directly correlated to the expression level of the foreign gene but not in soybean. In soybean (Townsend and Thomas 1994) and rice (Tabe et al. 2002) an underproportional Met increase indicated considerable depression in the formation of endogenous Met-containing compounds. Two questions emerged: 1) How can 100 percent FAO level be reached, and 2) how can limiting interactions with the formation of endogenous Met-containing compounds be overcome?

Increased level of seed Met was also reported for tobacco that had been engineered according to strategy B (Karchi et al. 1993). Tobacco plants had been transformed with the bacterial gene (*lysC*) encoding a feed-back regulation-insensitive aspartate kinase (AK). Gene expression was controlled by the seed-specific phaseolin promoter and the enzyme was targeted into the plastids of seed cells. Small but significant increases by 5 to 7 percent compared to the Met content of wild type tobacco seeds were reported for two transgenic lines. The increase resulted from raised soluble as well as globulin Met. No Met increase was found in barley and *Arabidopsis thaliana* transformed with the AK gene under control of the constitutive CaMV 35S-promoter (Brinch-Pedersen et al. 1996; Ben-Tzvi Tzschori et al. 1996) which is known for low expression activity in seeds, e.g. narbon bean (Saalbach et al. 1994, 1995b).

Here we report on experiments aimed at combining the effects of engineering the aspartate pathway of amino acid biosynthesis and of expressing the gene of the Met-rich BNA in the seeds of narbon bean double transformants. Homozygous AK and BNA gene transformants have statistically significant 10 to 12 and 80 percent, respectively, increases of seed Met. Double transformants were generated by retransformation of the homozygous BNA line B12 with the

AK gene or by crossing line AK18 with the BNA line B12. In both cases additive effects of both the AK enzyme and the BNA storage protein were achieved. The recently detected allergenicity of BNA prohibits its use in the engineering of agronomically important high Met grain legumes. *Vicia narbonensis* is closely related to *Vicia faba* but has no agronomic importance due to antinutritive compounds in their seeds. Nevertheless, our model experiments revealed a successful strategy for further increasing Met in the seeds of transgenic crops.

## Material and methods

### Plant material

Wild type seeds of *Vicia narbonensis* L. (narbon bean) were originally obtained from Dr G. Donn, Hoechst, Frankfurt/Main, Germany, and have been propagated at the experimental field of IPK Gatersleben. Homozygous transgenic line B12 of narbon bean with seeds containing 3.5% BNA in the total salt soluble protein was described previously (Pickardt et al. 1995; Saalbach et al. 1995b). Transgenic lines AK5, AK18, AK50, and AK54 with seeds containing the feed-back regulation-insensitive bacterial AK and used in the present research were propagated to homozygosity in the green houses of IPK Gatersleben.

Wild-type and transgenic plants were always simultaneously raised from seeds in pots with soil standing either in controlled growth chambers (12/12 hrs light/dark rhythm, 22°/18 °C day/night temperatures, 60 percent humidity), in green house compartments permitting control of temperature within certain limits or in normal green houses with supplementary illumination from September to May. Plants were dressed weekly with commercial NPK-fertilizer dissolved in water and supplemented with 2.5 g K<sub>2</sub>SO<sub>4</sub> per 10 l fertilizer solution. Whereas the sulphur dressing remained unchanged during plant growth NPK ratios were 16: 4: 8 before flowering and 8: 10: 8 after flowering. Mature seeds were harvested and immediately stored dry at 4 °C.

### Genes and vectors

The BNA gene (Altenbach et al. 1987) was under the control of the seed-specific legumin B4-promoter from *Vicia faba* L. (Bäumlein et al. 1991) as described by Pickardt et al. (1995). For generating AK

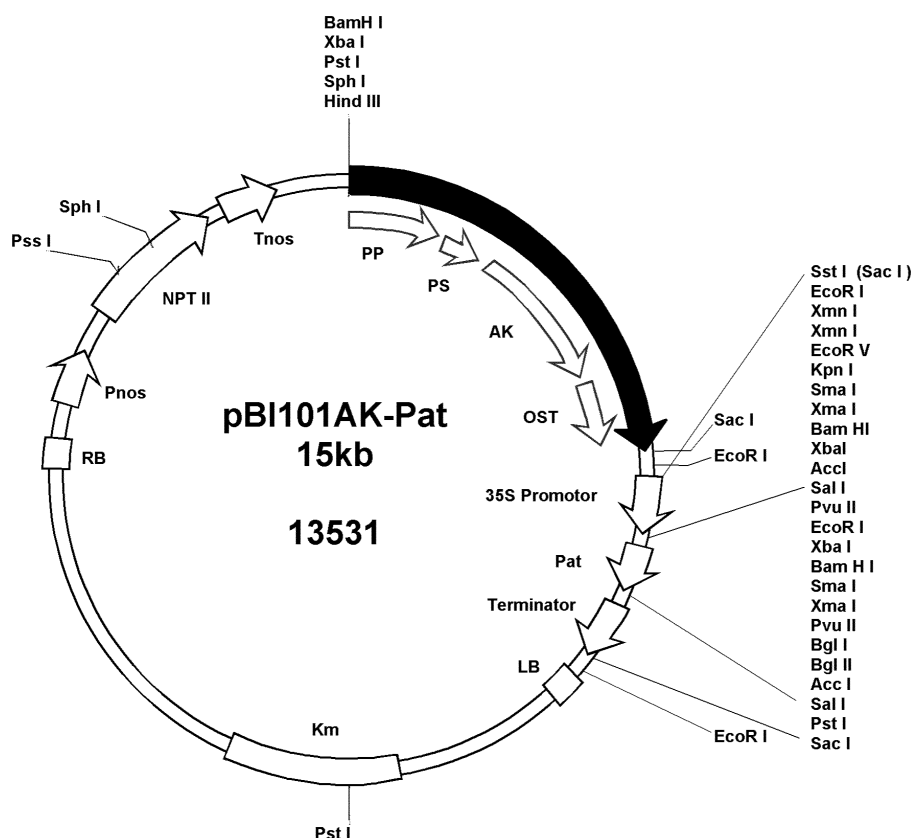


Figure 1. Physical map of the phosphinotricine resistance-conferring Ti-plasmid vector pBI101AK-PAT/NPTII harbouring a gene construct which encodes a plastid transitpeptide (PS) in front of a feed-back-insensitive bacterial aspartate kinase (AK) under control of the phaseolin promoter (PP) and octopine synthetase terminator (OST). Expression of the *pat* gene is directed by the constitutive CaMV 35S-promoter and terminator. The kanamycin-resistance conferring *nptII* gene is under control of the nos promoter (Pnos) and terminator (Tnos). RB and LB – right and left border of T-DNA. In addition different restriction and multicloning sites are indicated.

lines the gene construct of Karchi et al. (1993) was used which provides phaseolin promoter-mediated seed specific expression (Sengupta-Gopalan et al. 1985) of the AK gene and targeting of the enzyme into chloroplasts. Both, the BNA and the AK gene vectors confer kanamycin resistance as a selection marker. The *bar* gene (Hoechst, Frankfurt/Main, Germany) conferring phosphinotricine (PPT) resistance served as differential selection marker in our binary vector pBI101AK-Pat/NPTII (Figure 1) for retransformation of BNA lines with the AK gene. Constitutive gene expression which is mediated by the CaMV 35S-promoter permitted selection for PPT resistance already early during vegetative development of transgenic plants. Vector construction, e.g. plasmid isolation and DNA restriction, followed the methods described by Sambrook et al. (1989).

### Transformation

*Agrobacterium tumefaciens*-mediated transformation of *V. narbonensis* followed the protocol of Pickardt and Saalbach (2000). Double transformants were either generated by crossing line AK18 with the homozygous BNA line B12 of narbon bean or by retransformation of BNA line B12 with the AK gene. The vector for retransformation conferred phosphinotricine (PPT) resistance (see above) permitting differential selection in the background of the BNA line which expresses Km resistance. First selection was performed at the callus stage using medium with 15 mg PPT/l. PPT was omitted from the medium after embryogenesis took place since it inhibits further growth of the shoot. Epicotyl sections of young shoots were used for a) grafting onto epicotyls of wildtype seedlings in order to raise transgenic plants according to Pickardt et al. (1995), b) for recultiva-

tion in order to maintain the retransformant line, and c) for phosphinotricine acetyl transferase (pat) assays (see below). In addition, at later stages of growth PPT-resistant retransformants were selected using either leaf extracts for pat assay (see below) or by leaf painting.

#### *Single seed analysis for segregation*

The inheritance of both genes was followed by analysing single seeds for AK and BNA using enzyme activity test and SDS PAGE, respectively (see below). Extracts from small sections of cotyledons taken from 12 hrs imbibed seeds were used for AK activity screening in ELISA reader plates and for SDS PAGE analysis to detect BNA. The remaining part of the seed was germinated in order to raise the corresponding plant.

#### *Seed extraction*

##### *Extracts for AK determination*

Cotyledon sections were ground with a motor-driven rotating pistil in Eppendorf tubes at 4 °C. – Mature seeds were dehusked and embryo with cotyledons ground to flour using a ball mill. Extraction buffer according to Shaul and Galili (1992) was used (w/v = 1/10). For AK activity screening in ELISA plates, samples were only once extracted under shaking in Eppendorf tubes at 0 °C for 10 min. For quantitative AK activity analysis extraction was repeated twice and both extracts were pooled. Extracts were separated from sediments by 15 min centrifugation at  $23,000 \times g$  and 4 °C.

##### *Protein extraction*

Total salt-soluble proteins (albumins plus globulins) were extracted with phosphate-buffered saline (PBS = 0.1 M phosphate buffer, 0.5 M NaCl) at pH 7.0. For some purposes albumins and globulins were successively extracted from seed flour following the procedure of Lichtenfeld et al. (1979). All extractions were repeated 3 times under shaking in Eppendorf tubes at room temperature for 10 min each, and the extracts were pooled.

##### *Extraction of amino acids*

Amino acids were extracted according to Shaul and Galili (1992). The lyophilized extracts were used to quantify total soluble amino compounds and for amino acid analysis (see below).

##### *Extraction of soluble thiols*

Thiols were extracted with 60% hydrous ethanol which according to Enneking (1995) does not degrade  $\gamma$ -glutamyl-S-ethenyl-cysteine (GEC). Narbon bean seeds contain appreciable amounts of this sulphur-containing dipeptide (Enneking et al. 1998). It is degraded in 0.1 N HCl (Enneking 1995; Enneking et al. 1998) that usually is used to extract soluble sulphhydryl compounds (Lappartient and Touraine 1997). Neither chloroform-methanol-water extracts according to Shaul and Galili (1992) could be used since thiols are partially lost during lyophilisation. Seed flour was extracted once as described above for protein extraction.

##### *AK activity analysis*

For screening by micro assay in ELISA reader plates as well as for quantitation of AK activity the method of Shaul and Galili (1992) was followed with some modifications: a) Lys and Thr concentrations were doubled to suppress endogenous AK activity of narbon bean, but Asp concentration remained unchanged. b) DTT was used as a reducing agent instead of  $\beta$ -ME. c) Incubation took place under shaking at 37 °C overnight for subsequent AK activity screening in ELISA reader plates.

##### *Phosphinotricine acetyl transferase (pat) assay*

Screening for pat activity followed the procedure of Spencer et al. (1990) except that  $^{14}\text{C}$ -labelled PPT was used as substrate instead of  $^{14}\text{C}$ -labelled acetyl CoA in order to reduce background staining on autoradiographs.

##### *Protein quantitation*

Extract proteins were quantified according to Bradford (1976) using a reagent kit provided by Roth GmbH (Karlsruhe, Germany) with bovine serum albumin (BSA) as a standard.

##### *Electrophoresis*

SDS polyacrylamide electrophoresis (SDS page) according to Laemmli (1970) or according to Schägger and von Jagow (1987) was run in MiniproteanII or ProteanII apparatuses of BioRad (Hercules, CA, USA). The Schägger and Jagow system was preferred

to analyse extracts containing BNA since it better fractionates smaller polypeptides.

#### *Antibodies and immunoblot*

Antibodies raised against purified vicilin and legumin from *V. faba* (Saalbach et al. 1991, 1995a) were used for immunoblotting according to Towbin et al. (1979). Polyclonal antibodies raised against recombinant AK from *Escherichia coli* were kindly provided by Dr O. Shaul (Bar-Ilan University, Ramat Gan, Israel). Electrotransfer from gels to nitrocellulose membranes was performed using Minitransblot or Transblot cells from BioRad.

#### *Amino acid analysis*

Lyophilized soluble amino compounds were dissolved in 100 µl of double distilled water and appropriate aliquots used for amino compound quantitation with ninhydrin according to Mertz et al. (1974) with leucine as a standard.

The concentrations of Met and GEC in seed extracts were different by orders of magnitude. Therefore two variants of HPLC analysis (HPLC apparatus HP1100 from Hewlett Packard, Waldbronn, Germany) were applied to determine the amino acids of the extracts. Always lyophilized amino compounds of the extracts were dissolved in double distilled water prior to analysis. a) The small amounts of Met could be much better quantified using the post-column derivatisation with ninhydrin. 40 µl of the aqueous solution of soluble amino compounds were again lyophilized and subsequently redissolved in 80 µl of loading buffer. 40 µl of this solution were used for amino acid analysis in a Pickering Labs PCX post-column derivatization instrument according to the producers instructions. b) An HPLC-based method was applied to determine other free amino acids and GEC under conditions where the latter remained undegraded. Amino compounds of the aqueous solution were reacted with dansyl-Cl and fractionated according to Krause et al. (1995). Good separation of GEC from the flanking Asn and Gln was achieved by an appropriate increase of the original gradient slope. c) Analysis of the amino acid composition of seed flour or protein fractions took place after hydrolysis of the proteins with 6 N HCl. For Cys determination proteins were oxidized by performic acid before being hydrolysed (Moore 1963).

#### *Sulphate analysis*

Inorganic sulphate was directly determined using the aqueous solution of soluble amino compounds (see above). Soluble organic sulphur compounds of this extract and insoluble sulphur compounds in the extraction sediment were oxidized to sulphate using 65% suprapure HNO<sub>3</sub> (Merck, Darmstadt, Germany) at 170 °C in a pressure mineralization apparatus (Berghof Labortechnik, Eningen u. A., Germany) for 3 hrs with subsequent slow cooling down to room temperature overnight. Sulphate was determined by HPLC using an IonPac AG10 Guard Column followed by IonPac AS10 anionexchange column (both columns provided by Dionex Corporation, Salt Lake City, UT, USA) with isocratic ion elution by 80 mM NaOH and Postcolumn Suppressor Technique (Dionex) according to the manufacturers instructions.

#### *Analysis of soluble thiol compounds*

Since GEC is partially degraded by monobromobimane derivatisation with the thiol-specific fluorogenic reagent ammonium 7-fluoro-2,1,3-benzoxadiazol (SBD-F) according to Ling et al. (1990) followed by reverse-phase HPLC fractionation (Waters, Milford, MA, USA) was used.

#### *DNA analysis*

Number of inserts, zygosity and segregation of the AK gene were followed by DNA analysis. Total DNA was extracted from leaf tissue according to Dellaporta et al. (1985). DNA was restricted (Sambrook et al. 1989) with HindIII (MBI Fermentas Vilnius, Lithuania) which has only one cleavage site in the vector (Figure 1). Southern blotting was performed according to Sambrook et al. (1989). – PCR analysis of leaf DNA (Sambrook et al. 1989) was employed to screen segregating populations of transformants already during the early vegetative period. Primers of 30 nucleotides each corresponded to appropriate sequences of the BNA gene, the LeB4 promotor, and the AK gene, respectively.

#### *Statistics*

Samples from seeds of three different plants per line were analysed separately. In each case triple analyses were performed the mean of which represents the respective value per plant. Thus the number of values

Table 1. List of transformant lines used in the present experiments.

| Lines            | Genes                                                                         | Promoters                               | Targeting sequences                     | References                                         |
|------------------|-------------------------------------------------------------------------------|-----------------------------------------|-----------------------------------------|----------------------------------------------------|
| AK 5, 18, 50, 54 | <i>E. coli</i> gene ( <i>lysC</i> ) for deregulated aspartate kinase, homozyg | phaseolin ( <i>Phaseolus vulgaris</i> ) | chloroplast transit peptide             | Karchi et al. (1993)                               |
| BNA B12          | Brazil nut 2S albumin gene, homozyg                                           | legumin B4 ( <i>Vicia faba</i> )        | signal peptide, vacuolar targeting info | Pickardt et al. (1995) and Saalbach et al. (1995b) |
| Retr             | AK + BNA, retransformant, double homozyg                                      | see AK and BNA lines                    | see AK and BNA lines                    |                                                    |
| C2n              | AK + BNA, crossing, double homozyg                                            | see AK and BNA lines                    | see AK and BNA lines                    |                                                    |
| C1n              | AK homozyg + BNA hemizyg, crossing                                            | see AK and BNA lines                    | see AK and BNA lines                    |                                                    |

per line is  $n = 3$ . Standard deviations are not indicated in the figures since the significance of all differences has been evaluated statistically using the analysis of variance or Student's *t*-test.

## Results

### Transformants

Transformants are listed in Table 1. All three double transformant lines expressed the gene for the deregulated bacterial AK and the BNA gene encoding a Met-rich 2S storage albumin from Brazil nut. In all three cases the AK gene was brought into the background of narbon bean line B12 that was selected from a group of previously described similar homozygous BNA lines (Pickardt et al. 1995; Saalbach et al. 1995b, c). The AK gene was transferred into this background either by direct transfer (Retr) into line B12 or by crossing B12 with line AK18 (C2n and C1n). The latter represents a prototype selected from 4 similar AK lines described in the present paper. All 3 double transformants are homozygous for the AK gene. Retr and C2n are also homozygous for the BNA gene whereas C1n is BNA hemizygous. When compared with C2n it should reflect the known gene dosage effect of BNA gene expression. The AK as well as the BNA line have single inserts of the transgene. This and the zygosity state were documented by Southern blotting and segregation analysis. Segregation was not only followed by activity analysis of AK and immuno detection of BNA in seed extracts but also by PCR analysis of DNA from leaves at early

developmental stages of the tobacco plants (see Methods).

No phenotypical differences were observed between the 8 different transgenic lines and in comparison to the wildtype. Fruits contained on the average similar numbers of seeds and no significant differences were observed in weight, shape and size of the seeds (not shown).

### Aspartate kinase activity

Neither endogenous AK activity nor AK-specific immuno-reactive bands were found in extracts of cotyledons or total embryos of mature dry or 12 hrs imbibed wild type (Wt) and BNA line B12 seeds (Figure 2). Comparison of hemi- with homozygous AK lines revealed a gene dosage effect for AK activity (Figure 3). Segregation ratios of line AK18 did not significantly differ from the 1: 2: 1 ratio of monogenic Mendelian inheritance. This confirmed the presence of a single gene insert that was already observed previously by Meixner et al. (unpublished).

Independent of whether generated by crossing or by retransformation, all 3 double transformant lines being homozygous for the AK gene exhibited similar AK activities. These corresponded also with the activity of line AK18 used for crossing and with the average activity of the other homozygous AK lines. Transgenic lines with similar AK activities exhibited similar strength of AK bands on immunoblots, whereas line AK54 with lower AK activity showed also a weaker AK band (Figure 2).

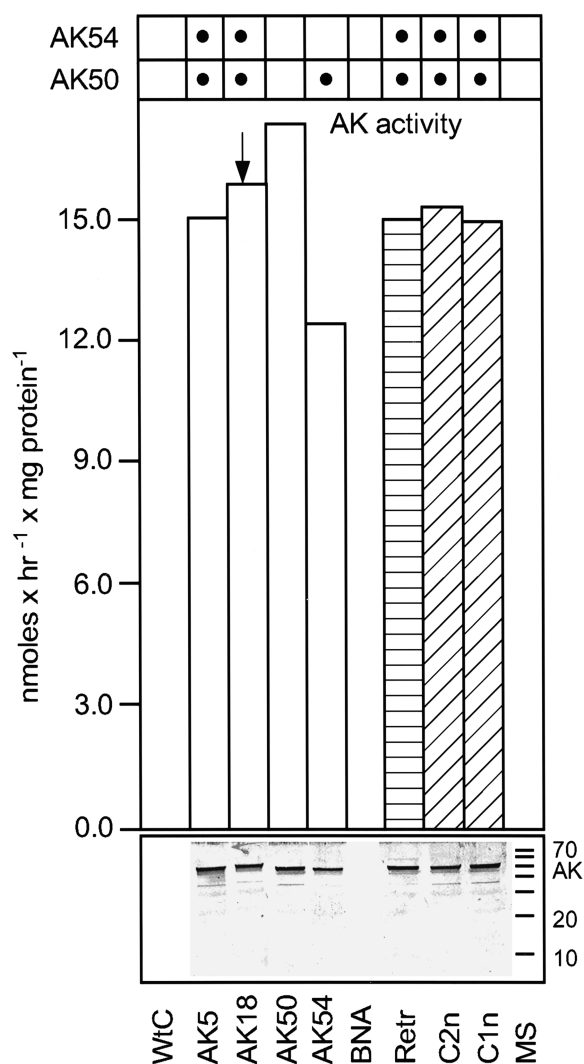


Figure 2. Activity of the feed-back-insensitive bacterial aspartate kinase (AK) in extracts from cotyledons of mature seeds of different transgenic narbon bean lines (*Vicia narbonensis* L.). Abbreviations of line names see Table 1. Results of statistical evaluation of differences between lines AK50 and AK54 and all other lines are indicated in a matrix in the upper part of the figure. Evaluation results are only once indicated when fields for reciprocal combinations of lines are available in the matrix. • – significant with at least 5% of probability of error. No significant differences were found between lines AK5 and AK18 and all other lines. Lower part: Immunoblot with polyclonal monospecific AK antibodies. Molecular weight markers are indicated ( $\times 10^{-3}$ ) on the right side of the blot. The arrow marks line AK 18 used for crossing with the BNA line.

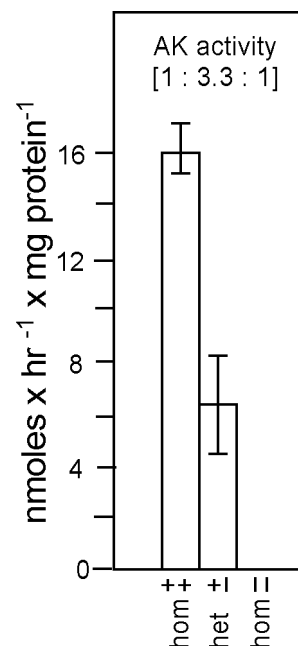


Figure 3. Segregation of aspartate kinase (AK) activity levels in seeds of a selfed T2 plant of narbon bean AK transformants. Sixteen seeds were analysed. The indicated ratio between AK homozygous (++), AK hemizygous (+-) and homozygous AK-free (--) seeds does not differ significantly from the 1: 2: 1 ratio of a monogenic Mendelian inheritance ( $X^2$ -test). Gene dosage effect is indicated by the AK activity levels.

#### Soluble amino acids

##### Total soluble amino compounds (Figure 4)

In contrast to the small but significant increases in AK lines the amount of total soluble amino compounds of BNA line B12 was decreased (Figure 4). The opposing effects of the two foreign genes were almost compensated in crossing-generated double transformants which did not show significant differences to Wt, AK lines and line B12. In C1n, which is hemizygous for the BNA gene, the compensating effect of this gene was correspondingly smaller. Only Retr showed an even stronger decrease in total soluble amino compounds than the BNA line B12.

##### Amino acids of the aspartate pathway (Figure 5)

All transgenic lines showed increased soluble Met per unit seed flour in comparison to the Wt. Double-homozygous double transformant lines contained approximately 3 times more soluble Met than Wt seeds. The effects of the two foreign genes on the levels of soluble Met were roughly additive in Retr and C2n seeds. This was also true for C1n taking into consid-

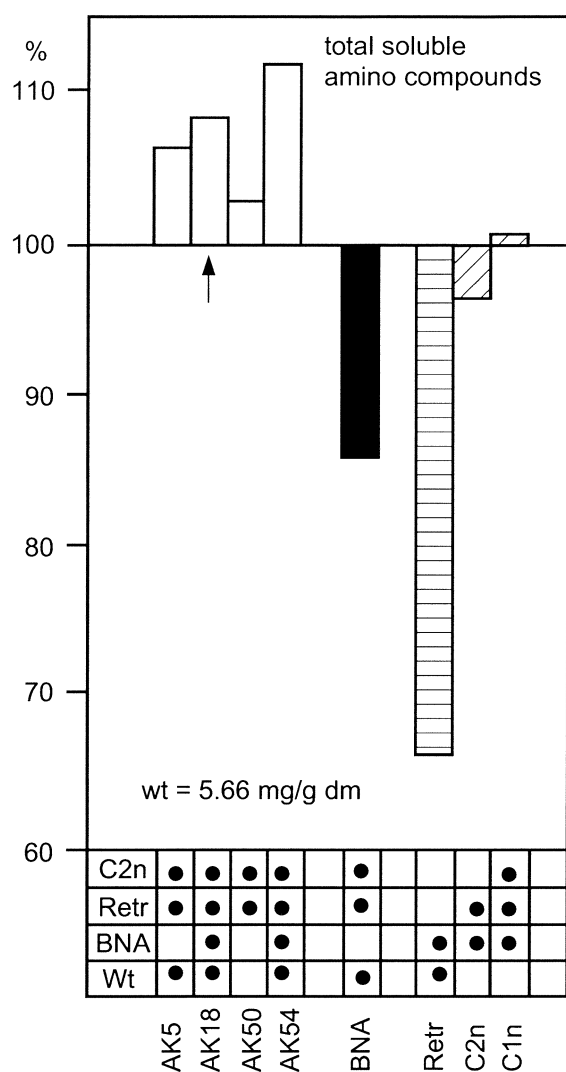


Figure 4. Percent changes in total soluble amino compounds in extracts from cotyledons of mature seeds from different transgenic narbon bean lines, *Vicia narbonensis* L. Abbreviations of line names see Table 1. The amount of total soluble amino compounds of Wt is indicated in the figure. Results of statistical evaluation are given in a matrix in the upper part of the figure. Evaluation results are only once indicated when fields for reciprocal combinations of lines are available in the matrix. ● – indicates significance with 5% or less probability of error. The arrow marks line AK 18 used for crossing with the BNA line.

eration that it is hemizygous for the BNA gene. All 7 transformants expressing the AK gene showed increased **Thr** amounts per g seed flour. In seeds of BNA line B12 **Thr** did not significantly differ from the Wt. Thus the BNA gene obviously does not exhibit an effect on the **Thr** levels of double transformants. Lines being homozygous for the AK gene had be-

tween 2.6 and 3.8 times higher **Thr** levels than the Wt. The **Thr** levels correlated with AK activities, except for line AK18. In contrast to **Thr** and **Met**, **Lys** showed significantly reduced amounts in all transgenic narbon bean lines in comparison to the Wt. No significant differences were observed between the 4 homozygous AK lines whereas the double transformants showed approximately additive decreases of **Lys** due to expressing both the AK and BNA gene. – Small increases of **Asp** were observed in all AK lines, but only AK18 had significantly more **Asp** than the Wt. In contrast, double transformant lines showed reduced **Asp** levels as compared to Wt.

#### Other amino acids

All lines homozygous for the AK gene had significantly increased **Gly** levels that reached 150 to 230% of the Wt whereas BNA line B12 was similar in **Gly** to the Wt (Figure 5). Small increases of **Leu** and **Ile** by 10 to 30% were also observed in AK gene expressing lines, whereas **Ser** and **Pro** were a little decreased (not shown).

#### Free thiols and $\gamma$ -glutamyl-S-ethenyl-cysteine (GEC)

Whereas **Met** was significantly increased in comparison to the Wt (see above) the amounts of soluble **Cys** were decreased in all transgenic lines (Figure 6). The absolute amounts of soluble **Cys** and **Met** were roughly similar in Wt seeds. But, the percentual decreases in soluble **Cys** were much smaller than the increases in soluble **Met** and therefore, the decrease in soluble **Cys** cannot compensate the increase in soluble **Met**. – Glutathione (**GSH**) and  $\gamma$ -glutamyl-cysteine ( **$\gamma$ GC**) were also significantly decreased in all transgenic lines. – Narbon bean seeds contain large quantities of **GEC** which is an S-ethenyl derivative of  **$\gamma$ GC**. Like  **$\gamma$ GC** also **GEC** was decreased in all transgenic lines compared to Wt but this decrease was not significant in AK5 and AK54. Taken together, the decreases in **Cys**, other free thiols and **GEC** compensated only partially the increases of sulphur-containing amino acids in proteins. Double transformants showed decreases in  **$\gamma$ GC**, **GSH** and **GEC** that roughly reflect additive effects of the two foreign genes.

#### Sulphate

Inorganic sulphate amounted to 1.8 percent of the total sulphur in cotyledons of mature Wt narbon bean. AK lines contained on the average 16 percent less in-

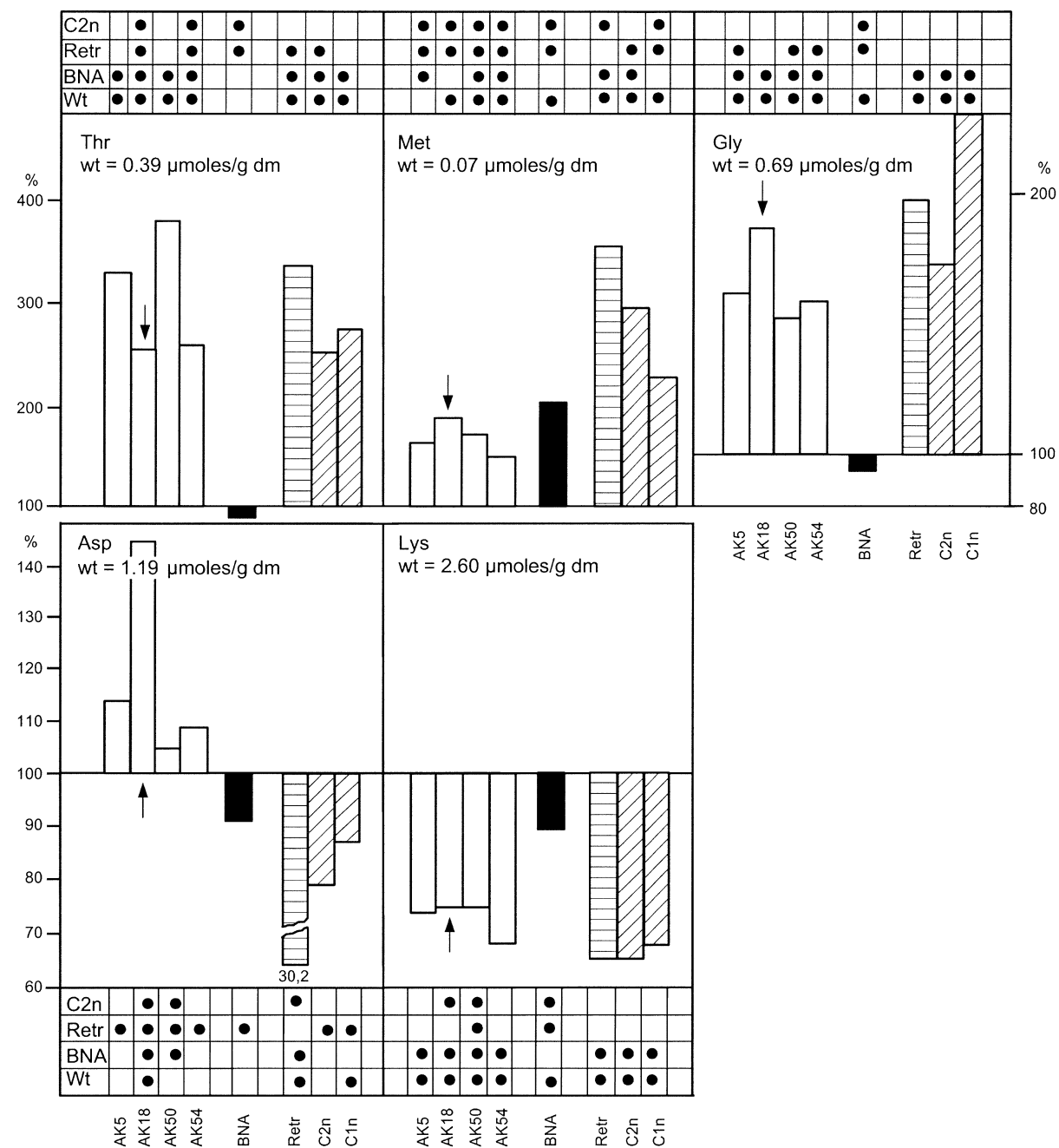


Figure 5. Percent changes in selected soluble amino acids of cotyledons of mature seeds from the different transgenic narbon bean lines, *Vicia narbonensis* L. Abbreviations for line names see Table 1. The amounts of the respective amino acid in Wt are indicated in the corresponding segments of the figure. The matrices in the different segments of the figure indicate results of statistical evaluation of differences. Evaluation results are only once indicated when fields for reciprocal combinations of lines are available in the matrix. ● – indicates significance with 5% or less probability of error. The arrow marks line AK 18 used for crossing with the BNA line.

organic sulphate the levels of which were even more decreased in mature seeds of the BNA line B12 and double-homozygous double transformants (Figure 7). In seeds of double transformant lines sulphate de-

creases reflected at maximum additive effects of the two foreign genes.

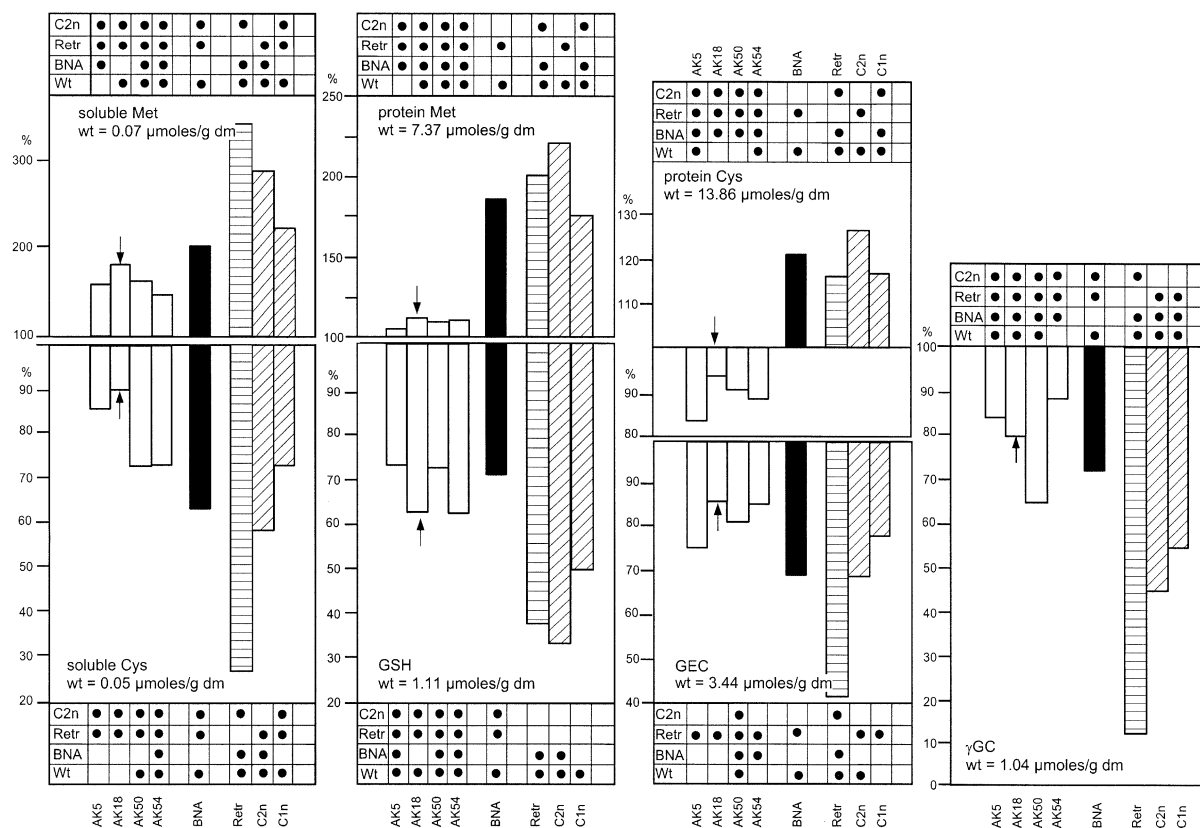


Figure 6. Percent changes in different soluble sulphur-containing compounds and in protein-bound sulphhydryl amino acids from cotyledons of mature seeds of the different transgenic narbon bean lines, *Vicia narbonensis* L. Abbreviations of line names see Table 1. The amounts of the respective compound in Wt are indicated in the corresponding segments of the figure. The matrices in the different segments of the figure indicate results of the statistical evaluation of differences. Evaluation results are only once indicated when fields for reciprocal combinations of lines are available in the matrix. ● – indicates significance with 5% or less probability of error. GSH – glutathione, GEC –  $\gamma$ -glutamyl-S-ethenylcysteine,  $\gamma$ GC –  $\gamma$ -glutamyl-cysteine. The arrow marks line AK 18 used for crossing with the BNA line.

### Protein-bound methionine and cysteine

Levels of protein-bound SH-amino acids were strongly increased in all transgenic lines expressing the BNA gene (Figure 6). BNA line B12 showed the already known almost doubled quantity of protein-bound Met as compared to the Wt (Saalbach et al. 1995b, c). Small significant increases in protein-bound Met were also observed in three of the homozygous AK lines including AK18 that was used to generate double transformant lines by crossing. Independent of whether generated by crossing or by re-transformation the double transformants reflected roughly additive effects of the two foreign genes on the levels of protein-bound Met in mature seeds. Protein-bound Cys was only increased in the BNA gene-expressing transgenic lines but was decreased in all AK lines. There the percentual decreases in protein-

bound Cys were on the average roughly similar to the percentual increases in protein-bound Met. However, the absolute decrease in Cys was twice as much as the Met increase in AK lines since the Wt cotyledon flour contains twice as much Cys than Met. Accordingly, absolute increases in Cys and Met were nearly similar in all BNA-gene expressing lines.

The amount of albumins was significantly increased above the Wt level in lines AK18, 50 and 54 whereas no significant increase in globulins was found in lines AK5, AK18 and AK54. The ratio of globulin and albumin quantities between the Wt and the average of all four AK lines was shifted from 4.53 to 4.20 reflecting the increase in albumins. The percentage of this increase roughly agreed to the average percentage of increase in protein-bound Met of the homozygous AK lines.

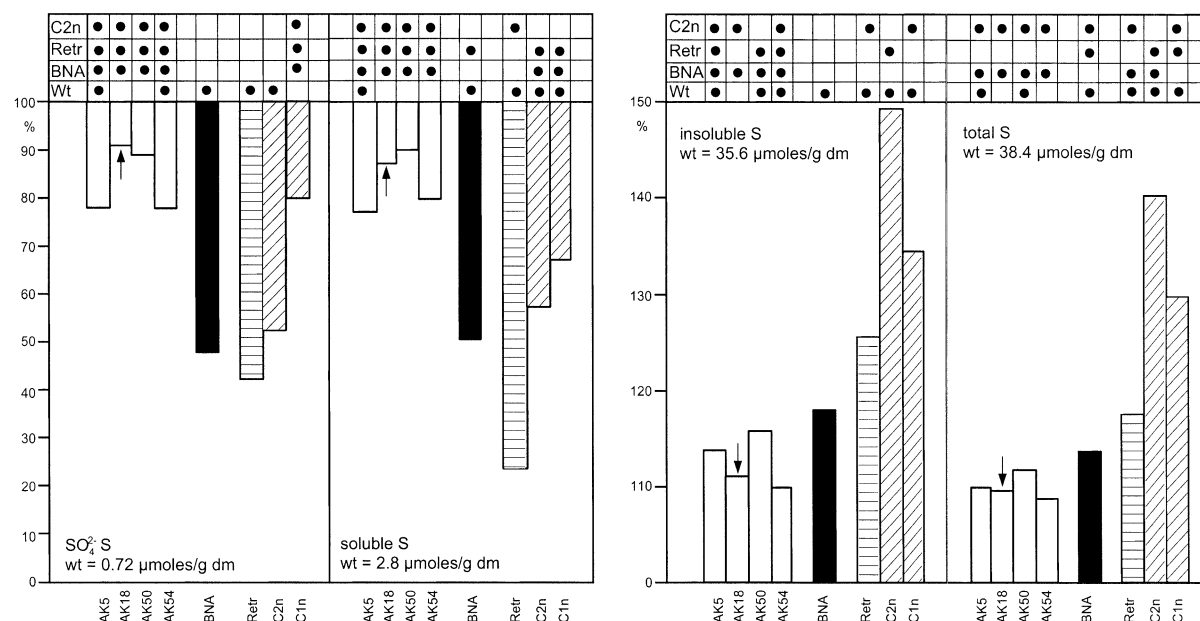


Figure 7. Percent changes in sulphate, soluble and insoluble sulphur in cotyledons of mature seeds from transgenic narbon bean, *Vicia narbonensis* L. Total sulphur was calculated by addition of soluble and insoluble sulphur. Abbreviations of line names see Table 1. The matrices in the upper part of the figure indicate the results of statistical evaluation of differences. Evaluation results are only once indicated when fields for reciprocal combinations of lines are available in the matrix. ● – indicates significance with 5% or less probability of error. The arrow marks line AK 18 used for crossing with the BNA line.

The amounts of albumins and globulins were on the average significantly increased by 50 and 18 percent, respectively, in the BNA and double transformant lines. The increase in albumins was due to the formation of BNA. Electrophoretic banding patterns did not show significant differences between Wt and the BNA lines, except for the additional BNA band. Stronger increases in albumins than in globulins were also observed in seeds of the AK lines 18 and 50. However, amino acid composition of albumin and globulin fractions of both lines did not show significant increases in the percentages of Met per unit protein. In addition, comparative one-dimensional electrophoretic analysis of polypeptide band patterns under detaturing and reducing conditions revealed no differences between albumins and globulins of the Wt and AK lines, respectively (not shown).

#### Sulphur balance

Due to its low amount in mature Wt seeds the observed decreases of sulphate in transgenic lines did not contribute appreciably to the increases in total organic seed sulphur. Soluble sulphur which includes sulphate corresponded to 8 percent of total sulphur.

Decreases in soluble sulphur were much stronger in double transformant than in AK lines but did not compensate more than one third of the increase in insoluble sulphur. This corresponds roughly to the percental portion of the increase in protein-bound sulphhydryl amino acids that in a sulphur balance is compensated by the decreases in soluble thiols and GEC.

#### Discussion

Due to the strategies of generation (see Results, section transformants), all double transformants must have similar insertion sites of the BNA gene. In addition, C2n and C1n must agree in the site of AK gene insertion, and this site is similar to that in line AK18. A divergent AK gene insertion site was to be expected for Retr. Nevertheless, similar AK activities were found in AK18 and all three double transformants (Figure 2) and only small differences in protein Met and Cys occurred between Retr and C2n (Figure 6). In these characters C1n agrees also to the double homozygous double transformants taking into consideration that C1n is hemizygous for the BNA gene. But,

significant percentual differences were observed for some soluble amino acids and thiols. In contrast to protein-bound Met and Cys they are present in small amounts. Therefore even large differences in percentages correspond only to small changes in absolute quantities.

Both transgenes were controlled by promoters that in grain legumes of their origin direct seed maturation-specific globulin gene expression. Phaseolin from kidney bean belongs to the class of 7S globulin, whereas legumin from field bean is a 11S globulin. Maximum gene expression of 7S globulin genes is known to precede maximum 11S globulin gene expression during legume seed development (Higgins 1984). This was also shown for *Vicia faba* (Bassüner et al. 1983) and *V. narbonensis* (Demidov, unpublished). The globulin gene promoters are known to direct similar developmental patterns of gene expression in seeds of transgenic plants (Sengupta-Gopalan et al. 1985; Pickardt et al. 1998). Therefore, the phaseolin promoter-controlled maximum AK gene expression should precede legumin promoter-driven maximum BNA gene expression in developing transgenic narbon bean, too. Due to this succession in maximum gene expression it was expected that the periods of Met overproduction and increased Met demand for BNA formation should approximately coincide. The overproduction of free and protein-bound Met in all our transgenic narbon bean lines is in accordance with this assumption.

Independent of how the double transformant lines were generated they showed additive effects of AK and BNA gene expression. Whereas in B12 seeds protein Met was elevated to 180 percent of the Wt, in Retr and C2n 200 and 240 percent, respectively, were reached. Best Met levels approximated the FAO standard for Met in balanced total protein of human food and non-ruminant animal feed. In absolute terms the increase in protein Cys equaled the elevation of protein Met although its relative increase was much less impressive due to the double quantity of Cys as compared to Met in Wt seed protein. Free Met and Cys amount only to 1 percent or less of the protein Met and Cys, respectively, in mature Wt seeds. Therefore, even the three-fold increase in free Met did not appreciably contribute to the increase in total Met in seeds of the double transformants.

No synergistic effect of the expression of the two foreign genes was observed in the double transformants. The availability of free Met might not have acted as a limiting factor since besides the strong in-

creases in protein-Met also free Met was appreciably increased in AK, BNA and double transformant lines. Increases in free Met and to a lesser extent also in protein-Met were reported similarly for seeds of homozygous AK transformants of tobacco (Karchi et al. 1993). Despite the strong percentual elevation of free Met the increases in protein-Met were limited to 7 to 10 and 10 to 12 percent in seeds of tobacco and narbon bean AK lines, respectively. This might indicate that gene expression but not supply of free Met limited further increase of protein-Met in seeds. Introduction of a foreign high-Met protein gene increased the capacity of seed proteins as a Met sink and generated the strong Met increase that was observed in BNA line B12. Expression of the BNA gene is the major factor determining the Met increases in seeds of double transformants, too.

Free Cys as well as Cys-containing di- and tripeptides were decreased in all transformants. But this decrease accounted only for approximately one third of the additional supply of organic sulphur for covering the increased demand due to the elevated protein Met and Cys levels. Seeds of narbon bean contain little inorganic sulphate. Therefore, its decrease in the transgenic seeds did not contribute appreciably to cover the additional sulphur expenses, either. The major part of these increased expenses must have been supplied by sulphur import from vegetative organs. As shown by *in vitro*-culture, maturing legume cotyledons can assimilate inorganic sulphate (Thompson and Madison 1990). This is also true for narbon bean (Wüstenhagen et al., unpublished). Cotyledons of mature lupins (*Lupinus angustifolius* L.) contain large amounts of sulphate. In transgenic lupins the increase in protein sulphur due to the expression of a high-Met protein gene goes on account of the stored sulphate (Molvig et al. 1997; Tabe et al. 1997; Tabe and Droux 2000). However, like narbon bean also soybean contains little sulphate. Strong reduction of endogenous protein sulphur was observed in soybean due to high level expression of the BNA gene (Townsend and Thomas (1994); Jung, personal communication). GSH is the major compound for sulphur import into developing soybean seeds (Surnapi 1997). Its degradation supplies Cys for various metabolic pathways including Met formation and protein biosynthesis (Hell 1997). The increases in free Gly that were observed might reflect GSH breakdown and preferential Cys metabolisation in the seeds of transgenic narbon bean. A complete set of enzymes for GSH degradation was shown to be present in narbon bean seeds (Demidov

2003). Similar to soybean in transgenic rice the formation of a sulphur-rich foreign 2S albumin induced a strong reduction of endogenous protein sulphur (Tabe et al. 2002). In our transgenic narbon bean the observed decreases in free Cys and Cys-containing peptides can be taken as reflecting limitation in Cys supply to the seeds. The increases in free and protein Met indicate that the channelling of Cys into Met biosynthesis might not be limited. However, the ratio between Cys and Met supply for protein formation might be decreased to a degree which does not permit synergistic effects of AK and BNA gene expression on Met increases in transgenic narbon bean. Therefore, combined engineering for high Cys supply from vegetative organs and expression of foreign high-Met protein in legume seeds might be suitable for generating synergistic effects.

Soybean double transformants that seed-specifically express the deregulated bacterial AK gene together with a deregulated dihydrodipicolinate synthase (DHDPS) gene were produced by Falco et al. (1995). No separate analysis of the effects of both enzymes could be performed in this legume. Therefore, our transgenic AK lines of narbon bean permitted for the first time an analysis of how the deregulated bacterial AK gene influences amino acid biosynthesis in the aspartate pathway of a legume seed. No endogenous AK activity was detected in seeds of Wt and BNA line B12. Seeds of the transgenic AK lines exhibited high enzyme activity. It was correlated to AK polypeptide levels and showed gene dosis effect. Not only free Met but also free Thr was strongly increased in comparison to Wt seeds. In contrast, free Lys was decreased in amount complementary to the increases in Met plus Thr. This might indicate that in AK lines aspartyl phosphate is channelled preferentially into Thr and Met formation, and that this goes on account of Lys biosynthesis in the aspartate pathway. An alternative explanation offers elevated Lys degradation. However this was so far found only in transgenic plants expressing a deregulated bacterial DHDPS (Karchi et al. 1994) but not in plants that over-express the deregulated AK gene. No decreases in Asp levels were observed that might limit amino acid biosynthesis in the aspartate pathway of AK lines. Except Met the changes in quantities of other free amino acids of the aspartate pathway are not reflected in proteins. The results with narbon bean AK lines agree to those published for seeds of tobacco AK lines with the exception that there no decrease of free Lys was described (Karchi et al. 1993).

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