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Arabidopsis loss-of-function mutant in the lysine pathway points out complex regulation mechanisms

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Abstract In plants, the amino acids lysine, threonine, methionine and isoleucine have L-aspartate-β-semialdehyde (ASA) as a common precursor in their biosynthesis pathways. How this ASA precursor is dispersed among the different pathways remains vague knowledge. The proportional balances of free and/or protein-bound lysine, threonine, isoleucine and methionine are a function of protein synthesis, secondary metabolism and plant physiology. Some control points determining the flux through the distinct pathways are known, but an adequate explanation of how the competing pathways share ASA in a fine-tuned amino acid biosynthesis network is yet not available. In this article we discuss the influence of lysine biosynthesis on the adjacent pathways of threonine and methionine. We report the finding of an *Arabidopsis thaliana* dihydrodipicolinate synthase T-DNA insertion mutant displaying lower lysine synthesis, and, as a result of this, a strongly enhanced synthesis of threonine. Consequences of these cross-pathway regulations are discussed. **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Dihydrodipicolinate synthase; Lysine; Threonine; Cross-pathway regulation; *Arabidopsis* knockout

1. Introduction

Considering the pathways for synthesis of lysine, threonine, methionine and isoleucine, three reaction points are crucial in controlling the flux of common precursors into the competing pathways (Fig. 1). L-Aspartate-β-semialdehyde (ASA) is the common precursor of all four pathways. Energetic activation of L-aspartate into L-β-aspartylphosphate irreversibly channels L-aspartate from the citrate cycle into one of the four pathways (Fig. 1, (1)). Aspartate kinase (AK) catalyses this reaction, which implies that this enzymatic step should be controlled by the needs for lysine, threonine, methionine and isoleucine as a function of protein synthesis, secondary metabolism and plant physiology. L-β-Aspartylphosphate, the product of AK, is subsequently reduced by aspartate-β-semialdehyde dehydrogenase (ASD) into ASA. Once ASA is formed, two enzymes compete for the substrate. Homoserine dehydrogenase (HSDH) transforms ASA into L-homoserine, which is subsequently converted into *O*-phospho-L-homoserine, the common precursor of the threonine, methionine and isoleucine biosynthesis pathways. Alternatively, dihydrodipicolinate synthase (DHDPS) condenses ASA and pyruvate into 4-hydroxy-2,3,4,5-tetrahydrodipicolinate, an intermediate of the lysine biosynthesis pathway. This means that the regulations controlling enzyme activities of DHDPS (Fig. 1, (2)) and HSDH (Fig. 1, (3)) discriminate whether ASA will be channelled into the threonine–isoleucine–methionine synthesis branch or into the lysine synthesis branch. If ASA is directed into L-homoserine synthesis, again competition arises between the threonine (and derived isoleucine) and methionine pathway branches for the common precursor *O*-phospho-L-homoserine. Here, the enzymes threonine synthase (TS), catalysing threonine synthesis, and cystathionine-γ-synthase (CGS), the first enzyme of the methionine synthesis pathway, determine whether ASA will serve threonine synthesis or methionine synthesis (Fig. 1, (4), (5)).

In recent decades, regulation studies performed on plant mutants altered for their synthesis of aspartate-derived amino acids have revealed some understanding of the aspartate pathway flux controls. However, questions remain unsolved and a profound understanding of how intermediates are channelled into the branching pathways is not yet available. In this article we describe the characterisation of a second *dhdps* gene (*dhdps-2*) found in *Arabidopsis*. We report the expression pattern of the *dhdps-2* gene and the DHDPS-2 enzyme activity properties as a function of lysine inhibition. In addition, through the isolation of an *Arabidopsis* T-DNA insertion mutant that is characterised by the lack of a functional *dhdps-2* gene, we show that lysine deficiency enhances the flux of the threonine biosynthesis pathway drastically. The aspartate pathway regulations are discussed in the context of these newly discovered flux controls.

2. Materials and methods

2.1. Isolation of the dhdps-2 knockout mutant

The genomic DNA collection of the *Arabidopsis* T-DNA insertion lines from Versailles was screened for T-DNA insertions in the *dhdps* genes. Sense and antisense primers from promoter and coding sequences of the *dhdps* genes were designed and used in combination with primers specific for the T-DNA flanking regions. Obtained PCR products were hybridised in duplicate with a T-DNA-specific and a *dhdps*-specific probe in order to reduce false positives. A positive amplification product was obtained from hyperpool 3 (T-DNA primer 5′-ctgataccagacgttgcccgcataa-3′; *dhdps-2* primer 5′-tggattaccagctttggtgacgacagtcgt-3′). Corresponding superpools and pools were subsequently screened to obtain the individual pool. Seeds corresponding to the 48 plant lines contained in this DNA pool were further screened and the individual plant remaining positive in the hybridisation tests and PCR amplifications was further characterised.

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2.2. Growth of plant material for biochemical analyses

Arabidopsis plants were grown in sterile conditions in a growth chamber at 20°C, 16 h light (Philips Hg-I lamps 400 W/m^2).

2.3. Amino acid analysis

Roots, leaves, stems and flowers from 20-day-old in vitro grown *Arabidopsis* plants were extracted for free amino acids. Extracts were hydrolysed with HCl to convert asparagine and glutamine into the respective acidic forms. Free amino acid content was determined using an ion exchange amino acid analyser (Beckman System Gold® 166 NM Detector) according to the manufacturer's specification.

2.4. Dihydrodipicolinate synthase extraction and activity tests

The DHDPS enzyme activity tests were performed as described [1].

2.5. GUS histochemical assay

Histochemical localisation of GUS activity was performed as described [2]. Histochemical analysis was done using a Wild Heerbrugge stereomicroscope and a Carl Zeiss Axiophot microscope.

3. Results

3.1. Two dihydrodipicolinate synthase genes are present in Arabidopsis thaliana

An *Arabidopsis* genomic sequence encoding a second DHDPS enzyme was found (AC002388) in the EMBL database. The corresponding cDNA (R90204) was obtained from the *Arabidopsis* EST database library. The *dhdps-2* coding sequence shows 84% identity at the nucleotide level with the previously cloned *dhdps* cDNA [3]. The corresponding genomic sequence including the promoter region was amplified from *Arabidopsis* DNA through PCR, and the sequence was determined. The genomic *dhdps-2* sequence is interrupted by two introns. The first intron is 355 bp long and is conserved in the *dhdps-1* gene (272 bp), the second intron is 119 bp long and is not present in *dhdps-1* (Fig. 2A). Comparison of the promoter regions of *dhdps-1* and *dhdps-2* did not reveal boxes with any significant conservation. The *dhdps-1* gene is located on chromosome III, the *dhdps-2* gene on chromosome II.

3.2. The dhdps-2 gene of Arabidopsis encodes a functional protein that is lysine-inhibited

The *dhdps-2* apoprotein coding sequence was amplified by PCR and inserted into the pUC18 vector to allow DHDPS-2 expression. The vector was transformed in the *Escherichia coli dapA*$^-$ strain AT997, which lacks a functional DHDPS activity. To allow growth of the strain, diaminopimelic acid was added to the medium [1]. Activity tests showed that a functional *Arabidopsis* DHDPS-2 enzyme is expressed. Adding lysine to the enzyme assays proved that the DHDPS-2 enzyme is strongly inhibited by lysine, with a 50% loss of activity at 30 μM lysine. This is slightly less sensitive compared to DHDPS-1, which has 50% inhibition at 10 μM lysine [1].

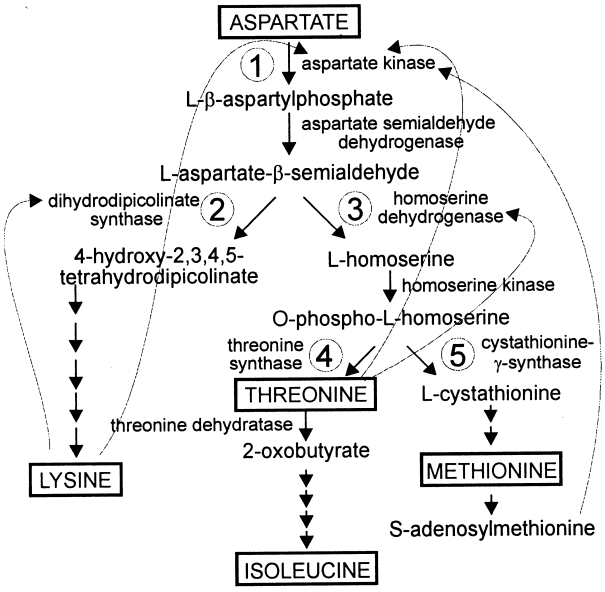

Fig. 1. Aspartate-derived biosynthesis pathways leading to the synthesis of lysine, methionine, threonine and isoleucine. Key pathway points determining flux into the different pathways are indicated with numbers.

3.3. Screening for T-DNA insertions in the dihydrodipicolinate synthase genes of Arabidopsis

An *Arabidopsis* T-DNA insertion bank was obtained from the INRA institute, Versailles, France [4]. Using primers specific for the *dhdps-2* gene and the T-DNA external sequences, we identified a T-DNA insertion mutant with a T-DNA insertion in the first exon of the *dhdps-2* gene, 61 bp downstream of the initiation codon of translation (Fig. 2B). The T-DNA is inserted in frame with the *dhdps* gene, rendering *gus* gene expression (located downstream of the T-DNA left border) driven under the control of the *dhdps-2* promoter. Southern blotting using the kanamycin coding sequence as radiolabelled probe and PCR-based techniques for detection of

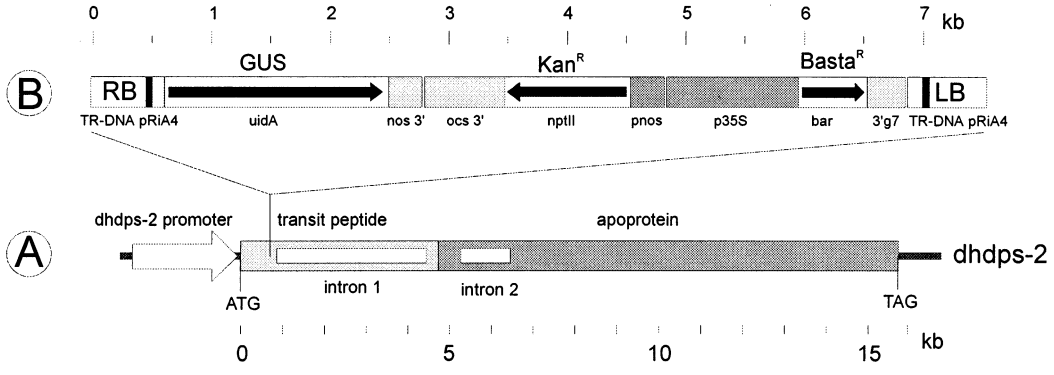

Fig. 2. A: Structure of the *dhdps-2* gene of *Arabidopsis*. The insertion site of the T-DNA into the *dhdps-2* gene is indicated (65 bp downstream of ATG). B: Structure of the T-DNA used to generate the *Arabidopsis* T-DNA insertion library (INRA, Versailles). A and B are proportionally drawn with respect to the given scales.

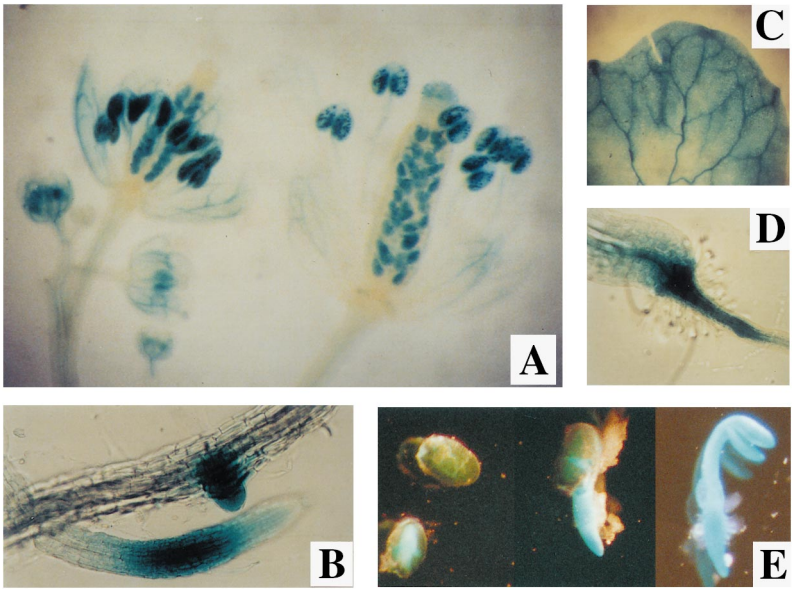

Fig. 3. GUS activity of the *Arabidopsis dhdps-2* promoter in different plant tissues. A: Flowers and developing seeds: mainly anthers and developing seeds are stained. B: Roots: root tips and lateral bud meristems are stained. C: Leaf: vasculature shows high *gus* expression. D: Strong staining at the zone between root and stem. E: Germination of a seed.

the T-DNA insert showed that the mutant is characterised by a single T-DNA insertion in the *dhdps-2* gene (data not shown).

3.4. GUS expression

GUS expression of the *dhdps-2* gene was observed from the second day after germination until seed setting (4 weeks). The expression pattern of the *dhdps-2* gene is striking similar to that observed for the *dhdps-1* gene [2], although the *dhdps-2* gene has in general a little stronger expression. In vegetative parts of the plants, GUS expression driven by the *dhdps-2* promoter was detected in meristems and vasculature (Fig. 3). Roots showed expression in the root tips, mainly in the elongation zone and in the meristems of emerging lateral roots (Fig. 3B). The zone between the main root and stem shows strong GUS expression (Fig. 3E). Expression of the *dhdps-2* gene is strongly detected in the vasculature of stems and leaves (Fig. 3C). There is also a slight expression in the mesophyll cells of the leaves, while *dhdps-1* expression was not detected in this tissue, except for cotyledons of young plantlets. In the reproductive organs, strong expression is observed in anthers and developing pollen, as was the case for *dhdps-1*. Expression of *dhdps-2* was also detected in carpels and developing seeds, and this expression is more pronounced than that in *dhdps-1* (Fig. 3A). Developing seeds show an overall weak expression (Fig. 3E).

3.5. Lysine starvation induces threonine overproduction

Arabidopsis wild-type plants and *dhdps-2* T-DNA insertion mutant plants were grown in vitro in culture room conditions. No differences could be observed in growth, development and seed production. From wild-type *Arabidopsis* plants and *dhdps-2* T-DNA plants, free amino acid content was determined from roots, leaves, stems and flowers. Due to the knockout of the *dhdps-2* gene, a slight decrease in lysine production could be observed in all organs. As this decrease was maximally 10% in roots, the effect of disrupting the DHDPS-2 function on lysine biosynthesis is relatively limited. Unexpectedly, a drastic effect was observed on threonine biosynthesis (Table 1). Threonine concentrations were significantly higher in all organs, ranging from a three-fold increase in roots to a six-fold increase in flowers. Aspartate and glutamate are slightly decreased, whereas other amino acids derived from the aspartate pathway are not affected. Amino acids derived from other metabolic pathways are not altered in their synthesis (data not shown).

Table 1
Total free amino acid content and percentage free amino acid content of the aspartate-derived amino acids, aspartate and glutamate, measured in wild-type and *dhdps-2* knockout *Arabidopsis* plants

Amino acid	Wild-type				*dhdps-2* knockout			
	Roots	Leaves	Stems	Flowers	Roots	Leaves	Stems	Flowers
Lysine	1.54	1.48	1.12	1.61	1.25	1.36	0.98	1.11
Threonine	5.96	3.57	7.20	4.20	19.28	16.50	36.42[a]	26.79[a]
Isoleucine	1.91	1.54	1.12	1.80	2.15	1.39	1.03	1.94
Methionine	0.40	0.52	0.69	0.15	0.84	0.67	0.12	0.58
Aspartate	13.83	15.39	15.87	18.89	12.23	14.46	12.53	16.29
Glutamate	37.17	45.66	51.82	47.06	35.52	39.36	36.41	35.97
Total nM/g FW	3 622	7 082	15 294	21 951	6 563	6 640	16 603	31 802

Measurements are mean values of two repetitions.
[a]Measurements for threonine and serine together (peak overlap).

4. Discussion

The metabolic pathways converting L-aspartate into the derived amino acids lysine, threonine, methionine and isoleucine have until now mainly been studied at the biochemical level. Through these studies, it has been shown that the end products of these pathways exert a negative feedback regulation on enzymes functional at key (branching) points of the pathways [5]. However, the biological relevance of these control mechanisms remains unclear and can be questioned. One of the most puzzling enzymes regarding this is AK. In plants, AK exists as a bifunctional isoform with functional domains corresponding to AK and HSDH activities and as a monofunctional isoform with AK activity only. *Arabidopsis* has at least two bifunctional isoforms, both threonine-inhibited (AKthr) ([6] and EMBL database), and two monofunctional isoforms (AKlys) [7,8], inhibited by lysine and synergistically by *S*-adenosylmethionine [9]. So far it is unclear which role each AK fulfils in the plant, and how the plant manages these functions not to be redundant. A similar problem has now arisen with the finding of a second isoform of DHDPS in *Arabidopsis*. We have shown that this *dhdps-2* gene encodes a functional DHDPS-2 protein with lysine inhibition properties and an expression profile very similar to the already known DHDPS isoform. Hence, an explanation for the presence of two *dhdps* isoforms is not available.

Even if expression profiles of the AK and DHDPS isoforms were complementary, there are still inconsistencies to be explained. The lysine-sensitive monofunctional AK is half-inhibited at a concentration of about 200–600 μM lysine [10–14]. DHDPS, which controls the lysine biosynthesis flux, is half-inhibited at values of 5–20 μM lysine [1]. These are values at least 25 times lower compared to those for AK inhibited by lysine. As both AK and DHDPS are plastid-located, how can lysine reach concentrations which can affect the activity of the lysine-controlled AK? However, lysine does influence the activity of the plant AKlys, as mutants with a lysine-insensitive AK [10,15–18] or transgenic plants expressing bacterial AK isoforms with no inhibition properties [19] overproduce high amounts of threonine. Moreover, our new results also indicate that lysine biosynthesis strongly modulates the AK activity. Knocking out of one of the two *dhdps* genes in *Arabidopsis* results in a slight decrease of lysine production, but in a drastic change in threonine synthesis. Whether the *dhdps-2* knockout mutant stimulates ASA synthesis through a relaxed inhibition upon the AKlys isoform, and hence promotes threonine synthesis, is unclear. If so, one has to conclude that a HSDH isoform exists in plants, which is not or low inhibited by threonine.

Considering the results from the *dhdps-2* knockout plants, it seems that free lysine has a tight control on the upstream synthesis of ASA, controlling flux through the other aspartate-derived pathways. The fact that HSDH is a weak competitor for DHDPS regarding their common substrate ASA indicates again that, when control on DHDPS is relaxed, lysine synthesis is favoured over threonine and methionine synthesis. This is evidenced by the analysis of plants obtained by crossing the lysine-overproducing mutant REAC-r1 with the threonine-overproducing mutant RLT1 [20] or by transgenic plants expressing bacterial-insensitive DHDPS and AK enzymes [19,21]. As these crosses or double transformants result in plants overproducing even higher levels of lysine, but never threonine, flux of ASA seems to be directed totally towards lysine synthesis.

A striking parallelism can be found with the branching point DHDPS–HSDH when considering the competition between TS and CGS for the common substrate *O*-phospho-L-homoserine (Fig. 1, (4), (5)). In abundance of the common precursor *O*-phospho-L-homoserine, it is TS that funnels all excess of substrate into threonine synthesis. This is also evidenced by the RTL1 mutant and by transgenic plants expressing a bacterial-insensitive AK. Both plants overproduce threonine while there is no increase in methionine synthesis. In addition, a decrease in threonine synthesis leads to a significant increase in methionine, a phenomenon comparable to the threonine overproduction in response upon lower DHDPS activity. This has been shown by the *Arabidopsis mto2-1* mutant characterised by a TS with lower enzyme activity [22].

Modification of the enzyme properties of AK, DHDPS or TS drastically affects synthesis of lysine, threonine or methionine. It should be investigated now how the enzyme activities of AK, DHDPS and TS control the carbon flow at their respective branching points. The activity of the TS enzyme has been reported to be important in determining flux rates between threonine and methionine synthesis [23] whereas the stability of the CGS mRNA has been found to regulate methionine synthesis [24]. Similarly, AK and DHDPS should be investigated for such regulation properties. A simple model of allosteric inhibition of lysine or threonine on the AK enzymes does not give an adequate explanation for the drastic increase of threonine synthesis observed in the *dhdps-2* knockout mutant. It should be considered seriously therefore that these inhibition mechanisms might be more complicated in vivo than is supposed from in vitro experiments. Enzymes such as AK and DHDPS might function in protein complexes associated with other proteins modulating the susceptibility of the allosteric site. Indeed, association of the GroEL chaperone with the *Escherichia coli* DHDPS enzyme has been reported to be necessary for proper DHDPS function [25]. Similarly, a FKBP12 protein has been shown to interact with the yeast AK protein, influencing threonine feedback inhibition on the enzyme [26]. In addition, protein motifs characteristic of the prokaryotic two-component system have been reported for AK [27]. At least, these preliminary findings give plausible explanations why in vitro measurements do not reflect the effective inhibition properties of the AK enzyme.

In conclusion, the observed phenomenon of a drastic increase in threonine synthesis as a result of knocking out one DHDPS isozyme strongly suggests that other, yet unknown regulations are superposed upon the feedback regulations of the key enzymes controlling carbon flow between the competing branches of the aspartate pathway. It should be investigated now if other proteins associate with these key enzymes, modulating their enzyme activities upon request of the pathway end products.

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