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Approaches towards understanding methionine biosynthesis in higher plants

Review Article

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Summary. Plants are able to synthesise all amino acids essential for human and animal nutrition. Because the concentrations of some of these dietary constituents, especially methionine, lysine, and threonine, are often low in edible plant sources, research is being performed to understand the physiological, biochemical, and molecular mechanisms that contribute to their transport, synthesis and accumulation in plants. This knowledge can be used to develop strategies allowing a manipulation of crop plants, eventually improving their nutritional quality.

This article is intended to serve two purposes. The first is to provide a brief review on the physiology of methionine synthesis in higher plants. The second is to highlight some recent findings linked to the metabolism of methionine in plants due to its regulatory influence on the aspartate pathway and its implication in plant growth. This information can be used to develop strategies to improve methionine content of plants and to provide crops with a higher nutritional value.

Keywords: Amino acids – Transgenic plants – Cystathionine gamma-synthase – Cystathionine beta-lyase – Methionine synthase – Methionine biosynthesis – Sulfur-rich proteins

Introduction

One of the goals of plant genetic engineering has been to create crops that are tailored to provide better nutrition for humans and their domestic animals. Methionine, lysine and threonine are synthesised from aspartate and belong to the aspartate family (Fig. 1). These amino acids are essentially required in the diets of nonruminant animals. Major crops, such as corn, soybean, and

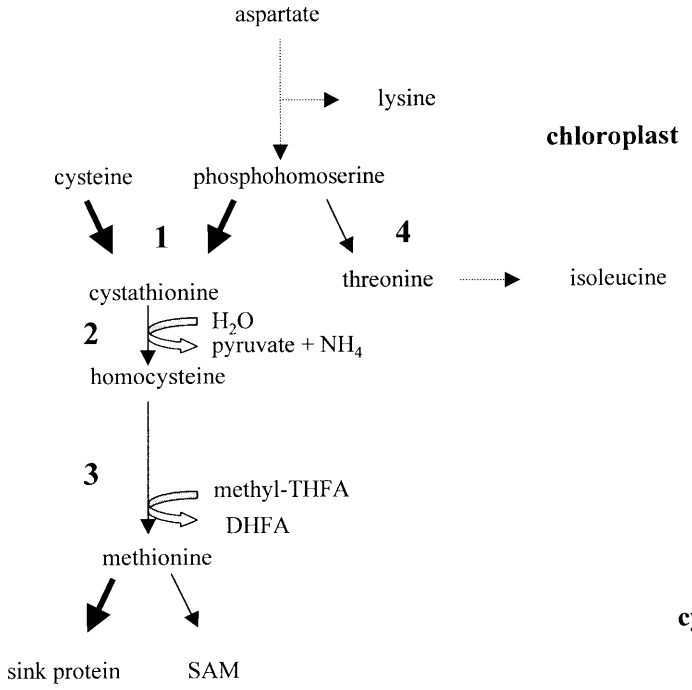

Fig. 1. Synthesis of the aspartate family of amino acids in plants. Bold arrows indicate traits to increase methionine content in plants. Numbers refer to the catalysing enzymes: *1*, cystathionine γ-synthase; *2*, cystathionine β-lyase; *3*, methionine synthase; *4*, threonine synthase. *THFA*, tetrahydrofolic acid; *DHFA*, dihydrofolic acid

rice, are low in one or more of these amino acids. Currently, these amino acids are supplemented to animal feed to allow optimal growth. Costs for the supplementation are direct expenses for farmers and therefore passed on to the consumers. Thus, increase of essential amino acids in crops has achieved a great deal of interest. In the last years transformation systems have been developed allowing the genetic manipulation of metabolic pathways in transgenic plants potentially leading to tailor made products. The introduction of novel functions or the repression of endogenous routes in transgenic plants enables the creation of metabolic configurations, which may be beneficial as long as cellular constituents necessary for growth and maintenance are not seriously affected. Thus, understanding the regulation of metabolic partitioning is a prerequisite for metabolic engineering. The use of mutants or transgenic plants altered with respect to the activity of a single enzyme allows studying the function of the target enzyme *in vivo*. Furthermore, ectopic expression of foreign enzymes enables the introduction of new pathways, allowing the manipulation of metabolite concentrations and/or end products.

Several reviews describe the regulation, expression, and manipulation of this pathway at the biochemical and genetic levels (Giovanelli, 1980; Bryan, 1990; Madison, 1990; Ravanel et al., 1998a; Matthews, 1999) and provide a comprehensive background to this review. This paper will focus on the more recent discoveries of the last few years, especially advances in understanding biochemistry, molecular biology, and applied aspects.

Manipulation of sink tissues

A major target has been the improvement of the amino acid composition of seed protein. Analysis of seed proteins revealed that in most cases the amino acid composition is unbalanced. Sulfur-rich proteins are thought to be very good targets for crop improvement, since important cereals and legume seeds often lack the agronomical desired amount of sulfur containing amino acids. The intention of plant genetic engineering has been to create crops that are tailored to provide better nutrition for humans and their domestic animals. In the past, several approaches have been started to improve crops via modifications of amino acid biosynthetic pathways, by protein modification, or by overexpression of suitable genes. A number of sulfur-rich plant seed storage proteins have been identified and their corresponding genes isolated (Altenbach et al., 1987; Higgins et al., 1986; Kirihara et al., 1988a,b; Pederson et al., 1986). One of the best known examples is the modification and hetorologous ectopic expression in seeds of a high-methionine 2S seed protein from Brazil nut in tobacco (Altenbach et al., 1989), canola (Altenbach et al., 1992), Narbon bean (Saalbach et al., 1995), and soybean (Sucki et al., 1984), which significantly increased the total sulfur amino acid content in seeds up to 30% (canola). Less accumulation of the Brazil nut protein was achieved when the gene was expressed in potato tubers (Tu et al., 1998). Only 0.2% of the total protein content was represented by the Brazil nut protein in tubers. In a second approach the expression of sulfur-rich proteins in leaves was possible by fusion of the seed proteins with an endoplasmatic retention signal (Wandelt et al., 1992; Khan et al., 1996). On the other hand designed protein molecules based on an a-helical coiled – coil structure by modification of the coding region have been expressed in transgenic tobacco plants which lead to an significant increase in methionine content in seeds (Keeler et al., 1997). However, all these approaches have one feature in common. Although all reports describe a significant increase in methionine content due to expression of methionine-rich proteins, the total amount of the sulfur-rich protein is not enough to reach the level of sufficient amount of approximately 5% of total protein.

Cystathionine γ-synthase catalysis the initial step of methionine formation

The first committed step of *de novo* methionine synthesis in plants is the formation of the thioether cystathionine catalysed by cystathionine γ-synthase (CgS, EC 4.2.99.9) from the substrates cysteine and phosphohomoserine. The reaction complies a transsulfurylation process via a γ-replacement reaction. This step separates methionine synthesis from the other amino acids belonging to the aspartate family because of its connection to the sulfur assimilation pathway. Furthermore, the carbon precursor of methionine synthesis is different than in yeast and bacteria. In yeast, methionine is synthesised by direct sulfhydration of O-acetylhomoserine while in bacteria in a different pathway with succinyl-homoserine as substrate. In microorganisms homoserine is the branch point intermediate leading to the synthesis of

methionine and threonine, whereas in plants phosphohomoserine is the last common intermediate to synthesise threonine and methionine (Datko et al., 1994). Therefore, cystathionine γ-synthase is the branch point enzyme leading to methionine synthesis competing with threonine synthase for the pathway intermediate, phosphohomoserine. Furthermore, for plant cystathionine γ-synthase enzymes a minor activity has been described accepting sulfide as substrate instead of cysteine (Kreft et al., 1994; Ravanel et al., 1995b). However, this alternative pathway seems to have only a minor physiological significance in plant cell metabolism for entry of reduced sulfur into methionine biosynthesis (MacNicol et al., 1981; Thompson et al., 1982a; Thompson et al., 1982b).

CgS enzyme is not feedback-inhibited by end products, but its expression is regulated by methionine. The feeding of *Lemna* with 2μM external methionine decreases CgS-specific activity to 15% of control, whereas supplementing with 36μM lysine and 4μM threonine to block methionine synthesis, increases CgS activity two- to threefold (Thompson et al., 1982a). Two lines of evidences have been published displaying the central role of cystathionine γ-synthase for the synthesis of methionine. On the one hand examples demonstrate that up-regulating enzymatic activity of CgS *in planta* by protein amount and messenger RNA stability, respectively, leads to an increase in methionine content. This has been shown by isolating an *Arabidopsis* (*mto* 1) mutant accumulating in a certain developmental stage up to 40-fold methionine (Inaba et al., 1994). Analysis of this mutant revealed that the CgS gene contains a mutation at position 81 leading to an exchange of Ser to Asn. In the recent publication, Chiba et al. (1999) could identify several mutants with higher methionine content resulting from mutated CgS genes. Analysis of one of the mutated genes revealed a more stable CgS transcript due to the introduced mutation when mutants were exposed to external methionine. Furthermore, they could demonstrate that the expression of the not mutated CgS gene is down-regulated by exogenously supplied methionine. Taken together, these findings demonstrate that CgS is transcriptionally and post-transcriptionally regulated by methionine or one of its metabolites and that the unregulated expression of CgS leads to an increase in methionine content. In a second approach Locke et al. (1997) expressed in a seed specific manner a maize cDNA in maize kernels. The analysis of seeds derived from transgenic plants showed an up to 5-fold higher methionine content than in control seeds indicating that CgS activity is limiting the flux towards methionine synthesis. Both examples are supported by the results obtained by Kim and Leustek (2000). In a *vice versa* experiment endogenous CgS mRNA and protein amount have been reduced by expression of a CgS antisense RNA in *Arabidopsis thaliana*. Transgenic plants with up to 9-fold less CgS activity revealed a methionine auxotrophy and developmental abnormalities resulting in severe growth stunting and an inability to flower. This is a further evidence for the essential role of CgS in methionine synthesis.

Cystathionine γ-synthase has been purified from various plants (Aarnes, 1980; Kreft et al., 1994; Ravanel et al., 1995b; Ravanel et al., 1998b) displaying sizes of the monomer between 34.5 kDa (wheat) and 53 kDa (spinach) with

native molecular masses between 155 kDa and 215 kDa, respectively for the CgS homotetramer. Enzyme activity reaches a pH optimum at a range of pH 7.5. The enzyme requires pyridoxalphosphate as a coenzyme for activity and operates by a hybrid ping-pong mechanism. cDNAs encoding CgS have been isolated from several plant species (Ravanel et al., 1995a; Kim and Leustek, 1996; Hesse et al., 1999; Hughes et al., 1999; Nam et al., 1999; Riedel et al., 1999) which are highly homologous to part of a genomic clone from *Arabidopsis thaliana* (Le Guen et al., 1994). The predicted proteins show high homology to each other and even to the corresponding bacterial genes. Southern blots suggests that soybean and potato CgS are encoded by single or low copy number genes, respectively (Hughes et al., 1999; Riedel et al., 1999).

Cystathionine β-lyase catalysis the cleavage of cystathionine to homocysteine

Cystathionine β-lyase (CbL, EC 4.4.1.8) catalysis the β-cleavage of cystathionine to homocysteine. CbL has been purified from spinach, *Arabidopsis thaliana*, and *Echinochloa colonum* (Droux et al., 1995; Ravanel et al., 1996; Turner et al., 1998). In anion-exchange chromatography two isoforms could be distinguished in extracts from spinach chloroplasts. One isoform is located in the chloroplast, whereas the other is cytosolic. This isoform turned out to be cysteine β-lyase, able to cleave cystathionine (Ravanel et al., 1998a). The native CbL protein with a molecular mass of 170 kDa consists of four identical subunits. CbL is PLP-dependent and maintains activity over a broad pH range, with an optimum between pH 8.3 and 9.0. CbL was cloned from *Arabidopsis* and potato by complementation for the *E. coli* methionine auxotroph GUC41, which lacks CbL activity (Ravanel et al., 1995a; Maimann et al., submitted) or by homologous screening (*A. thaliana*: Bork and Hell, 1997). Both predicted proteins contain a N-terminal extension showing features of a plastidial targeting sequence. There is evidence for only one gene encoding CbL in *Arabidopsis* and a low copy gene family in potato. The essential role has been demonstrated through the isolation of a Met-mutant from protoplast cultures of *Nicotiana plumbaginifolia* by Negrutiu et al. (1985). The mutant shows a severe phenotype, stunted in growth and development. Supply of homocysteine and methionine in spraying experiments is able to restore growth to wild type. A further indication for the essential role of cystathionine β-lyase are transgenic potato plants expressing antisense RNA (Maimann et al., submitted). Identified plants show the same severe phenotype as the tobacco mutant supporting the data obtained for the mutant. Intriguingly, metabolites of the aspartate pathway and sulfur assimilation are affected by this modification. Methionine decreases in content whereas cysteine, homoserine and cystathionine accumulate demonstrating the reduced flow of methionine precursors towards methionine synthesis. Unexpectedly homocysteine increases in content. About the reason we can only speculate.

Methionine formation is mediated by methionine synthase

The last step of methionine synthesis is localised in the cytosol (Wallsgrove et al., 1983) and catalysed by methionine synthase (MS, EC 2.1.1.14), which methylates homocysteine to form methionine, using N5-methyltetrahydrofolate as methylgroup-donor. The function of this enzyme is on the one hand the *de novo* synthesis of methionine and on the other hand the regeneration of the methyl group of S-adenosylmethionine. So far the molecular and biochemical characterisation of methionine synthase from plants is still limited. One of the reasons is the small amount of protein which is present in plants and on the other hand the substrate specificity of the enzyme. While bacteria are able to use monoglutameric methyltetrahydrofolate methionine synthase from *Catharantus roseus* (Eichel et al., 1995) accepts only the triglutameric isoform which is not freely available. Neither SAM nor cobalamin are required for activity of the cobalamin-independent methionine synthase as demonstrated for MS from *C. roseus*. The cDNA from *C. roseus* encodes for a protein with a molecular mass of 84.9 kDa, with no recognisable signal peptide.

Full-length cDNA has been described from *Coleus blumei* (Petersen et al., 1995), *Mesembryanthemum crystallinum* (U84889), *Chlamydomonas reinhardtii* (Kurvavi et al., 1995), and additionally to the EST sequences of MS (AA030695 and AA054818) from maize submitted to the GenBank by Basdorfer (1993).

In the last years a new debate is arising with respect to the localisation of the final step (Ravanel et al., 1998a). In a second model the plastidial synthesis of methionine is favoured. This assumption is supported by two indications. First, it was observed that pea chloroplasts and mitochondria are able to synthesise methionine *de novo* (Clandinin and Cossins, 1974; Shah and Cossins, 1970) and secondly, the photosynthetic protozoan *E. gracilis* Z. expresses besides a cytosolic cobalamin-independent methionine synthase, three isoforms of cobalamin-dependent enzymes located in chloroplasts, mitochondria, and cytosol (Isegawa et al., 1994). A similar distribution for higher plants could be assumed but has still to be proven for higher plants. The existence of additional methionine synthases would render plastids able to synthesise methionine *de novo*, as is the case for other aspartate-derived amino acids. The function of the current cytosolic isoform would be to recycle AdoMet during the transmethylation reactions.

Conclusion

In the last years an important progress has taken place to improve the knowledge about methionine synthesis in plants. The development of molecular probes and biochemical characterisation of the pathway enable us to investigate the regulation of methionine synthesis in more detail. Transgenic plants expressing genes from plant sources and *E. coli* can be used as a tool to give better insights into regulation of the pathway. Fundamental aspects concerning understanding regulatory patterns of methionine synthesis in plants is

crucial for improvement of nutritional quality of crops for food and feed. Several open questions are still existing and have to be answered. Examples presented in this paper suggest possible traits to increase methionine synthesis in plants in order to fulfil one of the aims of modern biotechnology to generate new and improved crops.

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