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Review Article

NUTRITIONAL QUALITY ENHANCEMENT OF PLANTS BY IMPROVING ITS METHIONINE CONTENT

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ABSTRACT

Human beings are only capable of synthesizing ten of twenty naturally occurring amino acids. The other ***essential amino acids*** are obtained from the diet. Cereal grains are often limiting for lysine, tryptophan and threonine, while the legume seeds have an adequate level of lysine but are limiting for the sulphur-containing amino acids, methionine and cysteine. Animals can convert methionine into cysteine, but not the reverse. Low level of methionine in plants diminishes their value as a source of dietary protein for human and animals. There are several attempts to improve the methionine level in plants. This study gives an overview of various technology for enhancement of methionine level in plants, including traditional plant breeding methods and selection of mutant; synthesis an artificial gene rich in methionine and cysteine residue; genetic modification to increase methionine storage in protein; genetic modification to increase methionine biosynthesis and co-expressing methionine-rich storage proteins with enzymes that lead to high soluble methionine level, with minimal interference on plant growth, phenotype and productivity. The studies have resulted in the identification of steps important for the regulation of flux through the pathways and for the production of transgenic plants having increased free and protein bound methionine. The goal of increasing methionine content, and therefore nutritive value, of plant protein is presently being achieved and will no doubt continue to progress in the near future.

Keywords: S-adenosylmethionine; Cystathionine gamma synthase; Methionine biosynthesis and metabolism; Methionine rich storage protein; Sulphur-containing amino acids; Nutritional improvement.

INTRODUCTION

Seeds are major sources of dietary protein for large vegetarian populations around the world and intensively farmed animals. Though, the protein in seeds can have a skewed amino acid composition due to the high abundance of a limited number of individual seed storage proteins. Deficiency of certain essential amino acids can be a cause of malnutrition in countries that are dependent on a diet of low diversity and can limit the efficiency of animal production [1]. Cereal grains are often limiting for lysine, tryptophan and threonine, while the legume seeds have an adequate level of lysine but are limiting for the sulphur-containing amino acids, cysteine and methionine. Animals can convert methionine into cysteine, but not the reverse, thus defining methionine as an essential amino acid. Methionine levels are generally not limited in human food in Western countries due to the significant consumption of livestock products, meat, eggs, and milk, which generally contain adequate levels of this essential amino acid. However, methionine deficiencies in plant-

derived feed for farm animals limit animal growth as well as animal products, such as reduced milk production by dairy animals, wool growth in sheep, and meat quality [2]. In developing countries plant-derived foods are predominant, up to 90% of food intake can be derived from a single crop species which is low in methionine content, leading to protein deficiency syndrome in humans, referred to as Protein-Energy Malnutrition (PEM). Due to importance of methionine in human food and animal feed, many efforts have been made to produce plants having higher methionine content. Recent developments in Recombinant DNA Technology, Plant tissue culture and *in vitro* regeneration are proposing new ways of increasing the level of essential amino acids, including methionine, by manipulating existing genes and/or introducing foreign genes into plants. Three important metabolic functions of methionine are: *1*) trans-methylation to form a primary methyl donor, *S*-adenosylmethionine (SAM), which methylates compounds to form products such as creatine and phosphatidylcholine, follow-on in the product of methylation homocysteine being produced; SAM can also be decarboxylated to form decarboxylated SAM, which then provides an aminopropyl group for polyamine synthesis after which methionine will be reproduced; SAM also influence DNA synthesis and repairs the expression of genes; its deficiency has been associated with DNA fragmentation and strand breaks. *2*) transsulfuration to form cysteine, which in turn is incorporated into glutathione or catabolized to taurine; and *3*) protein synthesis. Methionine can also go into the body pool by re-methylation of homocysteine or protein breakdown [3].

In developing countries in which plant-derived foods are predominant, low level of methionine can lead to non-specific signs of protein deficiencies in humans, such as decreased blood proteins, lowered resistance to disease and methylation retarded disorders, such as fatty liver, atherosclerosis, neurological disorders and tumorigenesis, retarded mental and physical development in young children. In animals, methionine depletion lowers the threshold of chemical-induced toxicity, suggesting that this may be significant in carcinogenesis processes [4].

A prominent role of methionine is its powerful antioxidant action against free radicals produced in the natural metabolic processes of the body. Methionine is also an excellent source for the essential mineral sulphur, which quickly inactivates free radicals produced in the body. Patients of Gilbert's syndrome, which results in an abnormality of liver functioning, are also benefited by supplements of the amino acid methionine. It is also required during the synthesis of collagen, nucleic acids and different proteins found in almost every cell of the human body as well as it is a constituent of many enzymes and proteins found in different parts of the body. The amino acid methionine reduces the level of histamine present in the body which is very useful for people affected by schizophrenia and related conditions, in which the levels of histamine are generally higher than those found in normal healthy adults. It also promotes the excretion of estrogen from the body of women. In the body, methionine can be converted into the amino acid cysteine, which itself is a precursor of the vital compound called glutathione. Glutathione is a vital neutralizer of toxins present in the liver; the chemical thus protects the liver from the damaging effects of toxic compounds produced as a result of general metabolism. Glutathione is thus afforded a level of protection by methionine, as levels of methionine inhibit the depletion of glutathione when the body becomes overloaded with accumulated toxins and chemicals.It is also believed that glutathione carries nutrients to lymphocytes and phagocytes, important immune system cells. The levels of the neuro-transmitting substances such as dopamine, nor-epinephrine and epinephrine are increased by methionine. Methionine is also used to bring relief from chronic pain, controlling hypertension, lower the potency of allergic symptoms, as an aid to reduce all kinds of inflammation, to lower cholesterollevel and to protect the person from the bad effects of aspirin and related chemicals.

Food sources which are abundant in methionine include foods such as beans, various lentils, eggs, fish, meat, onions, garlic, soybeans, seeds and yogurt. Methionine is used by the body to synthesize a particular molecular choline -a brain food. Diets must be supplemented either with choline or lecithin (another compound high in choline) so as to ensure an adequate supply of methionine at all times. Daily amino acid requirements of a person may be determined by their body weight and this requirement spans a range of values for different body types. About 800 - 1,000 mg of methionine is

required by an average sized adult per day; this is an amount of the amino acid that is exceeded by the total methionine intake found in the majority of diets in the western world. Methionine supplementation is therefore unlikely to be of great benefit to the majority of people in the West[5]

Methionine Biosynthesis and Metabolism in plant

In plants and micro-organisms, methionine is synthesized via a pathway that uses both aspartic acid (figure below) and cysteine. Methionine is derived from cysteine by the sequential action of three enzymes, the first of which, cystathionine gamma-synthase (CGS), combines O-phospho-homoserine from the aspartate amino acid pathway and cysteine. O-phospho-homoserine (OPHS), which is a common substrate for both threonine synthase (TS) and cystathionine g-synthase (CgS). OPHS is directly converted to threonine by TS, while methionine is synthesised in three steps. Condensation of cysteine and OPHS is catalysed by CgS resulting in cystathionine, which is subsequently converted to homocysteine by cystathione b-lyase, and methionine by methionine synthase[6].SMM is synthesized from Methionine by Met S-methyltransferase (MMT) and is recycled back to Methionine by homo-Cystein S-methyltransferase (HMT)[7].

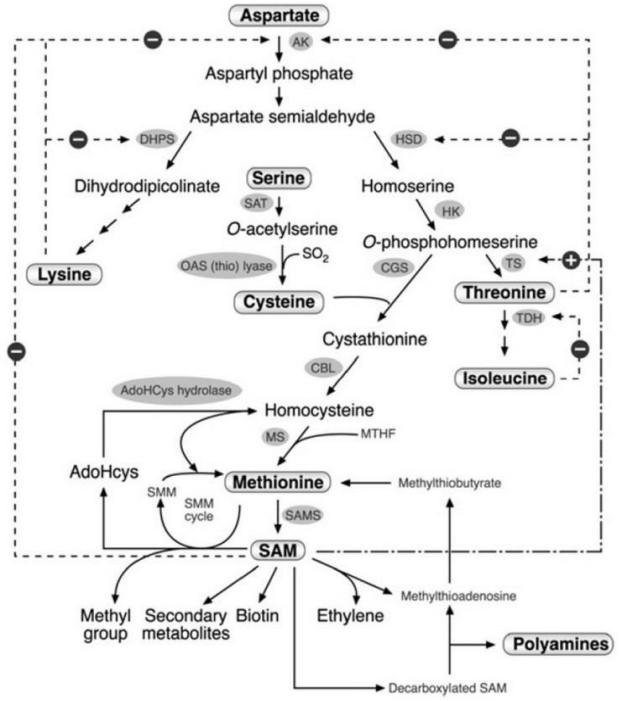

Figure: Methionine biosynthesis and Metabolism (Galili et al., 2008) Schematic diagram of the metabolic network containing the aspartate family pathway, methioninemetabolism, and last two steps in the cysteine biosynthesis. Only some of the enzymes and metabolites are specified. Dashed arrows with a "minus" sign represent feedback inhibition loops of key enzymes in the network. The dashed and dotted arrow with the "plus" sign represents the stimulation of TS activity by SAM.

Abbreviations: AK, aspartate kinase; DHPS, dihydrodipicolinate synthase; HSD, homoserine dehydrogenase; HK, homoserine kinase; TS, threonine synthase; TDH, threonine dehydratase; SAT, serine acetyl transferase; OAS (thio) lyase; O-acetyl serine (thio) lyase; CGS, cystathionineγ-synthase; CBL, cystathionineβ-lyase; MS, methionine synthase, SAM, S-adenosyl methionine; SAMS, S-adenosyl methionine synthase; AdoHcys, adenosylhomocysteine; SMM, S-methylmethionine; MTHF, methyltetrahydrofolate.

STRATEGIES TO ENHANCE METHIONINE CONTENT

By nucleotide modification:

One approach used has been to isolate the gene for a sulphur-poor protein and modify and enrich its nucleic acid sequence for the sulphur containing amino acids. This has been attempted for proteins such as Beta-phaseolin from *Phaseolus vulgaris* and Vicilin from *Viciafaba*, but the modified proteins were either unstable of contained too little methionine to make them useful. Another approach has been to construct totally artificial genes that code for proteins containing a high sulphuric amino acid content. One such totally synthetic protein, containing 13% methionine residues, has been expressed successfully in sweet potato (*Ipomoea batatas*)[8].

By mutant selection:

A number of plant mutants with increased levels of methionine have been isolated by selection on **ethionine**, *a toxic analogue of methionine*. Using this approach, three distinct groups of mutated genes have been characterized in *A. thaliana*, and have been found to define three enzymes from the methionine and S-adenosylmethionine biosynthetic pathways [9]. A soya bean mutant with increased total methionine in its mature seeds was recently isolated using an initial screen for ethionine resistance. The outcome of this work was a soya bean variant that was predicted to supply enough methionine for optimal animal nutrition without supplementation with synthetic amino acid [10]. A natural

maize mutant was identified by screening for germination on media containing lysine plus threonine, a combination that inhibits flux through the aspartate amino acid biosynthetic pathway, leading to methionine starvation. The mutant seeds had high levels of a specific, methionine-rich seed storage protein, the sulphur-rich δ-zein. The modified maize had methionine levels theoretically high enough to obviate the need for synthetic methionine in animal feed formulations containing the Genetically Modified (GM) seed[11].

By expressing methionine-rich storage proteins in transgenic plants: Several methionine rich proteins have been identified in **Maize** (21-kDa zein, 28% methionine), **Rice**(10-kDa prolamin, 20% methionine), **Sunflower(**2S sunflower seed albumin,16% meth.), **Brazil nut**(Brazil nut albumin, 18% methionine) and **Sesame** 2S albumin(1.656 methionine g/100g) [8].

With the aim of improving the nutritive value of rice a chimeric gene encoding a precursor polypeptide of sesamesulfur-rich seed storage protein- 2S albumin, was expressed in transgenic rice plants under the control of the glutelin promoter [12]. It was observed that rice grains harvested from the first generation of ten different transformed lines inherited the transgene, as well as the accumulated sesame 2S albumin was seemingly processed correctly as its mature form in sesame seed. This transgene was explicitly expressed in maturing rice seeds with its encoded sesame 2S albumin completely accumulated in the seeds. As a result, the crude protein content in rice grains from five putative homozygous lines was increased by 0.64-3.54%, and in these transgenic rice grainsthe methionineand cysteine contents of were respectively elevated by 29-76% and 31-75% compared with those of wild-type rice grains [13].For improving the nutritive value of legume crop, a chimeric gene specifying seed-specific expression transformed in lupin. The construct contained three chimeric genes: *35S-uidA*, encoding the reporter enzyme b-glucuronidase (GUS); ***vicssa***, encoding the sunflower seed albumin; and *35S-bar* encoding the selectable marker phosphinothricin-acetyltransferase. The transgenic seeds contained less sulfate and more total amino acid sulphur than the nontransgenic parent line. This was associated with a 94%increase in methionine content and a 12% reduction in cysteine content [14].

The Brazil nut 2S albumin has been expressed in a number of seeds including tobacco, narbon bean, soya bean and canola with increases in total seed methionine of 30–100% when compared with wild-type. The levels of seed methionine in the GM soya beans and narbon beans were predicted to be sufficient for optimal animal nutrition; on the other hand, the potential human allergenicity of the Brazil nut protein has prevented it from being used commercially [15].

The β-zein::3HA gene was overexpressed under the control of the constitutive promoter of the cauliflower mosaic virus(CaMV) 35S in transgenic tobacco and alfalfa plants. Results demonstrated that the added methionine enhanced the accumulation of the 15 kDazein::3HA in transgenic alfalfa and tobacco BY2 cells, but in not whole transgenic tobacco plants. Similar phenomena also occurred in Tobacco, but were significantly less pronounced. The result demonstrate that the accumulation of the 15 kDazein::3HA regulated in a species-specific manner and that soluble methionine plays a major role in the accumulation of the 15 kDazein in some plant species but less so in others [**16**].

Byengineering the methionine biosynthetic pathway in plants

Over-expression of Cystathionine gamma-synthase:- Sulphur is taken up from the soil in the oxidized form of sulphate and is subsequently reduced in the plastids of plant cells, then integrated into an amino acid backbone derived from serine via the action of the enzyme serine acetyl-transferase. Cysteine is the product of this reaction, the first stable reduced sulphur metabolite in the cell as well as a substrate for many other biochemical pathways. From cysteine, methionine is derived by the sequential action of three enzymes, the first of which, cystathionineγ-synthase (CGS), combines O-phosphohomoserine from the aspartate amino acid pathway and cysteine. Constitutive expression of a CGS enzyme from *A. thaliana* in Genetically Modified (GM) tobacco or GM alfalfa increased free methionine in the leaves, but had no significant effect on protein-bound methionine [17]. Conversely, in a rare exception to this generalization, expression of a mutated form of CGS in GM tobacco caused in not only a large increase in free methionine in the leaves, but also a twofold increase in protein-bound methionine in comparison to the controls. The high-methionine GM plants showed a severe, unusual phenotype[15].

Over-expression of Cystathionine gamma-synthase with Maize beta-zein a combined approach: Studies in *Arabidopsis* divulged that the activity of CgS, which is found in the chloroplast, is regulated at both the transcript and protein levels indirectly by methionine via SAM. CgSencompasses approx. 100 amino acids in the N-terminal that controls CgS activity. Lately, another C*gS*transcript was found in *Arabidopsis*, which contains an internal 90 nucleotide deletion at the N-terminal region (C*gS*Δ90). This was confirmed that transgenic tobacco plants over-expressing C*gS*Δ90 exhibit a significantly higher level of methionine than plants over expressing the full-length CgS. To increase the integration of free methionine into a storage protein the C*gS*Δ90 was co-transformed with the methionine-rich *15-kD* β-*zein*. Results demonstrated a two- to six-fold increase in the free methionine content and in the methionine content of the zein-containing protein fraction of the transgenic tubers. Furthermore, in line with high methionine content, the amounts of soluble isoleucine and serine were also increased.Though, all of the lines with high level of C*gS*Δ90 expression were phenotypically abnormal showing severe growth retardation, changes in leaf architecture and 40- to 60% reduction in tuber yield. In addition, the colour of the transgenic tubers was altered due to the reduced amounts of anthocyanin pigments. The mRNA levels of phenylalanine ammonia-lyase (*PAL*), the enzyme catalysing the first step of anthocyanin synthesis, were decreased. The level of *PAL* mRNA and consequently the amount of anthocyanin pigments are reduced in the C*gS*Δ90 transgenic tubers suggesting that production of anthocyanins and methionine synthesis is linked [24].

Reducing expression of Threonine synthase(TS) by Antisense inhibition approach: In plants, the branch point intermediate of methionine synthesis is O-phospho-homoserine (OPHS), which is a common substrate for both threonine synthase (TS) and cystathionineγ-synthase (CgS). OPHS is directly converted to threonine by TS, whereas methionine is synthesised in three steps. Condensation of cysteine and OPHS is catalysed by CgS resulting in cystathionine, which is subsequently converted to homocysteine by cystathioneβ-lyase, and methionine by methionine synthase [MS][6]. CgS and TS compete for the common substrate OPHS. In vitro activity measurements indicate that TS in plants has 250-500-fold higher affinity for OPH compared to AtCGS, causing reduced OPH availability for methionine synthesis. It is an interesting feature of plants that TS is activated through *S*-adenosyl-L-methionine (SAM), a metabolite derived from methionine[18]. TS are the major control point for methionine biosynthesis[19]. Reduction of TS activity to 6% of the wild-type potato by antisense inhibition led to a 2- to 240-fold increase in methionine content in leaves and a 2- to 30-fold increase in methionine content in tubers. Strong reduction of TS activity and/or substantial accumulation of methionine, however, were accompanied by severe phenotypic changes and acute reduction in tuber yield [20].

Expression of homo-Cystein S-methyltransferase (HMT): Another important metabolite regulating Met metabolism in plants is *S*-methyl-Met (SMM), a Met storage and phloem mobile metabolite that can be efficiently transported from leaves to developing seeds. SMM is synthesized from Met by Met *S*-methyltransferase (MMT) and is recycled back to Metby homo-Cysmethyltransferase. Though, despite the efficient transport capability of SMM, Arabidopsis and maize *mmt* mutants, which are incapable to synthesize SMM, grow and reproduce normally, suggesting a minimal regulatory role for SMM in sulfur transport of at least these two species. Nevertheless, a recent report showed that a mutant Arabidopsis plant overaccumulating Met in its seeds is due to a mutation eliminating the activity of HMT2, one of the three Arabidopsis HMT isozymes that recycle SMM into Met. The *HMT2* gene is expressed in vegetative tissues where it apparently shifts the balance from Met to SMM in these source tissues. The accumulated SMM in this mutant seemingly transports more efficiently than Met into the sink tissues, where it is converted back to Metheonine by the two other HMT isozymes [21]. Why plants need a subtle balance between Met and SMM is an interesting question, but surely the increased accumulation of Met in the seeds of the *HMT2* mutant suggests a novel approach to increase the nutritional value of crop plants [22].

Reducing expression of SAM synthase: The studies analysing the Arabidopsis mutant (mto3) in which one of its isozymes for SAM synthase exhibit a reduced expression level, have shown that methionine content increased significantly to over 200-fold compared to wild type plants.

SAM synthase silenced Arabidopsis plants show severe phenotype abnormalities but methionine level increased 250-fold. These results suggest that SAM has more importance for plant metabolism. Therefore it is not expected that this approach for increasing soluble methionine will be used in the future [9].

Reducing expression of methionine γ-lyase:

In Arabidopsis a sequence is similar to the bacterial gene encoding the methionine γ-lyase gene. cDNA of this gene cloning reveal that this cytosolic enzyme is abundant in all plant organs except in the seeds, and it catalyses the conversion of methionine into methanethiol, α-ketobutyrate and ammonia. This gene is expressed under standard growth condition and strongly induced when the cells accumulated methionine. Enzyme has a high K_m value for methionine, indicating that this pathway operates preferentially when methionine has accumulated above a certain value in the cytoplasm. SMM and Methionine content(9-fold) increased in Arabidopsis leaf when Knocked out the methionine γ-lyase gene, but did not affect methionine level under normal growth conditions. This finding suggest that this catabolic pathway play a role during sulphate starvation, but since the level of methionine is not altered under this stress, this situation is not clear and further studies are required[23].

CONCLUSIONS

In recent years many progress has been made to increasing methionine content in vegetative and seed tissues of plants, the factors that regulate the methionine level in these tissues is not completely known and further studies are required. Genomics approaches such as, micro-arrays, proteomics, metabolite profiling and flux analysis measurements, will give greatly contribute to our knowledge in the future and it is expected that they will strongly penetrate into the metabolism of amino acids and sulphur metabolites. Knowledge about plant metabolism will help in manipulating the methionine metabolism and in crop plants having higher levels of methionine, thus getting better nutritional quality of plants.

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