

Improving Nutritional Quality of Plant Proteins Through Genetic Engineering

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Abstract: Humans and animals are unable to synthesize essential amino acids such as branch chain amino acids methionine (Met), lysine (Lys) and tryptophan (Trp). Therefore, these amino acids need to be supplied through the diets. Several essential amino acids are deficient or completely lacking among crops used for human food and animal feed. For example, soybean is deficient in Met; Lys and Trp are lacking in maize. In this mini review, we will first summarize the roles of essential amino acids in animal nutrition. Next, we will address the question: “What are the amino acids deficient in various plants and their biosynthesis pathways?” And: “What approaches are being used to improve the availability of essential amino acids in plants?” The potential targets for metabolic engineering will also be discussed, including what has already been done and what remains to be tested.

Keywords: Protein nutritional quality, Essential amino acids, Lysine, Methionine, Tryptophan, Genetic engineering.

1. INTRODUCTION

One of the primary components of the living cells is protein, whose building blocks are amino acids. Animals, including humans, can only produce about half of the 20 common amino acids needed for life; the rest – known as essential amino acids – must be obtained via diet. Plants are the major source of dietary proteins for both humans and other animals. In plants, amino acids are synthesized from basic elements such as carbon and oxygen from the air, hydrogen from water and nitrogen from the soil. Recent estimates indicate that more than half of the world’s population suffer from diseases caused by inadequate supplies of minerals, vitamins and essential amino acids [1]. In developing countries, these problems are intensified because the food supplies mostly rely on a single crop, which often provides an imbalanced or inadequate nutritional profile [2]. Most plant proteins are incomplete sources of amino acids. Among essential amino acids, methionine (Met), lysine (Lys), and tryptophan (Trp) are present in limited quantity in a variety of plants, particularly in cereals and legumes, the major crops for food and feed. Increasing essential amino acid content in legumes and cereals is a key approach to fighting malnutrition in developing countries.

2. NUTRITIONAL ROLES OF ESSENTIAL AMINO ACIDS

S-adenosylmethionine (SAM), a substrate involved in epigenetics and in fatty acid oxidation, is made from Met. As a direct precursor of SAM, Met is known as an important

methyl donor indirectly (via SAM) in human metabolism [3, 4]. In most mammalian cells, homocysteine accumulation causes serious injury to endothelial cells, leading to atherosclerosis [4, 5]. However, the accumulated homocysteine can be reduced by re-methylation into Met [6, 7]. Several diseases in mice caused by methionine deficiency have been reported [4, 8]. Met residues in proteins, which are the most susceptible to oxidation by oxidative stress, are considered natural antioxidants when coupled with methionine sulfoxide reductases [9]. In animals, including humans, lack of Met also leads to methylation-related disorders such as fatty liver, tumorigenesis, neurological disorders and atherosclerosis [10-13]. The limited availability of Met leads to DNA strand breakage and fragmentation, which may be significant to the carcinogenic process [14]. In livestock, Met deficiency limits animal products such as quality of wool in sheep, milk and milk-derived products [15].

Lys plays several important roles in defense mechanism of animals, including humans. Lys deficiency could decrease the defense ability of mammalian cells to viruses [16, 17]. Lys deficiency is also the major cause of the osteoporosis in humans [18]. Stress-induced anxiety in mice was reportedly caused by the lack of Lys in their daily diets [19, 20]. A diet deficient in Met and Lys intake can reduce biological value of plant-based nutrition to 50-70%, compared to a balanced diet with high abundance of essential amino acids [21]. In humans, defects of Lys metabolism may result in familial hyperlysinemia due to genetic disorder. LKR-SDH gene mutation also causes familial hyperlysinemia [17].

Unlike other amino acids, Trp acts as a precursor to several neurochemicals, such as serotonin and melatonin. Dietary requirements for Trp in animals, including humans, were described [22]. Trp deficiency in daily diet leads to several symptoms in animals. Stresses caused by the loss of

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sleep were reportedly caused by the lack of Trp in daily food [23]. Loss of Met, Lys, and Trp in diet caused several symptoms, include weight loss, decrease in muscle mass and stress caused by losing sleep. These things can happen in both humans and animals. The quality of animal feed is determined by nutritional value of feed proteins, which could enhance the content of essential amino acids. It is possible to contribute a balanced diet for animals and, therefore, offers good growth and reproduction.

3. AMINO ACIDS LACKING IN PLANTS PROTEINS

Grains and grain legumes represent key sources of proteins for human and animal consumption. Grain legumes, such as soybean and soy products, beans, lentils, ground nuts, peas and chickpeas, are staple foods in many parts of the world. The world's total value of legume crops is estimated at US\$200 billion annually. Akibode and Maredia reported that many of the poorest countries in the world derive 10-20% of their total dietary energy supply from legumes [24]. In addition, cereals supply 68% food of nutritional calories worldwide. However, legumes and cereals contain limited levels of several essential amino acids. For example, pulse storage proteins are rich in Lys but lack sulfur-containing amino acids, mainly Met. In contrast, cereal crops are almost devoid of Lys and Trp [21, 25-27].

Lys and Trp levels, according to amino acid analysis and experiment results, are lowest in maize (*Zea mays*), lower than the minimum requirements for monogastric animals including humans [28]. Alcohol-soluble protein fractions of zein (prolamine), the most abundant protein in maize endosperm (40-60%), contain high levels of glutamine, proline, leucine and alanine but are deficient in Lys and Trp. Maize grains contain between 8.5% to 13.6% in proteins (*w:w*), of which Lys is 2.5 - 3.6% and Trp is 0.37 - 0.67% [29]. In 2005, Anjum and co-workers suggested that Lys is the most limiting amino acid in whole wheat flour [30].

In legumes, Met is considered the first limiting amino acid. Even soybean protein, considered the best plant protein, is not a complete protein because of the low level of Met content [31]. Scherz *et al.* [32] reported that stable foods, such as legumes, cereals, and nuts, are significantly deficient in Lys, Trp and Met content relative to animal-derived proteins (Table 1). In addition, according to a report issued in 1985 by WHO expert consultants, cereals supplied only 1.5-4.5 mol% of Lys compared to the requirement of 5.5 mol%, whereas protein from legumes supplied only 1-2 mol% sulfur-amino acid (Met and Cys) compared to the 3.5 mol % recommended by the WHO [33]. Another major crop for protein is potato. Although protein from potato is seen as better than that of cereal and legume crops, it is still suboptimal for Lys and several essential amino acids [34].

Deficiencies in several essential amino acids are partly due to the nature of their biosynthesis pathways and the long distance transportation from synthesis organs to storage organs. For examples, in plants, asparagine and glutamine were transported from leaves to developing seeds and were then converted to Lys. Another branch in the aspartate pathway can also synthesize Met [35]. In Lys biosynthesis pathway, dihydrodipicolinate synthase (DHDPS) plays an important role for Lys accumulation. The rate-limiting step in the

Lys catabolism *in vivo* is mainly catalyzed by DHDPS, the first unique enzyme in the pathway [36]. Pyruvate and aspartate semi-aldehyde are catalyzed to 4-hydroxy-2,3,4,5-tetrahydrodipicolinate by DHDPS. Recombinant DHDPS from several plant species were characterized and the enzyme was found to be extremely sensitive to feedback inhibition by Lys even at very low levels [36, 37]. Seed-specific overexpression of feedback-insensitive bacterial DHDPS in various plant species was shown to increase accumulation of Lys in seeds [36, 38]. In addition, enhanced Lys levels were also linked to the knockout of Lys catabolic enzymes; lysine-ketoglutarate reductase (LKR) catalyzes the formation of saccharopine and saccharopine dehydrogenase (SDH) hydrolyzes saccharopine to glutamate and α -amino adipic acid. These two enzymes are present in a single bifunctional polypeptide. The LKR-SDH knockdown *Arabidopsis* mutant was shown to increase Lys content in the seeds [38] and the combination of overexpressing a bacterial DHDPS and LKR-SDH knockdown resulted in 80-fold increases in free Lys in the seeds. In maize grains, *opaque2* transcription factor indirectly regulates the LKR-SDH gene expression as well as some classes of zein-coding genes. In developing grains, the LKR-SDH biofunctional enzyme is localized in the outer endosperm layer and little was detected in the embryo. Hence, there are a low levels of Lys in the outer endosperm layers where Lys may be transported from the embryo to be degraded [39]. Thus, Lys metabolic regulatory in the seeds may be different among plant species [21].

Table 1. Lys, Met and Trp content in the major protein sources worldwide.

Food	Lys*	Met	Trp
Beans	1870	260	230
Peas	610	100	100
Soybean	1900	580	450
Maize	290	190	70
Barley	380	180	150
Rice	290	170	90
Wheat	380	220	150
Potatoes	130	30	30
Nuts	750	330	450
Bovine meat	2310	650	290
Pig meat	2200	720	310
Freshwater fish	2020	700	260
Marine fish, other	2050	600	240

*According to [32]

*milligrams of limiting amino acid per 100 gram of food.

Met is generally regulated by its synthesis and metabolism as well as a complex network of interactions with the Lys biosynthesis pathway, because they are both derived from the aspartate family pathway. As a result, the *Arabi-*

dopsis mutant with increased Lys contains lower Met [38]. There is evidence that Met biosynthesis is regulated by the allosteric enzyme threonine synthase (TS) in competition with enzyme cystathionine synthase (CGS) for an important common substrate of the aspartate pathway, O-phosphohomoserine (OPH). OPH can be catalyzed to threonine by TS or cystathionine by CGS [35]. However, OPH is used by TS more than CGS due to higher TS activity in plants [36, 40]. In *Arabidopsis*, the TS level was found to be seven times greater than that of AtCGS, resulting in a four-fold threonine content compared to Met [40]. TS was higher in level and in activity measurements, indicating that its affinity for OPH is 250 to 500 times higher than that of CGS. This revealed that threonine is produced more than Met in the aspartate pathway [36, 40].

In the Trp biosynthesis pathway, Anthranilate synthase (AS) is an enzyme catalyzing the conversion of chorismate to anthranilate. It is the initial and important rate-limiting step. The enzymatic activity of AS is regulated by feedback inhibition by Trp [41]. Bypassing feedback inhibition was demonstrated to be feasible to increase dietary Trp in the seed of rice [42], in tobacco leaves [43] and in the roots of the forage legume *Astragalus sinicus* [44]. In addition, it was also found that the sufficiency of AS mRNAs tightly controls Trp accumulation in plants. AS mRNAs was induced under biotic and abiotic stress such as wounding, pathogen or fungal infection. Both AS enzymatic activity and protein level can influence Trp biosynthetic capacity [42].

4. BIOSYNTHESIS PATHWAYS OF ESSENTIAL AMINO ACIDS IN PLANTS AND MICRO-ORGANISMS

4.1. Lysine and Methionine Biosynthesis

Both Lys and Met are two well-known amino acids belonging to the aspartate pathway (Fig. 1) [26]. In the plant kingdom, these are almost always synthesized within the chloroplast and share the three initial steps as a common pathway. The first enzymatic step of the aspartate family is catalyzed by aspartate kinase (AK) which has multiple isoenzymic forms. At least two forms are found in plants. Using ATP and Mg^{2+} , the formation of phosphorylation of aspartate leads to the formation of β -aspartyl phosphate, which is subsequently oxidized to aspartate semialdehyde (ASD) by aspartate semialdehyde dehydrogenase (ASDH). In the last step of the common pathway, ASD forms either dihydrodipicolinate (DHDPS), a precursor of diaminopimelic acid and lysine, or O-phosphohomoserine (OPH). OPH may be channeled to threonine or Met [36].

Among the major regulatory steps controlling Lys synthesis in plants, DHDPS activity is the most prone to feedback inhibition by Lys. Moreover, DHDPS is about 100 times more sensitive to Lys inhibition in plants than in *Escherichia coli* [45]. Also, *E. coli* containing lysine-insensitive DHDPS contained increased levels of free Lys [45]. DHDPS cDNAs have been isolated from a range of plant species such as wheat, maize, tobacco, soybean, *Arabidopsis*, and rice [36, 37, 46]. In plants, diaminopimelate (DAP) has been known as a key intermediate in the Lys biosynthesis pathway. DAP also plays a central role in peptidoglycan biosynthesis, the main component of eubacterial cell walls.

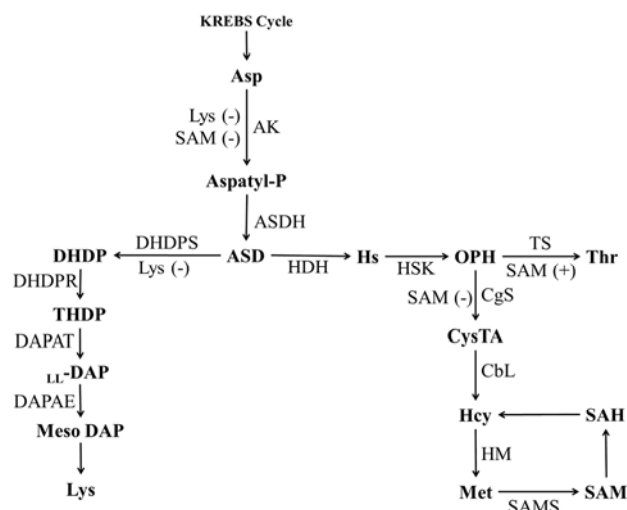


Fig. (1). The Aspartate pathway leading to the biosynthesis of Met and Lys. Asp: Aspartate, AK: Aspartate kinase, ASD: aspartate-semialdehyde, ASDH: aspartic semialdehyde dehydrogenase, DHDPS: dihydrodipicolinate synthase, DHDPS: dihydrodipicolinate, Hs: homoserine, HDH: homoserine dehydrogenase, OPH: O-phosphohomoserine, HSK: homoserine kinase, Thr: Threonine, TS: Thr synthase, CysTA: cystathionine, CgS: cystathionine γ -synthase, Hcy: Homocysteine, CbL: cystathionine β -lyase, SAH: S-adenosylhomocysteine, HM: homocysteine methyltransferase, SAM: S-adenosyl-methionine, SAMS: S-adenosyl-methionine synthetase, THDP: tetrahydro-dipicolinate, DAPAE: DAP epimerase, DAP: diaminopimelate, DAPAT: DAP-aminotransferase.

Recent studies indicate differences among pathways to Lys in plants, chlamydia and cyanobacteria [47-49]. After DHDPS formation, its reduction to tetrahydrodipicolinate (THDP) is catalyzed by DHDPS reductase. At that point, the pathways of plants and bacteria become distinct. In some bacteria including some *Bacillus* species, THDP directly transfers to meso-DAP by the enzyme DAP dehydrogenase. The majority of bacterial species use a multiple pathway to synthesis Lys: N-succinylation/acetylation followed by transamination and desuccinylation/deacetylation [48]. Plants convert THDP indirectly to L,L-DAP by DAP-aminotransferase. Both plants and bacteria convert L,L-DAP to meso-DAP by DAP epimerase. The final step is decarboxylated to form lysine [47-49].

In the plant kingdom, OPH appears in the common branch point intermediate with the synthesis of Met and threonine. OPH is a common substrate for both enzymes: threonine synthase (TS) and cystathionine gamma-synthase (CgS). Met is converted from OPH in three enzymatic steps: CgS catalyses the formation of the thioether cystathionine from substrates of cysteine, the sulfur atom donor and OPH by trans-sulfuration. This process is only found in plant plastids like *Arabidopsis thaliana* [35, 50]. The next step converts the intermediate to homocysteine and subsequently to Met. In this mechanism, reactions are catalyzed by the enzymes CgS, cystathionine β -lyase (CbL), and methionine synthase (MS), in that order. Almost 80% of Met is converted into SAM and the remainder takes part as a protein constituent [51]. However, in yeast and bacteria, the nitrogen/carbon precursor for Met synthesis is distinct. In yeast,

Met appears to be a direct result from sulphydration of O-acetylhomoserine. In bacteria, succinyl-homoserine as substrate has been investigated in Met synthesis. Another comparative observation between plants and microorganism is that homoserine is the intermediate compound which leads to Met and threonine in microbes. In plants, OPH is the last common intermediates of Met and threonine biosynthesis [52].

4.2. Tryptophan Biosynthesis

The Trp biosynthetic pathway in plants not only produces Trp but also associates with a range of secondary metabolites such as auxin, indole glucosinolates and other secondary compounds. Trp is synthesized from chorismate in chloroplast (Fig. 2). Anthranilate synthase (AS) catalyzes the first reaction of the Trp biosynthesis which converts chorismate and an amine donor (usually glutamine) to form anthranilate; its activity is subject to feedback inhibition by Trp. Microbial and plant anthranilate synthase is a holoenzyme consisting of two α and two β subunits [53]. The β subunits of AS were considered to catalyze glutamine as the donor of ammonia, used by α -subunits of AS to transfer chorismate to anthranilate. In the subsequent step, anthranilate phosphoribosylanthranilate transferase (PAT1) catalyses a conversion of anthranilate and phosphoribosylpyrophosphate to phosphoribosylanthranilate and inorganic pyrophosphate. The third enzyme in the biosynthesis of Trp is phosphoribosylanthranilate isomerase (PAI) activity converting phosphoribosylanthranilate to 1-(O-carboxyphenylamino)-1-deoxyribulose-5-phosphate (CDRP). Then, indole-3-glycerol phosphate synthase (IGPS) accepts CDRP as the substrate which is transferred to indole-3-glycerol phosphate [54]. IPGS in plants and micro-organisms exhibit different characteristics. In bacteria and fungi, IPGS is a polyprotein with multiple domains and each domain catalyzes a separate reaction. For examples, IPGS is fused with PAI to form a bifunctional enzyme in *E. coli*, *Salmonella typhimurium* and *Vibrio parahaemolyticus*. However, in plants IPGS is a monofunctional enzyme [54].

Trp synthase (TS) appears to be a heterodimer of α (TS α) and β (TS β) subunits. TS α cleaves indole-3-glycerol phosphate to form indole and glyceraldehyde-3-phosphate; this is called the α -reaction. Subsequently, TS β accepts indole as substrate and catalyzes the conversion of Ser into Trp. This is the β -reaction [55]. Unlike in bacteria and fungi, Trp in plants also serves as a precursor for the biosynthesis of various secondary metabolites, especially the phytohormone IAA [55].

5. CURRENT STATUS ON IMPROVING ESSENTIAL AMINO ACIDS CONTENT IN PLANTS

The success of the genetic approach has been mostly restricted to improving protein quality in model plants with enriched Lys, Trp and Met production (summarized in Tables 2 and 3). So far, this approach has had relatively limited success in crop species. Limited availability of crop genetic resources for plant breeding has been a significant limitation. Knowledge obtained from basic genetics and genetic engineering studies has also been successfully used to enrich the content of essential amino acids in crop plants. Enriching crop plants in essential amino acids has both economical and humanitarian interest. It helps humans and monogastric animals could obtain essentials amino acids through their diets. Here, we discussed several approaches for improving the content of Lys, Met, and Trp in plants.

5.1. Improving Met-content in Plants

Like carbohydrates and fats, protein is a “macronutrient”, which is needed in relatively large amounts for human and animals to be healthy. High quality protein sources are found in several crops, including beans, legumes, and nuts. Plants are the primary source of all protein consumed by human and livestock. Thus, improving the quality of plant proteins is an important step to enhance human nutrition. Thus, genetic engineering studies have been performed to increase Met-content in plant proteins by manipulating methionine synthesis.

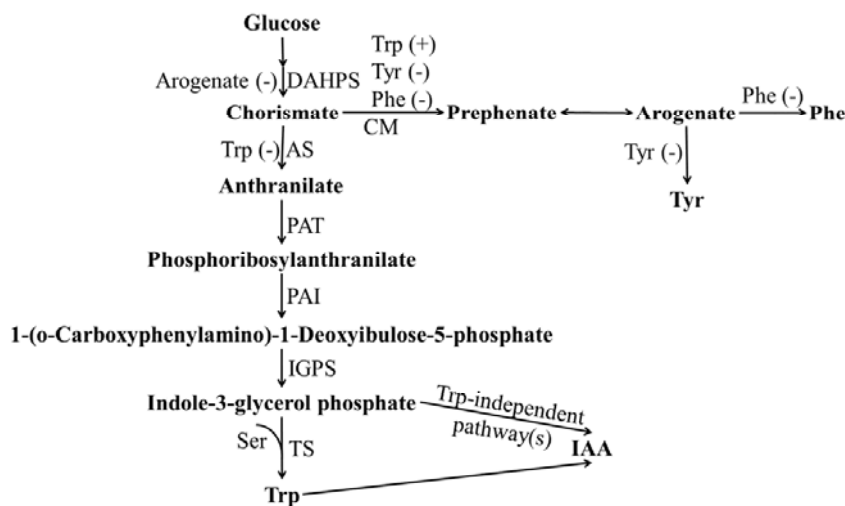


Fig. (2). Trp biosynthesis pathway. AS: Anthranilate synthase; PAT: Phosphoribosylanthranilate transferase; PAI: Phosphoribosyl anthranilate isomerase; IGPS: Indole-3-glycerol phosphate synthase; Trp: Tryptophan; TS: Trp Synthase; Tyr: Tyrosine; Ser: Serine; IAA: Indole-3-acetic acid; Phe: Phenylalanine; AH: Arogenate dehydro; DAHPS: DHP Synthase.

Table 2. Proteins rich in Lys and/or Met used to improve essential amino acids in crops.

Name	Protein Sizes	Amino Acid Content		Crops/Plants	Amino Acid Increases		Ref.
		Lys	Met		Lys	Met	
Zein	10 kDa		20 %	Maize (<i>Zea mays</i>)		22.5 %	[56]
Met-rich 2S seed protein	17 kDa		18 %	Tobacco (<i>Nicotiana tabacum</i>)		30 %	[57]
β -phaseolin	-		3 residues	Common bean (<i>Phaseolus vulgaris</i>)		9 residues	[58]
Prleg	-			Potato (<i>Solanum tuberosum</i> L.)		3 fold	[59]
SBgLR	-		8.7%	Maize (<i>Zea mays</i>)		30.43%	[60]
MB-16	11 kDa		11.11 mg/g total protein	Soybean (<i>Glycine max</i> L.)		12.91 mg/g total protein (16.2%)	[42]
GhLRP	24 kDa			Maize (<i>Zea mays</i>)	16.2-65%		[61]
lysine-rich sb401	240 aa	40 residues		Maize (<i>Zea mays</i>)	24%		[62]

Table 3. Crops modified to bypass feedback regulation of the Lys/Met/Trp biosynthetic pathways.

Amino Acids	Crops/Plants	Enzymes	Genes	Genebank Accession Number	Increase in Amino Acids Compared with WT (Fold)		Ref.
					Free	Total	
Lys	Soybean (<i>Glycine max</i> L.)	Lys insensitive DHDPS	<i>Corynebacterium dapA</i> gene	X53993.1	100	5	[76]
		Lys sensitive <i>E.coli</i> AK	<i>E. coli LysC</i> gene	M11812.1			
	Canola (<i>Brassica napus</i> L.)	Lys insensitive DHDPS	<i>Corynebacterium dapA</i> gene	X53993.1	>100	2	
	Corn (<i>Zea mays</i>)	Lys insensitive DHDPS	<i>Corynebacterium dapA</i> gene			>2	
Met	Thale cress (<i>Arabidopsis thaliana</i>)	TS	<i>mto2-1 TS</i> gene			22	[78]
	Thale cress (<i>Arabidopsis thaliana</i>)	Methionine γ -lyase (MGL)	<i>AtMGL</i> gene	At1g64660		9.2	[78]
	Alfalfa (<i>Medicago sativa</i>)	PEPC kinase (PPCK)	<i>GsPPCK3</i> gene			2.29-2.34	[79]
Trp	Astragalus (<i>Astragalus sinicus</i>)	Feedback-insensitive AS	<i>ASA2</i> gene		1.3-5.5		[65]
	Potato (<i>Solanum tuberosum</i> L.)	Mutant rice α -subunit of AS	<i>OASA1D</i> gene		2-20		[75]
	Rice (<i>Oryza sativa</i> L.)	Mutant rice α subunit of AS	<i>OASA1D</i> gene			80	[80]
	Tobacco (<i>Nicotiana tabacum</i>)	Feedback-insensitive AS	<i>ASA2</i> gene	M92353	3		[44]
	Adzuki bean (<i>Vigna angularis</i>)	Mutant rice α subunit of AS	<i>OASA1D</i> gene		6.5-16.5	1.3-2.1	[81]
	Rice (<i>Oryza sativa</i> L.)	AS	<i>OASA2</i> gene			230	[61]

Most enzymes in biosynthesis pathways leading to amino acids are inhibited by their end-products (allosteric regula-

tion). 2S-sunflower seed protein has been characterized for its IgE-binding capacity, the protein possesses a significant

amount of sulfur-containing amino acids [48]. This could be used to improve protein quality of other crops through genetic engineering. A chimeric gene encoding a methionine-rich Brazil nut (*Bertholletia excelsa*) protein contains over 18 % methionine, whereas most proteins contain only a few percent of methionine. The accumulation of the methionine-rich protein could make a significant enhancement in methionine levels in seed protein of transgenic tobacco [57] and potato [63]. Improving protein quality in the seed is another possibility. The expression of storage genes in seed crops directly affects the nutritional quality of the seed protein. Met-rich proteins were isolated to synthesize cDNA; among the proteins studied are 10kDa zein protein from maize [56], and MRNP34 hydrophobic protein [64]. Using genetic analysis, several genes encoded Met-rich protein in maize were recorded: Gene Zps10/(22) was found to be located on chromosome 9 [65], β -kafirin from sorghum grain [66], polymorphic cDNAs encoded for the Met-rich protein from *Manduca sexta* [67]. Basically, Met-rich proteins in plants were isolated and characterized to compare with major storage proteins. Another approach is to identify and characterize Met-rich protein from crops. A great deal of effort was focused on edible dry beans (*Phaseolus vulgaris* L.) [25], maize endosperm [68], *Canabis sativa* [69], peanuts [70], and soybean (*Glycine max*) [71].

Increasing biosynthesis of low-abundance, endogenous Met-rich protein was a strategy which could resolve the deficiency of sulfur amino acids in plants [72]. Using this approach, total seed methionine was increased by 6.8 % in transgenic soybean and canola plants. In addition, Gm2S-1 was recorded as a candidate gene for overexpression to improve nutritional quality of protein in soybean and other legumes [21]. Another strategy has been to isolate endogenous genes then modify the nucleotide sequence to accommodate more sulfur-containing amino acids. For instant, a 45-bp sequence encoding a Met-rich region from maize was inserted to a gene coding for β -phaseolin from *Phaseolus vulgaris* [58]. Using 2-D gel electrophoresis and *in vitro* labeling, George *et al.* detected several Met-rich proteins. The identification of a Met-rich protein in soybean seed and its composition were reported as a first important step to cloning its gene [73].

To increase Met levels in plants, several transgenic approaches have been used. Cystathionine γ -synthase (CGS), the first committed enzyme in the methionine biosynthesis pathway, was overexpressed in transgenic tobacco plants and showed that high expression level of T-AtCGS could lead to higher total Met contents in the transgenic plants [74]. The transformation of PrLeg gene into potato, which contains low amounts of sulfur-containing amino acids, was found to enhance Met content in the tubers [59]. To improve the quality of protein in soybean seed, research showed that M-16, a 11-kDa protein containing high sulfur amino acids, can be used [42]. On the other hand, the expression of some genes could enhance not only the plant stress tolerance but also its Met content, including genes encoding phosphoenolpyruvate carboxylase (PEPCs) and PEPC kinases (PPCKs) in alfalfa. The overexpression of PPCK could improve plant alkali tolerance as well as the Met content compared to wild type [75]. This approach might be an effective way to improve plant stress tolerance and methionine content.

In summary, several approaches for increasing Met content in plants have been attempted. But the most promising results of Met enhancement in plants came from the approach related to Met-rich proteins. Combining several methods, the improvement of Met content in plants could reach significantly improved levels and opportunity for use as food or feed.

5.2. Improving Lys-content in Plant

Lys plays an important role in nutrition because of the low Lys level in cereals and grains, which are major crops worldwide. Several approaches have been employed for improving Lys content in plants. Research to improve Lys content was conducted via plant breeding, intervention of amino acid metabolism, genetic engineering, and plant transformation techniques. Using a genetic selection for high lysine mutants in maize, barley, and sorghum led to the identification of mutants with high lysine content. Based on biochemical pathways of lysine biosynthesis, different combinations of genetic methods were used to increase synthesis of Lys.

Firstly, the improvement of Lys content could be done by increasing accumulation of Lys in plants. Reducing the activity of Lys-Ketoglutarate Reductase/Saccharopine Dehydrogenase (LKR/SDH) was reported to increase the accumulation of Lys in tobacco seeds [82, 83]. Lys is synthesized through Asp family pathway, which also leads to Met and Thr. Interestingly, dihydrodipicolinate synthase (DHDPS), an enzyme committed to Lys synthesis, is inhibited by Lys through an inhibition loop [84]. Mutant tobacco with high Lys content was developed by modulating the DHDPS gene. In maize, the transgenic lines carrying modified DHDPS exhibited increased Lys content [77]. LY038 transgenic maize line, which has twice as much Lys content as the control, was constructed by using a seed-specific promoter.

To improve Lys level in protein, efforts to synthesize Lys-rich proteins in plants via genetic engineering have been reported. Most of the time, the approaches employed were modifications of endogenous protein sequences or synthetic protein. Recently, the protein translation elongation factor 1 α (EF-1 α) from cereal embryo was reported to be rich in Lys. Singh *et al.* investigated the relationship between EF-1 α content and several proteins from wheat germ. Using immunochemical to detect Lys content of wheat, the authors could identify the correlation between EF-1 α levels and Lys content in plants [85].

Using a different method, genes encoding Lys-rich proteins, such as *SBgLR* from potato, were tested as a candidate for increasing Lys content in maize. Through gene transformation, a transgenic maize line harboring *SBgLR* gene had a significantly higher Lys content compared to the control. When treated with NaCl, the transgenic line also displayed enhanced tolerance to salt stress [60]. In maize, the *opaque2* mutant was a valuable material for breeding to enhancing the nutritional value of the crops. The accumulation of some Lys-rich protein, such as sorbitol dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase, was found in *opaque2* maize line [86]. *Sb401*, a gene encoding a Lys-rich protein, had been successfully integrated into the genome of maize. Compared to several conventional maize lines, the transgenic lines had a higher Lys content and content of total protein

[87]. Therefore, *sb401* can be another important gene for the improvement of nutritive value of maize.

In addition, Lys is thought to be the first limiting essential amino acid in rice. Thus, the creation of Lys-rich rice is of interest for the researchers. The first transgenic rice line was constructed from a gene encoding a Lys-rich protein cloned from *Psophocarpus tetragonolobus*. The transgene was found to express in leaves, stems and roots of the transgenic rice plants [44]. Another achievement was in *Brassica napus*, where a Lys-rich protein gene from the seeds of *Psophocarpus* was successfully transformed into the plant [88]. Three genes encoding Lys-rich proteins in *Arabidopsis*, including *AtAGP17*, *AtAGP18* and *AtAGP19* were reported, in which *AtAGP18* could be involved in a signal transduction pathway that regulates plant growth and development [89]. Other transgenic rice lines have been developed by over-accumulation of Lys-rich binding proteins [62]. Results from this research offer further approaches to improving Lys content in plants.

5.3. Improving Trp Content in Plants

Trp is the second most limiting essential amino acid in protein metabolism. As described above, Trp biosynthesis in plants is feedback-regulated by its end-products. The first research on Trp in plants was done by Kreps and colleagues, they found a mutant of *Arabidopsis*, *amt-1*, that had elevated Trp accumulation in the plants [90]. They suggested that elevated anthranilate synthase (AS) activity found in the mutant caused by an alteration in the α -subunit of the enzyme was responsible for the increased resistance to feedback inhibition by Trp. Following this approach, several studies were conducted to improve Trp content in crop plants. Feedback-insensitive AS and ASA2 isolated from a 5-methyl-tryptophan (5-MTP) resistant cell line were introduced into the forage legume *Astragalus sinicus* [65]. Results showed that the ASA2 transcript and high Trp were co-detected in the leaves, stems and roots of transgenic plants, indicating that ASA2 could be used to transform plants of different species for increased Trp levels. Maize seeds, deficient in Lys and Trp, become one of the most popular subjects to study. The accumulation of 19- and 22- kDa α -zeins in transgenic plants resulting in higher Lys and Trp content has also been reported [91]. The overexpression of *OAS1D*, a gene encoding a feedback-insensitive α -subunit of rice AS, was reported to accumulate Trp in calli and leaves of rice. The total Trp content in the seeds of the transgenic rice plants was also increased. Characterization of *OAS1D* transgenic rice lines demonstrated the feasibility of enhancing the Trp content in the seeds of rice and other crops [81]. The *OAS1D* transgene was also shown to raise Trp content in transgenic potato [41], *adzuki* bean (*Vigna angularis*) [81]. Several *OAS1D*-overexpressed rice lines were significantly correlated with over-accumulation of Trp [92]. Small changes were also observed in several minor metabolites in the *OAS1D*-overexpressed lines. AS activity was an important determinant in IAA biosynthesis as well as Trp accumulation [80]. In transgenic potato plants, the expression of *OAS1D* induced a significant increase in Trp [75]. This increase could be explained by a reduction in the sensitivity of AS to feedback inhibition by Trp.

According to the biosynthesis pathways for essential amino acids, a link between the synthesis of Phe and Trp was found. A mutant of *mtr1-D* gene encoded a form of prephenate dehydratase (PDT) in rice were created to elucidate the connection between Phe and Trp accumulations [72]. In tobacco, several transgenic lines with ASA2 had shown a significant increase in Trp level [44]. Furthermore, metabolic engineering of crops could be applied by a combination of computational biology and gene targeting. Saika *et al.* reported the mutation in OASA2, a key enzyme of Trp biosynthesis in rice, led to higher Trp accumulation in plants [61]. Gene targeting could reduce the risk of gene silencing relative to other conventional approaches.

6. FUTURE DIRECTIONS

Research summarized in this review has a significant potential for creating nutritionally enriched plants. The developments of molecular biology techniques will facilitate the improvement of protein quality in plants. Recombinant DNA techniques and plant transformation have become useful tools to determine the factors that could enhance essential amino acids in plants.

The efforts in enhancing the content of essential amino acids in plants had made significant progress. In the future, these results will be important for global agriculture. The approach of overexpressing genes encoding proteins rich in Met, Lys, and Trp, although making slower progress, is a key step for improving nutritional quality of plants. For example, the results in creating transgenic plants with Met-, Lys- or Trp-rich protein by overexpressing several specific genes would make a significant improvement in protein for use as food or feed. Met-, Lys- or Trp-rich proteins were characterized and found to be easily digested, and also safe for consumption [93].

Essential-amino-acids-rich proteins in plants must pass the test for their effects on the human and animal digestion systems before being continued to the next steps. Employing genetics transformation to improve protein quality in plants also encountered a trouble with the global acceptance of genetically modified (GM) crops. Like most GM crops, the development of plants with high levels of essential amino acids in protein depends on the public acceptance of genetically modified organism (GMO) [94]. Recently, there has been acceptance in some countries. For examples, the situation of LY038 maize, which has double the Lys content without changing the grain protein content, is recognized by the USDA to be as safe as other varieties bred through conventional methods. Then, LY038 maize was approved for commercial use in a number of countries [95]. In the future, production of GM crops with enhanced essential amino acids is also expected to combine two or three important amino acids, namely Met, Lys and Trp.

6.1. Genome Editing Techniques and the Improvement of Protein Quality in Crops

Targeted genome editing using synthetic nuclease or endonuclease enzymes has recently become a powerful tool in genetic engineering. Zinc finger nucleases (ZFNs) employing a zinc finger domain with FokI endonuclease domain, had been used successfully as genome editing tool in *Arabi-*

dopsis thaliana [96, 97], *Zea mays* [98] and legume [99]. More recently, researchers have developed new tools for plant genome editing, like the Transcription Activator-Like Effector Nucleases (TALENs), and the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/Cas9. In 2015, Clasen *et al.* used TALENs to knock-out vacuolar invertase gene (*VInv*), which encodes a protein that cleaves sucrose to glucose and fructose. These results improved the characteristics of commercial potato which eliminated reducing sugars, therefore reducing proportion of acrylamide [100]. In nature, CRISPR/Cas9 functions as a bacterial adaptive immunity system. CRISPR/Cas9 applications have been developed for gene modification in a range of plant species including soybean [101], rice, *Arabidopsis*, tobacco, sorghum [102] and sweet orange [103]. Genome editing techniques, especially TALENs and CRISPR/Cas9 system, are precise approaches to improve traits including enhanced nutritional quality and commercial valuable compounds. Given the high precision nature of genome-editing techniques, genes encoding feedback-sensitive enzymes in the amino acid biosynthesis pathways can be edited to become insensitive to feedback inhibition. Combined with conventional breeding, the transgenes encoding editing machinery can be removed and the “edited plant” with improved protein quality can be considered as “non-GMO” plants.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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